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Possible Application to Medium-term Organ Bioassays for Renal Carcinogenesis Modifiers in Rats Treated with N-Ethyl-N-hydroxyethylnitrosamine and Unilateral Nephrectomy

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The effects of the renal tumor promoters; β-cyclodextrin (β-C), DL-serine (DL-S), basic lead acetate (LA), trisodium nitrilotriacetate monohydrate (NTA) and potassium bromate (KB), and diethylene glycol (DEG) as a negative control, on early stage of renal carcinogenesis were investigated in unilaterally nephrectomized male Wistar rats after N-ethyl-N-hydroxyethylnitrosamine (EHEN) administration. Wistar male rats were fed 1000 ppm EHEN diet for 2 weeks and the left kidney was removed at week 3, then the animals were divided into 7 groups of 15 rats each. These groups received the following treatments: 1000 ppm LA, 10000 ppm NTA or 500 ppm KB diet for 18 weeks from week 3; 45 mg/100 g body wt./day of β-C injected sc for 7 days; 100 mg/100 g body wt. of DL-S injected sc biweekly for 6 weeks; 5% DEG in drinking water as a negative control for two days. Five rats in each group were killed at weeks 8, 12 and 20 and their kidneys were examined histologically. At week 20, the average numbers of adenomatous hyperplasias seen as preneoplastic lesions in the β-C, DL-S, LA, NTA or KB groups were significantly higher than those in the DEG or control groups. Thus within a relatively short period of 20 weeks, promoting effects of chemicals can be detected as a significant increase of adenomatous hyperplasias in this model.

Key words: N-Ethyl-N-hydroxyethylnitrosamine — Renal tumor — Promoter — Nephrectomy

Significant promoting effects of β-cyclodextrin (β-C[2]),[1] DL-serine (DL-S),[2] basic lead acetate (LA),[3] trisodium nitrilotriacetate monohydrate (NTA),[4] and potassium bromate (KB)[5] on the development of renal tubular cell tumors in rats pretreated with N-ethyl-N-hydroxyethylnitrosamine (EHEN) have been reported. Diethylene glycol (DEG), on the other hand, despite being a nephrotoxic chemical like β-C, DL-S, LA, NTA, and KB, did not show promotion potential.[6] In general, an experimental period of more than 30 weeks proved necessary for detection of the promoting effects of these chemicals on renal tubular cell tumor development.[7–11]

The purpose of the present studies was to assess an approach to shortening the necessary experimental duration by examining the effect of test chemicals on preneoplastic changes known to be involved in kidney neoplasia in rats treated with EHEN and unilateral nephrectomy as proliferation-enhancing stimulus. β-C, DL-S, LA, NTA and KB were used as known renal carcinogenesis-promoting agents and DEG as a negative (control) agent in the present studies.

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[2] Abbreviations: β-C, β-cyclodextrin; EHEN, N-ethyl-N-hydroxyethylnitrosamine; DL-S, DL-serine; NTA, trisodium nitrilotriacetate monohydrate; KB, potassium bromate; DEG, diethylene glycol; BrdU, bromodeoxuridine.

MATERIALS AND METHODS

Chemicals and diets EHEN [CAS: 13147-25-6, 2-ethylnitrosamine(ethanol), purity, 99.8%, liquid at room temperature], β-C, DL-S, LA, NTA, KB, and DEG were purchased from Nakarai Chemicals Ltd., Kyoto. The basal diet (CRF-1) was purchased from Charles River Japan, Inc., Atsugi. EHEN, LA, NTA, and KB were mixed at the concentrations of 1000, 1000, 10000 and 500 ppm, respectively, into the basal diet and β-C was injected sc at the dose of 45 mg per 100 g body weight per day for 7 days. DL-S was injected sc at the dose of 100 mg per 100 g body weight biweekly for 6 weeks. DEG was administered as a 5% solution in the drinking water for two days. These chemicals were used at the same doses as described in the previous reports.[1–7]

Animals A total of 110 male Wistar rats were purchased at 6 weeks of age from SLC Co., Shizuoka and maintained on basal diet for one week. They were housed in plastic cages, 5 rats per cage, and kept in an air-conditioned room at 24±1°C with a relative humidity of 55±5%.

All animals were fed on EHEN diet for 2 weeks and unilaterally nephrectomized (left kidney) at week 3. Animals were then divided into 7 groups of 15 rats each receiving the following treatments: group 1, β-C injection and basal diet; group 2, DL-S injection and basal diet; group 3, 1000 ppm LA diet; group 4, 10000 ppm NTA diet;

group 5, 1000 ppm KB diet; group 6, 5% DEG drinking water and basal diet; and group 7, basal diet alone. Five rats in each group were killed at weeks 8, 12 and 20. BrdU was injected ip at 2 mg per 100 g body weight into each animal one hour before death. Kidneys were immediately excised, weighed and fixed in 10% neutral formalin for histological studies and 70% alcohol for immunohistochemical studies. The numbers of simple hyperplasias, adenomatous hyperplasias and renal tubular cell tumors were counted microscopically in coronal kidney sections. BrdU-labeled tubular cells in the kidney cortex within and outside focal lesions were counted in ten 1 mm^2 areas per animal by light microscopy.

RESULTS

Average kidney weights Absolute kidney weights were significantly larger in groups 1, 3, 4, 5, and 6 than that in

Table I. Average Kidney Weights in Wistar Rats Treated with EHEN, Unilateral Nephrectomy and Test Chemicals

Exptl. groups	Treatment	8		12		20 (weeks)	
		kidney (g)	kidney/BW (%)	kidney (g)	kidney/BW (%)	kidney (g)	kidney/BW (%)
1	EHEN+β-C	1.37±0.16[a]	0.48	1.50±0.13[a]	0.47	1.55±0.12[a, b]	0.48
2	EHEN+DL-S	1.34±0.41	0.49	1.32±0.09	0.44	1.40±0.05	0.42
3	EHEN+LA	1.39±0.04[a]	0.48	1.63±0.12[a, b]	0.50	1.56±0.08[a, b]	0.46
4	EHEN+NTA	1.35±0.07[a]	0.49	1.44±0.11	0.49	1.51±0.15	0.51
5	EHEN+KB	1.39±0.13[a]	0.48	1.54±0.22	0.49	1.44±0.06	0.43
6	EHEN+DEG	1.31±0.06[a]	0.46	1.31±0.15	0.42	1.35±0.10	0.42
7	EHEN only	1.16±0.04[b]	0.38	1.31±0.05	0.31	1.26±0.25	0.36

EHEN, N-ethyl-N-hydroxyethylnitrosamine; β-C, β-cyclodextrin; DL-S, DL-serine; LA, basic lead acetate; NTA, trisodium nitrilotriacetate monohydrate; KB, potassium bromate; DEG, diethylene glycol; BW, body weight.
a) Significantly different ($P<0.05$) from Group 7.
b) Significantly different ($P<0.05$) from Group 6.

Table II. Average Numbers of BrdU-labeled Renal Cells per mm^2 in Rats Treated with EHEN, Unilateral Nephrectomy and Test Chemicals

Exptl. groups	Treatment	8		12		20 (weeks)	
		Cortical tubules	Interstitium	Cortical tubules	Interstitium	Cortical tubules	Interstitium
1	EHEN+β-C	16.20±4.32	5.56±2.36	15.33±0.58[a, b] (12.59±1.28)[b]	3.69±0.33	11.80±1.30[a] (7.08±2.89)	5.78±2.44
2	EHEN+DL-S	15.25±2.99	4.89±1.69	13.25±2.22[a, b] (13.26±2.88)[b]	6.67±2.65[a]	13.80±1.64[a] (6.18±2.25)	6.00±0.82[a]
3	EHEN+LA	13.40±1.82	5.22±2.14	14.40±4.58[a] (10.49±2.23)[b]	5.14±1.83[a]	18.40±2.88[a, b] (9.86±2.41)	9.44±1.42[a, b]
4	EHEN+NTA	17.60±5.86	4.89±1.72	19.40±4.16[a, b] (11.53±2.77)[b]	5.31±1.36[a]	22.20±4.09[a, b] (13.26±2.50)[b]	8.67±2.50[a, b]
5	EHEN+KB	13.40±2.07	4.00±1.00	12.25±1.89[a, b] (7.92±1.17)	3.89±1.58	13.60±1.34[a] (6.67±2.93)	3.19±1.15
6	EHEN+DEG	10.20±3.19	2.67±1.19	9.75±0.50[a] (6.46±1.10)	4.72±3.58	11.75±2.22[a] (6.39±1.96)	3.33±2.39
7	EHEN only	NA	NA	6.50±1.29	2.22±1.58	6.25±1.89	3.30±1.76

EHEN, N-ethyl-N-hydroxyethylnitrosamine; β-C, β-cyclodextrin; DL-S, DL-serine; LA, basic lead acetate; NTA, trisodium nitrilotriacetate monohydrate; KB, potassium bromide; DEG, diethylene glycol. ( ): Numbers of BrdU-labeled renal cells without adenomatous hyperplasia and adenomas. NA, not performed.
a) Significantly different ($P<0.05$) from Group 7.
b) Significantly different ($P<0.05$) from Group 6.

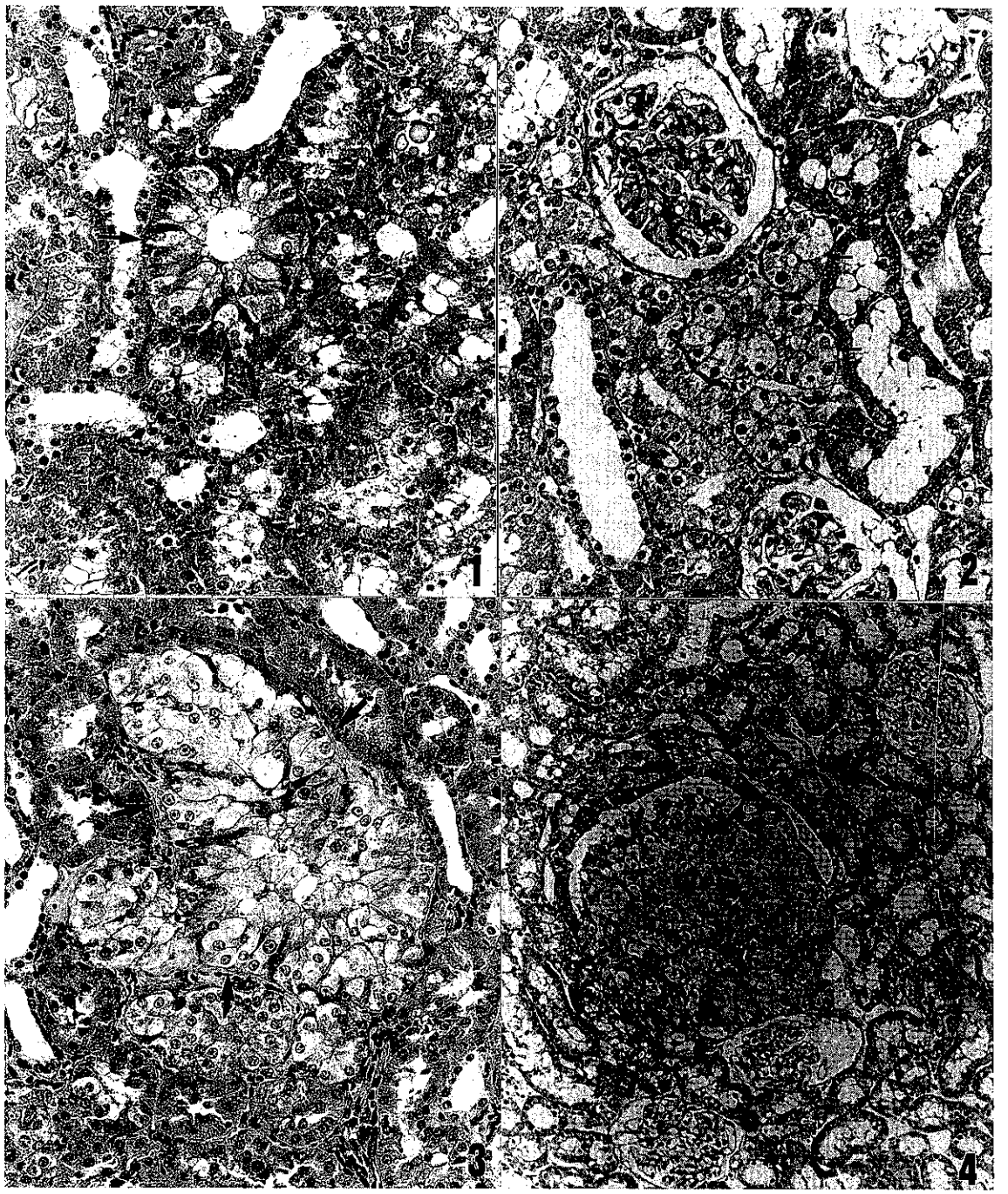

Fig. 1. Simple hyperplasias (arrows) in a rat treated with EHEN, unilateral nephrectomy and β-C at week 20, consisting of cells with abundant basophilic cytoplasm (HE, $\times 100$).

Fig. 2. Simple hyperplasias (arrows) in a rat treated with EHEN, unilateral nephrectomy and β-C at week 20, showing a tubular pattern (HE, $\times 100$).

Fig. 3. An adenomatous hyperplasia in a rat treated with EHEN, unilateral nephrectomy and β-C at week 20, consisting of cells with abundant cytoplasm. Note loss of tubular pattern (HE, $\times 100$).

Fig. 4. A renal tubular cell tumor in a rat treated with EHEN, unilateral nephrectomy and NTA at week 20 (HE, $\times 40$).

Table III. Histological Findings in Kidneys of Rats Treated with EHEN, Unilateral Nephrectomy and Test Chemicals

Exptl. groups	Treatment	Effective No. of animals	Simple hyperplasias (SH)						Adenomatous hyperplasias (AH)			Renal cell tumors (RCT)		
			No. of rats with SH (%)			No. of SH per rat			Total number (No. of rats)			Total number (No. of rats)		
		(weeks)	8	12	20	8	12	20	8	12	20	8	12	20
1	EHEN+β-C	5	5 (100)[a]	5 (100)[a]	5 (100)[a]	19.8 ± 1.0	5.8±2.5[a, b]	6.6±1.9[a, b]	0	2 (1)	10 (4)	0	0	1 (1)
2	EHEN+DL-S	5	4 (80)[a]	3 (60)	5 (100)[a]	12.4 ± 1.6	4.6±1.5[a, b]	8.4±1.1[a, b]	0	0	7 (5)	0	0	2 (2)
3	EHEN+LA	5	2 (40)	4 (80)[a]	5 (100)[a]	0.8±1.0	2.2±1.7	11.4±6.6[a, b]	0	2 (1)	6 (4)	0	0	1 (1)
4	EHEN+NTA	5	3 (60)	5 (100)[a]	5 (100)[a]	0.6±0.8	8.6±3.4[a, b]	12.2±7.2[a, b]	0	19 (4)	27 (4)	0	0	8 (3)
5	EHEN+KB	5	1 (20)	5 (100)[a]	5 (100)[a]	0.4±0.5	1.4±1.1	9.2±2.7[a, b]	0	3 (2)	8 (3)	0	0	1 (1)
6	EHEN+DEG	5	0	4 (80)[a]	5 (100)[a]	0	1.4±0.8	2.6±0.5[a]	0	0	1 (1)	0	0	0
7	EHEN only	5	0	0	2 (40)	0	0	0.6±0.5	0	0	0	0	0	0

EHEN, N-ethyl-N-hydroxyethylnitrosamine; β-C, β-cyclodextrin; DL-S, DL-serine; LA, basic lead acetate; NTA, trisodium nitriloacetate monohydrate; KB, potassium bromate; DEG, diethylene glycol.
a) Significantly different ($P<0.05$) from Group 7.
b) Significantly different ($P<0.05$) from Group 6.

group 7 at week 8. Values for groups 1 and 3 were also higher at weeks 12 and 20, a significant difference from group 6 being additionally evident at the latter time point. Relative kidney weights in all test compound-treated groups were increased as compared to the group 7 control at all time points (Table I).

BrdU-labeling indices Average numbers of BrdU-labeled cells within areas of adenomatous hyperplasia were significantly elevated in groups 1, 2, 3, 4, 5 and 6, as compared to the group 7 level at weeks 12 and 20. Indices of BrdU-labeled cells outside of tumor areas showed significant differences from control values in all treated groups and also between groups 1, 2, 3, 4, 5 and group 6 at week 12 (Table II).

Histological findings Simple hyperplasias present as small, single or multiple tubular structures characterized by increased basophilia. Two types of basophilic cells were encountered, one with more abundant cytoplasm than normal proximal tubular cells (Fig. 1) similar to that found in renal tubular cell tumors. The cytoplasm of the other was less abundant than in normal tubular cells, with an increase in nuclear size and therefore nuclear/cytoplasmic ratio (Fig. 2). Adenomatous hyperplasias were found to consist of basophilic cells with abundant cytoplasm like tumor cells, the normal tubular morphology being lost. Diagnosis of adenomatous hyperplasias (Fig. 3) was made for lesions less than 3 times glomerulus size, larger lesions being classified as renal tubular cell adenomas (Fig. 4).

Incidences of simple hyperplasias, adenomatous hyperplasias and renal tubular cell tumors Quantitative data for all lesions are summarized in Table III. Incidences of rats with simple hyperplasias were 100% in groups 1, 4 and 5, 80% in groups 3 and 6, 60% in group 2 and 0% in groups 7 at week 12, and 100% in groups from 1 to 6 and 40% in group 7 at week 20. At week 20, the average numbers of simple hyperplasias per animal in rats treated with β-C, DL-S, LA, NTA or KB after EHEN treatment and unilateral nephrectomy were significantly higher than that in rats treated with DEG or the controls after EHEN treatment and unilateral nephrectomy alone. Incidences of adenomatous hyperplasias were 20% (1/5) in groups 1 and 3, 80% (4/5) in group 4, 40% (2/5) in group 5, and 0% in groups 2, 6 and 7 at week 12, while at week 20 they were 100% (5/5) in group 2, 80% (4/5) in groups 1, 3 and 4, 60% (3/5) in group 5, 20% (1/5) in group 6 and 0% (0/5) in group 7. Numbers of adenomatous hyperplasias in groups 1–7 at week 20 were 10, 7, 6, 27, 8, 1 and 0, respectively. Incidences of renal cell tumors were 20% (1/5) in groups 1, 3 and 5, 40% (2/5) in group 2, 60% (3/5) in group 4 and 0% (0/5) in groups 6 and 7. Numbers of renal tubular tumors in groups 1–7 at week 20 were 1, 2, 1, 8, 1, 0 and 0, respectively.

DISCUSSION

The present paper describes a medium-term model capable of detecting promotion potential of test chemicals in terms of development of renal cell focal lesions in rats pretreated with EHEN and nephrectomy.

Induction of renal tubular cell tumors by EHEN,[12,13] nitrosomorpholine,[14] N-(4′-fluoro-4-biphenylyl) acetamide,[15] and lead acetate[16] is a long-term process and as a consequence it generally requires a long time for promoting activity to become evident. Previous reports which described β-C,[1] DL-S,[2] LA[3] and NTA[4] but not DEG[6] promotion of renal tubular cell tumor development in rats treated with EHEN, were based on experiments of 32 weeks' duration.

In the liver, the altered cell foci, which are believed to be preneoplastic in character, are easily recognizable because of their changed enzyme phenotype, particularly the strong increase in expression of the placental form of glutathione *S*-transferase (GST-P).[17,18] In the present study the analogous focal lesions, simple hyperplasias and adenomatous hyperplasias were therefore given special attention. Early proliferative lesions of tubular epithelial cell origin with slightly basophilic cytoplasm and atypical nuclei, classified as simple hyperplasia in the present study, partly correspond to the early neoplastic nodules,[8] atypical cell populations,[13] focal areas of dysplastic tubular epithelium,[19] atypical cell foci,[20] and altered tubules[21] of other authors, who gave no clear criteria for size definition. Our adenomatous hyperplasia corresponds to microscopic nodule,[22] small nodule,[23] or microadenoma[24] and may be viewed either as preneoplastic or neoplastic depending on morphological and biological growth characteristics, including lack of capsule and the presence or absence of compressive growth. Microscopic nodules were not seen in less than 24 weeks in rats treated with the renal carcinogen, N-(fluoro-4-biphenyl)acetoamide,[22] with small numbers being observed from this time onwards.

Administration of 2.5% DEG in the drinking water for 30 weeks was previously found to increase the incidence of renal tubular cell tumors in rats pretreated with EHEN, though not significantly.[6] Since treated animals died of nephrosis caused by 5% DEG in drinking water for more than 3 days in the present study, exposure was limited to 2 days. As a result, numbers of adenomatous hyperplasias induced by DEG did not increase significantly.

In conclusion, the present studies demonstrated that β-C, DL-S, LA, NTA and KB, known promoters of renal carcinogenesis, significantly increased the numbers of adenomatous hyperplasias (a preneoplastic lesion) at week 20 as compared with DEG. DEG did show a significant increase, but it was small. DEG may have weak promoting action. This medium-term renal bioassay system is capable of detecting strong promoters of renal carcinogenesis within 20 weeks.

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