SIGNIFICANT INDUCTION OF EPITHELIAL CELL PROLIFERATION IN THE URINARY BLADDERS OF NCI-BLACK-REITER MALE RATS BY *N*-BUTYL-*N*-(4-HYDROXYBUTYL) NITROSAMINE

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Abstract: The response of the urinary bladder epithelium in NCI-Black-Reiter (NBR) rats to administration of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), a potent carcinogen in rodents, was examined. Male NBR rats were exposed to 0.05% BBN in the drinking water for 8 weeks, or no added chemical. Microscopically, simple hyperplasia was observed in the urinary bladder epithelium of treated rats sacrificed at the end of week 4. Moreover, after 8 weeks administration of BBN, papillary or nodular (PN) hyperplasia and squamous metaplasia were produced. Levels of DNA synthesis, as indicated by incorporation of 5-bromo-2'-deoxyuridine, areas of simple and PN hyperplasia in rats treated by BBN were significantly increased than in non-treated ones at the end of week 8. These results indicate that the urinary bladder of NBR male rats is very responsive to BBN insult. (*J Toxicol Pathol* 8: 139~143, 1995)

Key words: NCI-Black-Reiter rat, Urinary bladder, Cell proliferation, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine

Introduction

Species and sex considerably influence the susceptibility to promotion of two-stage urinary bladder carcinogenesis in rodents. For example, sodium L-ascorbate does not exert promoting activity for mouse urinary carcinogenesis, in clear contrast to the case in the rat¹. Bladder tumor promoting activity of sodium saccharin is greater in male than in female rats² and can not be demonstrated in mice, hamsters, and monkeys^{3,4}. The

latter results concerning differences in response to sodium saccharin were recently explained on the basis of α 2u-globulin, a male rat specific low molecular weight protein that is synthesized in the liver of adult male rats, secreted into the blood and excreted into the urine⁵. Therefore, it is important to investigate the participation of α 2u-globulin in sodium salt promotion of rat urinary bladder carcinogenesis. NCI-Black-Reiter (NBR) rats were developed in 1966 from heterogeneous stock and the males are known not to synthesize α 2u-globulin^{6,7} and therefore of interest for such studies.

N-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) is widely used as an initiator for 2-stage rat urinary bladder carcinogenesis protocols^{1,2,8-13}. It has been reported that strain differences exist in

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the response of the urinary bladder epithelium to BBN^{12,13}. Since there have been no reports of BBN-treated urinary bladder carcinogenesis in NBR rats, we examined whether the urinary bladder of this strain is susceptible to BBN induction of morphological changes and/or DNA synthesis.

Materials and Methods

Animals

NBR rats were initially procured from the Laboratory of Animal Resources of the National Cancer Institute, U.S.A. The animals were housed 2-3 rats per cage in stainless-steel wire-mesh cages in a controlled environment, maintained on a 12-hour light/dark cycle (light on, 8:00-20:00), air-conditioned at $24 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ humidity. Food (CRF-1, Oriental Yeast Co., Tokyo) and water were available *ad libitum* throughout the experiment.

Experiment

A total of 12 male NBR rats, 39-weeks-old, were divided into 2 groups, and given drinking water with no additional chemical (control, 3 animals) or containing 0.05% BBN (Tokyo Kasei Co. LTD., Tokyo; total 9 animals) for 8 weeks. An interim sacrifice was performed for the BBN treated group (4 animals) at the beginning of week 5.

A daily health check was performed. Body weights were checked weekly. Food and water consumptions over 2 days were recorded during weeks 4 and 8.

For urine pH examination, fresh urine samples were obtained from almost all rats by forced urination in the morning at the end of weeks 4 and 8. The urinary pH was measured with a pH meter (Twin pH; Horiba, Kyoto). In addition, at the same time points, urine was collected over 24 hours from rats maintained in individual metabolic cages without food. Urine samples were applied for volume measurement and electrolyte analysis, namely of sodium, potassium and chloride with the aid of an automated analyzer (model NEO-5AT, Toa Electronics LTD., Tokyo).

At the end of week 4 and 8, all rats received a

single i.p. injection of 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co., MO, U.S.A.) at a dose of 100 mg/kg body weight, one hour prior to being killed under either anaesthesia. The urinary bladder was removed from each rat inflated by intraluminal injection of 10% phosphate-buffered formalin solution, and their immersion fixed in the same fixative. Each bladder was then cut into twelve strips, embedded in paraffin, sectioned routinely, and stained with hematoxylin and eosin.

To achieve immunohistochemical staining of BrdU, sections were reacted with anti-mouse BrdU antibody (DAKO A/S, Denmark) with visualization of binding sites performed using the avidin biotin complex method (Vectastain ABC kit, Vector Laboratories, Inc., CA, U.S.A.). The numbers of cells incorporating BrdU into the DNA per unit area (mm^2) and the basement membranous length (mm) of transitional epithelium in the urinary bladder were measured with the help of a color image analyzer (IPAP: Image Processor for Analytical Pathology; Sumika Technos Inc., Hyogo) under the light microscope. Transitional epithelium average heights were calculated as follows: the area of the transitional epithelium divided by the length of basement membrane.

Statistical analysis

Data for lesion incidences were analysed for statistical significance with the two-tailed chi-square test. Other data were analyzed using the two-tailed Student's *t* test.

Results

Clinical observation and values for food and water consumption did not reveal abnormalities or differences between the groups. No deaths occurred during the experimental period.

Urinary analysis data for weeks 4 and 8 are shown in **Table 1**. Treatment with BBN was not associated with changes of the urinary pH or volume, or the concentrations of Na^+ and K^+ . At week 8, the urinary chloride level was significantly higher in the BBN-treated group than in the control group.

Table 1. Data for Urinary Volume, pH, and Electrolytes

Treatment	Effective No. of rats	pH	Volume (ml/day)	Electrolytes (mEq/l)		
				Na	K	Cl
Examination at week 4						
Control	3	7.7±0.5	7.7±1.5	21.9±16.7	106.1±39.8	57.0±38.5
BBN treatment	7	8.1±0.2	8.0±1.1	40.0±17.0	114.1±15.8	75.1±21.7
Examination at week 8						
Control	3	8.0±0.2	7.3±0.6	9.6± 9.9	81.7±29.0	34.7±17.6
BBN treatment	5	7.7±0.5	8.3±1.2	18.3± 9.2	101.1± 9.9	67.4± 8.5*

BBN=N-butyl-N-(4-hydroxybutyl)nitrosamine

The asterisk indicates a significant difference from the corresponding control value (*P<0.05, Student's *t* test)

The data given are mean±SD values.

Table 2. Histopathological Findings and BrdU Labeling Indices for the Urinary Bladder Epithelium

Treatment	No. of rats	Hyperplasia		Squamous Metaplasia	Height of Epithelium (μm)	BrdU Labeling Index (No./mm ² epithelium)
		Simple	PN			
Terminal sacrifice ^a						
Control	3	0	0	0	9.2±0.5	7.6± 5.4
BBN treatment	5	5**(-#) ^c	5**(+) ^c	3(+) ^c	45.3±7.2***	127.6±15.8***
Interim sacrifice ^b						
BBN treatment	4	4(-#) ^c	0	0	46.1±5.7	171.9±31.3

BBN=N-butyl-N-(4-hydroxybutyl)nitrosamine

PN=Papillary or nodular

Asterisks indicate significant differences from the corresponding control values (**P<0.01; ***P<0.001, chi square or Student's *t* test).^a Sacrificed at the end of week 8.^b Sacrificed at the end of week 4.^c Grading of lesions: +, slight; #, moderate; #, severe.

The data for height of epithelium and BrdU indices are given as mean±SD values.

Histopathological findings and BrdU labeling index data for the urinary bladder epithelium are summarized in Table 2. Simple hyperplasia, papillary or nodular (PN) hyperplasia, a putative preneoplastic lesion¹⁴, and squamous metaplasia were seen in BBN-treated rats but not in control animals. The animals given BBN showed marked increases in height and BrdU labeling indices of the urinary bladder epithelium (Fig. 1). In particular, incidences of simple and PN hyperplasia were significantly higher in the BBN-treated group than in control group at the end of week 8.

Discussion

It is well known that carcinogen or promoting

chemical insult induces a rapid increase in cell division in the urinary bladder epithelium, resulting in epithelial hyperplasia as observed by light microscopy^{15,16}. The results of the present investigation provide clear evidence of such a response of the urinary bladder epithelium in NBR rats to BBN.

Concerning rat urinary bladder carcinogenesis, the Fischer 344 rat is the most commonly used strain^{1-11,14-16}. In Fischer 344 male rats treated with 0.05% BBN in the drinking water for 4 weeks, marked incorporation of BrdU and severe simple hyperplasia are seen in the urinary bladder epithelium¹⁶. The urinary bladder changes observed in the present study were estimated to be quantitatively and qualitatively

Fig. 1. BrdU immunohistochemical staining of urinary bladder epithelia from rats sacrificed at the end of week 8. $\times 160$.

(a) Control

Lack of BrdU positive cells in normal epithelium.

(b) BBN treatment

A large number of BrdU-incorporating cells are observed in an area of PN hyperplasia.

equally severe as those in the Fischer 344 strain. Therefore, BBN was confirmed to act typically on the NBR rat urinary bladder carcinogenesis indicating that NBR rats treated with this carcinogen can be regarded as initiated for the purpose of investigating the relation of $\alpha 2 \mu$ -globulin to the promotion stage.

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