Lack of Promotion Effects of Sodium L-Ascorbate on Urinary Bladder Carcinogenesis in NCI-Black-Reiter (NBR) Male Rats Initiated with N-Butyl-N-(4-Hydroxybutyl) Nitrosamine

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Abstract: Promotion effects of sodium L-ascorbate (Na-AsA) on two-stage urinary bladder carcinogenesis were examined in male NCI-Black-Reiter (NBR) rats, which lack α 2u-globulin-synthesizing ability, and F344 rats for comparison of the action. The animals were initiated with exposure to 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) via the drinking water for 4 weeks, and then treated with a basal diet containing a 5% supplement of Na-AsA or no added chemical for 32 weeks. The urinary pH and Na ion concentration in both strains of rats treated with BBN followed by Na-AsA (BBN $\rightarrow$ Na-AsA) were significantly higher than those in the matched controls (BBN alone). Microscopically, significantly increased incidences of papillary or nodular hyperplasia, papillomas, and transitional cell carcinomas were observed in F344 rats treated with BBN $\rightarrow$ Na-AsA, whereas the incidences of these lesions in NBR rats were comparable in the BBN $\rightarrow$ Na-AsA and BBN alone cases. These data indicate NBR rats to be resistant to Na-AsA-induced promotion of two-stage urinary bladder carcinogenesis. Since the elevation of urinary parameters generally associated with tumor promotion by Na-AsA was a feature with both strains, another factor such as α 2u-globulin, may also be active for enhancement of lesion development. (*J Toxicol Pathol* 1997; 10: 103~107)

Key words: Rat, Urinary bladder, Sodium L-ascorbate, α 2u-Globulin, Carcinogenesis

Introduction

It has been convincingly demonstrated that sodium salts play an important role in the promotion stage of two-stage urinary bladder carcinogenesis in the rat¹⁻³. The underlying mechanisms have not been completely elucidated but alkalization of urinary pH and an increase in urinary Na ion concentration have been demonstrated to play crucial roles⁴⁻⁶. Species and sex exert a considerable influence on promotion of two-stage urinary bladder carcinogenesis in rodents. For example, Na-AsA, a strong promoter of rat urinary bladder carcinogenesis, in clear contrast does not exert any enhancing effects on mouse urinary bladder carcinogenesis⁷. Furthermore, when given alone at high dose sodium saccharin itself demonstrates urinary bladder carcinogenicity in the rat, especially males, but not mice, hamsters, or monkeys^{8,9}. Species and sexes differences in such response to sodium salts have been recently explained on the basis of α 2u-globulin¹⁰⁻¹², calcium phosphate-containing amorphous precipitates formed in the urine^{13,14} or genetic background^{15,16}.

α 2u-globulin is a low molecular weight protein that is synthesized in large amounts in the livers of adult male rats, secreted into the blood, excreted into the urine, and reabsorbed by the kidney. There is approximately 120 times as

much α 2u-globulin in a male than in a female rat kidney^{17,18}. A variety of chemicals such as 2, 2, 4-trimethylpentane, d-limonene, and decaline have been reported to exert renal toxicity because they induce accumulation of α 2u-globulin in the male rat kidney, with no effects being observed in females because of their relative lack of α 2u-globulin synthesis^{17,18}.

NBR rats, which lack α 2u-globulin-synthesizing ability, were developed in 1966 from heterogeneous stock and subsequently maintained. The parent strains are now untraceable. While NBR male rats are known not to synthesize the androgen dependent α 2u-globulin, they do not demonstrate any endocrinological or reproductive abnormalities and the mRNAs for androgen-dependent other hepatic proteins are normally regulated in this animal¹⁹. Male NBR rats also do not develop renal disease when exposed to decaline²⁰. Previously, we reported that the urinary bladder epithelium of NBR rats does not respond to Na-AsA or sodium saccharin treatment despite elevation of urinary pH and Na ion concentration¹². However, male NBR rats are susceptible to BBN treatment, and the induced cell proliferation and morphological changes are quantitatively and qualitatively equal to those in F344 rats treated with BBN for the same period²¹.

Therefore, it is important to ascertain whether there is any sodium salt-induced promotion in this strain to cast further light on the participation of α 2u-globulin in rat urinary bladder carcinogenesis. In the present study, we therefore compared NBR and F344 rats using Na-AsA as a promoter in an established two-stage urinary bladder carcinogenesis model^{2,3}.

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Materials and Methods

Animals

NBR rats were initially procured from the Laboratory of Animal Resources of the National Cancer Institute, USA. F344 rats were purchased from Charles River Japan Inc., Atsugi. The animals were housed two or three rats per cage in stainless-steel wire-mesh cages in a controlled environment, air-conditioned to $24 \pm 2^\circ\text{C}$ and at a $55 \pm 10\%$ humidity with a 12-hour light-dark cycle (light on, 8:00–20:00). Rats aged 14 weeks were used for the experimentation.

Chemicals

BBN was purchased from Tokyo Kasei Co. Ltd., Tokyo and Na-AsA from Nakarai Tesque Ltd., Kyoto.

Experimental design

Thirty-one NBR and 33 F344 male rats were randomly divided into two groups for each strain. At the beginning of the study, all rats were given drinking water containing 0.05% BBN for four weeks. For the next 32 weeks, they received powdered basal diet (MF, Oriental Yeast Co., Tokyo) containing 5% Na-AsA or no-added chemical. Food and water were available *ad libitum* throughout the experiment. The total observation period was 36 weeks. A daily health check was performed. Body weights were measured biweekly during the BBN treatment period and every four weeks of the Na-AsA treatment period. Food and water consumption over one day was recorded every 12 weeks.

Urinalysis

For urine pH examination, fresh urine samples were obtained from 10 rats of each group at weeks 12, 24, and 36 by forced micturition in the morning. The pH was measured with a pH meter (Twin pH, Horiba, Tokyo).

In addition, at the same time points, 10 rats from all groups were housed individually in metabolic cages without food and water for 5 hr (from 8:30 to 13:30) for collection of urine samples. Urine specimens were measured for volume, osmolality, and concentrations of Na, K, and Cl ions with an automated electrolyte and osmolality analyzer (Neo-5AT, Toa Electronics, Ltd., Tokyo).

SDS-PAGE and immunoblotting analysis

$\alpha 2\mu$ -globulin in the remaining urine samples from each group collected at week 12, and in liver and kidney samples from each group after removal weighting at the terminal sacrifice were also measured using sodium dodecyl poly-

acrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Western blotting) analysis²².

Pathological examination

At the beginning of week 37, all rats were sacrificed under ether anesthesia and necropsied. The urinary bladder, liver and kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. Particular care was taken with the urinary bladders which were inflated by intraluminal injection of formalin solution and then cut into twelve strips. Tissues were processed routinely and stained with hematoxylin and eosin for histological examination. For quantitative analysis, urinary bladder lesions were counted under the light microscope, the total length of the basement membrane examined being measured with the help of a color image analyzer (IPAP, Sumika Technos Inc., Hyogo), and the numbers expressed per 10 cm of basement membrane.

To achieve immunohistochemical staining of $\alpha 2\mu$ -globulin, liver and kidney sections from each animal were reacted with anti- $\alpha 2\mu$ -globulin antibody²² with binding sites were visualized using the streptavidine-alkali phosphatase complex method (Histofine SAB-AP kit, Nichirei Co., Tokyo)²³.

Statistical analysis

Statistical analysis was conducted using the Yukms statistical library I (Yukms Cooperation, Tokyo). Body weights, organ-to-body weight ratios, values for urinary parameters, and numbers of urinary bladder lesions per 10 cm of basement membrane were analyzed using analysis of variance. With regard to parameters for which a significant difference was detected at the 5% level, the least significant difference (LSD) method was used as a further test for significant differences from the matched control values. Data for incidences of urinary bladder lesions were analyzed using the Fisher exact test.

Results

No deaths occurred during the treatment period. Final average body and relative organ weights are shown in Table 1. The final body weights of F344 rats treated with BBN followed by Na-AsA (BBN $\rightarrow$ Na-AsA) were significantly lower than those of the matched controls (BBN alone). The relative liver weights for F344 rats treated with BBN $\rightarrow$ Na-AsA and the relative kidney and urinary bladder weights for both strains rats treated with BBN $\rightarrow$ Na-AsA were significantly elevated as compared to the values for the matched controls. Data for average food and water con-

Table 1. Data for Body Weights, and Organ-to-Body Weight Ratios at Week 36 as well as Average Food and Water Consumption

Treatment	Strain	No. of rats	Final body weight (g)	Organ to Body Weight Ratios (%)			Consumption (g/rat/day)	
				Liver	Kidneys	Urinary bladder	Food	Water
BBN $\rightarrow$ Na-AsA	NBR	16	379 $\pm$ 20	2.46 $\pm$ 0.09	0.67 $\pm$ 0.04**	0.05 $\pm$ 0.01*	16.3	53.0
BBN	NBR	15	381 $\pm$ 11	2.44 $\pm$ 0.09	0.63 $\pm$ 0.02	0.04 $\pm$ 0.01	15.8	33.9
BBN $\rightarrow$ Na-AsA	F344	17	402 $\pm$ 27*	2.31 $\pm$ 0.09**	0.62 $\pm$ 0.03**	0.07 $\pm$ 0.04**	16.3	39.6
BBN	F344	16	416 $\pm$ 25	2.21 $\pm$ 0.08	0.58 $\pm$ 0.02	0.03 $\pm$ 0.01	15.1	21.0

The results are given as mean $\pm$ SD values.

Significantly different from the matched control values at *, $P < 0.05$; **, $P < 0.01$.

sumption at week 36 are also shown in **Table 1**, intake in both strains treated with BBN $\rightarrow$ Na-AsA being slightly higher than in the matched controls.

Urinalysis

The results of urinalysis at week 24 are shown in **Table 2**. Treatment with BBN $\rightarrow$ Na-AsA was associated with significant elevation of urinary pH, increased urine volume, and decreased urine osmolality in both strains of rats. Electrolytic analysis demonstrated significant increase in the Na ion concentration in both strains of rats, while the K ion concentration was significantly depressed in the F344 rat case. The Cl ion concentration was also significantly lower than in the matched controls. Results of urine analysis at weeks 12 and 36 in all groups showed essentially the same changes (data not shown).

SDS-PAGE and immunoblotting analysis revealed marked intensification of a protein band, approximately 19 kDa, in the liver, kidney, and urine samples from F344 rats. However, the same protein band was not detected in the specimens from NBR rats. The protein could be clearly identified as α 2u-globulin by immunoblotting analysis (Fig. 1).

Pathological Examination

The urinary bladder lesions were classified into simple hyperplasia, papillary or nodular (PN) hyperplasia, papil-

loma, and transitional cell carcinoma (TCC) categories²⁴. Simple hyperplasia was observed in all groups of rats with or without Na-AsA. The incidences and numbers of PN hyperplasia, papillomas, and TCCs per 10 cm of basement membrane of urinary bladder epithelium are summarized in **Table 3**. In F344 rats, the incidences and numbers of PN hyperplasia, papillomas, and TCCs in rats treated with BBN $\rightarrow$ Na-AsA were significantly higher than in the matched controls. In the NBR case, however, no significant inter-group differences were observed.

No toxic lesions of the liver and kidney which could be related to test chemical application were observed in any of the groups. Immunohistochemical examination of kidney sections revealed hyaline droplets in the proximal convoluted tubule epithelial cells positive for anti- α 2u-globulin in the F344 rats. The liver samples from F344 rats were also positive for anti- α 2u-globulin, but in neither tissue of NBR rats was any staining observed.

Discussion

It is well known that changes in urinary composition, including increases in both urinary pH and Na ion concentration may play important roles in the promotion stage of rat urinary bladder carcinogenesis. Thus they are elevated by a large number of sodium salts, like Na-AsA and sodium saccharin which strongly enhance bladder lesion develop-

Table 2. Analysis Data for Urine Samples Collected over a 5-hour Period at Week 24

Treatment	Strain	No. of rats	pH	Volume (ml/5 hour)	Osmolarity (mOsm)	Electrolytes		
						Na (mEq/l)	K (mEq/l)	Cl (mEq/l)
BBN $\rightarrow$ Na-AsA	NBR	10	7.59 $\pm$ 0.19*	2.6 $\pm$ 1.8*	1,276 $\pm$ 278**	160 $\pm$ 45**	93 $\pm$ 24	40 $\pm$ 17**
BBN	NBR	10	7.26 $\pm$ 0.17	1.8 $\pm$ 1.0	1,650 $\pm$ 282	81 $\pm$ 31	117 $\pm$ 24	87 $\pm$ 16
BBN $\rightarrow$ Na-AsA	F344	10	7.42 $\pm$ 0.09**	2.0 $\pm$ 0.4*	1,307 $\pm$ 164**	227 $\pm$ 40**	112 $\pm$ 14*	74 $\pm$ 21**
BBN	F344	10	6.96 $\pm$ 0.71	0.9 $\pm$ 0.3	2,127 $\pm$ 484	100 $\pm$ 49	245 $\pm$ 55	156 $\pm$ 59

Fresh urine specimens were obtained from rats by forced micturation for pH examination.

The results are given as mean $\pm$ SD values.

Significantly different from the matched control values at *, $P < 0.05$; **, $P < 0.01$.

Fig. 1. SDS-PAGE (A) and immunoblotting (western blotting) (B) analysis of urine samples at week 12, obtained from F344 rat treated with BBN $\rightarrow$ Na-AsA (lane 1) or BBN (lane 2), and NBR rat treated with BBN $\rightarrow$ Na-AsA (lane 3) or BBN (lane 4). Each urine sample for SDS-PAGE (2.5 mg/lane) or immunoblotting analysis (0.16 mg/lane) was analyzed in 18% resolving gels. Molecular weight markers (lane 5) are identified by numbers corresponding to their $M_r \times 10^{-3}$. The position of α 2u-globulin (α 2-G) is indicated ($\rightarrow$).

Table 3. Histopathological Findings in the Urinary Bladder

Treatment	Strain	No. of rats	Simple hyperplasia Incidence (%)	PN hyperplasia		Papilloma		Carcinoma	
				Incidence (%)	No./Unit	Incidence (%)	No./Unit	Incidence (%)	No./Unit
BBN→Na-AsA	NBR	16	16 (100)	8(50)	0.26±0.28	3(19)	0.14±0.33	1(6)	0.03±0.12
BBN	NBR	15	15 (100)	6(40)	0.30±0.43	5(33)	0.21±0.31	1(7)	0.04±0.16
BBN→Na-AsA	F344	17	17 (100)	16(94)**	1.99±1.04**	16(94)**	1.85±1.21**	9(53)**	0.53±0.61**
BBN	F344	16	16 (100)	8(50)	0.50±0.66	6(44)	0.26±0.39	1(6)	0.04±0.15

PN, Papillary or nodular.

No./Unit, number of lesions/10 cm of basement membrane.

The results are given as mean±SD values.

Significantly different from the matched control values at *, $P<0.05$; **, $P<0.01$.

ment in the rat¹⁻⁶.

In the present study, whereas both strains of rats treated with BBN → Na-AsA demonstrated elevation of the urinary pH and Na ion concentration, an increase in the incidence of the preneoplastic and neoplastic lesions of the urinary bladder was only observed in F344 rats. Previously we reported that the urinary bladder epithelium of NBR rats does demonstrate increased epithelial cell proliferation in response to Na-AsA treatment despite elevation of the urinary pH and the Na ion concentration¹². The results therefore suggest a link between cell proliferation and tumor promotion, which is in line with earlier findings for all categories of bladder cancer promoters⁶.

Male NBR rats are susceptible to BBN treatment, and the induced cell proliferation and morphological changes are quantitatively and qualitatively equal to those in F344 rats treated with BBN for the same period²¹. In the present study, there was also no difference in the development of preneoplastic and neoplastic lesions in the urinary bladder between NBR and F344 rats treated with BBN alone. The results thus clearly demonstrate that the lack of any tumor promotion effect by Na-AsA was not dependent on variation in susceptibility to BBN initiation.

With sodium saccharin, neither epithelial cell proliferation nor urinary bladder tumor promotion have been demonstrated in castrated male rats or females¹¹. A variety of urinary parameters considered to affect urinary bladder carcinogenesis, including pH, Na ion, and protein²⁵, differ between males and females. For example, total urinary protein in male rats is 10-times higher than in females. α 2u-globulin is one of the predominant urinary proteins in young males, with excretion rates 100-300 times higher than in females²⁶. Castration reduces the level of this protein in urine¹¹. The NBR rats reportedly does not synthesize the androgen-dependent α 2u-globulin¹⁹, and in the present study, it could not be detected in urine, liver, or kidney samples by using immunoblotting analysis or immunohistochemistry. Therefore, in the present study, the strain difference in the tumor promotion effect of Na-AsA correlated with the presence or absence of α 2u-globulin.

However, male ODS/Shi-*od/od* and WS/Shi rats, which synthesize α 2u-globulin to the same extent as F344 rats, also demonstrate resistance to the modifying effects of Na-AsA on two-stage urinary bladder carcinogenesis^{15,16}. The different susceptibility to the promotion effect of Na-AsA may be explained by most likely based on genetic difference among rat strains. The underlying genetic

difference remains to be elucidated but comparison of the NBR strain with these other non-susceptible rats should allow clarification of mechanistic parameters and hopefully also susceptibility genes.

A variety of hypotheses have been reported regarding the sodium salt-induced promotion effect²⁵. One possibility is that the rise in pH and the Na ion concentration directly or indirectly stimulates epithelial cell proliferation in the urinary bladder. Calcium phosphate-containing precipitates are readily formed at high urinary pH and these might affect the urinary bladder epithelium by direct mechanical stimulation^{13,14}. Na ions may cause cell proliferation through alteration of the Na⁺/H⁺ transport system in cellular membranes²⁷. However, from the present results a contribution of additional factors, such as α 2u-globulin appears essential. Further studies of this area appear warranted.

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