Table 3
Mutation spectra of *gpt* mutant colonies

Sex	Male				Female			
Treatment	Control		Dicyclanil		Control		Dicyclanil	
	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)
Base substitutions								
Transversions								
GC:TA	1[a] (5.0)	0.02	1 (4.8)	0.02	1 (5.0)	0.02	34 (32.7)	0.73
GC:CG	3 (15.0)	0.06	3 (14.3)	0.07	1 (5.0)	0.02	10 (9.6)	0.21
AT:TA	1 (5.0)	0.02	3 (14.3)	0.07	1 (5.0)	0.02	10 (9.6)	0.21
AT:CG	0	0	0	0	2 (10.0)	0.05	2 (1.9)	0.04
Transitions								
GC:AT	6 (30.0)	0.13	11 (52.4)	0.25	5 (25.0)	0.12	26 (25.0)	0.56
AT:GC	3 (15.0)	0.06	1 (4.8)	0.02	3 (15.0)	0.07	0	0
Deletions								
Single bp	5 (25.0)	0.11	2 (9.5)	0.05	6 (30.0)	0.14	15 (14.4)	0.32
Over 2 bp	0	0	0	0	0	0	2 (1.9)	0.04
Insertions	1 (5.0)	0.02	0	0	1 (5.0)	0.02	1 (1.0)	0.02
Complexes	0	0	0	0	0	0	4 (3.8)	0.08
Total	20	0.42 ± 0.20	21	0.48 ± 0.31	20	0.48 ± 0.29	104	$2.23 \pm 0.55^{*}$

[a] The number of colonies with independent mutations.
* $p < 0.01$ vs. Control.

3.3. Oxidative DNA damage and lipid peroxidation

The results for 8-OHdG and TBARS in the livers of *gpt* delta mice given DC are illustrated in Figs. 2 and 3, respectively. 8-OHdG levels in the males and females (males; 0.62 ± 0.06, $p < 0.01$, females; 0.65 ± 0.13 8-OHdG/10^5dG, $p < 0.01$) treated with DC were elevated compared with the relevant control values (male;

Table 4
Spi^- MFs in the livers of *gpt* delta mice given DC

Sex	Treatment	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi^-)	Mutant frequency (10^{-5})	Mean ± S.D.
Male	Control	1	10.4	4	0.39	0.27 ± 0.17
		2	13.3	2	0.15	
		3	19.4	3	0.16	
		4	14.2	2	0.14	
		5	11.7	6	0.51	
	Dicyclanil	6	20.4	10	0.49	0.42 ± 0.12
		7	17.1	4	0.23	
		8	10.1	4	0.40	
		9	12.5	7	0.56	
		10	11.6	5	0.43	
Female	Control	11	16.7	15	0.90	0.68 ± 0.29
		12	10.9	10	0.92	
		13	33.9	10	0.29	
		14	ND	ND	ND	
		15	19.4	12	0.62	
	Dicyclanil	16	18.9	14	0.74	0.83 ± 0.35
		17	22.6	29	1.28	
		18	17.4	7	0.40	
		19	15.4	10	0.65	
		20	16.0	17	1.06	

ND, not detected.

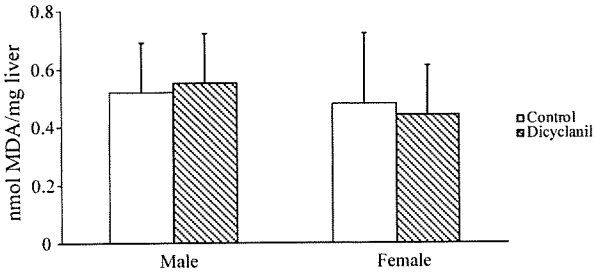

Fig. 2. Changes of TBARS levels in livers of male and female *gpt* delta mice fed DC in the diet at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals.

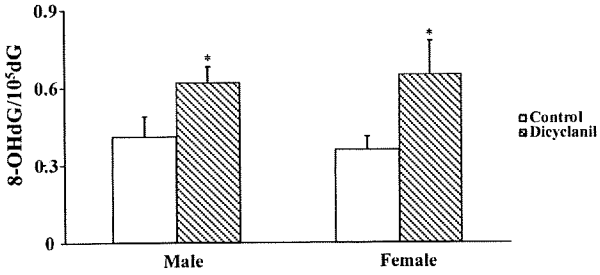

Fig. 3. Changes of 8-OHdG levels in liver nuclear DNA of male and female *gpt* delta mice fed DC in the diet at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals. Significant differences from the relevant control are shown by $^{*}p<0.01$.

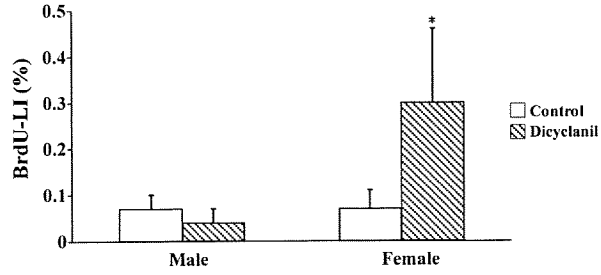

Fig. 5. Changes of BrdU-LIs in hepatocytes of male and female *gpt* delta mice fed DC at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals. Significant differences from the relevant control are shown by $^{*}p<0.05$.

0.41 ± 0.08, female; 0.36 ± 0.05 8-OHdG/10^5dG) with statistical significance. In contrast, there were no significant differences in TBARS levels among the groups.

3.4. Histopathology and immunohistochemical analysis of BrdU

Histopathologically, swelling of centrilobular hepatocytes was observed in the treated mice of both sexes without overt hepatocyte necrosis, the extents being almost equal in both genders (Fig. 4a and b). Fig. 5 summarizes changes in BrdU-LI for hepatocytes in male

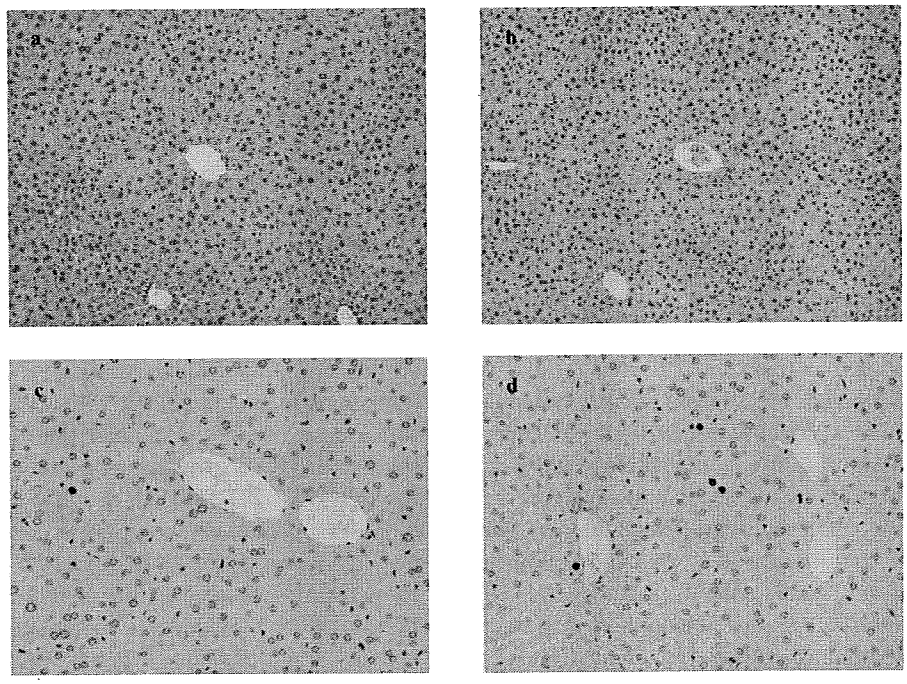

Fig. 4. Photomicrographs of livers of male (a and c) and female (b and d) *gpt* delta mice fed DC at a concentration of 0.15% for 13 weeks. Centrilobular hepatocyte hypertrophy is evident in both sexes (a and b). H&E staining at ×100 original magnification. In contrast to few BrdU-positive hepatocytes in a male (c), an appreciable number of the positive hepatocytes is evident in a female (d). BrdU immunohistochemical staining at ×200 original magnification.

and female *gpt* delta mice treated with DC. Although there were no differences between the male groups, BrdU-LIs in the treated females were elevated with significance ($0.30 \pm 0.17\%$, $p < 0.05$) as compared to the control value ($0.07 \pm 0.04\%$) (Fig. 4c and d).

4. Discussion

It has been reported that 18-month exposure of male and female mice to DC at a concentration of 0.15% in the diet caused hepatocelular adenomas and carcinomas with significantly elevated incidence only in the females, in spite of all negative outcomes in various genotoxicity studies [14]. In the present *in vivo* mutation assay, although there were no changes in Spi^- MFs, suggestive of large size of deletion mutations, among the groups, *gpt* MFs were significantly elevated in the females, but not the males. Their spectrum analyses revealed GC:TA transversion mutations to be predominant in the *gpt* mutations observed in the DC-treated females. To the best of our knowledge, this is the first report showing DC-induced genotoxicity, which was in good concordance with DC carcinogenicity in terms of the sex specificity.

It has been assumed that biotransformation of DC involves oxidative opening the cyclopropyl ring at various positions, followed by further oxidation and cleavage of the cyclopropyl-N-bond [14]. In the males, gene expression analysis using cDNA microarray and RT-PCR from the livers after DC treatment demonstrated upregulation of some metabolism-, reduction- and oxidation-related genes such as *CYP1A*, *Por* and *Txnrd 1*, suggesting a possible generation of reactive oxygen species (ROS) through P450-mediated metabolism of DC [22]. Hepatocyte hypertrophy was apparent in the treated mice of both genders and the present study revealed increases of 8-OHdG levels in liver DNA of female *gpt* delta mice given DC as well as the males, indicating that oxidative DNA damage due to ROS generated during DC metabolism is a phenomenon common to both sexes. By contrast, BrdU-LIs in hepatocytes were only significantly increased in the females, which were in line with the fact that significant increase in liver weights was observed in the treated females, but not the males. In the absence of overt cytotoxicity in the treated females, it seems unlikely that induction of cell proliferation resulted from a regenerative response, so that the underlying mechanisms remain unclear. It is well known that during cell replication, 8-OHdG primarily causes GC:TA transversion by mispairing with A bases [23,24]. Furthermore, the fact that regeneration of hepatocytes after partial hepatectomy does not affect 8-OHdG levels suggests that there is no replication coupled repair of preexisting 8-OHdG [25]. Consequently, high proliferation of cells with accumulated 8-OHdG lead to considerable increase in reporter gene MFs [25–27]. Accordingly, we hypothesized that the dual induction of 8-OHdG and cell proliferation due to DC exposure to female *gpt* delta mice might be responsible for the increment in the MFs. In addition, we have found that 4-week exposure of B6C3F1 mice, a back strain of *gpt* delta mice, to DC at the same dose was sufficient to induce significant elevation of 8-OHdG only in the females, but not the males, albeit without no overexpression of *OGG1*, *MYH* or *MTH* mRNA levels in the livers of both sexes (unpublished data). Therefore, it seems likely that the early onset of DNA oxidation is also responsible for the sex specificity.

As a matter of fact, 8-OHdG levels in nuclear genomic DNA may not always imply high levels of 8-OHdG at *gpt* loci specifically located at chromosome 17 [28]. It has been proposed that the distribution of 8-OHdG following exogenous oxidative stress is not random in the genome [29]. Nevertheless, abundant 8-OHdG modification at the *gpt* loci was reported to be observed in the kidneys of *gpt* delta mice treated with ferric nitrilotriacetate [30]. Partly due to the considerable number of copies of the transgene (approximately 80 copies) per haploid genome in the *gpt* delta mice [31], *gpt* loci indeed appear vulnerable to 8-OHdG modification [30]. Therefore, it is highly probable that DC exposure of the *gpt* delta mice caused accumulation of 8-OHdG at *gpt* loci judging from 8-OHdG levels in the genomic DNA. The present spectrum analysis of *gpt* mutants caused by DC exposure showed GC:AT transition mutations at the second highest incidence, despite this type of mutation being spontaneously observed with a certain incidence. In NIH3T3 cells transfected with the c-Ha-ras gene, which incorporates 8-OHdG at the first position of codon 12 (GGC), show mainly GC:TA transversions, while incorporation at the second position elicits GC:AT transitions to an appreciable extent [32,33]. In addition to our data, the results indicate that types of mutations other than GC:TA transversion mutations are induced by 8-OHdG in DNA [34]. We also found that 85.4% of base substitution mutations occurred at G:C pairs and 14.6% at A:T pairs. Although $1,N^6$-ethenoadenosine formed during lipid peroxidation induces AT:GC transition mutations [35], this type of mutation was not evident among the *gpt* mutants. This might reflect the apparent lack of lipid peroxidation despite oxidative DNA damage due to DC treatment.

In conclusion, DC hitherto categorized as a non-genotoxic carcinogen was here shown to have the

potential to induce gene mutations at target site of DNA, possibly due to 8-OHdG formation. The co-examined data strongly suggest that induction of cell proliferation is required to predispose cells harboring high amounts of 8-OHdG to generation of mutations. Thus, the fact that DC-induced genotoxicity is dependent on cell proliferation in addition to nuclear DNA damage by ROS generated through DC metabolism might provide a reason for why genotoxicity has not been detected previously in various mutation assays. The overall data suggest that examination of several parameters associated with carcinogenesis using reporter gene transgenic rodents is a powerful tool for risk assessment of so-called non-genotoxic carcinogens.

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Review

In vivo Approaches to Study Mechanism of Action of Genotoxic Carcinogens[1]

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Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. However, it is actually difficult to discriminate genotoxic and non-genotoxic carcinogens because both assays are basically independent each other, which raises a simple query as to how much the detected genotoxic potential can consequently contribute to carcinogenicity. To clarify this critical issue, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected as powerful tools for the simultaneous evaluation of both genotoxicity and carcinogenicity at the same organ level. A number of studies of genotoxic carcinogens using these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action. On the other hand, a known non-genotoxic carcinogen dicyclanil increased *in vivo* genotoxicity as well as oxidative DNA damage in female mice, consistently with the sex specificity of its carcinogenicity, albeit without clear evidence of direct DNA reactivity. In contrast, a genotoxic chlorinated water by-product MX failed to exert *in vivo* genotoxicity and carcinogenicity in mice. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity as compared with intact counterparts. These results thus indicate that understanding of the detailed mechanism of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose.

Key words: *in vivo* study, mechanism of action, genotoxic carcinogen

Introduction

Genotoxic carcinogens are chemicals or factors which not only induce neoplastic lesions in long-term animal bioassays but also test positive in genotoxicity assays *in vitro* or *in vivo*. In this context, test chemicals are classified into 4 categories, *i.e.*, genotoxic carcinogens, non-genotoxic carcinogens, genotoxic non-carcinogens and non-genotoxic non-carcinogens. Based on this classification, genotoxic and non-genotoxic carcinogens are evaluated without and with thresholds in current risk assessment procedures, respectively (1). However, it is actually difficult to discriminate genotoxic and non-genotoxic carcinogens because each assay is carried out separately. Namely, both assays are basically independent each other, which raises a simple query as to how much the detected genotoxic potential can contribute to carcinogenicity.

In this context, several possibilities are proposed for the critical issues that genotoxic carcinogens may also have the threshold. First, if a set of genotoxicity assays for a compound carcinogenic to rodents proved to be falsely positive, the compound is no more called as a genotoxic carcinogen, indicating the existence of true threshold as a non-genotoxic carcinogen. It may be difficult to confirm the genotoxic potential found in an assay as false positive, however, false reactions in a number of genotoxicity assays obviously exist judging from some discrepancy between *in vitro* and *in vivo* assays, as well as single dose and repeated dose *in vivo* studies. Second, it is unclear as to how much the detected genotoxicities contribute to the carcinogenicity found in long-term rodent assays. This point could be important to understand organ-, species- and sex-differences of carcinogenicity. Third, it is well known that carcinogenesis process *per* se involves multi-steps such as DNA adduct formation/repair, gene mutation, apoptosis, cell proliferation and immune suppression. If there is a threshold in some of these steps, it is likely that the carcinogenic compound may have the threshold in inducing carcinogenicity. Even in the simplest hypothesis, both genotoxic and non-genotoxic or epigenetic events are required for the completion of carcinogenesis, suggesting a possible threshold determined with non-genotoxic

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events. Finally, statistical or mathematical approaches are concerned with possible practical thresholds even for true genotoxic carcinogens.

Taken together, it can be emphasized that the mechanisms of action are crucial to determine as to whether the initiation of carcinogenicity is based on the direct DNA reaction, how much the genotoxicity contributes to the carcinogenicity, or if the carcinogenicity also fits to human risks. In this review article, our data using transgenic rodent mutation models/assays are shown, and the usefulness for investigating the mechanisms of carcinogenic action is discussed.

Transgenic Rodents Carrying Reporter Genes

Recently, a detailed review on several transgenic rodent mutation assays has been reported from OECD (2), in which Muta™ Mouse, Big Blue®, *lacZ* plasmid mouse, *gpt* delta rodents, use of the λ *cII* transgene and other transgenic systems such as *supF*, *lacI* (BC-1), *rpsL* and bacteriophage ΦX174 are shown as promising models (2). Among them, the *gpt* delta mouse was established by microinjection of λEG10 phage DNA (48 kb) into the fertilised eggs of C57BL/6J mice (3). Phage λEG10 carries about 80 copies of the transgene in a head-to-tail fashion at a single site of chromosome 17 and is maintained as a homozygote (*i.e.* the mouse carries about 160 copies of λEG10 DNA per diploid genome) (4). More recently, *gpt* delta rats have been developed in Sprague-Dawley (5) and F344 (6) backgrounds. The *gpt* delta rat has approximately 10 copies of the λEG10 vector integrated at position 4q24-q31. The transgenic rat is available as a hemizygote only (5). Mutation in the *gpt* delta mouse and rat can be assessed using 6-thioguanine and Spi^- selection, which respond primarily to point mutation and deletion, respectively (7).

To clarify the critical issues pointed out in the Introduction section, we have studied the mechanisms of action of carcinogens in transgenic rats or mice carrying reporter genes, which are expected to provide powerful tools for the evaluation of both genotoxicity and carcinogenicity at the same organ level.

Examples of Simultaneous Evaluation of Genotoxicity and Carcinogenicity in Transgenic Rodents

Our studies of genotoxic carcinogens such as environmental pollutants, nitrosamines and heterocyclic amines in these transgenic rodents have revealed good correlations between genotoxicity and carcinogenicity in terms of mechanism of action (6,8,10–16).

In order to cast light on carcinogen-specific molecular mechanisms underlying experimental hepatocarcinogenesis in rats, *in vivo* genotoxicity and mutation spectra of known genotoxic rat hepatocarcinogens *N*-nitrosopyrrolidine (NPYR), and 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), as well as the non-genotoxic hepatocarcinogen di(2-ethylhexyl)phthalate (DEHP) and the non-carcinogen acetaminophen (APAP) were investigated in *gpt* delta transgenic rats (8). After 13-week treatment, glutathione *S*-transferase placental form (GST-P)-positive liver cell foci were significantly increased in NPYR- and IQ-treated rats. In the DEHP-treated rats, marked hepatomegaly with centrilobular hypertrophy of hepatocytes occurred, although GST-P staining was consistently negative. There were no increases in GST-P positive foci in the APAP-treated rats. Positive genotoxicity was detected in IQ- and NPYR-treated rats of which mutant frequencies in the liver DNA were approximately 35-fold and 10-fold higher, respectively, than that of non-treatment control rats. There were no increases in mutant frequencies in the DEHP- or APAP-treated rats as compared to the non-treatment control value. IQ induced mainly base substitutions leading to G:C to T:A transversions and deletions of G:C base pairs. In contrast, NPYR primarily caused specific AT to GC transitions, which are very rare in the other groups. These data provided support for the conclusion that hepatocarcinogenesis by IQ and NPYR depends on genotoxic processes and specific DNA adduct formation while DEHP exerts its influence via a non-genotoxic promotional pathway. Our data also indicate that analysis of specific *in vivo* mutational responses with transgenic animal models can provide crucial information for understanding the molecular mechanisms underlying chemical carcinogenesis (8). In fact, thymine adducts were detected at levels as much as guanine adducts in the liver of rats given NPYR (9).

To clarify the role of 8-hydroxydeoxyguanosine (8-OHdG) formation as a starting point for carcinogenesis, we examined the dose-dependence and time-course of changes of OGG1 mRNA expression, 8-OHdG levels and *in vivo* mutations in the kidneys of *gpt* delta rats given $KBrO_3$ in their drinking water for 13 weeks (6). There were no remarkable changes in OGG1 mRNA in spite of some increments being statistically significant. Increases of 8-OHdG occurred after 1 week at 500 ppm and after 13 weeks at 250 ppm. Elevation of Spi^- mutant frequency suggestive of deletion mutations occurred after 9 weeks at 500 ppm although no mutations were increased before 5-weeks treatment (6). In a two-stage experiment, F344 rats were given $KBrO_3$ for 13 weeks then, after a 2-week recovery, treated with 1% nitrilotriacetate (NTA), a known kidney tumor-promoter, in the diet for 39 weeks. The incidence and multiplicity of renal preneoplastic lesions in rats given $KBrO_3$ at 500 ppm followed by NTA treatment were significantly higher than in rats treated with NTA alone. Results suggest that a certain period of time, more than

5 weeks in this experiment, might be required for 8-OHdG to cause permanent mutations. The two-step experiment shows that cells exposed to the alteration of the intranuclear status by oxidative stress including 8-OHdG formation might be able to form tumors with appropriate promotion (6).

On the other hand, dicyclanil, a mouse hepatocarcinogen showing all negative results in various genotoxicity tests increased *in vivo* genotoxicity as well as oxidative DNA damage in mice (17). Male and female *gpt* delta mice were given dicyclanil at a carcinogenic dose for 13 weeks. Significant increases in 8-OHdG levels and centrilobular hepatocyte hypertrophy were observed in the treated mice of both sexes. Bromodeoxyuridine-labeling indices and liver weights for the treated females, but not the males, were significantly higher than those for the controls. Likewise, the *gpt* mutant frequencies in the treated females were significantly elevated, GC:TA transversion mutations being predominant. The results for the transgenic mutation assays were consistent with dicyclanil carcinogenicity in terms of the sex specificity for females although it still remains unclear how much sex hormone contributes to these sex differences. Together with the early onset of 8-OHdG formation being observed 4 weeks after the treatment in the female B6C3F1 mice, a back strain of *gpt* delta mice, considering that 8-OHdG induces GC:TA transversion mutations by mispairing with A bases, it is likely that cells with high proliferation rates and a large amounts of 8-OHdG come to harbor mutations at high incidence. The results imply that examination of carcinogenic parameters concomitantly with reporter gene mutation assays is able to provide crucial information to comprehend the underlying mechanisms of so-called non-genotoxic carcinogenicity (17).

It is well documented that a chlorinated water by-product 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is strongly mutagenic in Ames assay without metabolic activation (18) and induces carcinogenicity targeting the liver, thyroid and lung in rats (19). In contrast to dicyclanil, MX exerted neither *in vivo* genotoxicity nor carcinogenicity in mice (20). Groups of male and female *gpt* delta transgenic mice were given MX at doses of 0–100 ppm in their drinking water for 12 weeks, and then killed to assess *in vivo* genotoxicity, and cell proliferative activity using immunohistochemistry for proliferating cell nuclear antigen (20). Further groups of *gpt* delta mice were given 0 or 100 ppm MX for 78 weeks, and a full necropsy with histopathological examination of all organs was conducted to detect neoplastic lesions. The 12-week MX treatment did not result in genotoxicity in the livers or lungs or cell proliferative activity in several organs of the mice, and the 78-week treatment did not cause carcinogenicity. These findings indicate that MX is not genotoxic, mitogenic or carcinogenic in mice, and suggest that the compound might exert epigenetic actions for carcinogenicity in rats although its *in vivo* genotoxicity remains unknown in rats (20).

Approaches for Genotoxic Carcinogens

Once a certain chemical was evaluated to be positive for both genotoxicity and carcinogenicity assays, the risk of such "genotoxic carcinogen" is assessed on non-threshold basis in the current risk assessment procedure. Based on properties of non-threshold, any genotoxic carcinogens are basically prohibited when intentionally added to food like food additives, or assessed with virtually safety dose (VSD) or margin of exposure (MOE) approaches when unavoidable from environment including food (1). For "genotoxic carcinogens", it may be critical to clarify the mechanism of action. As the first step, species specificity could be determined from the weight of evidence (21,22). For example, α_{2u}-globulin-related renal carcinogenicity in male rats is no more relevant to human risk (23). If the mechanisms are more or less relevant to human risk, then contribution of genotoxicity to carcinogenicity should be evaluated. For this purpose, transgenic rodents carrying reporter genes would be very useful for judging direct, indirect or no DNA reactivity *in vivo*. Taken together with other mechanisms of action such as cell proliferation, apoptosis and immunodeficiency, the key event for carcinogenicity would tell us as to whether there is any threshold. In some cases, other transgenic rodents such as *p53*, *nrf2* or constitutive active/androstane receptor (CAR) knockout mice might be helpful to elucidate the mechanisms of action. Finally, statistical or mathematical evaluation can provide VSD or MOE even for strictly defined genotoxic carcinogens like radiation.

Conclusions

These results clearly indicate that understanding of the detailed mechanisms of carcinogenic action could be crucial for more precise risk assessment, and bioassay systems using transgenic rodents carrying reporter genes would be extremely useful for that purpose. We also confirmed that such reporter gene-carrying rodents are not susceptible or resistant to carcinogenicity (10,14,20) as compared with intact counterparts. Taken together, we propose a combined subchronic toxicity/*in vivo* genoxicity study using such transgenic rodents (Fig. 1) as a rapid and advanced bioassay to detect genotoxic carcinogens. In terms of additional approaches to detect *in vivo* genotoxic potential at organ levels, our proposing bioassay system may be more promising than a bioassay system extended from subchronic toxicity study suggested by Dr. Cohen (24).

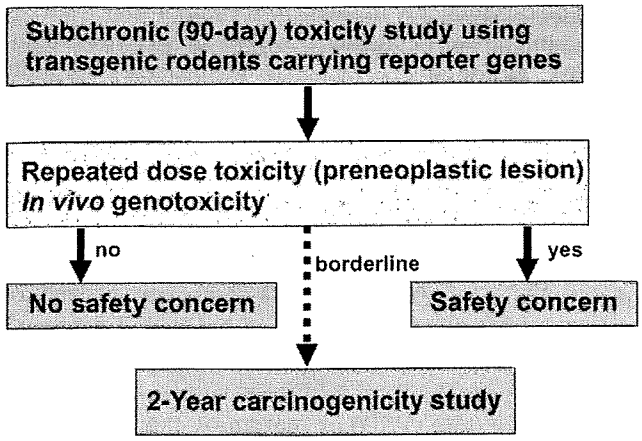

Fig. 1. Proposal of a combined subchronic toxicity/*in vivo* genoxicity study.

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Possible participation of oxidative stress in causation of cell proliferation and *in vivo* mutagenicity in kidneys of *gpt* delta rats treated with potassium bromate

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ABSTRACT

Clarifying the participation of oxidative stress among possible contributing factors in potassium bromate ($KBrO_3$)-induced carcinogenesis is of importance from the perspective of human health protection. In the present study, utilizing the antioxidative effects of α-tocopherol (α-TP) or sodium ascorbic acid (SAA) to attenuate oxidative stress, alterations in bromodeoxyuridine labeling indices (BrdU-LIs) and reporter gene mutations in kidneys of male and female *gpt* delta rats given $KBrO_3$ were examined. Five male and female *gpt* delta rats in each group were given $KBrO_3$ at a concentration of 500 ppm in the drinking water for 9 weeks, with 1% of α-TP or SAA administered in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Increases in 8-hydroxydeoxyguanosine levels in kidney DNA of both sexes of rats given $KBrO_3$ were significantly inhibited by SAA, but not α-TP. While BrdU-LIs in the proximal tubules of female rats were also significantly reduced by SAA, those in the males and *gpt* mutant frequencies in kidney DNA of both sexes were not affected by SAA or α-TP. Immunohistochemical and Western blot analyses for α_{2u}-globulin strongly suggested that induction of cell proliferation observed in the males might primarily result from accumulation of this protein, independent of oxidative stress. The overall data indicated that while oxidative stress well correlates with induction of cell proliferation in females, its role in males and in generation of *in vivo* mutagenicity by $KBrO_3$ in both sexes is limited.

1. Introduction

During the bread making process, bromate is considered to be converted to bromide (Kurokawa et al., 1990), so that use of potassium bromate ($KBrO_3$) has been permitted as a flour improver for bread making in Japan and the USA in spite of its carcinogenicity (Kurokawa et al., 1986; DeAngelo et al., 1998). However, since ozonation of surface water for disinfection yields $KBrO_3$ as a by-product (Cavanagh et al., 1992), there is still concern regarding the human hazard presented by its renal carcinogenicity. As is clear from the specific use as a food additive, $KBrO_3$ is a potent oxidizing agent. This property is responsible for changes in DNA bases as well as lipid peroxidation (LPO), in the kidneys of treated rats (Chipman et al., 1998; Umemura et al., 1998). Since 8-hydroxydeoxyguanosine (8-OHdG), a form of guanine oxidized at C-8 position, is known to be fairly stable (Kasai and Nishimura, 1991), elevation of this oxidized base following $KBrO_3$ exposure implies involvement of oxidative stress in $KBrO_3$-induced carcinogenesis (Umemura and Kurokawa, 2006; Delker et al., 2006). Simultaneous treatment with antioxidants is known to prevent elevation of 8-OHdG and LPO induced by $KBrO_3$ (Cadenas and Barja, 1999; El-Sokkary, 2000), but it remains unclear how oxidative stress contributes to $KBrO_3$-carcinogenesis.

In two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine as an initiator, $KBrO_3$ enhances renal tumorigenesis in both male and female rats (Kurokawa et al., 1985; Umemura et al., 1995). Also, short-term exposure to $KBrO_3$ in males was found to significantly elevate bromodeoxyuridine-labeling indices (BrdU-LIs) in proximal convoluted tubules (PCTs) in the same dose-dependent manner as evident in the promotion assay (Umemura et al., 1993). As a possible mode of action, we have proposed involvement of α_{2u}-globulin accumulation in $KBrO_3$-induced cell proliferation in males (Umemura et al., 2004). However, the fact that PCT BrdU-LIs in females exposed to $KBrO_3$ were also increased, albeit at higher doses than in males, implies the existence of other causal factors.

A two-stage model using nitrilotriacetate as a promoter has further shown that $KBrO_3$ possesses initiating activity for renal carcinogenesis in male rats (Umemura et al., 2006). In addition to previous positive results in several mutagenicity tests (Ishidate et al., 1984; Ishidate and Yoshioka, 1980; Hayashi et al., 1988), recent findings using isolated rat kidney cells (Nesslany et al., 2007) and

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human peripheral lymphocytes (Kaya and Topaktas, 2007) point to genotoxic potential. Also, in an *in vivo* mutation assay using reporter gene transgenic rats, $KBrO_3$ proved capable of elevating the transgene mutation frequency (Umemura et al., 2006; Yamaguchi et al., 2008). Although induction of micronuclei in rat peripheral blood reticulocytes by $KBrO_3$ was inhibited by antioxidants (Sai et al., 1992), there have been few reports demonstrating clear relationships between oxidative stress and its genotoxicity.

Assessment of the participation of oxidative stress in $KBrO_3$ carcinogenesis is clearly necessary for accurate estimation of its hazard risk to humans. In the present study, taking advantage of the inhibitory effects of two different types of antioxidants, α-tocopherol (α-TP) and sodium ascorbic acid (SAA), changes in BrdU-LIs and α_{2u}-globulin accumulation in PCT, and transgene mutations in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were investigated.

2. Materials and methods

2.1. Chemicals

$KBrO_3$, α-TP and SAA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaline phosphatase was obtained from Sigma Chemical (St. Loius, MO, USA) and nuclease P1 was from Yamasa Shoyu (Chiba, Japan). Anti-BrdU monoclonal and anti-α_{2u}-globulin polyclonal antibodies were from DakoCytomation (Glostrup, Denmark) and R&D Systems, Ltd. (Minneapolis, MN, USA), respectively.

2.2. Animals, diet and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female *gpt* delta F344 rats carrying about five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). They were housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility, maintained under conditions of controlled temperature (23 ± 2 °C), humidity (55 ± 5%), air change (12 times per hour), and lighting (12 h light/dark cycle) and were given free access to CRF-1 basal diet (BD; Charles River Japan) and tap water.

2.3. Animal treatments

Groups of 5 male and female *gpt* delta rats were administered $KBrO_3$ solution at a concentration of 500 ppm in the drinking water for 9 weeks. Additional subgroups of 5 male and female *gpt* delta rats were fed α-TP or SAA at a dose of 1% in the diet from 1 week prior to the $KBrO_3$ treatment until the end of the experiment. Further groups of 5 male and female rats each were given basal diet and distilled water (DW) throughout the experimental period as controls. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. At the end of each period, the animals were killed under ethyl ether anesthesia and a part of left kidney was homogenized in Isogen (Nippon Gene, Tokyo, Japan) and stored at −80 °C until use for isolation of total RNA. The remaining left kidney was also stored at −80 °C for 8-OHdG measurement, Western blot analysis and *in vivo* mutation assays. Portions of right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and immunostaining for α_{2u}-globulin.

2.4. Measurement of nuclear 8-OHdG

To prevent 8-OHdG formation as a byproduct during DNA isolation (Kasai, 2002), kidney DNA was extracted by a slight modification of the method of Nakae et al. (1995). Briefly, nuclear DNA was extracted with a commercially available DNA Extractor WB Kit (Wako Pure Chemical Industries, Ltd.) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of autooxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical Co.) was added to the lysis buffer (Helbock et al., 1998). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase, and levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, USA).

2.5. Immunohistochemical procedures

For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (1:100), biotin-labeled horse anti-mouse IgG (1:400), and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4N HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg et al. (1969) using L-glutamyl-4-methoxy-β-naphtylamide (Polysciences Ltd., Warrington, PA, USA) as the substrate in order to assist in distinguishing the three kinds of tubules, as previously described (Umemura et al., 1992). The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). For immunohistochemical staining of α_{2u}-globulin, sections were treated sequentially with normal goat serum, polyclonal rabbit anti-α_{2u}-globulin (1:100), biotin-labeled goat anti-rabbit IgG (1:400), and ABC after denaturation of DNA with 4N HCl. The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

2.6. Cell proliferation quantification

At least 3000 tubule cells in each kidney were counted and BrdU-LIs were calculated as the percentages of cells positive for BrdU incorporation.

2.7. Western blotting for α_{2u}-globulin

Kidney samples were homogenized with a Teflon homogenizer in ice-cold 50 mM Tris–HCl, pH 7.4 containing 0.25 M sucrose and a 1% protease inhibitor cocktail (Sigma Chemical Co.). The homogenate was centrifuged for 10 min at 10,000 × *g*, 4 °C, and the resulting supernatant was collected. Protein concentrations were determined with a BCA Protein Assay kit (Pierce Biotechnology Ltd., Rockford, IL, USA). The samples containing 20 μg protein were resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) and analyzed with anti-α_{2u}-globulin (1:200), as well as anti-β-actin as a loading control (1:8000, Sigma Chemical Co.). Appropriate peroxidase-conjugated secondary antibodies (1:2000, Dako Cytomation) were used to detect proteins with ECL Plus (Amersham Bioscience Corp., Piscataway, NJ, USA) reagents.

2.8. In vivo mutation assays

6-TG and Spi$^-$ selections were performed as previously described (Umemura et al., 2007) using the first three animals each group. Briefly, genomic DNA was extracted from the kidneys of the first 3 animals in each group, and lambda EG10 DNA (48 kb) was rescued as phages by *in vitro* packaging.

For 6-TG selection, packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and poured on plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For Spi$^-$ selection, packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. Real Spi$^-$ mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously (Nohmi et al., 2000). DNA sequencing was performed with the Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM™ 310 Genetic Analayzer (Applied Biosystems).

3. Results

As shown in Fig. 1, 8-OHdG levels in kidney DNA of male and female *gpt* delta rats given $KBrO_3$ were significantly increased as compared to the controls. Although the levels in *gpt* delta rats of both sexes co-treated with α-TP or SAA were still significantly higher than the controls, significant decreases in either sex of rats were evident as compared to $KBrO_3$-treated animals.

PCT BrdU-LIs in male and female gpt delta rats exposed to $KBrO_3$, with or without antioxidants, are shown in Fig. 2. In the males, $KBrO_3$ exposure induced prominent rise of BrdU-LIs with statistical significance, which was not affected by α-TP or SAA treatment. In the females, $KBrO_3$ significantly increased BrdU-LIs as in the males,

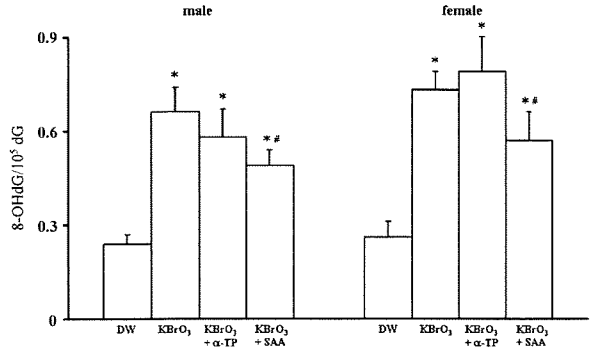

Fig. 1. 8-OHdG levels in kidneys of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. $^{*}p < 0.01$, significantly different from the controls (DW). $^{\#}p < 0.01$, significantly different from the $KBrO_3$ alone group.

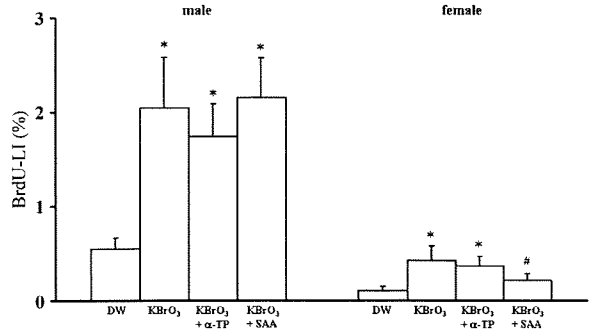

Fig. 2. BrdU-LIs for proximal convoluted tubules (PCT) of male and female *gpt* delta rats co-treated with $KBrO_3$ and α-TP or SAA. Values are means ± SDs of data for 5 rats. $^{*}p < 0.01$, significantly different from the controls (DW). $^{\#}p < 0.01$, significantly different from the $KBrO_3$ alone group.

but in this case, co-treatment with SAA, but not α-TP, was associated with suppression of the elevation.

Immunohistochemical data for α_{2u}-globulin are shown in Fig. 3(A–E). Because α_{2u}-globulin is a male rat-specific urinary protein, in controls scattered accumulation was limited to males (Fig. 3 A). $KBrO_3$ caused accumulation of the protein only in males (Fig. 3 B), which was not inhibited by any antioxidant treatments (Fig. 3 C and D), no binding being evident even in the $KBrO_3$-treated female rats (Fig. 3 E). These findings were directly in line with Western blot results for α_{2u}-globulin (Fig. 3 F).

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Tables 1 and 2. In the males, although statistical analysis could not be performed because no *gpt* mutant colonies were detected in one control rat, MFs in all the treated groups showed a tendency for increase (Table 1). Likewise, in the females, elevation of MFs in all the treated groups were found, the increase in the $KBrO_3$ alone group being statistically significant (Table 2). To characterize *gpt* mutations DNA sequencing was performed (Table 3). Among the groups in which the MFs were significantly increased, there were no common types of mutations. GC:AT transitions in α-TP treated males, GC:TA and AT:TA transversions in SAA treated males and single base deletions in $KBrO_3$ alone treated females showed the highest mutation frequencies. As shown in Table 4, there were no changes in Spi^- MFs in males. In all the treated females, a tendency for elevation of Spi^- MFs was apparent, with statistical significance in the α-TP treatment case (Table 5). However, co-treatment with the antioxidants did not appear to exert any effects on MFs for the *gpt* gene in the kidneys of rats given $KBrO_3$.

4. Discussion

In the present study, increases of 8-OHdG levels in kidney DNA of male and female rats following $KBrO_3$ exposure were significantly suppressed by SAA, but not α-TP. Although precise mechanisms responsible for the differences in efficacy between the two antioxidants remain to be determined, it has been reported that dietary ascorbic acid is capable of accumulating more effectively in renal cortical tissue of rats than is the case with dietary α-TP (Craven et al., 1997). In consideration of the fact that $KBrO_3$ is efficiently reduced by GSH at brush borders on the luminal surfaces of PCT cells (Murata et al., 2001), eventually yielding oxidative stress (Ballmaier and Epe, 1995, 2006), it is plausible that an aqueous antioxidant would exert preventive effects. Previous study demonstrated that dietary vitamin E inhibited 8-OHdG levels in kidney DNA induced by $KBrO_3$ at higher dose (Cadenas and Barja, 1999). The incompatible results might involve differences in the nature of damage to DNA produced by low vs. high doses of $KBrO_3$. In the present study, simultaneous treatment with SAA was in fact able to attenuate oxidative damage caused by $KBrO_3$.

$KBrO_3$ at a concentration of 500 ppm has been reported to promote tumor development in the rat kidney of both sexes (Kurokawa et al., 1985; Umemura et al., 1995). Induction of cell proliferation, regarded as a contributing factor, was observed even at 30 ppm of $KBrO_3$ in males, in contrast to the lowest effective dose in females being 250 ppm (Umemura et al., 2004). Interestingly, α_{2u}-globulin accumulation in the kidneys of male rats also occurred in a

Table 1
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
DW	BD	1	6.0	0[a]	0	
		2	9.3	1	0.11	0.12
		3	7.7	2	0.26	
$KBrO_3$	BD	6	9.2	3	0.33	
		7	98.8	2	0.20	0.43 ± 0.29
		8	6.6	5	0.76	
$KBrO_3$	α-TP	11	8.7	8	0.92	
		12	5.4	8	1.48	1.14 ± 0.30
		13	9.7	10	1.03	
$KBrO_3$	SAA	16	10.1	3	0.30	
		17	7.8	6	0.77	0.53 ± 0.24
		18	9.3	5	0.53	

DW: Distilled water, BD: basal diet.
[a] Two colonies were found on the plate, but neither harbored any *gpt* mutations.

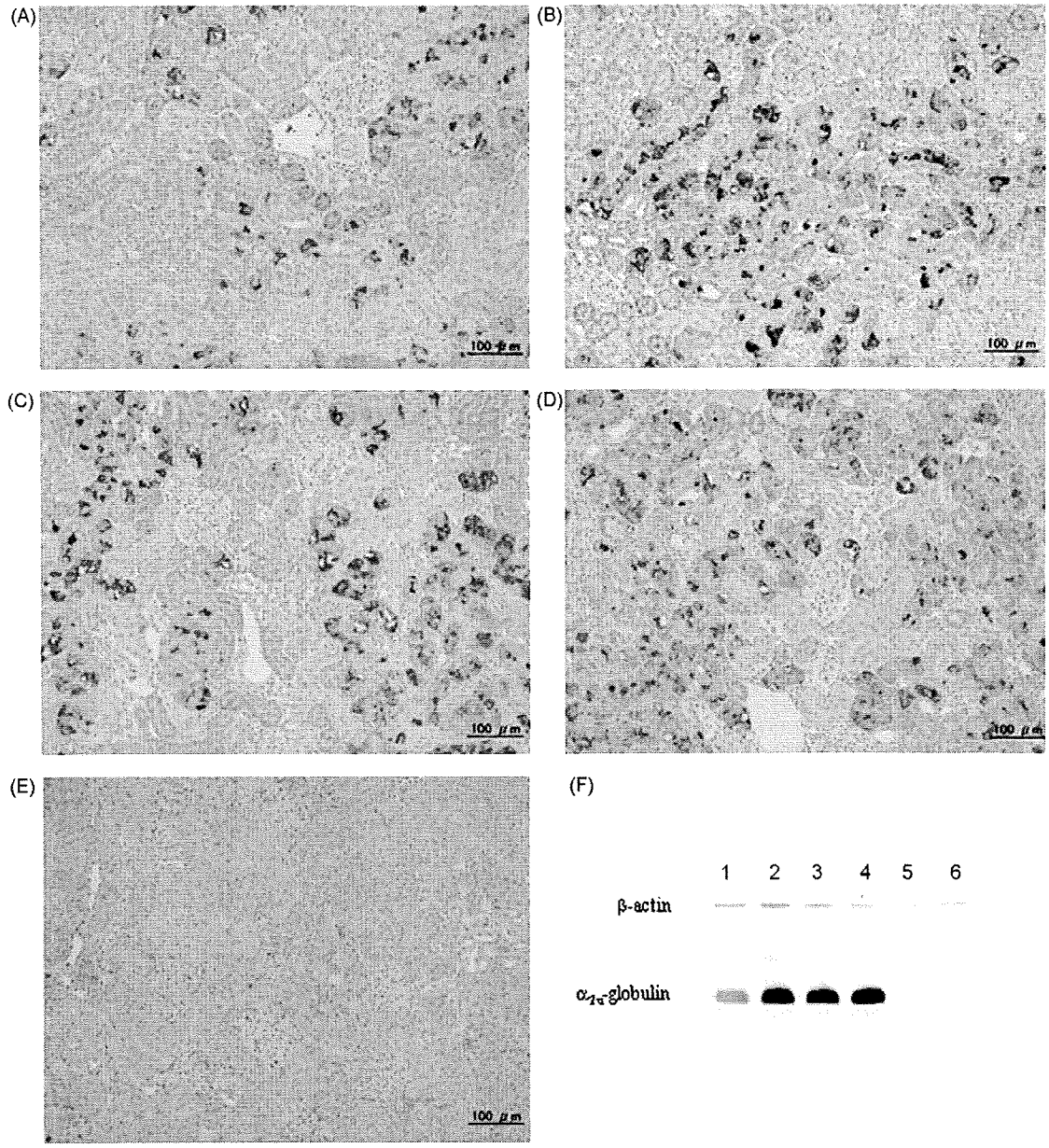

Fig. 3. Photomicrographs of immunohistochemical staining for α_{2u}-globulin in the kidneys of male (A–D) and female (E) *gpt* delta rats given DW (A), $KBrO_3$ (B), $KBrO_3$ and α-TP (C), $KBrO_3$ and SAA (D), or $KBrO_3$ (E). Western blot analysis of α_{2u}-globulin (F) from kidneys of male (lanes 1–4) and female (lanes 5 and 6) *gpt* delta rats given DW (lane 1), $KBrO_3$ (lane 2), $KBrO_3$ and α-TP (lane 3), $KBrO_3$ and SAA (lane 4), DW (lane 5), and $KBrO_3$ (lane 6). α_{2u}-globulin accumulation is more prominent in $KBrO_3$-treated male rats (B) as compared to the controls (A), and is not affected by α-TP (C) or SAA (D) treatment. Note the lack of accumulation in $KBrO_3$-treated females (E), in line with the Western blot analysis (F).

dose-dependent manner from 30 ppm, the protein levels being statistically significant at 125 ppm and above (Umemura et al., 2004). Therefore, it is very likely that this protein accumulation is involved in the cell proliferation observed in the males. In the present study, immunohistochemical and Western blot analysis of α_{2u}-globulin clearly demonstrated accumulation due to $KBrO_3$ exposure, which was not affected by either of antioxidants. This might account for the finding that simultaneous administration of SAA failed to block the rise in BrdU-LIs in males exposed to $KBrO_3$. In general, non-covalently binding of chemicals to the α_{2u}-globulin binding site, a highly aromatic region of the α_{2u}-globulin binding pocket (Huwe et al., 1996) seems to be an initial step, followed by accumulation of the protein in lysosomes of PCT because of resultant resistance to proteolysis. Alternatively, since activities of cystein proteases in lysosomes are prerequisite for degradation of α_{2u}-globulin (Saito et al., 1992), there might be the possibility of primary decrease of protease activity in lysosomes due to interaction of $KBrO_3$ with their thiols acting as a trigger for accumulation (Read, 1991). Although it remains uncertain whether $KBrO_3$ has affinity for the pocket or detrimental effects on lysomal functions, our data imply that induction of cell proliferation following accumulation of the protein in males occurs independently of oxidative stress. On the other hand, in the females lacking α_{2u}-globulin, the BrdU-LI elevation in PCT of rats given $KBrO_3$ was alleviated by SAA. Since mRNA levels of oxidative stress-related genes such as *c-fos*, *c-jun* and *NF-κB* were not elevated in kidneys of $KBrO_3$-treated female rats (data not shown), further studies appear warranted to determine links at the molecular level between oxidation and cell proliferation.

Exposure of female *gpt* delta rats with a genetic background of F344 to $KBrO_3$ at 500 ppm for 9 weeks induced significant elevation of *gpt* MFs along with Spi^- MFs. However, the antioxidants were unable to prevent any type of mutation. 8-OHdG is not only a

Table 2
Effects of antioxidants on *gpt* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
		51	9.9	3	0.30	
DW	BD	52	6.0	1	0.17	0.24 ± 0.07
		53	11.8	3	0.25	
		56	11.0	7	0.64	
$KBrO_3$	BD	57	9.6	4	0.42	0.53 ± 0.11*
		58	11.4	6	0.53	
		61	8.6	6	0.70	
$KBrO_3$	α-TP	62	9.5	4	0.42	0.48 ± 0.20
		63	13.0	4	0.31	
		66	6.8	2	0.29	
$KBrO_3$	SAA	67	10.2	2	0.20	0.44 ± 0.34
		68	8.4	7	0.83	

DW: Distilled water, BD: basal diet.
* $p < 0.01$ vs. DW/BD.

Table 3
Mutation spectra of *gpt* mutant colonies.

Sex	Male				Female			
Treatment (water/diet)	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA	DW/BD	$KBrO_3$/BD	$KBrO_3$/α-TP	$KBrO_3$/SAA
Base substitution								
Transversions								
GC:TA	0[a]	1(0.04)	4(0.17)	3(0.11)	2(0.07)	1(0.03)	0	2(0.08)
GC:CG	0	0	0	1(0.04)	0	1(0.03)	1(0.03)	1(0.04)
AT:TA	0	2(0.08)	4(0.17)	3(0.11)	0	3(0.09)	4(0.13)	2(0.08)
AT:CG	0	1(0.04)	2(0.08)	0	0	3(0.09)	0	0
Transitions								
GC:AT	2(0.09)	1(0.04)	8(0.34)	2(0.07)	2(0.07)	4(0.13)	6(0.19)	3(0.12)
AT:GC	0	1(0.04)	3(0.13)	1(0.04)	0	1(0.03)	1(0.03)	0
Deletion								
Single bp	0	1(0.04)	3(0.13)	1(0.04)	2(0.07)	6(0.19)	2(0.06)	1(0.04)
Over 2 bp	0	1(0.04)	2(0.08)	2(0.07)	1(0.04)	1(0.03)	0	1(0.04)
Insertion	0	1(0.04)	0	0	0	0	1(0.03)	1(0.04)
Complex	1(0.04)	1(0.04)	0	0	0	0	1(0.03)	0
Total	3(0.13)	10(0.39)	26(1.09)	14(0.51)	7(0.25)	20(0.63)*	14(0.51)	11(0.63)

DW: Distilled water, BD: basal diet. Values appearing in parenthesis indicates mutation frequency, $\times 10^{-5}$. * $p < 0.01$ vs. DW/BD.
[a] The number of colonies with independent mutations.

representative marker for oxidative stress but also a primary cause of GC:TA transversions due to mispairing with A (Cheng et al., 1992; Shibutani et al., 1991). Nevertheless, spectrum analysis of the *gpt* mutants induced by $KBrO_3$ did not indicate a majority of GC:TA transversions. Instead, deletions were most common in concord with the results of Spi^- mutation assays. In our previous study using male *gpt* delta rats with a Sprague–Dawley genetic background, significant elevation of Spi^- MFs was rather apparent (Umemura et al., 2006). Furthermore, in an *in vitro* genotoxicity assay for $KBrO_3$ using human lymphoblastoid TK6 cells (Luan et al., 2007) or mouse lymphoma cells (Harrington-Brock et al., 2003), $KBrO_3$ induced large deletions, including loss of heterozygosity at

Table 4
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of male *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi^-)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
		1	19.2	8	0.42	
DW	BD	2	20.3	13	0.64	0.82 ± 0.52
		3	17.8	25	1.40	
		6	15.3	36	2.36	
$KBrO_3$	BD	7	21.8	8	0.37	1.04 ± 1.15
		8	15.7	6	0.38	
		11	23.4	9	0.39	
$KBrO_3$	α-TP	12	26.0	11	0.42	0.43 ± 0.05
		13	14.3	7	0.49	
		16	17.8	9	0.51	
$KBrO_3$	SAA	17	14.5	8	0.55	0.57 ± 0.08
		18	19.5	13	0.67	

DW: Distilled water, BD: basal diet.

Table 5
Effects of antioxidants on *red/gam* mutant frequencies in the kidneys of female *gpt* delta rats given $KBrO_3$.

Treatment		Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi^-)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Water	Diet					
		51	15.2	2	0.13	
DW	BD	52	6.8	5	0.73	0.45 ± 0.30
		53	10.5	5	0.48	
		56	5.2	6	1.16	
$KBOrO_3$	BD	57	4.9	2	0.41	0.89 ± 0.42
		58	6.4	7	1.09	
		61	5.6	7	1.24	
$KBrO_3$	α-TP	62	8.4	8	0.96	1.02 ± 0.20*
		63	3.5	3	0.85	
		66	5.2	8	1.55	
$KBrO_3$	SAA	67	7.0	5	0.71	0.97 ± 0.51
		68	7.8	5	0.64	

DW: Distilled water, BD: basal diet.
* $p < 0.05$ vs. DW/BD.

TK locus, but not GC:TA transversions. On the other hand, in the present study, Spi^- MFs in males were not increased, in contrast to the previous study demonstrating significant increment. Although certain differences between genetic backgrounds should not be ignored, seemingly inconsistent results might reflect smaller increase of MFs following $KBrO_3$ exposure (2–3 fold) as compared to the case (10–30 fold) with potent genotoxic carcinogens (Kanki et al., 2005). In other words, as shown in microbial and the *Hprt* mutation assays in mammalian cells (Speit et al., 1999), the potential of $KBrO_3$ to induce mutations may be very weak (Harrington-Brock et al., 2003). Actually, we obtained negative data for transgene mutations at 250 ppm for 13 weeks (Umemura et al., 2006) and another group similarly reported negative findings with 125 ppm for 16 weeks (Yamaguchi et al., 2008). The hypothesis of weak mutagenicity is strongly supported by a bioassay showing $KBrO_3$ at 500 ppm for 13 weeks to be incapable of effecting tumor development with appropriate promotion, despite preneoplastic lesions being enhanced (Umemura et al., 2006).

In conclusion, oxidative stress generated by $KBrO_3$ might take part in induction of cell proliferation in PCT of female rats, leading to tumor promoting potential. In males, in contrast, α_{2u}-globulin accumulation independent of oxidative stress plays a major role in cell proliferation, which implies that the tumor promotion observed in males is not directly comparable to the human situation. Likewise, induction of reporter gene mutations by $KBrO_3$ is unlikely to be due to oxidative stress, the extent of which being much lower as compared to that of potent genotoxic carcinogens. The data overall allow us to speculate that the predominant contributing factor for $KBrO_3$-induced renal carcinogenesis is tumor promoting potential, which is only to a limited extent associated with oxidative stress.

Conflict of interest

None.

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Original article

Calculation of glomerular filtration rate in conscious rats by the use of a bolus injection of iodixanol and a single blood sample

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ABSTRACT

Introduction: To establish a simple and convenient procedure for the determination of glomerular filtration rate (GFR) in conscious rats, we developed a single-blood-sample method, in contrast to the conventional three-sample method, using a bolus injection of the nonionic contrast medium iodixanol. **Methods:** Iodixanol was intravenously administered at 1500 mg I/kg to healthy or renal-impaired rats, and blood was collected 60, 120, and 180 min later. Serum iodixanol concentrations were measured by HPLC, and serum urea nitrogen (UN) and creatinine concentrations were determined as renal function tests. **Results:** In rats subjected to 1/2 and 3/4 nephrectomies, GFR values decreased significantly without and with increases in serum UN and creatinine concentrations, respectively. In rats treated subcutaneously with gentamicin sulfate (GM) at 80 mg/kg/day or puromycin aminonucleoside (PAN) at 15 mg/kg/day for 10 consecutive days, the GFR values decreased or showed a tendency to decrease before increases in serum UN and creatinine concentrations. Accordingly, when the GFR decreased to more than 60% of the basal value, serum UN or creatinine concentrations became elevated. The GFR values obtained from the three-sample method were closely correlated ($r = 0.83$) with those calculated from the estimated distribution volume (V) and serum iodixanol concentration 120 min after iodixanol injection in the single-blood-sample method in which serum iodixanol concentrations ranged between 20 and 250 μg I/mL. **Discussion:** These results suggest that the single-blood-sample method with a bolus injection of iodixanol, allowing for the repeated use of the same animals, is an expedient procedure without ensuring accurate urine collection.

1. Introduction

In rats, although many reports have described the measurement of glomerular filtration rate (GFR) using inulin (Gabel, Ranaei, & Kivlighn, 1996; Onodera & Furuhama, 1983), contrast medium (Guesry et al., 1975), EDTA (Harvey, Jaffa, Loadholt, & Mayfield, 1988) and endogenous (Darling & Morris, 1991) or exogenous (Zager, 1987) creatinine, little information is available on a procedure that is easy, versatile and reliable, and required only a minimal number of animals without urine and/or repeated blood collections. In humans, the concentration of a tracer in a single plasma sample taken 180–240 min after injection is correlated with renal clearance (Groth & Aasted, 1981). Based on this information, Jacobsson (1983) devised a formula derived from a simple one-compartment model combined with the distribution (compartment) volume and optimum time for taking plasma using $^{99}Tc^{m}$-DTPA and accurately determined GFR. Later, based on this formula, it was reported that the clearance of iohexol, a nonionic monomeric X-ray contrast medium, was a simple, rapid and accurate alternative to that of inulin (Brown & O'reilly, 1991) or radiopharmaceuticals (Thomsen & Hvid-Jacobsen, 1991) for measuring GFR.

In an attempt to apply Jacobsson's formula to rats, we first measured GFR using a conventional three-sample method with iodixanol, an isotonic nonionic dimeric X-ray contrast medium, in experimental models with renal injuries such as partial nephrectomies and gentamicin sulfate (GM)- or puromycin aminonucleoside (PAN)-evoked nephropathy. By substituting these GFR values and serum iodixanol concentrations at 120 min into Jacobsson's formula, we sought the estimated distribution volume (V) in individuals. After confirming a negative relationship between the V values and serum iodixanol concentrations, we obtained a formula for calculating the V value. The GFR value in a single blood sample was obtained by substituting the V

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value and serum iodixanol concentration at 120 min in each animal into Jacobsson's formula once again. Because the *V* value is dependent on elimination kinetics of each substance (marker) and animal size, it is necessary to obtain it in the respective animals and species.

Iodixanol is rapidly excreted in urine without metabolic degradation and no or very little protein binding in rats (Heglund, Michelet, Blazak, Furuhama, & Holtz, 1995) or humans (Jacobsen, Blindheim, & Skotland, 1995). More recently, it has been reported that iodixanol is less nephrotoxic than iohexol in randomized, double-blind, prospective, multicenter studies using patients with chronic renal diseases (Aspelin et al., 2003; McCullough, Bertrand, Brinker, & Stacul, 2006), although iohexol has been used as a gold standard for measuring GFR so far (Brown & O'reilly, 1991). Additionally, the contrast effect of iodixanol was confirmed to be superior to that of iohexol because it possesses twice the amount of iodine in one molecule with a low osmolarity, when the equivalent iodine dose was administered to beagle dogs (Kishimoto et al., 2007, 2008).

Here, we selected iodixanol and Jacobsson's formula to establish a simple procedure for the determination of GFR in conscious rats.

2. Methods

2.1. Materials

Iodixanol (Visipaque 320®; 320 mg I/mL, 290 mOsm/kg H_2O) was purchased from Daiichi-Sankyo (Tokyo, Japan), gentamicin sulfate (GM) from Schering-Plough (Osaka, Japan), and puromycin aminonucleoside (PAN) from Wako Chemicals (Osaka, Japan). All other chemicals and reagents were of the highest grade available from commercial sources, unless otherwise stated.

2.2. Animals

Male Fischer 344 (F344) rats at 6–10 weeks of age were obtained from Japan SLC, Inc. (Shizuoka, Japan), and studies began after at least a 2-day acclimation period. Male F344 rats were chosen for the present investigations because they show a high susceptibility to nephrotoxicant (Tarloff, Goldstein, & Hook, 1989). The rats were housed in an air-conditioned facility (temperature, 22 ± 3 °C; relative humidity, 55 ± 25%; lighting, 8:00 AM to 8:00 PM with a 12-h light cycle) and fed commercial rodent chow (MEQ, Oriental Yeast Co., Ltd., Tokyo) and tap water *ad libitum*. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science (1987) and also approved by the Animal Experimental Ethics Committee of Iwate University (Morioka, Japan).

2.3. Optimum iodixanol dose

To identify the optimum dose for GFR measurement, iodixanol was administered intravenously at a dose of 375, 750, or 1500 mg I/kg to healthy 6-week-old F344 rats, and blood was collected 60, 120, 180, and 240 min later under conscious conditions by a previously reported procedure (Hatanaka, Kondoh, Kawarabayashi, & Furuhama, 1994). Iodixanol was diluted with a 0.9% saline solution and administered at 5 mL/kg to each animal. Before the iodixanol injection, a constant volume (5 mL) of distilled water was given orally by gavage to all animals to avoid dehydration due to repeated blood collection. The blood was centrifuged and sera were stored at −30 °C until assayed.

2.4. Measurement of GFR in healthy and renal-impaired rats

To measure the GFR in healthy rats, iodixanol was administered intravenously at 1500 mg I/kg to F344 rats at 6–9 weeks of age, weighing from 150 to 210 g, and blood was withdrawn 60, 120, and 180 min later using the aforementioned procedure (three-sample method). The GFR values were represented as mL/min/kg.

For partial nephrectomy models, 10-week-old F344 rats were fasted overnight with free access to water before the operation. After anesthesia with ether, a surgical ablation was performed on the right kidney (1/2 nephrectomy) or the right kidney with both poles of the left kidney (3/4 nephrectomy) according to the previous report (Sampaio-Maia, Serrao, Guimaraes, Vieira-Coelho, & Pestana, 2005). In 3/4 nephrectomized rats, the mean percentage of remnant renal mass was 32 ± 5%, based on the removed kidney weight. Animals subjected to sham surgery under the same conditions served as the sham control. The day the operation was performed was regarded as day 1. On day 2, the GFR was measured immediately after collecting the blood specimen to measure serum urea nitrogen (UN) and creatinine concentrations, and then all animals were euthanized by exsanguination under ether anesthesia.

For renal nephropathy models, GM and PAN were administered subcutaneously at 80 mg/kg/day and 15 mg/kg/day, respectively, to 11-week-old F344 rats for 10 consecutive days (days 1–10). Control animals received 0.9% saline solution (saline, 10 mL/kg) in the same way. The GFR was measured on days 0 (pre-dose), 6, and 11 in conjunction with the determination of serum UN and creatinine concentrations in the same animals. The dosage level and administration period of GM and PAN were selected based on the results of previous studies (Furuhama & Onodera, 1986; Onodera & Furuhama, 1983). On day 11, all animals were euthanized by exsanguination under ether anesthesia.

2.5. Laboratory tests and renal pathology

Serum UN and creatinine concentrations were measured with an autoanalyzer (Hitachi, Tokyo). In rats subcutaneously given GM, PAN or saline, the kidney was excised, fixed in 10% formalin, embedded in paraffin wax, cut at 3-μm thickness, stained with hematoxylin and eosin (H–E), and histopathologically examined.

2.6. Analysis of serum iodixanol concentrations

Serum iodixanol concentration was measured with reversed-phase high-performance liquid chromatography (HPLC) according to a previously reported procedure (Jacobsen et al., 1995), with some modifications. Serum specimens (0.1 mL) were deproteinized by adding 20% trichloroacetic acid (TCA; Wako Chemicals) at a ratio of 1:1 and placed at 4 °C for 30 min to complete precipitation before removal of the proteins by centrifugation (14,000×*g*, 10 min, 4 °C). The supernatant was centrifuged again under the same conditions. The HPLC system consisted of separation equipment (Alliance™ Waters 2690 Separations Module; Waters, Milford, MA, USA), a UV detector (Waters 996 Photodiode Array Detector; Waters) and analytical software (Millennium^{32}; Waters) equipped with a 250×4.6-mm C-18 reverse-phase column (RP-18 GP, 5 μm; KANTO CHEMICAL Co., Inc., Tokyo). The stepwise mobile phase profile was composed of distilled water followed by 80% acetonitrile in distilled water, and the flow-rate was maintained at 1 mL/min. The detection wavelength was 244 nm, which is the approximate absorbance maximum for iodixanol. The standard was prepared at known concentrations of iodixanol, and the results from the standard were used to calculate the concentration in each sample. A linear relationship existed between the summation of exso- and endo-isomers of iodixanol and the logarithm of iodixanol concentration. The qualification limit of serum iodixanol concentration was 6.25 μg I/mL.

2.7. Iodixanol clearance

In the three-sample method, the clearance calculations are based on the one-compartment model (Bröchner-Mortensen, 1972). Briefly,

the area under the iodine concentration curve (AUC) was calculated by the linear trapezoidal rule with extrapolation using the final three serum samples, and a clearance value (Cl_1) was calculated from the following formula.

$$Cl_1 = Q_{tot} / AUC,$$

where Q_{tot} is the dose of iodixanol injected. However, this formula overestimates clearance because it does not consider the serum concentration during the distribution phase. Therefore, the clearance (Cl_2) was corrected by the following empirically determined formula (Bröchner-Mortensen, 1972; Thomsen & Hvid-Jacobsen, 1991).

$$Cl_2 = 0.991 \times Cl_1 - 0.00122 \times (Cl_1)^2$$

The distribution volume (V_1) in each animal was back-calculated by substituting Cl_2 values and serum iodixanol concentrations (C_{t_2}) at 120 min (t_2) obtained from the three-sample method into the following Jacobsson's formula using the "Goal-Seek" command of Microsoft Office Excel (Microsoft 2007, Microsoft Co., Tokyo). In this function, when we know the result we want to return a formula, but we do not know the input values the formula needs to reach that result, we can use the command.

$$Cl_2 = 1 / (t_2 / V_1 + 0.0016) \times \ln[Q_{tot} / (V_1 \times C_{t_2})]$$

To seek the estimated distribution volume (V) in each animal, after the correlation between the V_1 value and C_{t_2} was assessed with a scatter diagram, a formula for calculating V_1 was determined. In this case, therefore, the V_1 value sought by using C_{t_2} was regarded as the V value ($V_1 = V$).

Finally, the Cl value in a single-blood-sample method was determined by substituting the V value and C_t at 120 min (t) from each animal into the following Jacobsson's formula once again. The Cl term was the GFR in the present work.

$$Cl = 1 / (t / V + 0.0016) \times \ln[Q_{tot} / (V \times C_t)]$$

2.8. Statistical analysis

Quantitative data are expressed as the mean ± SEM of the group. Statistical evaluation was occasionally performed by a one-way ANOVA, and differences between treatment and control groups were analyzed by Dunnett's test (among three groups) or Student's t-test (between two groups). A probability level of $p<0.05$ indicates statistical significance. Correlations were determined by Pearson's correlation coefficient (r).

3. Results

3.1. Disappearance of iodixanol from serum

In 6-week-old healthy male rats ($n=2$) given 1500 mg I/kg of iodixanol (Fig. 1), mean concentrations of iodixanol in serum had a linear disappearance until 240 min. At doses of 375 and 750 mg I/kg, however, serum concentrations decreased almost to the qualification limit (6.25 μg I/mL) 120 and/or 180 min after injection. In a subsequent three-sample method, therefore, a 1500 mg I/kg of iodixanol with sample times of 60, 120, and 180 min was chosen.

3.2. GFR obtained by the three-sample method in healthy or renal-impaired rats

The background GFR value in healthy male F344 rats at 6–9 weeks of age was 9.6 ± 0.6 mL/min/kg ($n=18$).

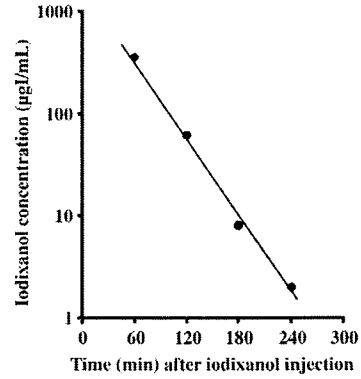

Fig. 1. Representative disappearance of iodixanol from serum of healthy 6-week-old male F344 rats given a bolus injection of iodixanol at 1500 mg I/kg. Blood was collected from the tail vein under conscious conditions 60, 120, 180, and 240 min after iodixanol injection, and serum iodixanol concentrations were measured by HPLC. Each plot shows the mean of two animals.

In rats subjected to partial nephrectomy (Fig. 2), the GFR values (mean: 4.5 mL/min/kg) decreased significantly without alterations in serum UN or creatinine concentrations in the 1/2 nephrectomy group, whereas significantly decreased GFR values (2.3 mL/min/kg) were observed with increases in serum UN (53.6 mg/dL) and creatinine (0.97 mg/dL) concentrations in the 2/3 nephrectomy group when compared to the sham control group (GFR, 6.8 mL/min/kg).

In rats subcutaneously administered GM or PAN for 10 days (Fig. 3), the GFR values showed a tendency to decrease in GM-treated rats (6.9 mL/min/kg) or statistically decreased in PAN-treated rats (5.6 mL/min/kg) without alterations in serum UN or creatinine concentrations on day 6. On day 11, however, markedly decreased GFR values (0.38–0.41 mL/min/kg) were noted with significant increases in serum UN and creatinine concentrations in both nephropathy groups. The GFR values in the corresponding saline control group ranged between 7.7 and 8.4 mL/min/kg throughout the experimental period. Renal histopathological examinations revealed that necrosis in the proximal epithelium and mitotic figures of epithelial cells with casts in the proximal tubule were observed in rats given GM, and that hydroponic vacuolated podocytes with hyaline casts were noted in rats receiving PAN.

3.3. Relationship between GFR values versus serum UN or creatinine concentrations

The relationship between the GFR values sought by the three-sample method versus serum UN or creatinine concentrations was assessed using a total of 48 animals (sample numbers, 125, where the number shows the sum total of the GFR values collected from the same animal on different days) from the preliminary and present (main) studies. When the GFR value decreased to more than 60% of the basal background level (9.5 mL/min/kg), serum UN or creatinine concentrations likely began to increase (Fig. 4A and B).

3.4. GFR by the single-blood-sample method

A formula for calculating the V value was determined from a scatter diagram (Fig. 5A) as follows:

$$y = -0.70x + 277 (n = 25;\ \text{sample numbers, } 47)$$

where y is the V value and x is serum iodixanol concentration at 120 min. This formula was valid only between 20 and 250 μg I/mL serum iodixanol concentrations, showing a close correlation ($r=0.55$; $p<0.01$). Within these concentration ranges, the GFR values were calculated as 2.9–13.7 mL/min/kg in a single-blood-sample method.