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Long-term treatment with L-isoleucine or L-leucine in AIN-93G diet has promoting effects on rat bladder carcinogenesis

Xiao-Li Xie, Min Wei, Takayuki Yunoki, Anna Kakehashi, Shotaro Yamano, Minoru Kato, Hideki Wanibuchi *

Department of Pathology, Osaka City University Graduate School of Medicine, Asahi-machi 1-4-3, Abeno-ku, 545-8585 Osaka, Japan

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ABSTRACT

In the present study, effects of L-leucine and L-isoleucine on rat bladder carcinogenesis were investigated using AIN-93G and MF basal diet. In Experiment 1, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine was used as an initiator of bladder carcinogenesis. In the AIN-93G diet groups, a significantly higher incidence and multiplicity of bladder tumors, accompanied by decreased final body weight, was observed in the L-leucine-supplemented group and a significantly higher incidence of papillomas and total tumors was observed in the L-isoleucine-supplemented group. In the MF diet groups, the multiplicity of papillary and nodular hyperplasia was significantly increased in the L-isoleucine-supplemented group. Urinary pH values were not affected by supplementing either type of diet with L-leucine or L-isoleucine. In Experiment 2, the amino acid was administered in the basal diets for 2 weeks without initiator. No pathological lesions were observed in the bladder urothelium in any of the groups, and no significant differences in urinary pH values, microcrystals or aggregates were observed between the amino acid-supplemented groups and their respective control groups. In conclusion, long-term treatment with L-leucine or L-isoleucine has a promoting effect on rat bladder carcinogenesis; therefore, their long-term use as a dietary supplement for bladder cancer patients should be avoided until more is known.

1. Introduction

According to the global cancer statistic, the incidence and mortality of human bladder cancers are generally higher in men than in women, and higher in developed countries than in developing countries (Parkin et al., 1999). Smoking, occupational exposure, and chronic infections with schistosoma are the most established risk factors for bladder cancer. At present, evidence that dietary factors may also contribute to bladder cancer incidence is accumulating. Epidemiologic studies suggest that a Western-style diet rich in proteins may contribute to bladder cancer incidence in developed countries (Nishio et al., 1986). High meat consumption has been associated with higher risk of several malignancies (World Cancer Research Fund American Institute for Cancer Research, 2007) and could plausibly increase the risk of bladder cancer.

Branched chain amino acids (BCAAs), which must be obtained from dietary sources (Norman et al., 2003; Baracos and Mackenzie, 2006), are widely used as dietary supplements by cancer patients, however, their effects on the efficacy of cancer treatments are controversial. BCAAs are simultaneously essential for tumor growth and for the physiological well-being of the tumor-bearing host (Baracos and Mackenzie, 2006). Currently, the cumulative effects of BCAAs and principles for their use as dietary supplements remain to be established.

In this study, we examined whether two BCAAs, L-leucine and L-isoleucine, when used as dietary supplements, could have an effect on bladder carcinogenesis in rats. Since the effects on rat bladder carcinogenesis might depend on the type of basal diet (Okamura et al., 1991) we used two different diets: AIN-93G, a semi-synthetic basal diet, and MF basal diet.

2. Material and methods

2.1. Chemicals and diets

N-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) was purchased from Tokyo Chemical Industry Co. Ltd, Tokyo, Japan. L-Leucine and L-isoleucine (purity of 99.9%) were provided by the Ajinomoto Co., Inc., Kanagawa, Japan. Basal diets (powder AIN-93G and MF; Oriental Yeast Co., Tokyo, Japan) and the diets containing L-leucine or L-isoleucine were prepared once a month by Oriental Yeast Co.

2.2. Animals

A total of 300 5-week-old male Fisher 344 rats were supplied by Charles River Japan, Inc. (Hino, Shiga, Japan). Animals were housed in polycarbonate cages (three per cage) in experimental animal rooms with a targeted temperature of 22 ± 3 °C, relative humidity of 55 ± 5% and a 12-h light/dark cycle. Diet and tap water were available ad libitum throughout the study. The animals were acclimatized for

* Corresponding author. Tel.: +81 6 6645 3735; fax: +81 6 6646 3093.
E-mail address: wani@med.osaka-cu.ac.jp (H. Wanibuchi).

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1 week prior to beginning the experiment. The experiment was conducted following approval of the Animal Care and Use Committee of the Osaka City University Graduate School of Medicine.

2.3. Treatment and processing

The dose (2%) of L-leucine or L-isoleucine used in the present study was based on the findings of previous studies which suggested that these two BCAAs were promoters of rat bladder cancer both *in vitro* (Kakizoe et al., 1982) and *in vivo* (Kakizoe et al., 1983; Nishio et al., 1986). While there are no standard doses of L-leucine or L-isoleucine which are administered to human patients, infusion with a BCAA-enriched (50% of the total amino acid content as BCAA) total parenteral nutrition (TPN) providing 1.2 g protein/kg body weight/day was found to be beneficial in a prospective randomized crossover trial (Tayek et al., 1986). Rats fed AIN-93G basal diet (1.73% leucine, 0.96% isoleucine, 1.14% valine) or MF basal diet (1.78% leucine, 0.89% isoleucine, 1.08% valine) supplemented with 2% L-leucine or 2% L-isoleucine ingested approximately 2 g total BCAA/kg body weight/day. Thus, ingestion of basal diet supplemented with 2% L-leucine or 2% L-isoleucine provides the body with approximately similar levels of BCAAs as infusion of BCAA-enriched TPN.

Experiment 1: 180 rats were administered 0.05% BBN in the drinking water for the first 4 weeks and then were randomly divided into AIN-93G (AIN-93G alone, 2% L-leucine, 2% L-isoleucine) and MF (MF alone, 2% L-leucine, 2% L-isoleucine) diet groups. Under deep ether anesthesia, all rats in the AIN-93G diet groups and 10 rats of each MF diet group were killed at week 29 and the remaining rats in the MF diet groups were sacrificed at week 36 after the commencement of the experiment (Fig. 1a): In the MF diet groups at sacrifice at week 29, the number of tumors, especially tumors with volumes over than 15 mm^3, was too small to evaluate the effects of L-leucine and L-isoleucine; therefore, 20 rats in each of the MF diet groups were not sacrificed at this time. Their urinary bladders were inflated by intraluminal injection of 10% phosphate-buffered formalin (PBF) and fixed in PBF at room temperature.

For macroscopic quantitative analysis (number and volume of tumors), fixed bladders were carefully opened and the lumen inspected for grossly visible lesions. The number of tumors per rat and the volume of each tumor were recorded to calculate the incidence and multiplicity of tumors per group. Tumor volumetry was performed by measuring three diameters using a formula for ellipsoid volumetry $V = d1 \times d2 \times d3 \times \pi/6$. A tumor was defined as a lesion of >0.5 mm in diameter.

Table 2
Body weight, food intake and water intake in the MF groups at week 36.

Group Number of rats	MF alone 20	MF + 2% Leu 20	MF + 2% ILe 20
Final body weight (g)	390.0 ± 25.1	394.9 ± 16.6	400.1 ± 11.0
Food intake (g/day/rat)	12.50 ± 0.61	12.87 ± 0.56	12.50 ± 0.65
Water intake (g/day/rat)	17.76 ± 1.22	18.78 ± 1.05	17.91 ± 1.35

For microscopic qualitative analysis (bladder histology), the bladders were cut into eight strips, routinely processed for embedding in paraffin, sectioned at 3 μm thickness and processed for histopathological analysis. After deparaffinization, slides were stained with hematoxylin and eosin (H&E) for histopathological examination. Papillary and nodular (PN) hyperplasia, papillomas and carcinomas were counted using a light microscope and categorized based on tumor size and presence of mitotic figures, according to the diagnostic criteria of Boorman et al. (1990).

For the detection of urinary pH values, fresh void urine samples were collected by forced urination from 15 rats in each group between 7:00 and 9:00 A.M before sacrifice at week 29. The urinary pH value was measured with a Compact pH meter < Twin pH > B-212 (No. 00715 Horiba Ltd., Japan).

Experiment 2: 120 rats were randomly divided into AIN-93G (AIN-93G alone, 2% L-leucine, 2% L-isoleucine) and MF (MF alone, 2% L-leucine, 2% L-isoleucine) diet groups. Under deep ether anesthesia, all rats were sacrificed at week 2 (Fig. 1b). The urinary bladders ($n = 5$ per group) were processed for pathological examination by scanning electron microscope (SEM, Hitachi model S-4700, Hitachi Co. Ltd., Japan) using the criteria of Shen et al. (2006). Urine was collected and prepared as described previously (Arnold et al., 1999) for measurement of urinary pH values and examination by SEM for the presence of calculi, microcrystals and/or precipitate.

2.4. Statistical analysis

All mean values were expressed as means ± standard deviations (SDs). Statistical analyses were performed using the Statlight program (Yukms Co. Ltd., Tokyo, Japan). Incidences of pathologic lesions were compared using the Chi-squared test.

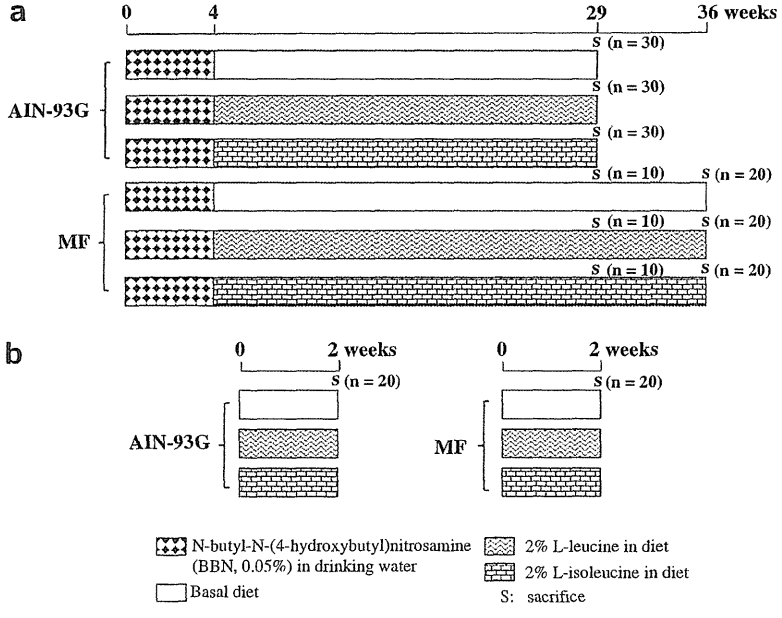

Fig. 1. Experiment design.

Table 1
Body weight, food intake and water intake at week 29.

Group Number of rats	AIN-93G alone 30	AIN-93G + 2% Leu 30	AIN-93G + 2% ILe 30	MF alone 10	MF + 2% Leu 10	MF + 2% ILe 10
Final body weight (g)	391.3 ± 20.7	368.3 ± 32.1**	385.4 ± 19.7	375.5 ± 15.4*	376.1 ± 19.5	379.7 ± 22.6
Food intake (g/day/rat)	11.75 ± 0.61	11.55 ± 0.53	11.65 ± 0.65	12.51 ± 0.65	12.86 ± 0.59	12.51 ± 0.69
Water intake (g/day/rat)	14.28 ± 1.40	15.87 ± 1.06	15.06 ± 1.18	17.93 ± 1.27	18.92 ± 1.09	18.14 ± 1.28

*$p < 0.05$, **$p < 0.01$ versus the AIN-93G alone group.

Table 3
Incidence and multiplicity of tumors by macroscopic observation at week 29.

Group Number of rats	AIN-93G alone 30	AIN-93G + 2% Leu 30	AIN-93G + 2% Ile 30	MF alone 10	MF + 2% Leu 10	MF + 2% Ile 10
Incidence (%)						
1–15 (mm3)	11 (37)	18 (60)	19 (63)	2 (20)	4 (40)	4 (40)
>15 (mm^3)	2 (7)	11 (37)*	6 (20)	0 (0)	1 (10)	0 (0)
Multiplicity						
1–15 (mm^3)	3.2 ± 4.5	2.0 ± 3.2	4.0 ± 3.9	0.3 ± 0.7	0.5 ± 0.9	0.6 ± 0.8
>15 (mm^3)	1.9 ± 7.2	81.4 ± 212.6	44.1 ± 129.7	0 ± 0	1.9 ± 6.0	0 ± 0

* $p < 0.05$ versus the AIN-93G alone group.

Table 4
Incidence and multiplicity of tumors by macroscopic observation at week 36.

Group Number of rats (%)	MF alone 20	MF + 2% Leu 20	MF + 2% Ile 20
Incidence (%)			
1–15 (mm^3)	8 (40)	8 (40)	7 (35)
>15 (mm^3)	0 (0)	0 (0)	0 (0)
Multiplicity			
1–15 (mm^3)	2.8 ± 4.9	3.6 ± 6.5	0.8 ± 1.1
>15 (mm^3)	0 ± 0	0 ± 0	0 ± 0

Homogeneity of variance was tested by the *F* test in the basal diet groups and each treatment group. Differences in mean values between the control and treatment groups were evaluated by the two-tailed Student *t*-test when variance was homogeneous and the two-tailed Aspin–Welch *t*-test when variance was heterogeneous. *P* values less than 0.05 were considered significant.

3. Results

3.1. Promoting effects of long-term treatment with L-isoleucine and L-leucine on rat bladder carcinogenesis (Experiment 1)

Tables 1 and 2 show the body weight, food and water intake in Experiment 1. Neither L-leucine nor L-isoleucine supplementation affected food or water intake in any of the groups. At week 29, the body weight of the AIN-93G diet group supplemented with L-leucine was significantly decreased compared to the control; the body weight of the AIN-93G diet group supplemented with L-isoleucine was also decreased compared to the control, but the difference was not significant. The final body weight of the MF alone group was significantly lower than that of the AIN-93G alone group, however, neither L-leucine nor L-isoleucine supplementation had an affect on the body weight of the MF diet groups at either week 29 (Table 1) or week 36 (Table 2).

Tables 3 and 4 show the incidence and multiplicity of macroscopic tumors in Experiment 1. The incidence of larger tumors (volume greater than 15 mm^3) at week 29 in the AIN-93G diet group supplemented with L-leucine was significantly increased compared to the control (Fig. 2a–c and Table 3); L-isoleucine supplementation had no affect on macroscopic tumor incidence in the AIN-93G diet group. Neither L-leucine nor L-isoleucine supplementation had an affect on macroscopic tumor multiplicity in the AIN-93G diet groups. Neither L-leucine nor L-isoleucine supplementation had an affect on macroscopic tumor incidence or multiplicity in the MF diet groups at either week 29 (Fig. 2d–f and Table 3) or week 36 (Fig. 3 and Table 4).

Tables 5 and 6 show the incidence and multiplicity of PN hyperplasia, papillomas, carcinomas, and total tumors detected by

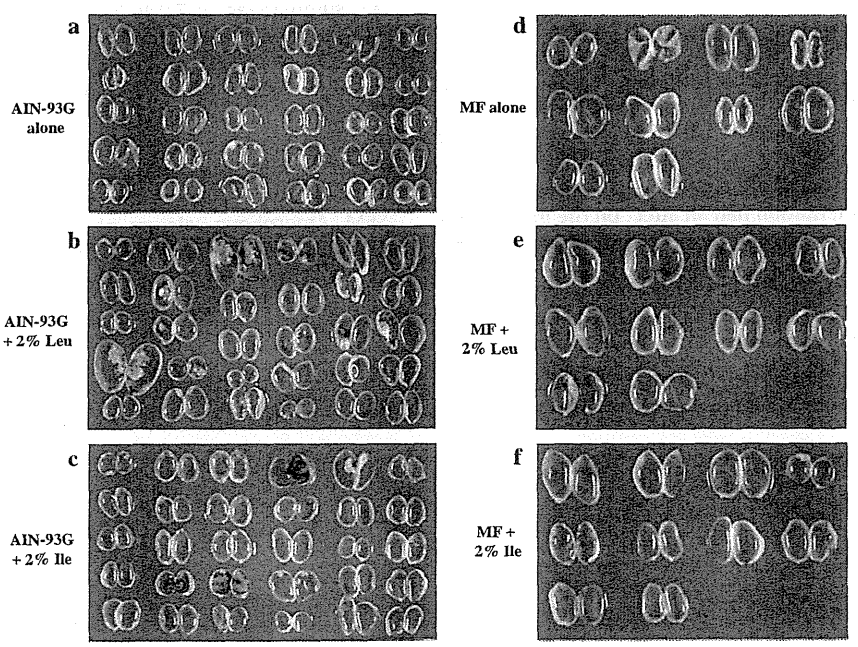

Fig. 2. Macroscopic images of rat bladders at week 29 of animals fed the two different basal diets (Experiment 1). (a–c) AIN-93G basal diet groups ($n = 30$); (d–f) MF basal diet groups ($n = 10$). Leu, L-leucine; Ile, L-isoleucine. Compared with the AIN-93G control group (a), the number of macroscopic tumors was increased by dietary supplementation with Leu (b) or Ile (c). In the MF basal diet groups, no macroscopic tumors were observed in rats fed MF alone (d) or the Ile supplemented group (f); two macroscopic tumors were observed in the Leu supplemented group (e).

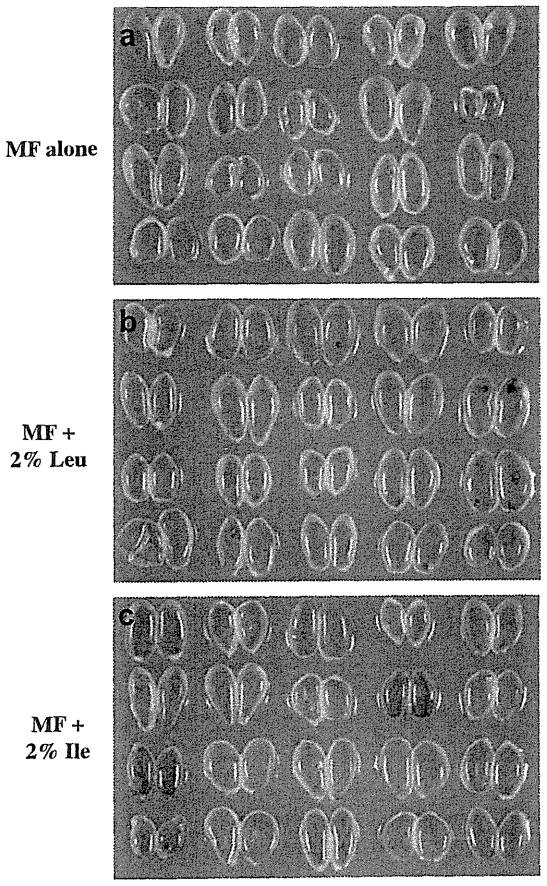

Fig. 3. Macroscopic images of rat bladders at week 36 of animals fed the MF basal diet (Experiment 1). Compared with the MF control group (a) no significant increase in the number of macroscopic tumors was observed in either the Leu-supplemented (b) or Ile-supplemented (c) groups. $n = 20$ per group.

microscopic examination in Experiment 1. The incidence of PN hyperplasia and carcinomas and the multiplicity of PN hyperplasia at week 29 in the AIN-93G diet group supplemented with L-leucine was significantly increased compared to the control. L-isoleucine supplementation significantly increased the incidence of papillomas and total tumors in the AIN-93G diet group. Neither L-leucine nor L-isoleucine supplementation had an affect on the incidence of PN hyperplasia, papillomas, carcinomas, or total tumors in the MF diet groups at either week 29 (Table 5) or week 36 (Table 6). However, at week 36, L-isoleucine supplementation significantly increased the multiplicity of PN hyperplasia in the MF diet group (Table 6). Typical microscopic pictures of normal bladder urothelium, PN hyperplasia, papilloma and carcinoma are shown in Fig. 4.

Table 6
Histopathological changes at week 36 in the bladder urothelium of the MF groups.

Group (number of rats)	MF alone 20	MF + 2% Leu 20	MF + 2% ILe 20
Incidence (%)			
PN hyperplasia	16 (80)	14 (70)	18 (90)
Papillomas	8 (40)	8 (40)	10 (50)
Carcinomas	4 (20)	8 (40)	4 (20)
Total tumors	11[a] (55)	13[b] (65)	12[c] (60)
Multiplicity			
PN hyperplasia	1.35 ± 0.93	1.70 ± 1.34	2.05 ± 1.15*
Papillomas	0.40 ± 0.50	0.55 ± 0.76	0.70 ± 0.80
Carcinomas	0.20 ± 0.41	0.50 ± 0.69	0.20 ± 0.41
Total tumors	0.60 ± 0.60	1.05 ± 1.10	0.90 ± 0.91

* $p < 0.05$ versus the MF alone group.
[a] One rat had both papillomas and carcinomas.
[b] Three rats had both papillomas and carcinomas.
[c] Two rats had both papillomas and carcinomas.

Urinary pH values at week 29 from animals in Experiment 1 are summarized in Table 7. The number of rats from which urine was successfully collected in the AIN-93G alone, AIN-93G + L-leucine, AIN-93G + L-isoleucine, MF alone, MF + L-leucine and MF + L-isoleucine groups was 15, 13, 10, 12, 14 and 13, respectively. No significant differences of pH values were detected between the basal diet groups and the amino acid supplemented groups.

3.2. Urinary pH values and sediments were not related to the effects of amino acid supplementation on the bladder urothelium (Experiment 2)

Urinary pH values at week 2 from animals from Experiment 2 are summarized in Table 7. No significant differences in urinary pH values were detected between the basal diet groups and the amino acid supplemented groups.

Fig. 5 shows bladder urothelium observed by SEM in Experiment 2. No pathological lesions were observed in the bladder urothelium in any of the groups by light microscope (data not shown) or SEM. Fig. 6 presents urine sediment observed by SEM. No obvious urine sediment was observed in any of the AIN-93G diet groups by SEM. Amorphous calcium phosphate, Ca/Mg phosphate crystals and aggregates were observed in the MF diet groups, however, no

Table 5
Histopathological changes at week 29 in the bladder urothelium of the AIN and MF groups.

Group (number of rats)	AIN-93G alone 30	AIN-93G + 2% Leu 30	AIN-93G + 2% ILe 30	MF alone 10	MF + 2% Leu 10	MF + 2% ILe 10
Incidence (%)						
PN hyperplasia	16 (53)	26 (87)*	23 (77)	6 (60)	5 (50)	7 (70)
Papillomas	10 (33)	16 (53)	17 (57)*	4 (40)	3 (30)	3 (30)
Carcinomas	7 (23)	15 (50)*	11 (37)	0 (0)	2 (20)	0 (0)
Total tumors	16[a] (53)	22[b] (73)	24[c] (80)*	4 (40)	5 (50)	3 (30)
Multiplicity						
PN hyperplasia	1.00 ± 1.20	2.10 ± 2.12*	1.40 ± 1.07	1.10 ± 1.29	1.00 ± 1.41	1.10 ± 0.99
Papillomas	0.50 ± 0.90	0.73 ± 0.83	0.70 ± 0.70	0.50 ± 0.71	0.50 ± 0.97	0.30 ± 0.48
Carcinomas	0.33 ± 0.66	0.50 ± 0.51	0.57 ± 0.77	0 ± 0	0.20 ± 0.42	0 ± 0
Total tumors	0.83 ± 1.09	1.23 ± 1.07	1.27 ± 0.98	0.50 ± 0.71	0.70 ± 0.95	0.30 ± 0.48

* $p < 0.05$ versus the AIN-93G alone group.
[a] One rat had both papillomas and carcinomas.
[b] Nine rats had both papillomas and carcinomas.
[c] Four rats had both papillomas and carcinomas.

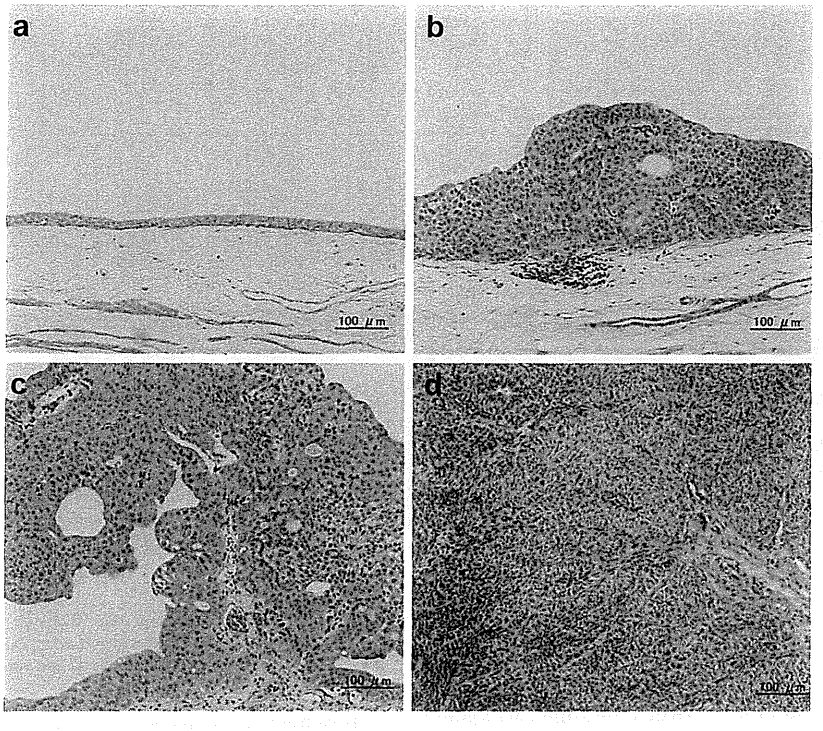

Fig. 4. Microscopic observations by H&E staining (Experiment 1). (a) Normal bladder urothelium; (b) papillary or nodule (PN) hyperplasia; (c) papilloma; (d) carcinoma.

Table 7
Urinary pH values.

Groups	AIN-93G alone	AIN-93G + 2% Leu	AIN-93G + 2% ILe	MF alone	MF + 2% Leu	MF + 2% ILe
Experiment 1 (at week 29)						
Number of rats	15	13	10	12	14	13
pH value of urine	6.19 ± 0.16	6.16 ± 0.20	6.10 ± 0.15	7.54 ± 0.19	6.88 ± 0.33	7.30 ± 0.22
Experiment 2 (at week 2)						
Number of rats	10	10	10	10	10	10
pH value of urine	6.30 ± 0.20	6.30 ± 0.20	6.50 ± 0.40	7.50 ± 0.10	7.40 ± 0.10	7.50 ± 0.20

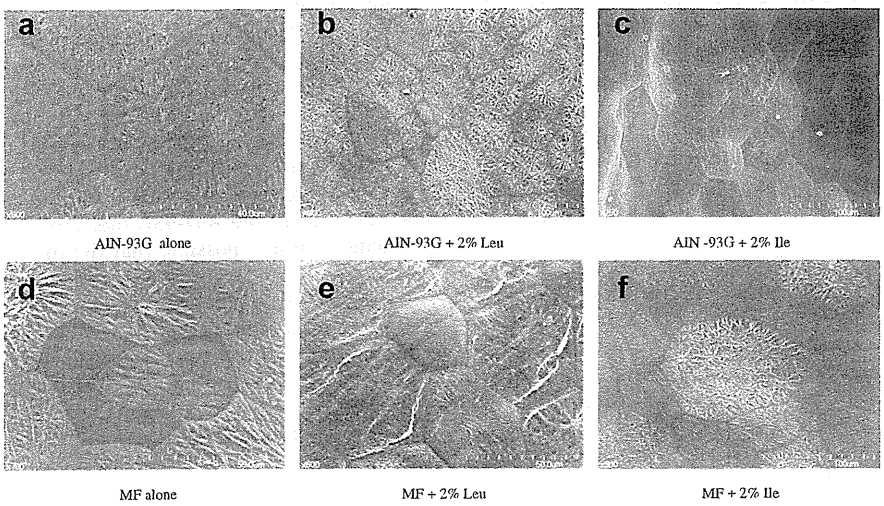

Fig. 5. Rat bladder urothelium observed by scanning electron microscopy (Experiment 2). (a–c), AIN-93G diet groups ($n = 5$); (d–f) MF diet groups ($n = 5$). Leu, L-leucine; Ile, L-isoleucine. No significant differences were observed between the amino acid-treated groups and the basal diet control groups.

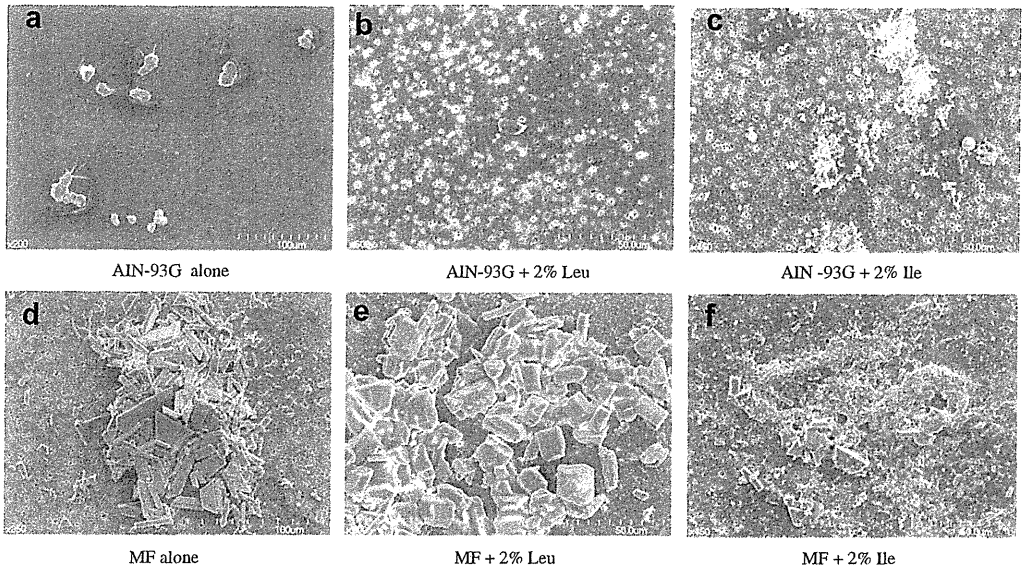

Fig. 6. Urine sediments observed by scanning electron microscopy (Experiment 2). (a–c) AIN-93G diet groups ($n = 5$); (d–f) MF diet groups ($n = 5$). Leu, L-leucine; Ile, L-isoleucine. No obvious urine sediment was observed in the AIN-93G diet groups (a–c). Although amorphous calcium phosphate, Ca/Mg phosphate crystals and aggregates were observed in the MF diet groups (d–f), no significant differences were observed between the amino acid-treated groups and the MF control group.

significant changes were observed between the amino acid-supplemented groups and the control.

4. Discussion

BCAAs are commonly used in food and drink as additive and nutritional supplements. BCAAs have also been administered to individuals recovering from certain diseases, such as cancer cachexia (Siddiqui et al., 2006), hepatic cirrhosis (Moriwaki et al., 2008; Muto et al., 2005, 2006), and renal insufficiency (Doering et al., 2000). However, a study using rats suggested that L-leucine and L-isoleucine are promoters of bladder cancer in these animals. (Nishio et al., 1986). The authors of this study suggest that there may be a relationship between the high incidence of human bladder cancer in Western countries with a protein rich diet and the tumor promoting effects of these amino acids.

In Experiment 1, a promoting effect on bladder cancer by L-leucine and L-isoleucine was observed in the AIN-93G diet groups at week 29 and by L-isoleucine in the MF diet group at week 36. Overall, there were some differences in the incidence and multiplicity of papillomas, carcinomas and total tumors between the L-leucine and L-isoleucine-supplemented groups; however, no significant differences were observed between the AIN-93G + L-leucine and AIN-93G + L-isoleucine groups or between the MF + L-leucine and MF + L-isoleucine groups. Therefore, the promoting effect on bladder carcinogenesis by L-leucine and L-isoleucine was similar in the present study.

The basal diet had a marked effect on bladder cancer promotion by L-leucine and L-isoleucine. In the AIN-93G diet groups, a significantly higher incidence and multiplicity of bladder tumors was observed in the L-leucine-supplemented group and a significantly higher incidence of papillomas and total tumors of the bladder was observed in the L-isoleucine-supplemented group. In the MF diet groups, L-leucine did not promote bladder carcinogenesis and in the L-isoleucine-supplemented group only the multiplicity of papillary and nodular hyperplasia in the bladder was significantly increased.

The factors in the AIN-93G and MF diets which affect bladder cancer promotion by L-leucine and L-isoleucine remain to be investigated, however, polyphenols are candidate factors. The Oriental Yeast Co. states that the AIN-93G basal diet does not contain polyphenols while the MF basal diet contains leguminosae, a source of polyphenols. Consumption of polyphenols from lemon has been shown to suppress weight gain in a high-fat diet-induced obesity mouse model (Fukuchi et al., 2008), and the final body weight of the AIN-93G alone group was significantly higher than that of the MF alone group at week 29, in accordance with the reported activity of polyphenols. Supplementation of BCAAs, at doses comparable to human usage, significantly down-regulated the expression of some antioxidant genes in the cortex of mice and may have indirectly altered some oxidative stress pathways in the brain (Piscopo et al., 2011); notably, the anti-carcinogenesis activity of polyphenols is associated with their antioxidant activity and their ability to decrease oxidative stress (Cai et al., 2006; Henning et al., 2012). Therefore, these data together with our study suggest the possibility that the presence of polyphenols may repress the promoting effects of L-leucine and L-isoleucine on bladder carcinogenesis.

In the AIN-93G basal diet group, supplementation with L-leucine significantly reduced body weight. Importantly, in this group, supplementation with L-leucine also significantly increased the incidence of larger tumors (volume greater than 15 mm^3) in the bladder. It is possible that the increased tumor burden resulted in the lower body weight observed in this group. There was also a non-significant decrease in body weight at week 29 in the AIN-93G diet group supplemented with L-isoleucine, and it is possible that the increased incidence of papillomas and total tumors in this group may have affected the body weight of these animals.

High urinary pH caused by certain chemicals may contribute to their tumor promotion activity (Fukushima et al., 1991; Shibata et al., 1992; Clayson et al., 1995). In our study, in both Experiments 1 and 2, rats fed the MF diet had urinary pH values as high as 7.5; in contrast, rats fed AIN-93G diet had urinary pH values less than or equal to 6.5. Furthermore, no significant differences of pH values were detected between the amino acid-supplemented groups and the basal diet control groups. Our results suggest that the promoting activity of L-leucine or L-isoleucine is not related to urinary pH.

Urinary microcrystals and other solid precipitates dispersed in the urine have also been considered relevant to rodent bladder carcinogenesis (Clayson et al., 1995). In Experiment 2, no pathological lesions were observed in the bladder urothelium in any of the groups by SEM, and no obvious urine sediments were observed in the AIN-93G diet groups. Although several kinds of urine microcrystals and aggregates were observed in MF diet groups, no significant differences were observed between the amino acid-supplemented groups and the basal diet control groups. Our results suggest that formation of urine sediments do not account for the promoting activity of L-leucine or L-isoleucine.

Thus, our results suggest that neither urinary pH nor urinary sediments mediate tumor promotion by L-leucine or L-isoleucine. Possibly, factors such as amino acid transporters might take part in the progress of bladder cancer. Another consideration, as reported by Piscopo et al. (2011), is that supplementation of BCAAs, at doses comparable to human usage, down-regulates the expression of some antioxidant genes and may be able to indirectly alter some oxidative stress pathways in the mouse brain. It is possible that modulation of these carcinogenesis-associated pathways may occur in the rat bladder as well. Finally, as suggested by Baracos and Mackenzie (2006), supplementation of the diet with these essential BCAAs may create a better growth environment for initiated cells. The mechanism of tumor promotion by BCAAs is an important area for future research.

In summary, our results indicate that long-term treatment with L-leucine or L-isoleucine, especially when combined with a diet low in polyphenols, has tumor-promoting activity on bladder carcinogenesis in rats. Therefore, long-term use of BCAAs as dietary supplements should be avoided until more is known about their effects on carcinogenesis in humans. This is particularly applicable to patients with bladder cancer. Further investigations are needed to determine the mechanism of the tumor promoting activity of BCAAs and to ascertain safe levels of BCAA supplementation for bladder cancer patients.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Dammar resin, a non-mutagen, inducts oxidative stress and metabolic enzymes in the liver of *gpt* delta transgenic mouse which is different from a mutagen, 2-amino-3-methylimidazo[4,5-*f*]quinoline

Xiao-Li Xie, Min Wei, Anna Kakehashi, Shotaro Yamano, Kyoko Okabe, Masaki Tajiri, Hideki Wanibuchi*

Department of Pathology, Osaka City University Graduate School of Medicine, Asahi-machi 1-4-3, Abeno-ku, 545-8585 Osaka, Japan

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ABSTRACT

Dammar resin has long been used in foods as either a clouding or a glazing agent. In a recent study, 2% Dammar resin showed significant hepatocarcinogenicity in a rat 2-year bioassay. Therefore, for an accurate estimate of human risk, it is necessary to understand whether Dammar resin induces liver genotoxicity and the underlying mechanisms of its hepatocarcinogenicity. Modifying effects of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), a typical genotoxic carcinogen produced during cooking of protein-rich foods, was also studied in the present study. Exposure of *gpt* delta mice to Dammar resin at a dose of 2% for 12 weeks did not induce any obvious mutagenicity in the liver. However, the index of cell proliferation, the level of 8-OHdG, and *bax*, *bcl-2*, *p53*, *cyp1a2*, *cyp2e1*, *gpx1* and *gstm2* gene expression were all significantly increased when compared with the control group. In the IQ treatment group, at a dose of 300 ppm, mutagenicity was readily detected, the index of cell proliferation increased, and *p53*, *cyp2e1* and *gpx1* gene expression was down-regulated in the liver. Down-regulation of *p53*, *P450s*, and *gpx1* in the livers of IQ treated mice are consistent with its genotoxic mechanism of carcinogenicity observed in a 675-day study. In contrast, our results using *gpt* delta mice suggest that Dammar resin is not genotoxic. Instead, the Dammar resin-induced hepatocarcinogenicity seen in our previous 2-year study with rats may have been mediated by non-genotoxic mechanisms, including increased P450 enzyme activity, increased oxidative stress, altered gene expression, and promotion of cell proliferation.

1. Introduction

Carcinogens can be classified into two categories, genotoxic and non-genotoxic. The former indicates a chemical capable of producing cancer by directly causing irreversible genetic damage, while the latter represents a chemical capable of producing cancer by some secondary mechanisms not related to direct gene damage [1].

Heterocyclic aromatic amines are the major class of genotoxic hepatocarcinogens in rodents [2]. 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is a genotoxic and carcinogenic heterocyclic amine formed by high-temperature cooking of proteinaceous food [2,3]. Long-term treatment (675 days) with 300 ppm IQ has been shown to induce tumors in the liver, lung and forestomach of CDF1 mice [4]. The In vivo mutagenicity and the mutation spectrum of IQ has been examined in commercially available transgenic rodent models such as BigBlue and guanine phosphoribosyltransferase (*gpt*) transgenic rats [3,5]. On the basis of these results, IQ was chosen as a positive mutagen for mutation assays.

Currently, little is known about Dammar resin metabolism. Dammar resin, isolated from plants belonging to the family Dipterocarpaceae, contains dammarane type triterpenes [6], which was reported to possess antiviral activities and to be protective against in vitro low density lipoprotein (LDL) oxidation [6,7]. Dammar resin is widely used in the food industry as a stabilizer and thickener. However, in a rat 2-year bioassay, the incidence of liver tumors was significantly increased in rats administered 2% Dammar resin when compared with the control group (unpublished data). The mechanism by which it exerts carcinogenic activity in rats has not been fully clarified.

The capability of a carcinogen to directly induce genetic mutations is routinely evaluated. However, in a recent study, up to 90% of rodent non-carcinogens tested positive in one or more of the standard in vitro assays used to determine the mutagenic capability of potential carcinogens, resulting in an unacceptably high number of false positive results [8]. Therefore, an in vivo genotoxicity test of Dammar resin and exploration of its mechanisms of hepatocarcinogenicity are required to help to evaluate the risk of this potential human carcinogen.

* Corresponding author. Tel.: +81 6 6645 3735; fax: +81 6 6646 3093.
E-mail address: wani@med.osaka-cu.ac.jp (H. Wanibuchi).

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8-hydroxydeoxyguanosine (8-OHdG) is well established as a representative biomarker for oxidative stress [9]: Oxidative damage to DNA is considered to be important in the mutagenesis and carcinogenesis process. The cytochrome P450s (P450s) act on a wide variety of chemicals, and their involvement in the production of reactive oxygen species during substrate oxidation is well documented [10,11]. Various chemicals with P450-inducible properties demonstrate hepatocarcinogenicity without overt genotoxicity [12–14], which suggests that reactive oxygen species resulting from increased activity of P450s enzymes may play a role in the carcinogenic activity of these chemicals. Researchers have hypothesized that non-genotoxic hepatocarcinogens with P450-inducible capability may cause oxidative damage to liver DNA, which might subsequently take part in inducing carcinogenesis [15].

The *gpt* delta mouse was established by microinjection of λ EG10 phage DNA (48 kb) into the fertilized eggs of C57BL/6J mice [16]. Progeny, carrying 80 copies of the transgene in a head-to-tail fashion at a single site of chromosome 17, were obtained and are maintained as homozygotes. These homozygous mice have been useful in the assessment of the in vivo genotoxicity and the estimation of carcinogenic risk of environmental chemicals [17].

In the present study, the in vivo genotoxicity of Dammar resin and IQ were examined using *gpt* delta mice and their possible mechanisms of hepatocarcinogenicity are discussed.

2. Materials and methods

2.1. Animals, diet and housing conditions

Eighteen male 5-week-old *gpt* delta C57BL/6J transgenic mice were obtained from Japan SLC and housed in plastic cages (six animals/cage) in our animal facility; animals were maintained under standard conditions (room temperature, 23 ± 1 °C; relative humidity, 44 ± 5%; and light/dark cycle, 12 h) and given free access to powdered diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. Dammar resin was kindly provided by San-Ei Gen F.F.I., Inc., Osaka, Japan. The animals were acclimatized for 1 week prior to beginning the experiment. The experiment was conducted following approval of the Animal Care and Use Committee of the Osaka City University Graduate School of Medicine.

2.2. Animal treatments

Eighteen *gpt* delta transgenic mice were randomly divided into three groups (6 in each group) and were fed a basal diet or diet containing 2% Dammar resin or 300 part per million (ppm) of IQ for 12 weeks followed by 2-week basal diet, as described previously [18]. The dose levels of Dammar resin and IQ used in the present study are consistant with the carcinogenic levels which were studied in previous long-term carcinogenesis studies. In the present study, 12 weeks feeding with Dammar resin or IQ followed by a 2-week treatment-free period was employed because in previous transgenic studies using the *gpt* delta transgenic mouse [18,19], a positive response could be readily detected after a 12-week exposure to genotoxic carcinogens, and, in transgenic assays, it is important to choose an appropriate expression time, i.e., the time after the last exposure to mutagen which is required for the mutant frequency to stabilize in a given tissue [20]. An appropriate expression time for liver is 14 days [21].

All surviving animals were killed under deep anesthesia at the end of the experiment. The livers were isolated from each animal and immediately excised, weighted, and dissected into halves. One half was immediately frozen in liquid nitrogen and stored at −80 °C for mutation assays and gene expression analyses. The other half was cut into 2–3-mm thick slices. The slices were fixed in 10% buffered formalin solution and routinely processed to paraffin blocks for histopathological examination and immunohistochemistry. Hematoxylin and eosin (H&E)-stained tissue cut from the blocks were examined by light microscopy.

2.3. Immunohistochemistry staining for ki-67

Paraffin blocks of the livers were sectioned at 3-μm thickness. After deparaffinization, sections were incubated with 30% hydrogen peroxide to block endogenous peroxidase and antigen retrieval was performed by microwaving at 95 °C for 30 min. After blocking, sections were incubated with ki-67 primary antibody (1:500, 550690, BD Pharmingen, USA) overnight at 4 °C. Antigen visualization was done with 3,3′-diaminobenzidine tetrahydrochloride (DAB). To investigate proliferative activity, at least 1000 hepatocyte nuclei were counted in each liver; labeling indices were calculated as the percentage of cells positive for ki-67 staining.

2.4. Measurement of 8-OHdG

Nuclear DNA was extracted with a DNA Extractor WB kit (Wako Pure Chemical Industries) containing an antioxidant NaI solution to dissolve cellular components. For additional prevention of auto-oxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical, St Loius, MO, USA) was added to the lysis buffer. The DNA was digested into deoxynucleotides by treatment with nuclease P1 (Yamasa Shoyu, Chiba, Japan) and alkaline phosphatase (Sigma Chemical), and levels of 8-OHdG (8-OHdG/10^5 dG) were measured by high-performance liquid chromatography with an electrochemical detection system (Coulochem II; ESA, Bedford, MA, USA).

2.5. DNA isolation and in vitro packaging of λ phage DNA

High-molecular-weight genomic DNA was extracted from liver tissue using the RecoverEase DNA Isolation kit (Stratagene, La Jolla, CA, USA). λ EG10 phages were rescued using Transpack Packaging Extract (Stratagene).

2.6. gpt mutation assay

The assay was conducted according to previously published methods [16]. All the confirmed *gpt* mutants recovered from the livers were sequenced; identical mutations from the same mouse were counted as one mutant. The mutant frequency (MF) of the *gpt* gene in the liver was calculated by dividing the number of confirmed 6-thioguanine (6-TG)-resistant colonies by the number of rescued plasmids. DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Inc., Carlsbad, CA, USA) on an Applied Biosystems PRISM 310 Genetic Analyzer.

2.7. Spi⁻ assay

The Spi⁻ assay was conducted according to previously published methods [16]. Packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. The next day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes and the agar plugs were suspended in SM buffer. The Spi⁻ phenotype was confirmed by spotting the suspensions on three types of plates in which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains were spread with soft agar. True Spi⁻ mutants, which made clear plaques on all of the plates, were counted. Spi⁻ mutant lysates were obtained by infecting E. coli LE392with the recovered Spi⁻ mutants.

2.8. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of p53, bax, bcl-2, caspase-3, cyp1a1, cyp1a2, cyp2e1, cyp2r1, cyp7b1, glutathione peroxidase 1 (gpx1) and glutathion S-transferase mu 2 (gstm2) mRNA

Total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA copies of total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan Ltd.). Primers and probes (Taqman Gene Expression Assay) were purchased from Applied Biosystems, Inc., Carlsbad, CA, USA. The PCR program cycles were set as follows: initial denaturing at 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. PCR reactions were performed as described previously [22], with primers for mouse *p53*, *bax*, *bcl-2*, *caspase-3*, *cyp1a1*, *cyp1a2*, *cyp2e1*, *cyp2r1*, *cyp7b1*, *gpx1* and *gstm2*. β-Actin mRNA was employed as an internal standard, and the mRNA levels of the target gene were normalized to the β-actin mRNA level. The Values in each treatment group were expressed as fold increases compared to the mean value in the control group, which was given an arbitrary value of 1.

2.9. Statistical analysis

All mean values were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the Statlight program (Yukms Co., Ltd., Tokyo, Japan). Homogeneity of variance was tested by the F test between the treatment and control groups. Differences in mean values between the treatment and control groups were evaluated by the two-tailed Student's *t*-test when variance was homogeneous and the two-tailed Aspin–Welch *t*-test when variance was heterogeneous. *p* values less than 0.05 were considered significant.

3. Results

3.1. Body and liver weights, water intake, food consumption and compound intake

The results for body and liver weights, water intake, food consumption and compound intake of the mice are presented in Table 1. In the Dammar resin group, one mouse died during the

Table 1
Final body weight, liver weight, water intake, food consumption and compounds intake of *gpt* delta transgenic mice.

Groups Number of effective mice	Control 6	2% Dammar resin 5	300 ppm IQ 6
Final body weight (g)	34.67 ± 1.87	28.76 ± 0.09*	30.7 ± 1.27*
Absolute liver weight (g)	1.20 ± 0.13	1.06 ± 0.08	1.38 ± 0.09*
Relative liver weight (g/g body weight)	0.035 ± 0.005	0.037 ± 0.003	0.045 ± 0.002*
Average water intake (g/g body weight/day)	0.24 ± 0.05	0.22 ± 0.03	0.26 ± 0.02
Average food intake (g/g body weight/day)	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.02
Average compound intake (mg/g body weight/day)	-	2.43 ± 0.32	0.04 ± 0.01

* $p < 0.05$ versus the control group.

second week. Since the cause of death was unclear, it was not included in the final analysis.

Dammar resin and IQ did not affect average water or food intake, but significantly suppressed final body weight. No significant changes of absolute or relative liver weights were observed in the Dammar resin-treated group. The absolute and relative liver weights were significantly increased in the IQ-treated group ($p < 0.05$).

3.2. Histopathological evaluation and hepatocyte proliferation analysis

There were no obvious pathologic changes in the livers of the Dammar resin group; hypertrophy and vacuolar degeneration of hepatocytes were observed in some livers of IQ-treated mice (data not shown).

Fig. 1 shows the results of cell proliferation analysis by ki-67 staining. Proliferation of hepatocytes was significantly elevated in both Dammar resin and IQ treatment groups compared with the control group.

3.3. Examination of oxidative stress by 8-OHdG measurement

Fig. 2 shows the 8-OHdG levels in the livers. Significant increases of 8-OHdG were present in the Dammar resin-treated group, but not in the IQ treatment group, compared with the control group.

3.4. In vivo mutation assay

Data for *gpt* MF analyzed by 6-TG selection are summarized in Table 2. There was no difference in the *gpt* MF in the Dammar resin-treated mice (3.01×10^{-6}) compared with the control group (5.39×10^{-6}). In contrast, in the IQ-treated mice, *gpt* MF (32.49×10^{-6}) was significantly increased, approximately 6-fold higher than the control group.

To characterize the *gpt* mutations in the livers, DNA sequencing was performed (Table 3). In Dammar resin-treated mice, the predominant types of base substitutions were G:C to A:T transitions (5/12 = 41.7%) and G:C to T:A transversions (3/12 = 25%), neither of which differed significantly from the control group. On the other hand, the predominant type of base substitution in the IQ treatment group was the G:C to T:A transversion (81/117 = 69.2%), which was significantly increased compared to the control group.

The results of the Spi$^-$ mutation assay are shown in Table 4. There was no difference in Spi$^-$ mutant frequency in the mice administered Dammar resin (5.94 ± 3.00, $p > 0.05$), but Spi$^-$ mutant frequency in mice fed IQ (21.69 ± 10.44, $p < 0.01$) is significantly elevated compared to the control (6.21 ± 1.05).

3.5. mRNA expression levels of p53, bax, bcl-2, caspase-3, cyp1a1, cyp1a2, cyp2e1, cyp2r1, cyp7b1, gpx1 and gstm2 in mouse liver

RT-PCR was performed on all the mice livers. The results are shown in Fig. 3. Expression of *p53*, *bax*, *bcl-2*, *cyp1a2*, *cyp2e1*, *gpx1* and *gstm2* were significantly increased in Dammar resin-treated mice ($p < 0.05$). Expression of *p53*, *cyp2e1* as well as *gpx1* were

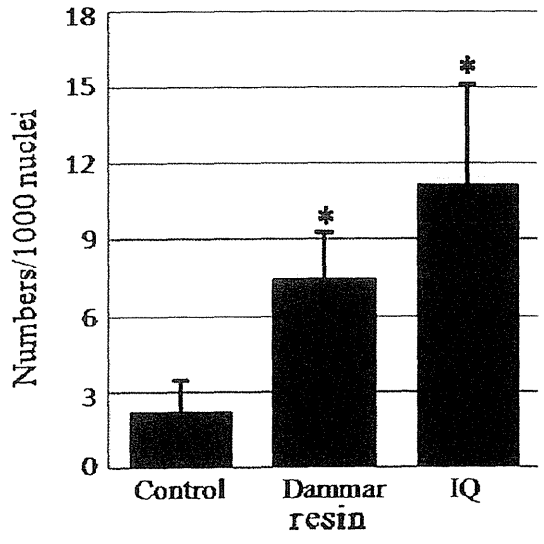

Fig. 1. Cell proliferation index. Compared with the control group, hepatocyte proliferation was significantly elevated by administeration of Dammar resin or IQ, at doses of 2% and 300 ppm, respectively. Significant compared with the control group (*$p < 0.05$).

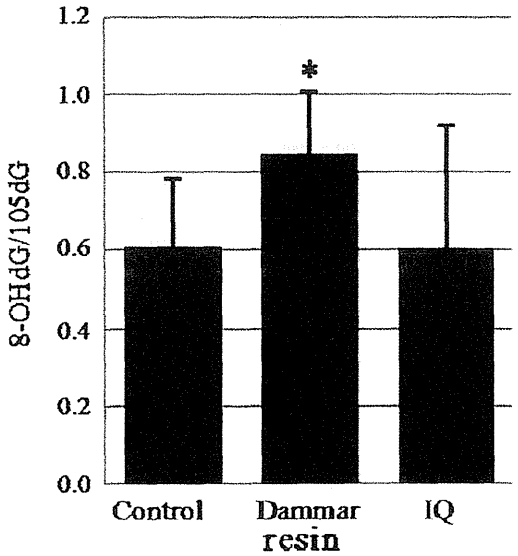

Fig. 2. Formation of 8-OHdG in the liver. The level of 8-OHdG was significantly increased by treatment with 2% Dammar resin, while no change was observed with 300 ppm IQ treatment. Significant compared with the control group (*$p < 0.05$).

Table 2
gpt mutation frequency in mouse livers.

Treatment	Mouse number	Cm^R colonies ($\times 10^5$)	Independent *gpt* mutation	*gpt* MF ($\times 10^{-6}$)	Mean ± SD
Control	11	7.80	3	3.85	5.39 ± 2.78
	12	7.08	4	5.65	
	13	7.29	3	4.12	
	14	6.27	6	9.57	
	15	5.64	1	1.77	
	16	5.40	4	7.41	
2% Dammar resin	21	9.60	2	2.08	3.01 ± 1.19
	22	5.52	1	1.81	
	23	7.48	2	2.67	
	24	5.10	2	3.92	
	26	10.96	5	4.56	
300 ppm IQ	31	6.51	14	21.51	32.49 ± 11.14*
	32	5.13	19	37.04	
	33	6.99	22	31.47	
	34	6.99	17	24.32	
	35	6.36	18	28.30	
	36	5.16	27	52.33	

* $p < 0.01$ versus the control group.

Table 3
Classification of *gpt* mutations in *gpt* delta mouse livers.

Types of *gpt* mutation	Control		2% Dammar resin		300 ppm IQ	
	Number (%)	Mutant frequency ($\times 10^{-6}$)	Number (%)	Mutant frequency ($\times 10^{-6}$)	Number (%)	Mutant frequency ($\times 10^{-6}$)
Base substitution						
Transition						
G:C to A:T	3(14.3)	0.71 ± 0.79	5(41.7)	1.18 ± 1.19	8(6.8)	2.25 ± 1.80
A:T to G:C	2(9.5)	0.49 ± 0.77	1(8.3)	0.39 ± 0.88	3(2.6)	0.76 ± 1.30
Transversion						
G:C to T:A	5(23.8)	1.25 ± 1.07	3(25)	0.68 ± 0.97	81(69.2)	22.56 ± 7.17*
G:C to C:G	1(4.8)	0.24 ± 0.58	0	0 ± 0	7(6)	1.92 ± 2.53
A:T to T:A	0	0 ± 0	0	0 ± 0	2(1.7)	0.65 ± 1.59
A:T to C:G	2(9.5)	0.57 ± 0.89	0	0 ± 0	1(0.9)	0.26 ± 0.64
Deletion						
Single bp	2(9.5)	0.45 ± 0.70	0	0 ± 0	6(5.1)	1.65 ± 1.83
Over 2bp	1(4.8)	0.31 ± 0.76	0	0 ± 0	2(1.7)	0.49 ± 0.77
Insertion	5(23.8)	1.36 ± 1.70	2(16.7)	0.57 ± 0.87	6(5.1)	1.63 ± 1.59
Others	0	0 ± 0	1(8.3)	0.18 ± 0.41	1(0.9)	0.32 ± 0.79
Total	21 (100)	5.39 ± 2.78	12 (100)	3.01 ± 1.19	117 (100)	32.49 ± 11.14*

* $p < 0.01$ versus the control group.

significantly decreased in IQ-treated mice ($p < 0.05$). The ratio of *bax* versus *bcl-2* as well as expression of *caspase-3*, *cyp1a1*, *cyp2r1* and *cyp7b1* did not differ in the treated groups compared with the control group.

4. Discussion

IQ is known to be a potent mutagen for the liver [23], and it was therefore used as a positive control mutagen for validating

Table 4
Spi^- mutant frequency in mouse livers.

Treatment	Mouse number	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaque within WL95 (P2)	Mutant frequency ($\times 10^{-6}$)	Mean ± SD
Control	11	13.06	6	4.59	6.21 ± 1.05
	12	10.22	7	6.85	
	13	11.24	7	6.23	
	14	10.58	8	7.56	
	15	10.92	6	5.49	
	16	24.39	16	6.56	
2% Dammar resin	21	15.96	4	2.51	5.94 ± 3.00
	22	13.72	13	9.48	
	23	14.44	6	4.16	
	24	10.38	9	8.67	
	26	10.18	5	4.91	
300 ppm IQ	31	10.16	15	14.76	21.69 ± 10.44*
	32	12.24	20	16.34	
	33	14.26	49	34.36	
	34	14.12	29	20.54	
	35	21.68	21	9.69	
	36	11.04	38	34.42	

* $p < 0.01$ versus the control group.

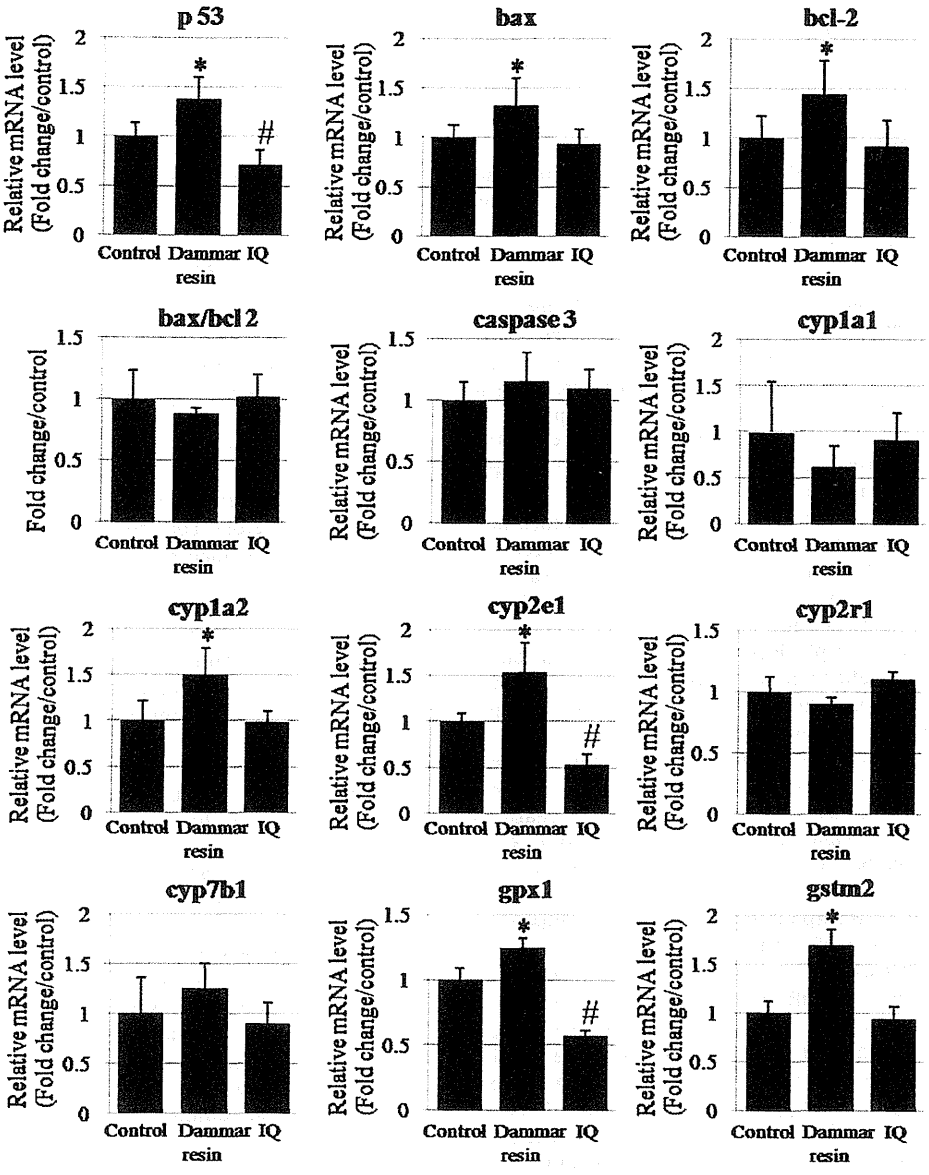

Fig. 3. mRNA expression of *p53*, *bax*, *bcl-2*, *bax/bcl-2*, *caspase-3*, *cyp1a1*, *cyp1a2*, *cyp2e1*, *cyp2r1*, *cyp7b1*, *gpx1* and *gstm2* in the livers of *gpt* delta mice. Significantly increased *p53*, *bax*, *bcl-2*, *cyp1a2*, *cyp2e1*, *gpx1* and *gstm2* gene expression was observed in the Dammar resin-treated group, while significantly decreased *p53*, *cyp2e1* and *gpx1* gene expression was observed in the IQ-administered group. The ratio of *bax* versus *bcl-2* as well as the mRNA expression of *caspase-3*, *cyp1a1*, *cyp2r1* and *cyp7b1* did not differ in the treated and control groups. Significantly up-regulated compared with the control group ($^{*}p < 0.05$); significantly down-regulated compared with the control group ($^{\#}p < 0.05$).

the mutagenicity assay system. In the present study, the mutation assay clearly showed that exposure to IQ at a dose of 300 ppm for 12 weeks was mutagenic. Significant increases in *gpt* MF, for the detection of point mutations, and Spi$^-$ MF, mainly attributable to deletion mutations, were found in IQ-treated mice. In the *gpt* assay, IQ primarily caused transversion of G:C to T:A, which is consistent with previous studies [3,5].

On the other hand, exposure to Dammar resin at a dose of 2% for 12 weeks was not mutagenic, which was consistent with the results of our mutation assay conducted in *gpt* delta transgenic rats (manuscript in preparation). There was no change in either the *gpt* MF or Spi$^-$ MF between the Dammar resin and control group. In the Dammar resin-administered group, the predominant types of base substitutions were G:C to A:T transitions and G:C to T:A transversions, neither of which were significantly different from the control group.

Ki-67 is a nuclear marker of cell proliferation and is detectable in the cell at all phases of the cell cycle except G_0 [24]. The ki-67 labeling index is associated with liver cancer outcome [25]. In the present study, proliferation of hepatocytes was significantly augmented by administration of both IQ and Dammar resin, suggesting the possibility that enhanced cell proliferation might be at least partly responsible for the carcinogenic activity of these agents.

Oxidative DNA damage with 8-OHdG formation is reported to be induced by 2-amino-imidazo[4,5-*f*]quinoline [23] and is thought to play a critical role in IQ-mediated carcinogenesis. However, in the present study, IQ did not induce 8-OHdG formation. Our result was consistent with the studies of Kitamura et al. [26] and Wei et al. [22]: the level of 8-OHdG was not significantly changed in the livers of rats treated with 300 ppm IQ.

It is generally recognized that oxidative stress occurs in a cell or tissue when the concentration of reactive oxygen species (ROS)

generated exceeds the antioxidant capability of that cell [27]. ROS generation is related to P450 enzyme activity. Multiple sources of ROS may contribute to persistent oxidative stress, resulting in higher 8-OHdG levels, and subsequently resulting in pathophysiologic changes that allow for the selective growth of preneoplastic initiated cells [27]. In the present study, levels of P450 enzymes and 8-OHdG levels were increased in the Dammar resin fed group, suggesting that Dammar resin may induce the expression and activity of P450 enzymes which would result in increased ROS production and 8-OHdG generation. However, while the Dammar resin fed group had increased 8-OHdG levels, there was no evidence suggestive of direct DNA damage, i.e., the Dammar resin fed group did not have increased DNA mutations. Therefore, our results suggest that the production of reactive oxygen species resulting from increased expression and activity of P450 enzymes, while causing chemical damage to DNA bases, is insufficient to cause permanent gene mutations; this result is consistent with a previous study [15].

Disruption of apoptosis can promote tumor initiation and progression [28], and the induction of apoptosis is central to the tumor-suppressive activity of p53 [29]. Bax is a proapoptotic Bcl-2 family member that binds to the anti-apoptotic Bcl-2 protein and antagonizes its function [30]. The *bax* gene is known to be transcriptionally regulated by p53 during induction of apoptosis [31,32]. Thus, up-regulated *bax* is one mechanism whereby p53 induces apoptosis [33]. Furthermore, p53 loss and Bcl-2 overexpression can have virtually identical effects on the pathology of some tumors [34]. Caspase-3 is a key mediator of apoptosis and can be activated through both extrinsic and intrinsic signaling pathways [35,36]. In the present study, treatment with IQ significantly down-regulated expression of *p53* expression, but did not affect expression of *bax*, *bcl-2* or *caspase-3* or change the ratio of *bax* versus *bcl-2*. Treatment with Dammar resin significantly increased expression of *p53*, *bax* and *bcl-2* genes, but did not change the ratio of *bax* versus *bcl-2* or change expression of *caspase-3*. This suggests that neither IQ nor Dammar resin affected basal apoptotic/anti-apoptotic signaling in hepatocytes, but expression of *p53*, the upstream inhibitor of *bcl-2* and activator of apoptosis [34], was affected by these agents: IQ suppressed expression of *p53* mRNA while Dammar resin induced expression of *p53* mRNA. This suggests that in the livers of *gpt* delta mice, the genotoxic agent IQ suppresses apoptotic removal of mutated cells with carcinogenic potential while the non-genotoxic agent Dammar resin does not suppress apoptotic removal of cells with carcinogenic potential. This also explains why Dammar resin increased the formation of 8-OHdG but did not cause an increase in mutation frequency in the livers of Dammar resin treated mice.

P450s are the predominant catalysts of phase I metabolism in the liver. It has been generally accepted that the various forms of P450s show different rates of activation as well as detoxication of chemical carcinogens [37]. Gpx1 is a phase II enzyme: Gpx1 is one of the most important of the antioxidant phase II metabolic enzymes which protect cells and tissues from damage caused by reactive oxygen species by helping to maintain the balance between prooxidant and antioxidant forces [38]. Gstm2 is another phase II enzyme: Gstm2 is a member of the Glutathione S-transferase (GST) family which represents a major group of detoxification enzymes [39].

In our study, mRNA levels of *cyp2e1* were significantly down-regulated by treatment with IQ. In addition, IQ itself is able to affect the activity of P450s enzymes depending on the treatment regimen [40]. Low P450s enzyme activity would delay the metabolic conversion of IQ into a substrate for GST enzymes and this may be one factor in the low mRNA levels of *gpx1* after treatment with IQ. Morgan et al. [41] suggest that low levels of P450s mRNA and proteins in tumor-bearing mice and repression of hepatic transport proteins is linked with reduced drug clearance and the resultant toxicity of the drug. Taken together, these data suggest that the delay of metabolic transformation of IQ and its metabolites may be involved in its toxicity.

In the Dammar resin treatment group, mRNA levels of *cyp1a2*, *cyp2e1*, *gpx1* and *gstm2* were significantly up-regulated compared to the control group. While induction of detoxification and antioxidant enzymes (*gpx1* and *gstm2*) is protective [41,42], induction of these enzymes also indicates production of reactive metabolites by phase I enzymes. Importantly, induction of P450s by chemicals can induce hepatocarcinogenicity in rodents without overt genotoxicity [13,14]. Induction of P450s (*cyp1a2* and *cyp2e1*) by Dammar resin administration might play a role in carcinogenicity through mechanisms related to oxidative stress [43]: Klaunig et al. [27] have shown that chronic sublethal oxidative injury can alter cellular metabolic pathways and gene expression, leading to altered cell growth in the absence of genetic mutations. Thus, we hypothesize that intranuclear oxidative stress could play a role in Dammar resin hepatocarcinogenicity by altering gene expression as a result of 8-OHdG adduct formation, but not by inducing DNA mutation. Therefore, consistent with a previous study by Tasaki et al. [15], it is reasonable to suggest that the hepatocarcinogenicity of Dammar resin observed in our previous long-term experiment with rats (unpublished data) may not be caused by DNA mutations, but rather by non-genotoxic mechanisms: Promotion of cell proliferation, induction of P450s, detoxification and antioxidant enzymes, increased intranuclear oxidative stress and other gene products. Although the mechanisms responsible for the carcinogenicity of Dammar resin remain to be further defined, our findings support the suggestion that its carcinogenicity does not necessarily correlate with in vivo mutagenicity [1].

In a recent study, 20 terpenoids from Dammar resin are pointed to have cytotoxic activity against human leukemia (HL60) and melanoma (RL1579) cells in vitro, which suggest that triterpenoids isolated from Dammar resin and its derivatives are valuable as potential cancer chemopreventive agents [7]. However, although no genetoxicity was observed in Dammar resin-treated group in the present study, considering its hepatocarcinogenesis in rats, Dammar resin should be used with care, if used at all, as a cancer chemopreventive agent until further investigations are conducted focusing on its metabolism and the underlying mechanisms of its hepatocarcinogenicity. Furthermore, the results of the present study suggest that Dammar resin has potential carcinogenic activity in mice.

In summay, administration of the known genotoxic hepatocarcinogen IQ results in increased liver cell proliferation and a characteristic mutation spectra with a predominant occurrence of G:C to T:A transitions in the *gpt* sequence analysis. Decreased expression of *cyp2e1* and *gpx1* increases the genotoxicity of IQ and decreased expression of p53 allows the survival and proliferation of cells with mutated DNA, suggesting that the hepatocarcinogenicity of IQ is initiated by DNA mutation. Like IQ, administration of Dammar resin to *gpt* delta mice also promotes liver cell proliferation; however, unlike IQ, Dammar resin administration does not cause DNA mutation. Rather, Dammar resin promotes cell proliferation, induces expression metabolic enzymes, and increases intranuclear oxidative stress. These results suggest that Dammar resin is a potential hepatocarcinogen in mice, although this remains to be verified. Dammar resin does not increase DNA mutation frequency, indicating that it is non-genotoxic in mice, which is consistent with our results of mutation assay conducted in *gpt* delta rats (manuscript in preparation). Importantly, the possible mechanism of Dammar resin-mediated carcinogenicity suggested by the results of the present study is also active in humans, indicating that the use of Dammar resin or its constituents in foods or as a food supplement for humans should be carefully monitored.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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RESEARCH COMMUNICATION

Effects of Pinocembrin on the Initiation and Promotion Stages of Rat Hepatocarcinogenesis

Charatda Punvittayagul[1], Wilart Pompimon[2], Hideki Wanibuchi[3], Shoji Fukushima[4], Rawiwan Wongpoomchai[1]*

Abstract

Pinocembrin (5,7-dihydroxyflavanone) is a flavanone extracted from the rhizome of *Boesenbergia pandurata*. Our previous studies demonstrated that pinocembrin had no toxicity or mutagenicity in rats. We here evaluated its effects on the initiation and promotion stages in diethylnitrosamine-induced rat hepatocarcinogenesis, using short- and medium-term carcinogenicity tests. Micronucleated hepatocytes and liver glutathione-S-transferase placental form foci were used as end point markers. Pinocembrin was neither mutagenic nor carcinogenic in rat liver, and neither inhibited nor prevented micronucleus formation as well as GST-P positive foci formation induced by diethylnitrosamine. Interestingly, pinocembrin slightly increased the number of GST-P positive foci when given prior to diethylnitrosamine injection.

Keywords: *Boesenbergia pandurata* - cancer chemoprevention - diethylnitrosamine - liver micronucleus test

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Introduction

Cancer chemoprevention is defined as the use of chemical agents to reverse, suppress, or prevent multistage carcinogenesis (Surh, 2003). Nowadays, many dietary phytochemicals can be considered as chemopreventive agents because they have been shown to inhibit carcinogenesis (Debersac et al., 2001). The mechanism of chemical protection against the initiation stage involves the induction of phase I and phase II xenobiotic-metabolizing enzymes (Tan & Spivack, 2009). Moreover, the chemopreventive activity also influences cell proliferation, differentiation and apoptosis (Chen & Kong, 2004), preventing the accumulation of damaged cells.

Flavanones are a subclass of flavonoids that naturally occur in various plant species, including spices and condiments, cereals, vegetables and fruits. There have been many reports indicating their effects on multistep carcinogenesis (Galati & O'Brien, 2004). Hsiao et al. (2007) showed that flavanone and 2'-OH flavanone inhibited the invasion and metastasis of lung cancer cells in both *in vitro* and *in vivo* models. In 2009, Aranganathan and Nalini demonstrated that hesperetin had anti-carcinogenic potential against DMH-induced colon cancer. In addition, naringenin reduced tumor size and weight in N-methyl-N'-nitro-N-nitrosoguanidine-induced rat gastric carcinogenesis (Ekambaram et al., 2007), and also inhibited glial tumor cell proliferation in rat C6 glioma models (Sabarinathan et al., 2011).

Pinocembrin is a flavanone found in rhizomes of *B. pandurata* or "Kra-chai" in Thai (Jaipetch et al., 1982). The chemical structure of this compound is shown in Figure 1. Previous investigations have demonstrated that pinocembrin has various pharmacological activities, including anti-oxidant and anti-inflammatory (Pepeljnjak et al., 1985; Santos et al., 1998; Tuchinda et al., 2002; Hwang et al., 2003; Sala et al., 2003; Liu et al., 2008). Moreover, it exhibited a strong antimutagenic activity against mutagenic heterocyclic amines (Trakoontivakorn et al., 2001). Our previous study indicated that pinocembrin had no toxicity or mutagenicity in male rats (Charoensin et al., 2010). In addition, it could inhibit activities of P450 isozymes involved in carcinogen metabolism (Siess et al., 1995) and also induced the activity of heme oxygenase in rat liver (Punvittayagul et al., 2011).

Based on these observations, we hypothesized that pinocembrin may help protect against chemical-induced hepatocarcinogenesis. However, the in vivo

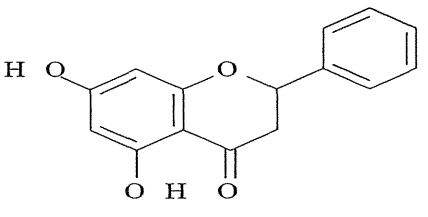

Figure 1. Structure of Pinocembrin

[1]*Department of Biochemistry and Center for Innovation in Chemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai,* [2]*Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Lampang Rajabhat University, Lampang, Thailand,* [3]*Department of Pathology, Osaka City University Medical School, Osaka,* [4]*Japan Bioassay Research Center, Hirasawa, Hadano, Kanagawa, Japan* **For correspondence: rpuatana@mail.med.cmu.ac.th*

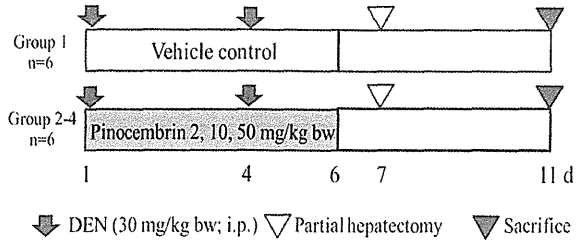

Figure 2. The Protocol for Examining the Inhibitory Effect of Pinocembrin on DEN–Induced Initiation Stage of Rat Hepatocarcinogenesis

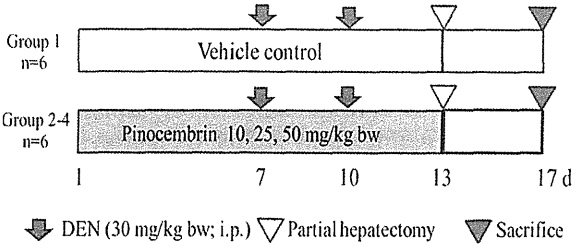

Figure 3. The Protocol for Investigating the Preventive Effect of Pinocembrin on DEN–Induced Initiation Stage of Rat Hepatocarcinogenesis

carcinogenic and anticarcinogenic effects of pinocembrin have not previously been investigated. Therefore, rat models are needed to determine whether administration of pinocembrin could inhibit hepatocarcinogenesis. Hence, the rat liver micronucleus and medium-term carcinogenicity tests were performed to determine the effect of pinocembrin on the initiation and promotion stages of rat hepatocarcinogenesis, respectively.

Materials and Methods

Animals

Male Wistar rats were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn-Prathom, Thailand and were kept in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Rats were given an acclimatization period of one week before each experiment. They were housed at a maximum of three per cage with a light–dark cycle 12–12 hours, at temperatures of 21–25 °C and relative humidity 50–60% throughout the study. Each animal had free access to diet and tap water. The experimental protocols were approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University.

Chemicals

Pinocembrin was obtained from Assoc. Prof. Wilart Pompimon, Faculty of Science, Lampang Rajabhat University, Thailand; collagenase type IV and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen, USA; diethylnitrosamine was purchased from Tokyo Kasei Kogyo Co. Ltd., Japan; diaminobenzidine was from Dojindo, Japan; primary rabbit polyclonal antibodies against rat GST-P was obtained from MBL, Japan; Vectastrain ABC kit was obtained from Vector

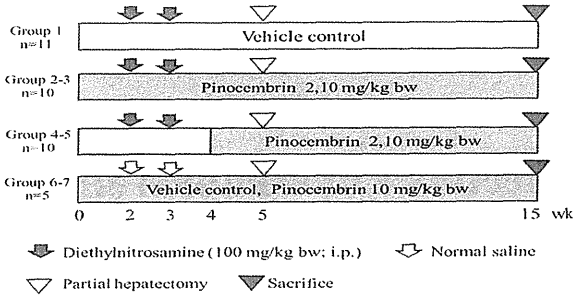

Figure 4. Medium-Term Carcinogenicity Protocol

Laboratories, Inc., USA.

Short-term carcinogenicity test

The first experiment investigated the inhibitory and preventive effects of pinocembrin on diethylnitrosamine (DEN)–induced initiation stage of rat hepatocarcinogenesis. This study was performed using 2 protocols. All rats were intraperitoneal (i.p.) injected with DEN on day 0 and day 3. In the first protocol, rats were divided into 4 groups orally receiving various concentrations of pinocembrin, 0, 2, 10, and 50 mg/kg bw for 6 days on day 0 to day 5. In the latter protocol, rats were classified into 4 groups receiving various dosages of pinocembrin, 0, 10, 25 and 50 mg/kg bw for 12 days, 6 days before DEN injection on day 6 of the experiment. The incidence of micronucleated hepatocytes was determined 4 days after partially hepatectomy, as shown in Figures 2 and 3, respectively. Hepatocytes were isolated from anesthetized rats by the 2-step collagenase perfusion method according to Puatanachokchai et al. (Puatanachokchai et al.,1996). Then hepatocyte suspensions were mixed with DAPI stain solution, and analyzed under a fluorescent microscope. The micronucleated hepatocytes (MNHEPs) and mitotic cells were recorded based on analysis of 2000 hepatocytes from each animal.

Medium-term rat liver carcinogenicity test

To determine the effect of pinocembrin on the promotion stage in DEN-induced hepatocarcinogenesis, a modified method of the medium term bioassay system of Ito based on the two-step model of hepatocarcinogenesis (Ito et al., 2003; Tsuda et al., 2010) was developed in our laboratory for detection the carcinogenic and anticarcinogenic activities of chemical compounds. In this experiment, male Wistar rats were divided into 7 experimental groups (Figure 4). At weeks 3 and 4 of the experiment, groups 1 to 5 were given a double i.p. injection of DEN to initiate hepatocarcinogenesis, while groups 6 and 7 were i.p. administered a normal saline solution. Before 2 weeks of injection, groups 2 and 3 received oral pinocembrin at 2 and 10 mg/kg bw, respectively. Groups 4 and 5 were fed with pinocembrin at 2 and 10 mg/kg bw, respectively, after injections for 1 week. Groups 1 and 6 were treated with a vehicle control, while group 7 was fed pinocembrin at 10 mg/kg. All animals were 2/3 partial hepatectomized at week 6 to stimulate the hepatocytes into mitosis using the technique described by Higgins and Anderson (1931) and were sacrificed at week 15. Blood samples were collected and analyzed for serum alanine aminotransferase, aspartate aminotransferase and alkaline

Table 1. Inhibitory Effect of Pinocembrin on Diethylnitrosamine-Induced Micronucleus Formation in Rat Liver

Treatment	Body weight (g)		MNHEPs/1,000	Mitotic
(mg/kg bw)	Initial	Final	hepatocytes	index (%)
DEN	197.7 ± 5.4	216.7± 7.5	31.8 ± 9.0	3.38±1.12
DEN+PC2	206.3 ± 11.1	228.8± 8.5	27.9 ± 12.4	3.73±1.50
DEN+PC10	200.0 ± 7.9	226.0± 13.9	28.5 ± 4.8	2.94±0.62
DEN+PC50	201.7 ± 8.2	218.3± 12.5	27.6 ± 11.9	2.81±0.99

*Values expressed as mean ± SD, MNHEPs = micronucleated hepatocytes, DEN = diethylnitrosamine, 30 mg/kg bw; i.p.

Table 2. Preventive Effect of Pinocembrin on Diethylnitrosamine-Induced Micronucleated Hepatocyte Formation in Rats

Treatment	Body weight (g)		MN-	% Inhi-	Mitotic
(mg/kg bw)	Initial	Final	HEPs[a]	bition	index%
DEN	176.0±8.2	248.0±18.2	26.8±5.3	-	2.1±0.4
DEN+PC10	168.8±6.3	235.0±7.1	20.1±3.1	25.1	1.9±0.3
DEN+PC 25	171.7±9.3	243.3±10.3	26.5±7.3	1.3	2.3±0.7
DEN+PC 50	173.3±5.2	235.0±12.1	23.2±4.2	13.6	2.1±0.2

*MNHEPs = micronucleated hepatocytes, [a]MNHEPs/1,000 hepatocytes

Table 3. Relative Organ Weight and Blood Biochemical Analysis of Rats in the Medium-Term Carcinogenicity Experiment

Treatment	Exposure period	Relative organ weight (%)			Enzyme activity (IU/L)		
	of pinocembrin	Liver	Spleen	Kidney	AST	ALT	ALP
DEN	-	2.75±0.14	0.20±0.02	0.55±0.02	101.4±18.6	53.1±11.1	122.0±20.6
DEN[+]PC 2 mg/kg bw	week 1-15	2.95±0.21	0.19±0.02	0.56±0.06	81.4±14.8	42.6± 8.7	133.3±26.7
DEN[+] PC 10 mg/kg bw	week 1-15	2.84±0.19	0.20±0.02	0.56±0.04	108.1±15.9	73.0±20.2	137.7±34.3
DEN[→]PC 2 mg/kg bw	week 5-15	2.85±0.27	0.19±0.01	0.54±0.10	96.8±11.5	57.4±20.5	152.3±55.8
DEN[→]PC10 mg/kg bw	week 5-15	2.78±0.23	0.19±0.02	0.52±0.04	92.2±13.1	41.8±10.2	139.3±32.4
NSS	-	2.68±0.37	0.20±0.04	0.51±0.04	103.0±16.0	47.6±13.2	140.4±61.3
NSS+ PC10 mg/kg bw	week 1-15	2.78±0.34	0.20±0.03	0.52±0.04	99.6±24.3	42.2± 9.7	124.0±43.2

*AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase, Values expressed as mean ± SD, DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p., [+]Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment), [®]Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

phosphatase activities. The liver samples were fixed in 10% formalin and embedded in paraffin. They were used for immunohistochemical examination of glutathione-*S*-transferase placental form (GST-P), which is the end point marker of rat hepatocellular carcinoma.

Immunohistochemical assessment of GST-P positive foci was performed using the avidin–biotin complex method according to Puatanachokchai et al. (Puatanachokchai et al., 2006). The numbers and areas of GST-P positive foci greater than 0.2 mm^2 in area and the total areas of the liver sections were measured using a color image processor to give values per cm^2 of liver section.

Statistical methods

Data are reported as means ± SD of each variable for each group. Differences between treated groups and control groups were determined by Welch's t-tests after application of a preliminary F-test for equal variance and $P<0.05$ was considered as significant.

Results

Effect of pinocembrin on initiation stage of rat hepatocarcinogenesis

In the inhibitory study, rats treated with 2, 10 and 50 mg/kg bw of pinocembrin showed no significant effect on the number of micronucleus formation induced by DEN (Table 1), indicating that pinocembrin did not inhibit the micronucleus formation induced by DEN.

Due to the initial observation of pinocembrin lacking inhibitory effects, the next study was designed to increase the concentration of pinocembrin and duration of treatment. Rats were orally administered with 10, 25 and 50 mg/kg bw of pinocembrin 6 days before the first injection of 30 mg/kg bw of DEN. The number of micronucleated hepatocytes and mitotic index are summarized in Table 2. Ten mg/kg bw of pinocembrin showed a slight decrease in micronucleated hepatocytes, but there were no significant differences between groups. These finding suggested that pinocembrin did not prevent micronucleus formation induced by DEN in rat liver.

Effect of pinocembrin on promotion stage of rat hepatocarcinogenesis

Glutathione-*S*-transferase placental form formation in rat liver was used to evaluate the effect of pinocembrin on promotion stage in DEN – induced rat hepatocarcinogenesis. In this study, we evaluated the effect of pinocembrin concentrations of 2 and 10 mg/kg bw after and before diethylnitrosamine injections for 10 and 15 weeks, respectively. There were no significant differences in water and food intake among the investigated groups (data not shown). The general observations, including relative organ weights and the serum AST, ALT and ALP activities, are summarized in Table 3. There were no significant differences between groups, demonstrating that pinocembrin at concentrations 2 and 10 mg/kg bw had no toxic effects in rats.

The quantitative data for GST-P positive foci are summarized in Table 4. Pinocembrin 10 mg/kg bw did not induce GST-P positive foci formation. It is evident that pinocembrin did not present carcinogenicity in

Table 4. Quantitative Values for GST-P Positive Foci of Rats in the Medium-Term Carcinogenicity Experiment

Treatment (mg/kg bw)	Exposure period of pinocembrin (week)	Body weight (g) Initial	Final	GST-P positive foci No./cm²	Area (mm²/cm²)
DEN	-	65.6±1.7	436.7±32.2	3.17±1.15*	0.23±0.09*
DEN+PC 2	1-15	67.5±2.6	429.5± 46.5	2.38±1.77	0.24±0.26
DEN+PC 10	1-15	67.0±3.5	427.5±35.3	5.85±4.39	0.64±0.54
DEN+PC 2	5-15	65.0±4.1	432.0± 28.8	4.34±3.09	0.41±0.35
DEN+PC10	5-15	68.0±2.6	422.0±20.8	2.89±0.99	0.31±0.13
NSS	-	68.5±2.2	429.3±35.3	0.00±0.00	0.00±0.00
NSS+PC10	1-15	69.4±7.9	421.3±41.6	0.06±0.14	0.00±0.00
NSS+PC10	1-15	69.4±7.9	421.3±41.6	0.06±0.14	0.00±0.00

*significantly different from negative control group, p<0.05

rats. Moreover, rats treated with 2 and 10 mg/kg bw of pinocembrin received before or after DEN injection did not show a significant decrease in the number of GST-P positive foci. Interestingly, pinocembrin at 10 mg/kg bw slightly increased the number of GST-P positive foci relative to the positive control (84%) when administered before DEN injection. These results indicated that pinocembrin did not inhibit or promote the DEN-induced promotion stage of rat hepatocarcinogenesis.

Discussion

Pinocembrin exhibited a strong antimutagenic activity against mutagenic heterocyclic amines in vitro using the Ames test (Trakoontivakorn et al., 2001). In this study, the carcinogenic and anticarcinogenic activities of pinocembrin on rat hepatocarcinogenesis were evaluated by short- and medium-term carcinogenicity tests using DEN as a hepatocarcinogen. We found that pinocembrin did not induce micronucleus formation. Moreover, it did not decrease the number of micronucleated hepatocytes in the DEN – induced initiation stage of hepatocarcinogenesis. We also found that 10 mg/kg bw of pinocembrin tended to decrease the number of micronuclei more than 25 and 50 mg/kg bw. Subsequently, an analysis of the reduction of mutagenic potency of DEN and prolonged administration of pinocembrin exposure were performed. Rats were orally fed with 10 mg/kg bw of pinocembrin for 21 days, 14 days before 20 mg/ kg bw of DEN injection. We found that, oral administration of 10 mg/kg bw of pinocembrin reduced micronucleus frequency by 30% in rat liver when compared to positive control, but the difference was not statistically significant (data not shown). The results of the present investigation clearly showed that pinocembrin did not present either mutagenic or antimutagenic potential on diethylnitrosamine-induced mutagenesis in rat liver.

In the promotion stage, pinocembrin at 10 mg/kg bw did not induce GST-P positive foci formation. Moreover, rats treated with 2 and 10 mg/kg bw of pinocembrin had no significant decrease in the number of GST-P positive foci for treatments given before or after DEN injection. These results are relevant to previous studies showing that propolis, which contains pinocembrin, did not protect against DEN-induced GST-P positive foci formation in rat liver (Said et al., 2010). Interestingly, pinocembrin at 10 mg/kg bw slightly increased the number of GST-P positive foci higher compared to positive control (84%) when administered before DEN injection. The present study clearly indicated that high doses of pinocembrin (10 mg/kg bw) promoted the development of preneoplastic lesions in the rat livers. Our results are relevant to previous findings that *Boesenbergia pandurata* significantly increased the number of GST-P positive foci (Tiwawech et al., 2000) in 2-amino-3, 8-dimethylimidazo (4, 5-f) quinoxaline induced rat hepatocarcinogenesis. It should be emphasized that pinocembrin is one of compounds in *B. pandurata* that promoted hepatocarcinogenesis. In addition, Satoh et al. (2001) demonstrated that end-products of lipid peroxidation can induce the expression of GST-P in rat liver. In this study, we also found that administration of pinocembrin 10 mg/kg bw before DEN injection slightly induced lipid peroxidation relative to positive control (data not shown). This is one result supporting the suggestion that the promoting effect of pinocembrin might be due to lipid peroxidation.

In this study, extraction of 1 kg of dried *B. pandurata* yielded 69 mg of pinocembrin. Based on the average consumption, the doses of pinocembrin that we used in these experiments corresponded to dried *B. pandurata* 6 – 145 g/day in the short-term and 6 and 29 g/day in medium-term carcinogenicity tests. As a result, the concentrations of pinocembrin may not have been suitable for inhibiting DEN-induced rat hepatocarcinogenesis. In addition, pharmacokinetic study of pinocembrin in rats indicated that the plasma concentration of pinocembrin rapidly decreased due to either fast excretion and/or extensive metabolism (Yang et al., 2009). Thus pinocembrin might rapidly conjugate with either glucuronide or sulfate and then be excreted from the body. This may be one of the major reasons why pinocembrin did not present anticarcinogenic activity in rat liver.

Recently, our laboratory studied the effects of pinostrobin (5-hydroxy-7-methoxyflavanone), a flavanone compound found in *B. pandurata* rhizome, in DEN-induced initiation of rat hepatocarcinogenesis. We demonstrated that pinostrobin prevented the initiation stage of rat hepatocarcinogenesis induced by DEN (Charoensin, 2008). Even though pinostrobin inhibited hepatocarcinogenesis, pinocembrin did not; this may be associated with the structure and the position of functional groups of this compound. According to a previous study, free hydroxyl groups of the polyphenols are rapidly excreted from the body after conjugation with glucuronide and/or sulfate. In addition, flavonoids containing methoxyl groups in their structure may not only increase hepatic metabolic stability but also increase their intestinal absorption. These effects could be due to greatly increased oral bioavailability, and thus methoxylated flavonoids had greater chemopreventive potency than unmethylated flavonoids or polyphenols (Wen & Walle 2006; Walle et al., 2007).

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