Differences Among Three Sister Strains of NON/Shi Mice in Sensitivity to Urinary Tract Carcinogenesis by *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine

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Abstract: In this study we examined the susceptibility of three sister inbred strains of mice, NON/Shi, CTS/Shi, and NOD/Shi to the urinary bladder carcinogen, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in an attempt to optimize our model for tumor induction and spontaneous metastasis. The animals were given BBN at a dose of 0.05% in drinking water for 8 weeks and then maintained without further treatment until week 24 when the renal pelvis and urinary bladder were examined microscopically. The incidences of renal pelvic and urinary bladder carcinomas in NON/Shi, CTS/Shi, and NOD/Shi were 13/25 (52%) and 11/25 (44%), 3/19 (16%) and 12/19 (63%), and 0/24 (0%) and 1/24 (4%), respectively. Metastasis to lungs was only observed in renal pelvic carcinoma-bearing NON/Shi mice at an incidence of 77%. Invasion of the prostate and/or intraperitoneal dissemination were observed in urinary bladder carcinoma-bearing NON/Shi and CTS/Shi mice at incidences of 18% and 25%, respectively. BBN intake was highest in NON/Shi mice but urinary concentrations of the carcinogen and its ω -oxidative metabolite, *N*-butyl-*N*-(3-carboxypropyl)nitrosamine did not differ among the three strains. These results indicate that NON/Shi is the most sensitive of the three strains to carcinogenic effects of BBN on the urinary tract, particularly renal pelvis, and therefore the most suitable for our spontaneous metastasis model. (J Toxicol Pathol 2000; 13: 225~229)

Key words: Urinary tract, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine, renal pelvic carcinogenesis, metastasis

Introduction

Animal models featuring cancers which spontaneously metastasize are indispensable for the development and understanding of the underlying mechanism of anti-metastatic agents. For this purpose transplantation is artificial and only very few animal models which reliably reproduce all steps of metastasis *in vivo* have so far been reported¹⁻³.

The NON/Shi (non-obese non-diabetic) mouse is highly sensitive to the carcinogenic effects of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), targeting both the upper and the lower urinary tract⁴⁻⁸. Particularly, BBN treatment in this strain induces primary renal pelvic carcinomas, which metastasize to distant organs, mainly the lung, within a relatively short period⁴. To establish a spontaneous metastasis model, it is necessary to increase the incidence of malignant renal pelvic carcinoma, which metastasize to

distant organs. Previously we showed that the pretreatment of an analgesic phenacetin, which has carcinogenicity to the covering epithelium of renal pelvis, is one way to increase the incidence of BBN-induced malignant renal pelvic carcinoma⁵. However, the sensitivity in the sister strains of NON/Shi mice, which may have higher sensitivity to BBN than NON/Shi, has remained to be elucidated.

This NON/Shi mouse was derived together with the CTS/Shi (cataract Shionogi) mouse from a colony of Jcl:ICR mice as inbred sister strains of the NOD/Shi (non-obese diabetic) mouse which is as good as an animal model for type I diabetes mellitus⁹. CTS/Shi mouse was established as an inbred mutant strain with cataracts from a mouse with cataracts and small eyes found among outbred ICR mice purchased from Clea Japan, Inc. NOD mouse was established as an animal model for type I diabetes mellitus from a mouse with euglycemia separated at sixth generation during the course of developing the CTS strain. We have found the incidence of hydronephrosis, which is one of the factors to induce renal pelvic carcinoma^{4,5,10,11}, is approximately 30% in NON/Shi mouse⁴. However, the incidences of hydronephrosis in the other two strains have not yet been reported.

Since the CTS/Shi and NOD/Shi might also be sensitive to BBN because of the genetic relationship as described above, their susceptibility was here elucidated in an attempt to optimize our urinary tract cancer model of spontaneous metastasis. To cast light on the mechanistic aspect, urinary excretions of *N*-butyl-*N*-(3-carboxypropyl)nitrosamine (BCPN), a proximate metabolite regarded to have a causal

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Abbreviations used

N-butyl-*N*-(4-hydroxybutyl)nitrosamine: BBN

N-butyl-*N*-(3-carboxypropyl)nitrosamine: BCPN

role in bladder carcinogenesis, were measured in the three strains.

Materials and Methods

Animals

Five-week-old male NON/Shi, CTS/Shi, and NOD/Shi mice were obtained from Aburahi Laboratories, Shionogi Co., Ltd., Shiga. CA-1 pelleted diet (Japan CLEA Co., Ltd., Osaka) was used as the basal diet throughout the experiment. The animals were housed 5–8 mice per cage (RT-2 type, Charles River Japan, Inc.) in a room maintained under a 12-h (7:00–19:00) light-dark cycle, at a constant temperature of $25 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$. All mice were given basal diet and water *ad libitum* until they were used for the following experiments at 8 weeks of age. Mortality and general condition were checked daily.

Chemicals

The carcinogen, BBN was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo. BCPN was synthesized in our laboratory according to the method of Okada *et al.*¹². Purity of BCPN was confirmed to be $>99\%$ by high-performance liquid chromatography (HPLC).

Experiment 1

Experimental design. Thirty-two NON/Shi, 26 CTS/Shi, and 31 NOD/Shi mice were used in this experiment. They were given drinking water containing 0.05% BBN by weight per volume for 8 weeks and then maintained without treatment for a further 16 weeks. Subgroups of 7 mice of all strains were sacrificed to examine the incidence of hydronephrosis at experimental week 8. The remainder were kept until the end of week 24. Body weights were measured every 4 weeks. The amount of water containing BBN consumed was measured throughout on a per-cage basis. Moribund mice during the experimental period and all survivors at the end of the experiment were killed under ether anesthesia for pathological examination.

Histopathological examination. Kidney, urinary bladder, and sites of metastatic lesions were fixed in 10% phosphate-buffered formalin (pH 7.4) and the preserved organs were routinely processed for embedding in paraffin. Sections were stained with hematoxylin and eosin (HE) for light microscopic examination.

Experiment 2

Experimental design. Five male mice of each strain were given drinking water containing 0.05% BBN as described above for seven days. On administration day 6 each animal was kept in an individual urine collection cage and given food and water containing BBN *ad libitum*. Urine collection was performed under light protection and 24-hr samples were used for determination of urinary concentrations of BCPN and BBN. Treatment of the urine samples, and extraction, separation, and determination of BBN and BCPN by thin-layer chromatography and HPLC were performed as described in detail previously¹³.

Data evaluation

Statistical analysis of incidences of histopathological lesions was performed with the Fisher's exact probability test (program by Shionogi Co., Ltd.). The other data were evaluated by Student's *t*-test (program by Shionogi Co., Ltd.).

Results

Experiment 1

Data for survival rates, average changes in body weights, and average BBN intakes of mice are summarized in **Table 1**. In NON/Shi mice, the survival rates were lowest and the amount of BBN intake was significantly highest among the three strains, although the survival period was the same with CTS/Shi mice. Some mice which showed weight loss and piloerection died or were killed on and after experimental week 15 in NON/Shi strain, experimental week 18 in CTS/Shi strain, and experimental week 22 in NOD/Shi strain, mainly due to the development of urinary tract tumors. In NOD/Shi mice the occurrence of marked polyuria and emaciation that are the sign of onset of diabetes was not observed during experiment. The incidences of hydronephrosis in NON/Shi, CTS/Shi, and NOD/Shi at experimental week 8 were 4/7 (57%), 7/7 (100%), and 0/7 (0%). The degree in all CTS/Shi mice examined was severe, while that in NON/Shi mice was mild to moderate. In the CTS/Shi case hydronephrosis generally was unilateral and the affected kidney featured an extremely extended cavity of the renal pelvis with very thin parenchyma, never seen in NON/Shi kidneys (Fig. 1). At the termination of experiment the hydronephrosis was observed in all mice of these two strains and the degree of hydronephrosis tended to become aggravated with the occurrence of urinary tract tumors. However,

Table 1. Survival rates, body weights, and BBN intake

Strain	No. of animals used	Survivors at wk 24 (%)	Duration of survival period (days) ^a	BBN intake (mg/kg/day) ^a	Body weight ^a		
					Initial (g)	Final (g)	Increase ratio (%)
NON/Shi	25	9/25 (36)*	145 $\pm$ 23 [‡]	98 $\pm$ 2 ^{‡,‡}	32 $\pm$ 2	36 $\pm$ 2	14 $\pm$ 4 [‡]
CTS/Shi	19	10/19 (52)*	145 $\pm$ 34 [‡]	82 $\pm$ 2	33 $\pm$ 2	45 $\pm$ 6	40 $\pm$ 17
NOD/Shi	24	23/24 (96)	167 $\pm$ 2	83 $\pm$ 5	24 $\pm$ 1	29 $\pm$ 3	21 $\pm$ 9 [‡]

^a Mean $\pm$ SD

* Significantly different from the NOD/Shi case at $P < 0.05$ (Fisher's exact probability test)

[‡] Significantly different from the NOD/Shi case at $P < 0.05$ (Student's *t*-test)

[‡] Significantly different from the CTS/Shi case at $P < 0.05$ (Student's *t*-test)

a

b

Fig. 1. Appearance of representative hydronephrosis: a, NON/Shi mouse; b, CTS/Shi mouse. In NON/Shi mouse mild to moderate hydronephrosis is generally seen, on the other hand, severe hydronephrosis with an extremely extended cavity of renal pelvis and very thin parenchyma is seen in CTS/Shi mouse. HE, $\times 12$.

Table 2. Incidences of urinary tract carcinomas and metastases

	Incidence (%)		
	NON/Shi	CTS/Shi	NOD/Shi
No. of animals examined	25	19	24
Number of animals bearing carcinomas	20 (80) [*]	13 (68) [*]	1 (4)
Renal pelvis			
Carcinoma	13 (52)*	3 (16)	0
Metastasis (lung) ^a	10 (77)*	0	0
Urinary bladder			
Carcinoma	11 (44) [*]	12 (63) [*]	1 (4)
Direct invasion (prostate) ^a	2 (18)	3 (25)	0

^a Percentage incidences were calculated as numbers of metastasis bearing-animals per numbers of carcinoma bearing-animals.

^{*} Significantly different from the NOD/Shi case at $P < 0.05$ (Fisher's exact probability test).

^{*} Significantly different from the CTS/Shi case and the NOD/Shi case at $P < 0.05$ (Fisher's exact probability test).

the histology of parenchyma was not different from the case at experimental week 8.

Yields of urinary tract carcinomas and metastases are summarized in **Table 2**. Renal pelvic carcinomas were more frequent in NON/Shi than in CTS/Shi mice and lacking in the NOD/Shi strain. The high mortality in NON/Shi strain is attributed to this high incidence of malignant renal pelvic carcinoma. In NON/Shi mice eight carcinomas (62%) were classified as transitional cell carcinoma and five (38%) as squamous cell carcinomas, while in CTS/Shi mice they were all classified as transitional cell carcinomas. The growth pattern of carcinomas observed in all carcinoma-bearing NON/Shi mice was non-papillary, invasive type and the all carcinoma observed in NON/Shi strain invaded into the parenchyma as shown in Fig. 2. The growth pattern of three carcinoma-bearing CTS/Shi mice was papillary, non-invasive type, non-papillary, non-invasive type, and non-papillary, invasive type, each. These were all seen in the kidney without hydronephrosis but not in another kidney with severe hydronephrosis. Metastases from renal pelvic carcinomas were only in the lungs of 77% of renal pelvic carcinoma-bearing NON/Shi (Fig. 3). All cases with lung metastases were observed in mice bearing only renal pelvic carcinoma. Urinary bladder carcinomas were observed in all strains used, but the incidence was extremely low in NOD/Shi mice as compared with the other two strains. No significant difference in incidences of urinary bladder carcinomas and invasion into the prostate or intraperitoneal dissemination were evident for the other two strains. The histological type of all carcinoma observed in the present study was classified as transitional cell or squamous cell type. The incidences of transitional cell carcinoma observed in NON/Shi, CTS/Shi, and NOD/Shi mice were 8/11 (73%), 10/12 (83%), and 1/1 (100%), respectively. The growth pattern of carcinomas observed in all carcinoma-bearing NON/Shi mice was non-papillary, invasive type and the all carcinoma observed in NON/Shi strain invaded beyond the muscle layer. The growth pattern and the degree of invasion of urinary bladder carcinomas observed in CTS/Shi mice were similar to those of NON/Shi mice but two of them did

Fig. 2. Transitional cell carcinoma developing in renal pelvis of NON/Shi mouse. Carcinoma emerged from the covering epithelium of renal pelvis invades into parenchyma. HE, $\times 98$.

Fig. 3. Lung metastasis of a renal pelvic carcinoma in the same NON/Shi mouse as shown in Figure 2. Similar histological type to primary carcinoma is noticed. Carcinoma invades into parenchyma of lung. HE, $\times 98$.

not invade beyond the submucosa. In NOD/Shi only one carcinoma *in situ* was observed. Direct invasion from urinary bladder carcinoma was observed in the prostate in two of NON/Shi and three of CTS/Shi mice.

Experiment 2

Values for concentrations of BCPN or BBN in the urine are summarized in **Table 3**. No significant differences were seen among the three strains. The urine output per body weight also did not vary with the strain.

Discussion

In the present study, NON/Shi mice exhibited a significantly higher incidence of renal pelvic carcinomas than the sister strains examined, with very frequent metastasis. With induction of carcinogen, our previous experiment revealed hydronephrosis to be an important predisposing factor^{4,5,10,11}. While NOD/Shi mice did not show any hydronephrosis in the present study, the incidence in CTS/Shi mice was 100% and the degree was severer than in the NON/Shi case, so that no clear link with neoplasia was immediately apparent. However, the hydronephrosis in CTS/Shi strain may have impaired production and output of urine in the affected kidney so that exposure of the damaged tissue to proximate carcinogen in the urine might have been reduced, because the intake of BBN was not different from NOD/Shi and no

Table 3. Total urinary concentration over 24 hr of BCPN and BBN in three sister strains of mice after treatment with 0.05% BBN for 1 wk

Strain of mouse	Effective no. of mice examined	Concentration in urine ($\mu\text{g}/\text{ml}$) ^a	
		BCPN	BBN
NON/Shi	4	23.1 $\pm$ 6.0	14.2 $\pm$ 2.0 ^b
CTS/Shi	4	16.0 $\pm$ 2.3	16.4 $\pm$ 5.8
NOD/Shi	4	20.1 $\pm$ 5.3	13.0 $\pm$ 2.6

^a Mean $\pm$ SD

^b $n=3$

carcinoma was observed in the affected kidney. The occurrence of hydronephrosis is not related to the BBN treatment, because we recently found a similar incidence and degree of hydronephrosis in intact CTS/Shi mice. Our previous study suggested that the cause of hydronephrosis might be due to partial obstruction or stenosis of the urinary passage⁵, although the mechanism of spontaneous occurrence of hydronephrosis is not obvious. Alternatively, genetic factors would have played a role in the occurrence of spontaneous hydronephrosis.

The incidences of urinary bladder carcinomas and metastasis in NON/Shi and CTS/Shi mice were similar,

although the degree of invasion was slightly less in CTS/Shi than NON/Shi mice. Survival period was also the same for both strains. Recently, Higashi *et al.*¹⁴ obtained evidence that differences in survival may be related to at least two quantitative trait loci mapped on chromosomes 2 and 6 by a linkage analysis of 248 C57BL/6J × (C57BL/6J × NON/Shi)F1 back-crosses. Genes that regulate the susceptibility of the covering epithelium of urinary tract to BBN might be shared in common by NON/Shi and CTS/Shi strains.

Interestingly, NOD mice almost had no susceptibility to BBN in the urinary bladder, although urinary concentrations of BBN or BCPN, the latter as the ultimate carcinogen of BBN, did not differ as compared with the other two strains. Therefore, mechanisms other than metabolism of BBN may underlie this resistance. Similar phenomena have been found in *p53*(+/-) knockout or nude mice with regard to susceptibility to BBN or *N*-ethyl-*N*-(4-hydroxybutyl)-nitrosamine but the reason remains unknown^{11,15}. Resistance genes or examples involving diabetes may affect the susceptibility, but these have yet to be elucidated. Further experiments to determine their influence is now in progress.

BBN-induced renal pelvic carcinoma tended to metastasize to the lung, while urinary bladder carcinoma tended to invade directly to the prostate. In humans, renal pelvic carcinoma has the propensity to metastasize to the lung as compared with urinary bladder carcinoma. As in the previous experiment the metastasis to vena cava was also observed in renal pelvic carcinoma, the metastatic route may be mainly hematogenous.

In conclusion, mouse models that reliably reproduce the entire process of metastasis for a specific tumor type are very limited. The renal pelvic carcinomas induced in our NON/Shi mice demonstrate a propensity for metastasis in a relatively short latency period. Therefore, this system can be used to establish an optimal metastasis model in mice by improving the incidence of renal pelvic carcinomas induced in BBN.

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