Induction of Renal Pelvic Carcinoma by Phenacetin in Hydronephrosis-bearing Rats

of the SD/cShi Strain

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ABSTRACT

Carcinogenicity of phenacetin (PH) to the urinary tract was tested with the use of spontaneously hydronephrosis-bearing rats. In Experiment 1, 55 SD/cShi male rats were fed with 2% PH-containing diet for 85 weeks, and 32 SD/cShi male rats fed basal diet for 85 weeks served as controls. Forty-three of 53 rats fed with PH had renal pelvic carcinoma with lung metatases in three. The mean induction time was 78 weeks. Ureteral carcinoma and urinary bladder carcinoma were observed in 2 and 6 of 53 rats given PH, respectively. No urinary tract carcinoma was found in control animals. In Experiment 2, early lesions of the kidney affected by PH were also evaluated with the use of SD/cShi and Sprague-Dawley (SD) rats. Two groups of animals containing 6 SD/cShi or 6 SD male rats per group were fed 2% PH-containing diet for 8 weeks. Control animals containing 6 SD/cShi rats or 6 SD rats were fed basal diet for 8 weeks. Simple hyperplasia was found in 5 of 6 SD/cShi rats given PH and 2 of 6 SD/cShi control rats. Papillary necrosis was seen in 4 of 6 SD/cShi and 2 of 6 SD rats given PH. SD/cShi rats, especially those treated with PH, showed higher but not significant 5-bromo-2'-deoxyuridine labeling indices in the covering epithelium of the renal pelvis and papillae. In this short term experiment PH and its metabolites, N-hydroxyphenacetin and N-acetyl-p-aminophenol, were measured in urine and plasma by using high performance liquid chromatography. Significantly higher PH and slightly higher metabolites were detected in urine and plasma of SD/cShi rats compared to SD rats. These results indicated that the renal pelvis of SD/cShi rats had more sensitivity to PH carcinogenicity. This paper provides experimental proof of PH carcinogenicity toward the renal pelvis in an animal model.

INTRODUCTION

Since PH\(^1\) was introduced as an analgesic in 1897 (1), PH alone or PH mixed with caffeine and aspirin or phenazone has been used widely as an analgesic and antipyretic agent. But its chronic excessive use is associated with toxic side effects, such as interstitial nephritis and renal papillary necrosis (2). The development of urinary tract tumors, especially renal pelvic carcinoma, after levels of abuse of PH with consumption of at least 1 g/day for at least 1 year is a more serious complication, although carcinogenicity of PH remains the subject of debate (3–6).

Experimental studies revealed that PH had a carcinogenic effect on kidneys of C57BL/6 × C3H F, mice and nasal cavity and urinary bladder in Sprague-Dawley (SD) rats (7–9). The increase of labeling index of renal pelvic and renal papillary epithelium in rats fed PH and the promoting capability of PH in urinary tract carcinogenesis were reported (10–12). However, it has not been satisfactorily proven that PH induces renal pelvic carcinoma in experimental animals as PH appears to do in humans (13, 14).

Previous reports indicated that PH might have some genotoxic potential. PH was mutagenic in a bacterial mutagenesis assay performed with S9 fractions of rodent liver homogenates (15). PH was usually positive in a forward mutation test in V79 cells in the presence of hamster liver S9 (16). PH caused damage to chromosomes as evident from the increased levels of sister chromatid exchange (17). Multiple dosing of PH induced micronuclei in polychromatic erythrocytes in CD-1 mice (18). S9-mediated metabolism of PH resulted in the induction of weak morphological transformation in C3H/10T\(^{1/2}\) clone 8 mouse embryo cells (19).

An appropriate animal model to detect renal pelvic carcinogenicity of chemical substances does not yet exist. Actually, there are only a few cases of chemically induced renal pelvic carcinoma in experimental animals in which ureter ligation was performed with p.o. administration of BBN, or N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide was administered in the diet with or without mechanical perforation of the renal pelvis (12, 20). Recently we reported that a clear positive correlation was present between renal pelvic carcinogenesis and the stagnation of proximate carcinogen-containing urine, using a variety of rats and mice which had hereditary hydrenephrosis. In these studies we used SD/cShi rats and NON/Shi mice which had hereditary hydrenephrosis and showed high incidences of renal pelvic carcinoma following p.o. administration of BBN (21, 22).

Bilateral hydrenephrosis-bearing SD/cShi rats, which are recessively inherited, have been established by full-sibling mating of SD rats supplied in 1961 to Aburahi Lab., Shionogi & Co., Ltd., from Charles River Breeding Laboratory in the United States. The cause of hydrenephrosis is due to stenosis of ureteric orifice.

We, therefore, hypothesized that use of SD/cShi rats might make it possible to induce carcinoma in the renal pelvis following p.o. administration of PH. In this study we report the induction of renal pelvic carcinoma by means of p.o. administration of PH to SD/cShi rats.

MATERIALS AND METHODS

Chemicals

PH, Japanese Pharmacopoeia grade, was purchased from Maruishi & Co., Ltd., Tokyo, Japan. BrdUrd was purchased from Sigma Chemical Co., St. Louis, MO. NHP, used as internal standard, was synthesized by NARD Institute, Ltd., Amagasaki, Japan, as described previously (23). AAP, helicase (β-glucuronidase plus arylsulfatase) and 4-methylacetanilide were obtained from Wako Pure Chemical Industries, Tokyo, Japan.

Animals

Ninety-nine male SD/cShi rats, 4 weeks old, were obtained from Aburahi Lab. of Shionogi Co., Shiga, Japan, and 12 male SD (Crj:SD) rats, 4 weeks old, were obtained from Charles River Japan, Inc., Atsugi, Japan. At 6 weeks old, all rats were used for the experiments. These animals were housed 2 rats per cage on dry chip bedding in an air-conditioned room at 24 ± 1°C and 50 ± 10% humidity with a 12-h light, 12-h dark cycle. Body weights and food intakes were measured every week from the start to experimental week 20. From experimental week 21 to the end...
of the experiment, body weights were measured every other week and food
intakes were determined once a month.

Experimental Design

Experiment 1. Eighty-seven male SD/cShi rats were randomly divided into
2 groups of 55 (Group 1) and 32 (Group 2) rats, respectively. Group 1 was
given 2% PH-containing diet for 85 weeks. The 2% PH-containing diet was
specially prepared by CLEA Japan, Inc., Tokyo, Japan. Dose selection of PH
was based on body weight changes of rats fed 1.25 or 2.5% PH in preliminary
experiments.2 The control rats in Group 2 were given CA-1 pellet food (CLEA
Japan, Inc.) as a basal diet.

Experiment 2. Twelve SD/cShi rats and 12 SD rats were each divided into
groups, respectively. Group 1 (6 SD/cShi rats) and Group 3 (6 SD rats) were
fed with 2% PH-containing diet for 8 weeks. Group 2 (6 SD/cShi rats) and
Group 4 (6 SD rats), controls, were used as intact controls and were given
CA-1 diet.

Pathological Examination

In Experiment 1, survivors in each group were anesthetized and killed at the
core of the experimental period, and animals found dead or were killed when
moribund were dissected and all organs were inspected macroscopically. The
renal pelvis and urinary bladder were inflated with 10% phosphate-buffered
(pH 7.4) formalin. The nose of each rat was flushed antegrade with 10%
phosphate-buffered (pH 7.4) formalin, removed from the head, trimmed of
external tissue, fixed for approximately 2 weeks, decalcified for approxi-
ately 4 days in Plank-Rychlo solution, and sectioned into three levels, in-
cluding level 1, 2, and 3 as described by Young (24). All organs were taken
from the body, and the kidneys, ureters, urinary bladder, liver, and nose, as well
other grossly abnormal tissues, including spleen, were examined microscopi-
cally. Tissues were embedded in paraffin and stained with hematoxylin and
eosin for histological examination.

The epithelial lesions in the renal pelvis, ureter, and urinary bladder were
histopathologically classified as described previously (25, 26) into three cat-
cegories: papillary and nodular hyperplasia, papilloma, and carcinoma. To dis-
tinguish carcinoma multilocentric, carcinoma-bearing rats were classified into
six categories according to a modification of the system described previously
(21): A, carcinomas arising only in one kidney; B, those in one kidney and in
the ureter; C, those in one kidney and in the urinary bladder; D, those in both
kidneys and in the urinary bladder; E, those in both kidneys without urinary
bladder carcinoma; and F, those only in the urinary bladder. The severity of
renal papillary necrosis was graded into 6 grades according to criteria de-
scribed previously (27). The degree of hydronephrosis was subjectively clas-
sified as described previously (28) into three categories, mild, moderate, and
marked.

DNA Synthesis Examination

In Experiment 2 at week 8, BrdUrd (50 mg/kg) dissolved in saline was
administered I.P. to all rats 1 hr prior to sacrifice between 9 and 10 a.m. In order
to avoid any variance between groups by circadian rhythms demonstrated in a
number of cell cycle components (29), the animals were sequentially killed so
that one rat from each of the groups was simultaneously killed by exsanguin-
ation under ether anesthesia. Tissues, kidney, and nasal cavity were processed
for both histological examination and immunohistochemical BrdUrd staining
(Vectorstain ABC Kit, Vector Laboratories Inc., Burlingame, CA) to assess
DNA synthesis (30). The labeling index percentage was microscopically de-
ned as the number of cells incorporating BrdUrd into DNA per the total
number of urothelial cells counted (3000-6000 cells/slide), including both the
covering epithelium of the renal pelvis and the renal papillae. In the nasal
cavity, the three regions of respiratory mucosa mentioned above were also
examined for cell proliferation as described previously (31). Each 1000 cuboi-
dal cells in regions 1 and 2 and each 1000 ciliated and nonciliated respiratory
cells in region 3 were counted microscopically.

Measurement of Concentration of PH, NHP, and AAP in Urine and
Plasma

PH can be metabolized to N-hydroxylated (NHP) and O-deethylated (AAP)
PH. These are carcinogenic compounds in rodents (32, 33). Therefore, these
two metabolites of PH in urine and plasma were measured.

The rats were placed in individual metabolism cages with deprivation of
food and water during the 4-h urine collection period (from 9 a.m. to 1 p.m.)
3 days before sacrifice. Blood was collected via the abdominal aorta by
heparinized syringe, and plasma was obtained after centrifugation. These urine
and plasma samples were treated as described previously (34). One ml of 0.3
m acetate buffer at pH 5.2 and 250 µl of helicase were added into each 1-g
portion of sample and incubated at 37°C overnight to hydrolyze conjugated
metabolites, and 4-methylacetanilide (400 µl) was added to the hydrolysate
and diluted to 8 ml with 50% ethanol. After centrifugation, the upper layer was
applied to HPLC as described previously (35). The HPLC system was
equipped with a commercially available reverse-phase column (4.6 x 250 mm
inside diameter) filled with TSK-gel ODS-80TM (Tosoh, Tokyo, Japan), a
recording integrator (Shimadzu type CR4A, Kyoto, Japan), and a 254 nm
detector (Shimadzu type SPD-10AV), and was operated at a flow rate of 1.5
ml/min at 50°C. For measurement of the metabolite, a mobile phase of 1% phos-
phoric acid-methanol (8:2) was used for HPLC. The synthesized NHP and
purchased PH and AAP were used as internal standards.

Measurement of Concentration of PH, NHP, and AAP in 2%
PH-containing Diet

Samples of 2% PH-containing diet were obtained once a month and were
ground to powder; then 40 ml of ethanol were added. The mixture was shaken
for 30 min. After centrifugation, the upper layer was transferred to a 100-ml,
volumetric flask. The residue was extracted one more time in the same way,
and the solution in the flask was diluted to 100 ml with ethanol and applied to
the HPLC as described above.

Statistical Analysis

Data evaluation of histopathological lesion incidences was done by using
Fisher’s exact probability test. Other data were evaluated by analysis of vari-
ce (36, 37).

RESULTS

Measurement of Amount of PH, NHP, and AAP in PH-contain-
ing Diet. Analysis of the food samples showed actual levels of PH in
the range of 1.9 to 2.0%, but there was no detectable amount of NHP
or AAP (lower limit of detection was 0.01%).

Experiment 1. The survival rates, changes in average body
weights, and average PH intake of rats are summarized in Table 1. Two rats in Group 1 and 2 rats in Group 2 were dead or were sacrificed early when moribund, due to unknown causes, through experimental
week 52. All dead or killed rats which showed weight loss and
piloerection during the experiment had abscesses in preputial glands
or the parotid. Animals surviving more than 53 weeks were regarded
as the effective number of animals because renal pelvic carcinoma
was found for the first time in a PH-treated rat (Group 1) that died at
experimental week 53. The numbers of rats surviving through the
experimental period of 85 weeks in groups 1 and 2 were 21 of 55
(38%) and 19 of 32 (59%), respectively. The rats in Group 1 showed
marked weight loss. The body weight gain in Group 1 was 20% less

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Body wt (g)</th>
<th>PH intake (mg/kg/day)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>Final</td>
<td>Start</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>21</td>
<td>212 ± 29</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>19</td>
<td>210 ± 35</td>
</tr>
</tbody>
</table>

* Mean ± SD.

b P < 0.001, Group 1 versus Group 2.

2 Unpublished data.
compared to that of control rats in Group 2. Average daily PH intake was 1030 mg/kg body weight/day and average total PH intake was 612 g/kg body weight.

Almost all renal pelvic tumors were recognized as white or yellowish masses macroscopically (Fig. 1). Some of the tumor-bearing kidneys were enlarged, accompanied with severe hydronephrosis. Hydronephrosis was recognized macroscopically as slight enlargement of the kidney or as cystic kidney with a thin rim of renal parenchyma. Hydronephrosis and hydroureter were seen in all rats of both groups and almost all of them occurred bilaterally. The majority of the hydronephrosis was ranked as mild to moderate as described previously (Fig. 1).

Yields of renal pelvic lesions are summarized in Table 2. Carcinoma was observed in 43 rats (81%) of Group 1. In Group 1, 24 of 34 rats (71%) which were dead or killed during experiment had carcinomas. The mean induction period was 78 weeks. Papilloma was observed in 10 rats (19%) and PN hyperplasia in 42 rats (79%) of Group 1. One rat in Group 1 had basophilic renal cell adenoma. No carcinoma, papilloma, or PN hyperplasia was observed in Group 2. Histopathological examinations revealed 43 renal pelvic carcinomas classified as 41 (96%) transitional cell carcinomas (Fig. 2) and 2 (4%) squamous cell carcinomas. Twenty-seven of the transitional cell carcinomas (Fig. 3) and one of the squamous cell carcinomas were invasive and the others were noninvasive. They occurred in the covering epithelium of the renal pelvis rather than that of the renal papillae. Three of these invasive transitional cell carcinoma-bearing rats developed metastases to distant organs, including the lung and liver (Fig. 4).

Table 2. Incidence of urinary tract lesions in SD/cShi rats given phenacetin

<table>
<thead>
<tr>
<th>Location and histology</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH treated rats (53)²</td>
<td>Control (30)³</td>
</tr>
<tr>
<td>Renal pelvis</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>43 (81)³</td>
</tr>
<tr>
<td>Papilloma</td>
<td>10 (19)²</td>
</tr>
<tr>
<td>PN hyperplasia</td>
<td>42 (79)³</td>
</tr>
<tr>
<td>Ureter</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>2 (4)³</td>
</tr>
<tr>
<td>Papilloma</td>
<td>6 (11)³</td>
</tr>
<tr>
<td>PN hyperplasia</td>
<td>7 (13)³</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>6 (11)³</td>
</tr>
<tr>
<td>Papilloma</td>
<td>7 (13)³</td>
</tr>
<tr>
<td>PN hyperplasia</td>
<td>6 (11)³</td>
</tr>
</tbody>
</table>

* Effective number of animals.
² p < 0.001.
³ p < 0.01.
⁴ p < 0.05, PH-treated rats versus control.

Fig. 1. Left, gross appearance of tumors of the renal pelvis in a SD/cShi rat fed PH for 85 weeks; right, gross appearance of hydronephrosis in a control SD/cShi rat fed basal diet for 85 weeks without tumor. The degree of hydronephrosis is moderate.

Fig. 2. A, noninvasive renal pelvic carcinoma occurred in rats fed PH-containing diet. H&E, × 40. B, higher magnification of a. Mitoses were seen frequently in the carcinoma. H&E, × 200.

Fig. 3. Invasive transitional cell carcinoma of renal pelvis. Note the extensive invasion of transitional cell carcinoma into renal cortex. H&E, × 100.

All rats fed PH-containing diet had renal papillary necrosis in various degrees from grade 1 to 5. The majority of these lesions were ranked as grade 3, which is confluent necrosis of the tip or distal portion of the papillae.

Ureteral carcinoma was observed in 2 rats of Group 1 (Table 2). Papilloma was observed in 6 Group 1 rats, and PN hyperplasia in 7 rats of Group 1. No ureteral tumor was observed in Group 2.

Urinary bladder lesions are listed in Table 2. Carcinoma was observed in 6 rats in Group 1. Based on histological type and growth pattern, these were all papillary, noninvasive carcinomas. Five had
transitional cell carcinoma and one had squamous cell carcinoma. Papilloma was observed in 7 rats, and PN hyperplasia in 6 rats of Group 1. No urinary bladder tumor was observed in Group 2.

The 43 carcinoma-bearing rats were categorized as follows: A, 25 (57%); B, 1 (2%); C, 2 (5%); D, 2 (5%); E, 11 (26%); and F, 2 (5%). Two rats in category A and one rat in category E had metastases to distant organs.

Transitional cell carcinomas of the nasal cavity were observed in 2 of 23 rats in Group 1. No tumors in the nasal cavity were observed in Group 2. Lymphoma was observed in spleen, liver, and prostate in 6, 1, and 1 rat, respectively, in Group 1. No hepatocellular carcinoma was observed.

**Experiment 2.** Incidences of renal pelvic lesions and hydroureter, and BrdUrd labeling indices of renal pelvis and papillae are listed in Table 3. Simple hyperplasia was observed in 5 rats of Group 1 and 2 rats of Group 2. Papillary necrosis was observed in Groups 1 and 3, particularly in Group 1. Hydroureterohia was observed in almost all rats in Groups 1 and 2. BrdUrd labeling index of Group 1 was 2 times higher than that of Group 2 (SD/cShi control) (but not significantly different), which was 3 times higher than that of Groups 3 and 4.

Nasal mucosa in SD/cShi and SD rats fed PH showed apparently normal histology. No degeneration of respiratory epithelium, migration of inflammatory cells into the epithelium, or necrosis of Bowman’s glands described previously was observed (38). BrdUrd labeling index of nasal mucosa in both rats fed PH was not significantly different from each control, and the increase of BrdUrd labeling index of SD/cShi was not significantly different compared with SD rats (Table 4).

PH and its metabolites in urine and plasma were measured and are listed in Table 5. Rats fed PH-containing diet excreted PH, NHP, and AAP in urine in both strains used, but NHP was not detected in plasma. PH in urine and plasma was significantly higher in SD/cShi rats than in SD rats, and the other two metabolites of PH were also slightly higher, but not significantly.

**DISCUSSION**

The main concern of past studies were to prove experimentally that the target tissues of PH carcinogenesis were the upper urinary tract, especially the renal pelvis, which is epidemiologically the major target tissue in humans (3–6). Unfortunately, these studies did not show significant PH carcinogenicity toward the renal pelvis (8, 9, 14). We experimentally induced renal pelvic carcinoma in SD/cShi rats given PH-containing diet in the present study. This paper is, therefore, the first experimental proof of PH carcinogenicity toward the renal pelvis.

This study differed from previous studies in that it used hydroureterohia-bearing rats. Mori et al. (22) indicated that this SD/cShi strain is a suitable animal for renal pelvic and ureteral carcinogenesis by using the urinary tract carcinogen, BBN, and that urine stagnation by hydroureterohia and hydroureterohia plays an important role in renal pelvic and ureteral carcinogenesis (20, 22). In the present short term experiment the BrdUrd labeling index in the renal pelvis of SD/cShi rats fed PH was higher but not significantly so than in control SD/cShi rats. In SD rats the labeling index was not different between PH treatment and nontreatment groups. These results also indicated that stagnation of proximate carcinogen-containing urine due to hydroureterohia caused the increased DNA synthesis. Consequently, stagnation of urine containing carcinogen may play an important role in this study.

Almost all rats with renal pelvic tumors had renal papillary necrosis, but the location of tumor development was not always at the renal papillae. The majority of tumors occurred in epithelial cells in the pelvic thin area. It appears that renal papillary necrosis is not necessary for the development of renal pelvic tumors in rats treated with PH, as indicated previously (8, 39).

The concentration of PH in the PH-containing diet was selected as the MTD for these rats based on preliminary experiments. This high dose induced renal pelvic carcinoma and is relevant to humans. The daily dose of PH to these rats is calculated at 1030 mg/kg body weight. This dose is about 240 times higher than the amount of human daily intake epidemiologically considered to cause carcinoma in humans (40). Ames et al. commented on the fact that a carcinogen at the MTD in rodents provides no information about low-dose risk to humans without studies of the mechanism of carcinogenesis.
PH PELVIC CARCINOGENICITY IN SD/cShi RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Phenacetin treatment</th>
<th>Urine (µg/kg body wt/h)</th>
<th>Plasma (µg/g plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SD/cShi</td>
<td>+</td>
<td>47.5 ± 27.1(^a)</td>
<td>19427 ± 11,997</td>
</tr>
<tr>
<td>2</td>
<td>SD/cShi</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>SD</td>
<td>+</td>
<td>24.5 ± 10.1</td>
<td>11678 ± 3,511</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD.

| 1     | SD/cShi  | +                    | 7.2 ± 3.1\(^b\)         | ND\(^e\)             |
| 2     | SD/cShi  | –                    | ND                      | ND                   |
| 3     | SD       | –                    | ND                      | ND                   |

\(^b\) P < 0.05, Group 1 versus Group 3.

\(^e\) ND, not detected.

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REFERENCES


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