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 metastases 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 with 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 PH1 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 intestinal 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 F1, 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/10T1/2 clone 8 mouse embryonic 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-thiazoyl]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 hydronephrosis. In these studies we used SD/cShi rats and NON/Shi mice which had hereditary hydronephrosis and showed high incidences of renal pelvic carcinoma following p.o. administration of BBN (21, 22).

Bilateral hydronephrosis-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 hydronephrosis 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 Maruiishi & 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. 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 2 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 end 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% formalin. The extraneous tissue, fixed for approximately 2 weeks, decalcified for approximately 4 days in Plank-Rychlo solution, and sectioned into three levels, including level 1, 2, and 3. All 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 categories: papillary and nodular hyperplasia, papilloma, and carcinoma. Carcinoma multicientricity, 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 described previously (27). The degree of hydronephrosis was subjectively classified 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 h 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 exsanguination after ether anesthesia. Tissues, kidney, and nasal cavity were processed for both histological examination and immunohistochemical BrdUrd staining (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA) to assess DNA synthesis (30). The labeling index percentage was microscopically defined as the numbers 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 evaluated for cell proliferation as described previously (31). Each 1000 cuboidal 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) metabolites. 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 heparinase were added into each 1-g portion of sample and incubated at 37°C overnight to hydrolyze conjugated metabolites, and 4-methylcetanolide (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 (Toyko, Japan), a recording integrator (Shimazu 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% phosphoric acid-methanol (8:2) was used for HPLC. The synthesized NHP and purchased PH and AAP were used as internal standards.

**Results**

**Measurement of Amount of PH, NHP, and AAP in PH-containing 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 moribundness were dissected and all organs were inspected macroscopically. 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 than the control group.

<table>
<thead>
<tr>
<th>Body wt (g)</th>
<th>PH intake (mg/kg/day)</th>
</tr>
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<tbody>
<tr>
<td>Group</td>
<td>Start</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
</tr>
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</table>

* a Mean ± SD.
* P < 0.001, Group 1 versus Group 2.

2 Unpublished data.

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**Table 1 Survival rates, phenacetin intake, and average body weights**

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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).

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 hydronephrosis, 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. Hydronephrosis 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 2 to 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. 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 hydronephrosis-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 hydronephrosis and hydrourter 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 hydronephrosis 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.

<table>
<thead>
<tr>
<th>Table 4 Effect of phenacetin on cell proliferation in nasal respiratory mucosa</th>
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<tr>
<td>Group</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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<td>4</td>
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*Strains were described under "Materials and Methods."*

**Table 3 Incidence of renal lesions and BrdUrd labeling index of renal urothelium**

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Phenacetin treatment</th>
<th>No. of rats</th>
<th>Simple hyperplasia</th>
<th>Papillary necrosis</th>
<th>Hydronephrosis</th>
<th>BrdUrd labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SD/cShi</td>
<td>+</td>
<td>6</td>
<td>5°</td>
<td>4°</td>
<td>5°</td>
<td>0.29 ± 0.23€</td>
</tr>
<tr>
<td>2</td>
<td>SD/cShi</td>
<td>–</td>
<td>6</td>
<td>0°</td>
<td>0°</td>
<td>0°</td>
<td>0.11 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>SD</td>
<td>+</td>
<td>6</td>
<td>0°</td>
<td>2°</td>
<td>0°</td>
<td>0.05 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>SD</td>
<td>–</td>
<td>6</td>
<td>0°</td>
<td>0°</td>
<td>0°</td>
<td>0.06 ± 0.04</td>
</tr>
</tbody>
</table>

* P < 0.01, Group 1 versus Groups 3 and 4.

**Table 4 Effect of phenacetin on cell proliferation in nasal respiratory mucosa**

**Table 3 Incidence of renal lesions and BrdUrd labeling index of renal urothelium**

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**Figure 4. Lung metastasis from a renal pelvic carcinoma. H&E, X 100.**
(41). But carcinogens such as PH which are sometimes abused at high levels of consumption by humans suggest that the use of the MTD is still appropriate in the identification of rodent tumors as relevant to humans. In addition, the use of an appropriate model and the MTD is very useful to assess the multiple low-dose risk assessment of chemicals to humans. If toxicologists use an appropriate model such as SD/cShi strain in a rodent carcinogenesis bioassay of PH, important information about cancer risk to humans who abused PH can be obtained, although epidemiological studies already indicated the carcinogenicity of PH (3–6).

The metabolites of PH are found in urine and plasma, although NHP could not be detected in plasma by us. The reason why we could not detect it in plasma is not known, although NHP is produced in liver from PH (42, 43). A previous report showed that the capacity of PH in human liver to elaborate the putative carcinogenic metabolite, NHP, was similar to that from rat liver microsomes (42). The amount of NHP in plasma might be below the limit of detection that we used. NHP and AAP in urine were significantly or slightly higher in SD/cShi rats than in SD rats. A putative carcinogen, NHP, was identified in the urine of humans and rats, but it appeared to be more extensive in humans than in rats (43). Stagnation of urine containing these metabolites might successfully produce renal pelvic carcinoma even though rats produced relatively small amounts of the putative carcinogen as compared to humans, because total exposure time of the epithelium to a carcinogen may be important, as suggested previously (21). In such a sense, the SD/cShi rats might be an excellent model for detection of chemical carcinogenicity in the renal pelvis.

Tumor arising from the nasal cavity as described previously (7, 8) was also seen in the long-term experiment (Experiment 1), but no lesion as described previously (31) was seen in the nasal mucosa in the short-term experiment (Experiment 2). Early cell proliferation of nasal mucosa in SD/cShi rats fed PH-containing diet for a short term was not significantly different from SD rats fed PH. Because nasal mucosal lesions described in a previous report (31) were produced by intragastric administration of PH once a day, the blood concentration of PH might be relatively high compared to feeding PH-containing diet. Under intragastric administration conditions, nasal mucosal lesions might be produced. Longer exposure to PH may produce somewhat different results (7, 8). The sensitivity to PH or metabolites of PH in the nasal mucosa of this SD/cShi rat may also be somewhat different from rats tested previously (31, 38, 44).

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