STRAIN AND SEX DIFFERENCES IN KIDNEY CARCINOGENESIS IN RATS TREATED WITH *N*-ETHYL-*N*-HYDROXYETHYLNITROSAMINE AND URACIL

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We earlier demonstrated that simultaneous administration of EHEN and uracil for 3 weeks resulted in enhancement of renal carcinogenesis in F344 female rats. Therefore, to establish a model of renal carcinogenesis in rats that can induce advanced renal carcinoma at a high incidence, differences in the susceptibility to *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN) and uracil of the kidneys in male and female rats of two strains were examined. Group 1 (male Wistar rats), group 2 (female Wistar rats), group 3 (male F344 rats) and group 4 (female F344 rats) received a 3-week simultaneous administration of 0.05% EHEN in the drinking water and 3% uracil in the diet after one week's acclimation. In all the above four groups, the rats were thereafter given a basal diet and water without chemical addition for a 29-week period. Group 5 (male Wistar rats), group 6 (female Wistar rats), group 7 (male F344 rats) and group 8 (female F344 rats) received no chemicals for the entire 33 weeks. At the end of the experiment, renal adenocarcinomas were found in 85, 68, 14 and 0% of the rats in groups 1, 2, 3 and 4, respectively. The incidence of adenomas and adenocarcinomas in Wistar rats were significantly greater than in F344 rats ($p<0.0001$). These findings indicate strain and possibly sex differences in kidney carcinogenesis in rats treated with EHEN and uracil, and simultaneous administration of the two agents to male Wistar rats might have an advantage for models to induce advanced renal carcinoma at a high incidence.

(Acta Urol. Jpn. 41: 781-787, 1995)

Key words: EHEN (*N*-ethyl-*N*-hydroxyethylnitrosamine), Uracil, Kidney carcinogenesis

INTRODUCTION

Several chemicals are known to induce renal epithelial tumors in rats, including *N*-ethyl-*N*-hydroxyethylnitrosamine (EHEN)[1], dimethylnitrosamine[2], *N*-nitrosomorpholine[3], *N*-(4-fluoro-4-biphenylyl) acetamide[4], and streptozotocin[5]. EHEN in particular induces renal epithelial cancers selectively in rats[6] and several substances have been found to enhance EHEN-initiated lesion development.

Lalich first reported that uracil, a component of ribodeoxynucleic acid, induces urolithiasis in rats when given by p.o. administration[7]. Shirai *et al.* demonstrated that mucosal papillomatosis of the urinary bladder is associated with this urolithiasis and that severe epithelial hyperplasia is caused in F344 rats receiving 3% uracil in the diet[8]. Furthermore, Shirai *et al.* reported that 3% uracil given by p.o. administration strongly promotes urinary bladder carcinogenesis after *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) initiation, suggesting that prolonged stimulation by uracil-induced urolithiasis results in chronic cell proliferation of the bladder epithelium[9]. We earlier demonstrated that simultaneous administration of EHEN and uracil for 3 weeks resulted in enhancement of renal carcinogenesis in F344 female rats[26]. We conducted the present investigation to compare kidney lesion induction in male and female Wistar and F344 rats treated with EHEN and uracil.

MATERIALS AND METHODS

1. Animals and chemicals

A total of 240 animals, equal numbers

of male and female F344 and Wistar rats, 4 weeks old, were obtained from Charles River Japan, Inc. (Kanagawa, Japan). The animals were kept in a room at 25±2°C, with a relative humidity of 55±5% and a 12-h light 12-h dark cycle, and were given basal diet (Oriental M, Oriental Yeast Co., Tokyo, Japan) and water. EHEN (Sakai Research Laboratories, Fukui, Japan) was dissolved in the drinking water at a concentration of 0.05% and administered in light-opaque bottles. Uracil (Yamasa Shoyu Co. Ltd., Chiba, Japan) was mixed in a powdered basal diet at a concentration of 3%.

2. Experimental schedule

The animals were kept on the basal diet for a oneweek acclimation before the beginning of the experiment. They were divided into eight groups of 30 rats each. Group 1 consisted of male Wistar rats, Group 2 female Wistar rats, Group 3 male F344 rats. Group 4 female F344 rats, Group 5 male Wistar rats, Group 6 female Wistar rats, Group 7 male F344 rats, and Group 8 female F344 rats. The rats in groups 1,2,3 and 4 were simultaneously given 0.05% EHEN and 3% uracil for 3 weeks. The rats in groups 1~4 were given the basal diet and water without chemical addition for a 29 week period after these treatments. The rats in groups 5~8 received no chemicals in the diet and water for the entire 33 weeks.

Body weights and consumption of diet and water were recorded weekly for the first 4 weeks. At the end of week 33, all surviving animals were killed for examination under ether anesthesia, and all major organs examined for macroscopic change. Body, kidney, and liver weights and organ per body weight ratios were determined for each group. The kidneys, liver, and other organs were removed and fixed in 10% phosphate-buffered formalin. The urinary bladder was inflated *in situ* with 10% phosphate-buffered formalin, then removed and fixed in the same fixative. These organs were processed routinely and sections stained with hematoxylin and eosin for histological examination.

Kidney lesions were classified into microadenomas, adenomas, and adenocarcinomas, as described previously[10,11]. Lesions less than 0.5 mm in diameter, approximately three times the diameter of a glomerulus, were tentatively classified as microadenomas and those more than 0.5 mm in diameter as adenomas, the latter showing clear compression of surrounding tissue. The lesions diagnosed as carcinomas were composed of irregularly arranged cells with occasional mitotic figures.

3. Statistical analysis

Data were expressed as mean values ± SD and analyzed using the Student's *t*-test. Differences in incidence of lesions were evaluated using the chi-square test or Fisher's exact probability test when indicated.

RESULTS

1. General observations

During the experimental period a total of 21 rats died. The final number of animals in each group are shown as the effective numbers of rats in Table 1. Fig. 1 illustrates the changes of body weights in

Table 1. Mean final body, kidney and liver weights

Group	Strain and sex	Treatment	Effective no. of rats	Final body weight, g	Right and left kidney weight, g (% of body weight)	Liver weight, g (% of body weight)
1	Wistar, male	EHEN+uracil	20	546±63.9**	8.02±10.5** (1.5 ±1.9)	25.3 ±5.65** (4.64±0.92)
2	Wistar, female	EHEN+uracil	22	313±43.2*	4.21± 4.48** (1.45±1.6)	15.07±4.47** (4.79±1.24)
3	F344, male	EHEN+uracil	28	343±26.7**	2.53± 0.21** (0.74±0.06)	19.57±8.85** (5.84±3.46)
4	F344, female	EHEN+uracil	30	195±13.7	1.49± 0.17 (0.76±0.06)	8.57±1.76** (4.38±0.73)
5	Wistar, male	None	30	671±84.3	3.83± 0.44 (0.58±0.06)	21.3 ±3.89 (3.16±0.31)
6	Wistar, female	None	30	335±29.3	2.27± 0.17 (0.68±0.04)	9.62±1.01 (2.88±0.23)
7	F344, male	None	30	378±21.8	2.46± 0.18 (0.65±0.03)	11.65±0.98 (3.08±0.15)
8	F344, female	None	29	194±10.5	1.42± 0.09 (0.74±0.03)	5.99±0.48 (3.09±0.18)

All values are mean±SD. Significantly different from the respective control group at $p<0.05$* or 0.01**.

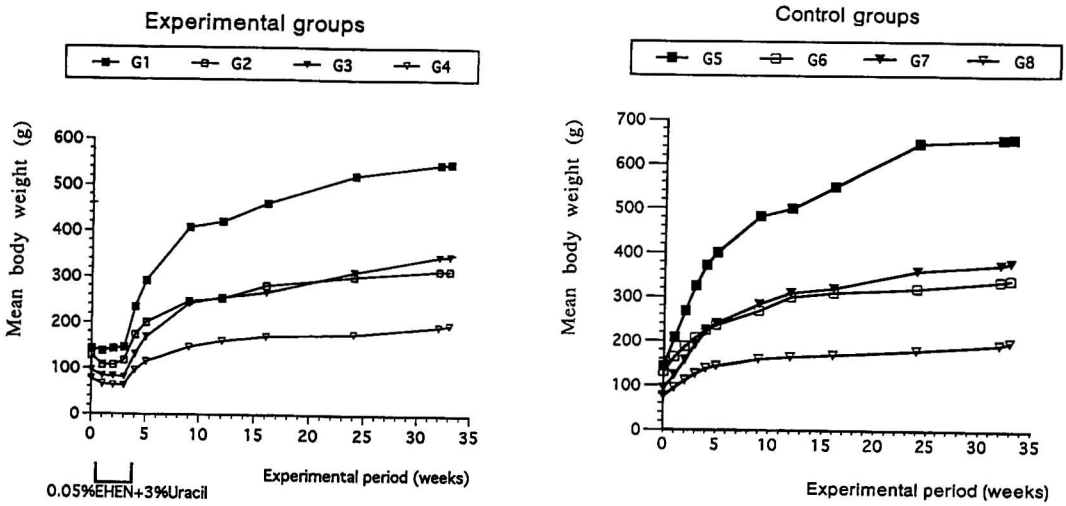

Fig. 1. Changes of body weights in rats of experimental and control groups

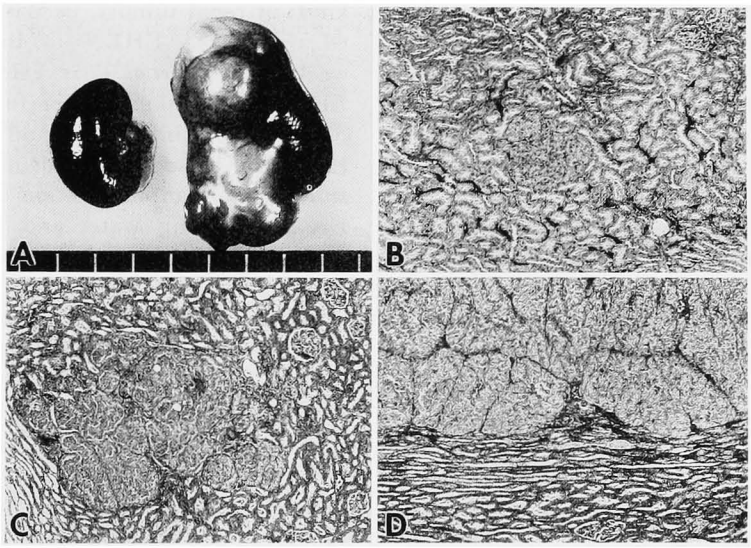

Fig. 2. (A) Gross appearance of a tumor of the left kidney in a rat treated with 0.05% EHEN and 3% uracil (group 1) (B) A microadenoma of the kidney (HE staining, ×75) (C) An adenoma of the kidney (HE staining, ×70) (D) An adenocarcinoma of the kidney (HE staining, ×70)

rats treated with 0.05% EHEN and 3% uracil for 3 weeks (groups 1~4). At the start of the experiment no differences in body weights of rats were found among groups. Administration of EHEN and uracil for 3 weeks suppressed the growth of the rats. After returning to normal water and basal diet, rats gained body weight normally. Table 1 shows the final body weights for each group. Rats in groups 1~3 still had significantly lower body weights than the respective control rats in groups 5~7.

Total doses of EHEN ingested over 3 weeks, calculated from water consumption data, were 2.25 mg/g (body weight) in group 1, 1.63 mg/g in group 2, 1.75 mg/g in group 3, and 1.58 mg/g in group 4. Total amounts of uracil given over 3 weeks, obtained from food consumption data,

Table 2. Incidences of neoplastic lesions in the kidneys of treated rats

Group	Strain and sex	Effective no. of rats	Total no. of neoplastic lesions No. (%)	Neoplastic lesions		
				Microadenoma No. (%)	Adenoma No. (%)	Adenocarcinoma No. (%)
1	Wistar, male	20	20 (100)	1 (5)	2 (10)	17 (85)
2	Wistar, female	22	21 (95)	0 (0)	6 (27)	15 (68)
3	F344, male	28	20 (71)	7 (25)	9 (32)	4 (14)
4	F344, female	30	18 (60)	11 (37)	7 (23)	0 (0)

Each rat is tabulated in the column of the most advanced lesion present in the kidneys as determined by histopathological examination. No neoplastic lesions were found in control rats in groups 5-8.

were 51.5 mg/g (body weight) in group 1, 55.0 mg/g in group 2, 34.9 mg/g in group 3, and 37.2 mg/g in group 4.

2. Kidney

Table 1 shows the kidney weights and kidney to body weight ratios. Kidney to body weight ratios in groups 1, 2, and 3 were significantly higher than those in the respective control groups. Renal tumors were observed in groups 1, 2, 3 and 4. Most were grayish-white, round, and located in the renal cortex. Fig. 2A shows the gross and microscopic appearance of a typical renal tumor.

Histologically, renal tumors were composed of tubular, cord-like or papillary structures made up of cells with basophilic or sometimes clear cytoplasm. Table 2 shows the incidence of renal lesions classified into microadenomas, adenomas, and adenocarcinomas (Fig. 2B~2D). Each rat is tabulated in the column of the most advanced lesion present in its kidney. The incidence of adenocarcinomas in Wistar rats (group 1 or 2) were significantly higher than that in F344 rats (group 3 or 4), ($p<0.0001$). No significant difference was found in the incidence of neoplastic lesions between the male and female rats in the same strain. No kidney mesenchymal tumors were observed.

3. Liver

Liver weights and liver to body weight ratios are also shown on the right side of Table 1, values in groups 1,2,3 and 4 being significantly higher than those of control rats in groups 5, 6, 7 and 8 respectively. Histologically, the livers of rats treated with EHEN and uracil had multiple lesions including hepatocellular carcinomas.

DISCUSSION

Druckrey *et al.*[1] first reported that EHEN induces tumors in the kidneys, liver, and ovaries of rats. Hiasa *et al.*[6] showed that renal tubular cell tumors selectively develop at a high incidence, without other kinds of renal tumors, in Wistar rats treated with 0.1% EHEN in the diet for 2 weeks. Hirose *et al.*[12] revealed that 0.01% EHEN in drinking water for 2 weeks induces tumors in kidneys and liver of F344 rats. The present study demonstrated that simultaneous administration of 0.05% EHEN in drinking water and 3% uracil in diet for 3 weeks causes renal carcinomas in rats at a high frequency.

Several substances have been shown to exert promotive effects on renal carcinogenesis in rats treated with EHEN. Ohshima *et al.*[13] showed that injection of β-cyclodextrin, which is known to induce injury of renal tubules, for one week resulted in an increased incidence of renal cell tumors in rats treated with 0.01% EHEN for 2 weeks. Kurata *et al.*[14] also reported that para-aminophenol stimulated the mitotic activity of preneoplastic tubular lesions in the kidney. Thus, many nephrotoxic substances including *N*-(3,5-dichlorophenyl) succinimid[15], citrinin[16], basic lead acetate[17], trisodium nitrilotriacetate[18] have been reported as promoters of renal carcinogenesis. Presumably nephrotoxic effects causing tubular injury and consequent stimulation of reactive tubular proliferation are involved.

Uracil by p.o. administration causes urolithiasis in rats[7]. Uracil has no mutagenic properties[19] and no marked influence

on the urinary pH or Na ion concentration[20]. Several studies which have showed that uracil-induced calculi strongly promote BBN induced urinary bladder carcinogenesis add support to the hypothesis that enhancement of cell proliferation within the urothelium, associated with irritant effects, is an important event for promotion of bladder chemical carcinogenesis in rats. Since uracil has no mutagenic properties, its carcinogenic activity is thought to rely on chronic mechanical stimulation. The schedule applied in the present study featured simultaneous administration of EHEN and uracil but it is unlikely that uracil would chemically interact with EHEN or alter its metabolism in rats *in vivo*. Its influence is concluded to be primarily due to an increase in the susceptibility of the target cells to carcinogen. We recently have shown that simultaneous administration of BBN and uracil induces transitional cell carcinomas and squamous cell carcinomas in the renal pelvis of rats at high rates[21].

Differences in the susceptibility of certain organs in various animal strains and species to carcinogens have been widely examined. In urinary bladder carcinogenesis, Fukushima *et al.* reported a higher tumor incidence in male ACI rats than in male Wistar, F344 and Sprague-Dawley rats treated with sodium saccharin[25]. Biochemical studies on susceptibility factors have focused on genetic differences in carcinogen metabolism. Thus differences in the metabolic pathways for activation of carcinogens may account for the differences in response among strains.

The present study indicates that there are differences in the susceptibility to EHEN and uracil of the kidney in two strains of rats, with Wistar animals being extremely sensitive. Despite a tendency for lower values in females, no significant variation in the incidence of renal tumors in different sexes was evident. We previously demonstrated that the incidence of renal adenocarcinomas at the end of week 52 was 53% in female F344 rats treated with EHEN and uracil for 3 weeks[26]. In the present case, the observed incidence was 0%. One of the causes of these different results might be the short duration of the experimental period. Previous studies documented incidences of renal adenocarcinomas in rats treated with EHEN as an initiator to be 0~13% at best[14,22-24]. In the present study, the incidence of renal adenocarcinoma in male Wistar rats treated with EHEN and uracil for only 3 weeks was 85% after only a relatively short-term observation. Thus, simultaneous administration of the two agents to male Wistar rats might have an advantage for models requiring induction of advanced renal carcinoma at a high incidence.

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(Received on April 27, 1995
Accepted on July 3, 1995)
(迅速掲載)

和文抄録

EHEN とウラシルの同時投与によるラット腎腫瘍の性差および系統差

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N-ethyl-*N*-hydroxyethylnitrosamine（EHEN）とウラシルの同時投与によるラット腎腫瘍の発生率について性差，系統差を検討した．1．実験動物：第1群，第5群（Wistar ラット，雄），第2群，第6群（Wistar ラット，雌），第3群，第7群（F344 ラット，雄），第4群，第8群（F344 ラット，雌）の8群で，各群30匹，合計240匹のラットを用いた．2．実験方法：第1群から第4群までが薬剤投与群，第5群から第8群までが薬剤非投与の対照群とした。薬剤投与群には飲料水中に0.05% EHEN を，粉末飼料中に3%ウラシルを混入して，これを3週間同時に投与した．全実験期間は33週間とした．3．結果：実験終了時の有効動物匹数は，第1群から第8群までそれぞれ，20，22，28，30，30，30，30，29匹であった．腎腫瘍の発生率は，第1，2，3，4群でそれぞれ100%，95%，71%，60%であり，Wistar ラットで F344 ラットよりも有意に高率に腎腫瘍の発生がみられた。性差による発生率の差については，雄に高率に発生する傾向があったが，有意差はなかった．対照群には腎腫瘍は発生しなかった．Wistar 雄ラットに，EHEN とウラシルを同時に投与すれば，短期間に高頻度に腎腫瘍を誘発させうることが示された．

（泌尿紀要　**41**：781-787，1995）

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