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# Strain Differences in Sensitivity to the Promoting Effect of Sodium L-Ascorbate in a Two-stage Rat Urinary Bladder Carcinogenesis Model

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**Rat strain differences in sensitivity to the promoting effect of sodium L-ascorbate (SA) on the development of urinary bladder tumors were investigated. In experiment 1, WS/Shi (WS), ODS/Shi-od/od (ODS), and LEW/Crj (LEW) rats were initiated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN) in their drinking water and subsequently given basal Oriental MF diet (M) with or without a 5% SA supplement. In LEW rats the SA treatment increased the induction of neoplastic lesions in the urinary bladder, whereas WS and ODS animals proved unresponsive to its promoting effects. In experiment 2, WS and F344 rats were maintained on two kinds of commercial basal diets, M and CLEA CA-1 (C), during administration of SA, since dietary factors can influence promoting effects. Feeding M during the promotion period in F344 rats yielded significantly more neoplastic lesions than feeding C, but in WS rats no such dietary influence was apparent. In experiment 3, strain differences in biosynthesis of $\alpha$-2u-globulin ($\alpha_{2u}$-g) were assessed because both $\alpha_{2u}$-g in the urine and administration of sodium salts of organic acids such as SA have been reported to be involved in tumor promotion. Immunohistochemical analysis of renal tubules and Western blotting analysis of urine revealed the presence of $\alpha_{2u}$-g in all three strains examined. These data suggest that differences in susceptibility to promotion are due to genetic factors rather than dietary factors and the ability to synthesize $\alpha_{2u}$-g.**

Key words: ODS/Shi-od/od rat — WS/Shi rat — Sodium L-ascorbate — Urinary bladder carcinogenesis — $\alpha_{2u}$-globulin

Several reports of family, race and population variation in human urinary bladder cancer have emphasized that genetic factors play an important role.[1,2] Urinary bladder carcinogenesis in experimental animals is also reported to be dependent on the species and strain.[3–11]

The development of neoplasia in the urinary bladder has been demonstrated to be divisible into at least two stages, initiation and promotion.[12,13] The susceptibility to sodium L-ascorbate (SA) promotion differs among strains of rats, with F344/DuCrj (F344) and LEW/Crj (LEW) animals being sensitive, while ODS/Shi-od/od (ODS, Osteogenic Disorder Shionogi, genotype: od/od) rats, which lack L-ascorbate (AA) synthesizing ability, are not.[14] Heterozygotes (+/od) and normal (+/+) ODS/Shi rats, which can synthesize AA, also showed resistance to SA promotion, like homozygotes (od/od), when compared with F344 and LEW rats.[14] This suggested involvement of genes other than that at the od locus in ODS rats. The ODS rats were developed from a sister strain of the WS/Shi (WS) rats which have been established by full-sibling mating of Wistar rats introduced in 1952 to Aburahi Lab., Shionogi & Co., Ltd., from the Faculty of Agriculture of Tokyo University.[15–17] Therefore, some loci of biochemical markers in ODS/Shi rats are shared by the WS strain.[17]

Dietary factors could also play important roles in determining susceptibility, as well as genetic factors. In F344, but not ODS, rats fed CLEA CA-1 diet (C) containing 5% SA, the cell proliferation rate in the urinary bladder epithelium was increased, although C was less effective in terms of SA promoting action than Oriental MF diet (M) in F344 rats.[7,18] Therefore, we can speculate that WS rats may also show resistance to the promoting effect of SA under certain dietary conditions, assuming genetic commonality with the ODS strain.

It was previously suggested that the promoting effect of sodium saccharin (SS) on rat urinary bladder carcinogenesis is related to mechanical injury by urolithiasis due to silica and $\alpha$-2u-globulin ($\alpha_{2u}$-g) deposits, these materials being formed in urine at the relatively high pH brought about by ingestion of SS.[19,20] Recently Uwagawa *et al.*[21] reported that $\alpha_{2u}$-g might also be involved in SA promotion of rat urinary bladder carcinogenesis, on the basis of their work with NCI-Black-Reiter

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(NBR) rats which lack $\alpha_{2u}$-g-synthesizing ability. If WS rats, like the ODS strain, show resistance to SA promotion, it might be for a similar reason.

The purpose of the present study was to ascertain which of the possibilities described above is actually responsible for rat strain differences in susceptibility to SA promotion of two-stage rat urinary bladder carcinogenesis. The questions of whether WS rats are resistant to SA effects, whether dietary factors generally affect susceptibility to promotion, and whether $\alpha_{2u}$-g synthesizing ability is directly related to resistance, were therefore investigated.

## MATERIALS AND METHODS

**Animals** The following 4 strains of male rats were obtained at 4 weeks of age. LEW and F344 animals were purchased from Charles River Japan, Inc., Hino, Shiga. The WS and ODS strains were obtained from Aburahi Laboratories of Shionogi & Co., Ltd., Shiga. Two kinds of diets, C (containing 250 ppm TAA; Japan CLEA Co., Ltd., Osaka) and M (containing 50 ppm TAA; Oriental Yeast Co., Tokyo), were used; their compositions were given in our previous report.[18] C was found to be a suitable commercial diet to prolong the life of ODS rats up to and beyond 36 weeks.[14] The animals were housed, 2–3 rats per cage (RT type: Charles River Japan, Inc.), in a room maintained on a 12-h (7:00–19:00) light-dark cycle, with constant conditions of temperature, 25±1°C, and relative humidity, 55±10%. All strains of rats were given C and water *ad libitum* up to 6 weeks of age, and then used for experiments. Mortality and condition were checked daily.

**Chemicals** *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN, Tokyo Kasei Kogyo Co., Ltd., Tokyo) was used as the initiator and SA (Takeda Chemical Industries, Ltd., Osaka), food additive grade (99.8% pure), was evaluated as the promoter. Rabbit anti-$\alpha_{2u}$-g-sera and purified rat $\alpha_{2u}$-g were kindly provided by Dr. K. Saito (Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd.).[22]

**Experiment 1** Forty-six LEW, 46 WS, and 46 ODS rats were fed the C diet throughout the experiment. Each strain was randomly divided into 3 groups (LEW rats: groups 1, 2 and 3; WS rats: groups 4, 5 and 6; ODS rats: groups 7, 8 and 9) as shown in Table I. In the first 4 weeks of the experiment, the 20 rats in each of groups 1, 4, and 7 were given drinking water containing 0.05% BBN by volume, and then they were given C containing 5% SA for 32 weeks. The matched control groups 2, 5, and 8, were treated with 0.05% BBN for the first 4 weeks and then fed the C diet alone. The other control groups 3, 6, and 9, each containing 6 rats, were maintained on drinking water without BBN for the first 4 weeks and then received the C containing 5% SA. The total observation period was 36 weeks. Body weights, food consumption and water intake were measured weekly up to week 4 and every other week thereafter. The amounts of food and water consumed during two consecutive days of a week were measured on a per-cage basis. Samples of urine for urinalysis were obtained from 4–11 rats in each group during experimental week 24. At the end of the experiment, all survivors were killed for pathological examination.

**Experiment 2** Eighty F344 and 80 WS rats were ran-

Table I. Data for Survival, Average Body and Urinary Bladder Weights and Intakes of BBN (wks 1–4), Water(wks 5–36), Sodium (wks 5–36) and Total Ascorbic Acid (wks 5–36) (Experiment 1)

| Group | Strain | Chemicals | Survivors | Body weight (g) | | Urinary bladder weight (mg) | BBN intake (mg/kg/day) | Water intake (ml/kg/day) | Sodium intake (mg/kg/day) | Total ascorbic acid intake (mg/kg/day) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Initial | Final | | | | | |
| 1 | LEW | BBN-SA | 20/20[a)] | 161±4[b)] | 481±25[c)] | 237±107[c,d)] | 58.6 | 71[c)] | 1,036[c)] | 4,330[c)] |
| 2 | | BBN | 19/20 | 160±4 | 524±27 | 130±17 | 58.6 | 55 | 196 | 10 |
| 3 | | SA | 6/6 | 160±4 | 486±26 | 136±8 | 0 | 68 | 906 | 3,791 |
| 4 | WS | BBN-SA | 20/20 | 185±9 | 409±24[c)] | 199±119 | 72.1 | 143[c)] | 910[c)] | 3,807[c)] |
| 5 | | BBN | 20/20 | 186±11 | 436±16 | 191±161 | 71.5 | 82 | 210 | 11 |
| 6 | | SA | 6/6 | 188±12 | 421±8 | 148±13 | 0 | 132 | 931 | 3,892 |
| 7 | ODS | BBN-SA | 19/20 | 164±22 | 486±39 | 251±154[c)] | 84.4 | 132 | 697[c)] | 2,918[c)] |
| 8 | | BBN | 17/20 | 163±19 | 517±32 | 148±37 | 84.1 | 102 | 224 | 12 |
| 9 | | SA | 6/6 | 179±10 | 502±49 | 173±37 | 0 | 145 | 848 | 3,548 |

*a*) Survivors: No. alive at the end of experiment/No. at the start.
*b*) Mean±SD.
Values for the following groups were significantly different:
*c*) 1 vs. 2, 4 vs. 5, and 7 vs. 8, $P<0.05$;
*d*) 1 vs. 3, $P<0.05$.

domly divided into 4 groups of 20 rats each (F344 rats: groups 1–4; WS/Shi rats: groups 5–8) as shown in Table V. In the first 4 weeks of the experiment, all rats were fed diet C and given drinking water containing 0.05% BBN by volume. During the experimental weeks 4–36, groups 1, 2, 5, and 6 were fed diet M, and groups 3, 4, 7, and 8 received diet C. Groups 1, 3, 5 and 7 were given the 5% SA supplement for 32 weeks, with groups 2, 4, 6, and 8 being the matched controls. Body weights, food consumption and water intake were measured as for experiment 1. Samples of urine for urinalysis were obtained from 20 rats in each group during experimental week 24. At the end of the experiment, all survivors were killed for pathological examination.

**Urinalysis** Fresh urine samples obtained by forced urination in the morning (9:00–11:00 a.m.) were used for measurement of pH. To determine urinary volume, sodium ion concentration, total ascorbic acid (TAA) concentration and sediment, rats were individually housed in metabolic cages without diet and water for 4 h (9:00–13:00). The TAA concentration was estimated by the 2,4-dinitrophenylhydrazine colorimetric method[23] and the sodium ion concentration by atomic absorbance chemical analysis (AA-175 atomic absorption spectrophotometers; Varian Techtron Co., Canberra, Australia).

**Pathological examination** In experiments 1 and 2, rats were killed by decapitation and the urinary bladders were inflated and fixed with 10% phosphate-buffered formalin (pH 7.4). They were weighed, cut into 12 pieces and routinely embedded in paraffin. Sections were stained with hematoxylin and eosin (HE) for light microscopic examination. Histopathological lesions of urinary bladder epithelium were classified into 3 categories: papillary or nodular (PN) hyperplasia, papilloma and carcinoma, as described previously.[24] For quantitative analysis, numbers were counted under the light microscope, and the total length of the basement membrane examined was measured with an image processor (COSMOZONE-S; Nippon Kogaku K.K., Tokyo).[18]

In experiment 1 both kidneys of all survivors and of some of the animals which died were also removed and fixed in 10% buffered formalin (pH 7.4), and midsagittal sections were processed for paraffin embedding. Sections were stained with HE for microscopic assessment of nephropathy, which was graded from 1+ to 4+ in severity according to the criteria described by Rao *et al.*[25]

**Experiment 3**

*Analysis of the existence of $\alpha_{2u}$-g in renal tubules and urine*: Kidney (from 5 animals each of the WS and F344 strains) and urine (from 3 animals each of the WS, ODS and F344 strains) samples from ten week-old males were respectively used for immunohistochemical and western blotting investigation of $\alpha_{2u}$-g. After urine had been individually collected by forced micturition, WS and F344 rats were anesthetized with pentobarbital and perfused via the aorta with 10% phosphate-buffered (pH 7.4) formalin. The kidneys were removed, fixed by immersion in 10% phosphate-buffered (pH 7.4) formalin and routinely embedded in paraffin, sectioned and stained with HE for histological examination. Immunohistochemical staining was carried out to confirm the existence and localization of $\alpha_{2u}$-g using the streptavidinbiotin-alkaline phosphatase complex method (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA).

Western blotting was performed as described by Matsuda *et al.*[26] Urine samples from experiment 3 containing 1 mg protein were loaded and electrophoresed on a 12.5% (w/v) polyacrylamide slab gel containing 0.1% (w/v) sodium dodecyl sulfate (SDS) according to the method of Laemmli after treatment with 4% (w/v) SDS and 10% (v/v) 2-mercaptoethanol.[26, 27] Purified rat $\alpha_{2u}$-g (10 $\mu$g) was used as a control marker for SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes (125 mA/cm$^2$, 90 min) and incubation in TPBS (10 m*M* sodium phosphate, 0.9% sodium chloride, pH 7.5, 0.05% Tween 20) for 30 min, rabbit anti-$\alpha_{2u}$-g serum was added in TPBS containing 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (1 : 10000). The blots were incubated for an additional 1 h at room temperature, then washed in TPBS, and specific reactive proteins were detected using a Vectastain ABC Kit.

**Data evaluation** Statistical analysis of histopathological lesion incidences was performed with the Fisher exact probability test. The other data were evaluated by analysis of variance.[28, 29]

## RESULTS

**Experiment 1** There were no symptoms of toxicity (such as diarrhea) in LEW rats given 5% SA in diet, but WS and ODS rats occasionally showed loose feces. Scurvy was, however, not observed in ODS rats fed basal diet without 5% SA throughout the experiment. Data for survival, body weights, urinary bladder weights, and intakes of BBN, water, sodium and TAA are summarized in Table I. No significant variation in mortality was observed among the groups, although there was a tendency for fewer survivors in the ODS strain. All rats which died had pyelonephritis without urinary bladder tumors. The final average body weights of rats given BBN-SA, were reduced by approximately 5–10%, as compared to those of matched controls given BBN alone, the difference often attaining significance. The urinary bladder weights in LEW and ODS, but not WS rats given BBN-SA were significantly higher than the BBN-alone values (groups 1 versus 2, and 7 versus 8). Intakes of BBN were the same for the BBN alone and BBN-SA

Table II. Data for Urinary Bladder Lesions in Rats Treated with BBN Followed by Sodium L-Ascorbate (SA) (Experiment 1)

| Group | Strain | Chemicals | Effective number of rats | No. of rats with urinary bladder lesions | | | |
|---|---|---|---|---|---|---|---|
| | | | | PN hyperplasia | Papilloma | Carcinoma | |
| | | | | (%) | (%) | (%) | No./10 cm of BM |
| 1 | LEW | BBN-SA | 20 | 17 (85)[a)] | 17 (85)[a)] | 13 (65)[a, b)] | 1.1±1.5 |
| 2 | | BBN | 19 | 2 (11)[c)] | 0[c)] | 0 | 0 |
| 3 | | SA | 6 | 0 | 0 | 0 | 0 |
| 4 | WS | BBN-SA | 20 | 16 (80) | 16 (80) | 5 (25) | 0.2±0.4 |
| 5 | | BBN | 20 | 15 (75) | 11 (55) | 3 (15) | 0.1±0.2 |
| 6 | | SA | 6 | 0 | 0 | 0 | 0 |
| 7 | ODS | BBN-SA | 19 | 13 (68) | 16 (84) | 8 (42) | 0.4±0.6 |
| 8 | | BBN | 17 | 14 (82) | 12 (71) | 3 (18) | 0.2±0.4 |
| 9 | | SA | 6 | 0 | 0 | 0 | 0 |

PN, papillary or nodular; BM, basement membrane; data are mean values±SD.
Values for the following groups were significantly different:
*a*) 1 vs. 2, $P<0.05$;
*b*) 1 vs. 4, $P<0.01$;
*c*) 2 vs. 5, and 8, $P<0.01$.

Table III. Severity of Nephropathy (Experiment 1)

| Group | Strain | Chemicals | Number of rats examined | Pathological grade | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Negative | 1+ | 2+ | 3+ | 4+ |
| 1 | LEW | BBN-SA | 20 | 6 | 14 | 0 | 0 | 0 |
| 2 | | BBN | 20 | 14 | 6 | 0 | 0 | 0 |
| 3 | | SA | 6 | 0 | 6 | 0 | 0 | 0 |
| 4 | WS | BBN-SA | 20 | 0 | 0 | 5 | 13 | 2 |
| 5 | | BBN | 20 | 0 | 0 | 1 | 12 | 7 |
| 6 | | SA | 6 | 0 | 0 | 1 | 5 | 0 |
| 7 | ODS | BBN-SA | 20 | 9 | 9 | 2 | 0 | 0 |
| 8 | | BBN | 18 | 4 | 13 | 1 | 0 | 0 |
| 9 | | SA | 6 | 1 | 5 | 0 | 0 | 0 |

1+, slight; 2+, mild; 3+, severe; 4+, marked.

treated rats, the amounts being in the order ODS > WS > LEW rats. Administration of SA was associated with increased water intake (WS > LEW > ODS). The intakes of sodium and TAA in rats given SA alone or BBN-SA were higher than those in rats given BBN alone.

Data for histopathological lesions in the urinary bladders are summarized in Table II. LEW rats given BBN alone (group 2) had no carcinomas, whereas WS and ODS rats (groups 5 and 8) demonstrated approximately 20% incidences. SA administration significantly increased the development of carcinomas only in the LEW case. Similarly, the incidences of PN hyperplasia and papillomas were only increased by SA in the LEW strain, the WS and ODS animals having high values independently of promoter treatment.

Data for severity of nephropathy are summarized in Table III. The lesions were slight to mild in LEW and ODS rats and severe to marked in WS rats.

The results of urinalyses at experimental week 24 are summarized in Table IV. The urinary pH and the urinary concentrations of sodium ions were increased by the administration of SA without any inter-strain differences, although the absolute $Na^+$ levels achieved were highest in the LEW case. The TAA concentrations were also highest in LEW rats and very much lower in the ODS strain, with SA-associated elevation noted for all strains. No consistent changes in urinary volume were observed in response to SA. Increase of sediment crystals due to SA treatment was larger in LEW than in ODS and WS rats (data not shown) but at autopsy, no urolithiasis was found in any group.

**Experiment 2** There were no symptoms of toxicity such as diarrhea in F344 or WS rats given 5% SA in the diet. Data for survival, body weights, urinary bladder weights, and intakes of BBN, water, sodium and TAA are summarized in Table V. One spontaneous death occurred, of a WS rat with pyelonephritis but no urinary bladder tumor. Final average body weights were significantly lower in WS rats given BBN-SA than in the matched controls given BBN alone. Urinary bladder weights of F344, but not WS were clearly increased by SA treatment, especially in animals receiving the C–M dietary schedule. No intergroup variation in BBN intake was noted. The administration of SA increased both water and sodium intake: the order of increase was group 7 > group 5 > group 3 > group 1. The increase in TAA intake due to SA was less in F344 than in WS rats.

Table IV. Urinalysis Data at Experimental Week 24 for Rats Treated with BBN Followed by Sodium L-Ascorbate (SA) (Experiment 1)

| Group | Strain | Chemicals | pH | Sodium ion (mg/100 ml) | Total ascorbic acid (mg/100 ml) | Urinary volume (ml/4 h) |
|---|---|---|---|---|---|---|
| 1 | LEW | BBN-SA | 7.5±0.4[a, b)] | 648±509[b)] | 113±107[b)] | 2.3±1.3 |
| 2 | | BBN | 6.6±0.1 | 245±99 | 15±8 | 2.7±1.6 |
| 3 | | SA | 7.3±0.2 | 537±154 | 89±27 | 1.5±0.9 |
| 4 | WS | BBN-SA | 7.4±0.3[b)] | 436±333[b)] | 76±49[b)] | 1.9±1.4 |
| 5 | | BBN | 6.5±0.2 | 198±42 | 5±2 | 2.3±0.6 |
| 6 | | SA | 7.3±0.2 | 341±70 | 71±17 | 3.6±1.7 |
| 7 | ODS | BBN-SA | 7.4±0.3[b)] | 312±83[b)] | 17±6[c)] | 7.8±2.8[c)] |
| 8 | | BBN | 6.9±0.3 | 192±60 | 1±1[d)] | 6.5±1.6[d)] |
| 9 | | SA | 7.5±0.2 | 309±68 | 16±5[e)] | 4.6±1.2[f)] |

*a*) Mean±SD: Numbers of samples were 9–11 for groups 1, 2, 4, 5, 7, and 8 and were 4–6 for groups 3, 6, and 9. Values for the following groups were significantly different:
*b*) 1 vs. 2, 4 vs. 5, and 7 vs. 8, $P<0.05$;
*c*) 7 vs. 1, and 4, $P<0.05$;
*d*) 8 vs. 2, and 5, $P<0.05$;
*e*) 9 vs. 3, 6, $P<0.05$;
*f*) 9 vs. 3, $P<0.05$.

Table V. Data for Survival, Average Body and Urinary Bladder Weights, and Intakes of BBN (wks 1–4), Water (wks 5–36), Sodium (wks 5–36) and Total Ascorbic Acid (wks 5–36) (Experiment 2)

| Group | Strain | Diet[a)] | Chemicals | Survivors | Body weight (g) | | Urinary bladder weight (mg) | BBN intake (mg/kg/day) | Water intake (ml/kg/day) | Sodium intake (mg/kg/day) | Total ascorbic acid intake (mg/kg/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Initial | Final | | | | | |
| 1 | F344 | C-M | BBN-SA | 20/20[b)] | 130±4[c)] | 415±21 | 707±512[d, e, f)] | 72.6 | 73[d, e, f)] | 375[d, e, f)] | 1,934[g)] |
| 2 | | C-M | BBN | 20/20 | 130±4 | 422±16 | 111±8 | 72.8 | 48[f)] | 89[e)] | 1 |
| 3 | | C-C | BBN-SA | 20/20 | 130±5 | 411±15 | 296±210 | 71.9 | 87[d, f)] | 432[d, f)] | 1,881[h)] |
| 4 | | C-C | BBN | 20/20 | 131±4 | 409±16 | 122±15 | 73.9 | 60 | 184 | 10 |
| 5 | WS | C-M | BBN-SA | 19/20 | 198±13 | 422±16[d)] | 153+33 | 69.1 | 117[d, e)] | 471[d, e)] | 2,429 |
| 6 | | C-M | BBN | 20/20 | 194±14 | 476±18 | 129±27 | 70.0 | 66[e)] | 102[e)] | 1 |
| 7 | | C-C | BBN-SA | 20/20 | 196±11 | 405±12[d)] | 185±57 | 70.0 | 146[d)] | 539[d)] | 2,348 |
| 8 | | C-C | BBN | 20/20 | 193±11 | 440±20 | 150±50 | 70.4 | 82 | 214 | 12 |

*a*) Diet: (0–4 wks)-(5–36 wks); C, CA-1 diet; M, MF diet.
*b*) Survivors; No. alive at the end of experiment/No. at the start.
*c*) Mean±SD.
Values for the following groups were significantly different:
*d*) 1 vs. 2, 3 vs. 4, and 5 vs. 6, $P<0.05$;
*e*) 1 vs. 3, 2 vs. 4, 5 vs. 7, and 6 vs. 8, $P<0.05$;
*f*) 1 vs. 5, 2 vs. 6, and 3 vs. 7, $P<0.05$;
*g*) 1 vs. 5, 7, $P<0.01$;
*h*) 3 vs. 5, 7, $P<0.01$.

Data for histopathological lesions of the urinary bladder are summarized in Table VI. F344 rats treated with BBN alone had no carcinomas, whereas WS rats had an approximately 10% incidence with either diet. However, this latter was not influenced by SA-treatment whereas strong promotion was noted in the F344 strain. Similar data were obtained for papillomas and, to a lesser extent, PN hyperplasia.

The results of the urinalysis at experimental week 24 are summarized in Table VII. SA-treatment caused an elevation of urinary pH and increased concentrations of sodium ion and TAA in both WS and F344 rats. Urolithiasis was not found.

**Experiment 3** The results of immunohistochemical staining using anti-$\alpha_{2u}$-g-sera are illustrated in Fig. 1. The kidneys of rats used in Experiment 3 were all anti-$\alpha_{2u}$-g

Table VI. Data for Urinary Bladder Lesions in Rats Treated with BBN Followed by Sodium L-Ascorbate (SA) in Different Diets (Experiment 2)

| Group | Strain | Diet[a)] | Chemicals | Effective number of rats | No. of rats with urinary bladder lesions | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | PN hyperplasia | Papilloma | Carcinoma | |
| | | | | | (%) | (%) | (%) | No./10 cm of BM |
| 1 | F344 | C-M | BBN-SA | 20 | 20 (100)[b)] | 18 (90)[c)] | 19 (95)[b)] | 1.9±0.9[d)] |
| 2 | | C-M | BBN | 20 | 8 (40) | 4 (20) | 0 | 0 |
| 3 | | C-C | BBN-SA | 20 | 15 (75) | 15 (75)[e)] | 12 (60) | 0.9±1.1[e,f)] |
| 4 | | C-C | BBN | 20 | 11 (55) | 4 (20) | 0 | 0 |
| 5 | WS | C-M | BBN-SA | 19 | 9 (47) | 14 (74) | 2 (11) | 0.1±0.2 |
| 6 | | C-M | BBN | 20 | 11 (55) | 10 (50) | 3 (15) | 0.1±0.3 |
| 7 | | C-C | BBN-SA | 20 | 12 (60) | 13 (65) | 5 (25) | 0.2±0.4 |
| 8 | | C-C | BBN | 20 | 7 (35) | 7 (35) | 2 (10) | 0.1±0.2 |

PN, papillary or nodular; BM, basement membrane; Values are mean±SD.
*a*) Diet: (0–4 wks)-(5–36 wks); C, CA-1 diet; M, MF diet.
Values for the following groups were significantly different:
*b*) 1 vs. 2, 4–8, $P<0.01$;
*c*) 1 vs. 2, $P<0.01$;
*d*) 1 vs. 2–8, $P<0.01$;
*e*) 3 vs. 4, $P<0.01$;
*f*) 3 vs. 7, $P<0.05$.

Table VII. Urinalysis Data at Experimental Week 24 for Rats Treated with BBN Followed by Sodium L-Ascorbate (SA) with Different Diets (Experiment 2)

| Group | Strain | Diet[a)] | Chemicals | pH | Sodium ion (mg/100 ml) | Total ascorbic acid (mg/100 ml) | Urinary volume (ml/4 h) |
|---|---|---|---|---|---|---|---|
| 1 | F344 | C-M | BBN-SA | 8.6±0.7[b,c)] | 258±93[c)] | 51±15[c)] | 4.5±1.0[c)] |
| 2 | | C-M | BBN | 6.8±0.7 | 79±56 | 3±1 | 3.0±1.0 |
| 3 | | C-C | BBN-SA | 8.9±0.4 | 217±71 | 40±13[c)] | 4.2±1.0 |
| 4 | | C-C | BBN | 7.4±0.9 | 123±85 | 3±1 | 4.4±1.0 |
| 5 | WS | C-M | BBN-SA | 7.6±0.7 | 166±74 | 43±21[c)] | 3.7±1.2 |
| 6 | | C-M | BBN | 7.7±0.5 | 122±39 | 2±1 | 4.6±0.8 |
| 7 | | C-C | BBN-SA | 8.3±0.8[c)] | 230±107[c)] | 41±26[c)] | 2.8±1.1[c)] |
| 8 | | C-C | BBN | 6.8±1.0 | 149±48 | 2±1 | 4.6±1.2 |

*a*) Diet: (0–4 wks)-(5–36 wks); C, CA-1 diet; M, MF diet.
*b*) Mean±SD: Number of samples was 20 in each group.
Values for the following groups were significantly different:
*c*) Groups 1 vs. 2, 3 vs. 4, 5 vs. 6, and 7 vs. 8, $P<0.05$.

positive, without any marked differences in the degree of immunohistochemical staining or nephropathy. Fig. 2 shows the results of western blotting of urine samples from WS, ODS and F344 rats. The $\alpha_{2u}$-g-positive bands migrated to the same position as purified $\alpha_{2u}$-g in all cases, with no differences in the densities of the bands being observed among the strains.

## DISCUSSION

The present work clearly demonstrated that WS rats, which are related to the ODS strain, are also resistant to SA-promoting effects on urinary bladder carcinogenesis, independently of the diet given in the post-initiation period. Furthermore, we revealed using immunohistochemical and western blot analyses that WS and ODS rats, unlike NBR rats, can synthesize $\alpha_{2u}$-g. The observed strain differences among ODS, WS, F344 and LEW rats are similar to those reported earlier with regard to promotion of hepatocarcinogenesis.[30,31)] The available data strongly suggest that genes other than that at the od locus in ODS rats may be responsible for the differences in susceptibility to promotion by SA, rather than dietary factors or the influence of $\alpha_{2u}$-g. The remarkable strain

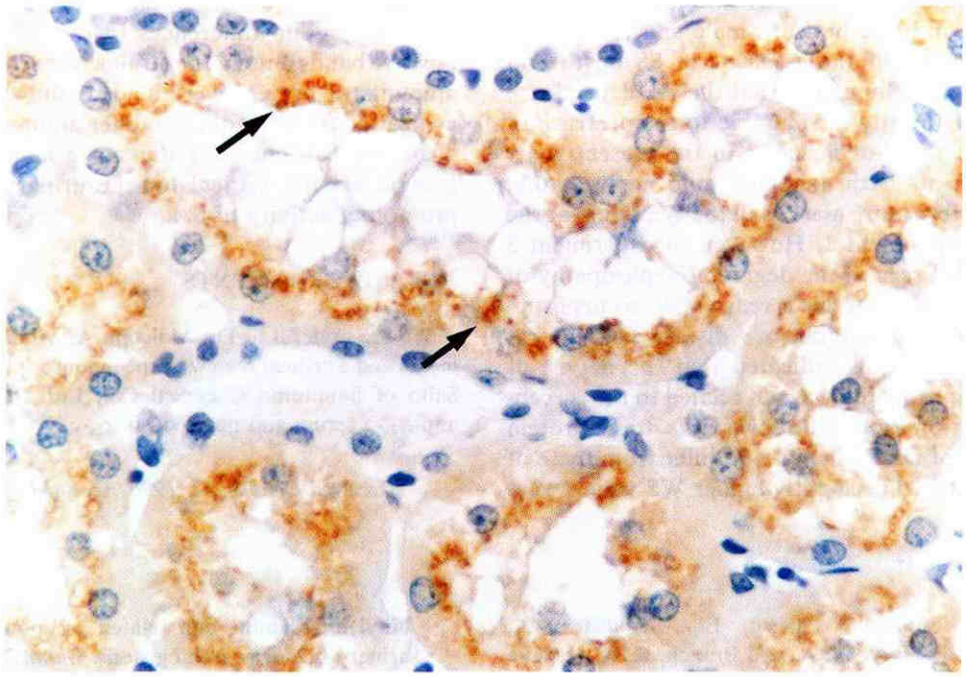

Fig. 1. Section through the cortex of WS rat kidney immunostained for $\alpha_{2u}$-g. ×400. Droplets (arrows) are stained positively.

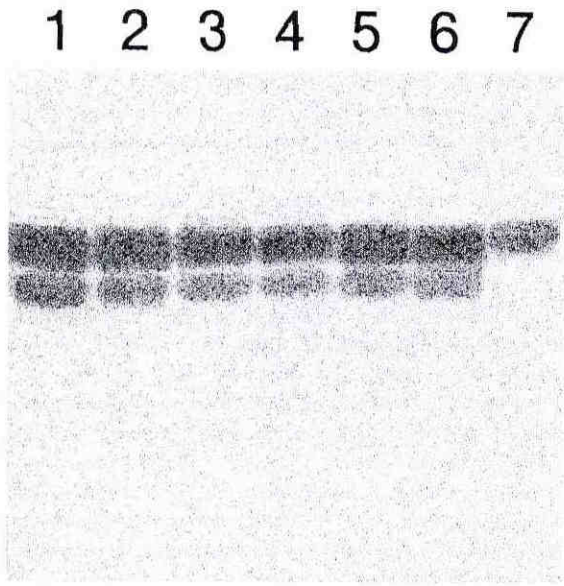


Fig. 2. Western blot of rat urine. Lanes 1 and 2, F344; lanes 3 and 4, ODS; lanes 5 and 6, WS; lane 7, $\alpha_{2u}$-g used as standard protein.

difference in susceptibility to the promoting effect of SA is most likely based on genetic difference among rat strains. Our parallel study (Kamoto *et al.*, to be published elsewhere) revealed that the strain difference in promoter effect of SA between F344 and WS strains involves at least two host genes.

Urine samples from WS, ODS, F344 and LEW rats treated with SA all showed increased pH and concentrations of sodium ion and TAA, in accordance with previous reports.[32] Sodium salts of organic acids such as sodium saccharin, sodium *O*-phenylphenate, sodium erythorbate, trisodium nitrilotriacetate and sodium succinate share the potential to promote urinary bladder carcinogenesis in male F344 rats fed on M, as well as to similarly affect these parameters.[33–38] Previous studies focusing on urinary contents have indicated that urinary $Na^+$ and $K^+$ concentrations, as well as pH, play important roles in promotion as well as DNA synthesis and cell proliferation.[39,40] Urinary $Na^+$ can be transported into the transitional epithelial cells of urinary bladders in the rat and rabbit,[41,42] but the degree of permeability may differ among species and strains. If so, this might play a role in the susceptibility to SA promotion. Further studies of proliferation and other intracellular biological parameters are clearly warranted to assess this possibility.

Previous reports have demonstrated that different levels of protein in urine between male and female rats are related to the proliferative effects of SS on the rat urinary bladder epithelium, and that the sex dependence of nephropathy is mostly attributable to the presence of $\alpha_{2u}$-g only in the urine of males.[20] In the present study LEW and ODS rats both exhibited mild nephropathy, whereas lesions were more marked in the WS strain at the termination of experiment 1. However, in experiment 3 no differences were seen in the degree of nephropathy of the ten week-old rats used; this is reasonable, as nephropathy is an age-related spontaneous disease in most rat strains. The findings thus indicated that the degree of progression of nephropathy was not related to the susceptibility to SA promotion. Moreover, the total protein level in the urine of ODS rats did not differ from these of F344 or LEW rats, while slightly higher WS values were found using test sticks (data not shown). On western blot analysis no differences in the density of the $\alpha_{2u}$-g positive bands among the strains was seen, when identical amounts of protein were loaded, showing that the concentrations of $\alpha_{2u}$-g did not differ among the strains used. Thus, we can conclude that $\alpha_{2u}$-g and protein levels in general are not critical for the urinary bladder tumor-promoting activity of SA.

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REFERENCES

1) Kaisary, A., Smith, P., Jaczq, E., McAllister, B., Wilkinson, G. R., Ray, W. A. and Branch, R. A. Genetic predisposition to bladder cancer: ability to hydroxylate debrisoquine and mephenytoin as risk factors. *Cancer Res.*, **47**, 5488–5493 (1987).
2) Silverberg, E. Statistical and epidemiologic data on urologic cancer. *Cancer*, **60**, 692–717 (1987).
3) Ito, N., Arai, M., Sugihara, S., Hirao, K., Makiura, S., Matayoshi, K. and Denda, A. Experimental urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Gann Monogr. Cancer Res.*, **17**, 367–381 (1975).
4) Ito, N. Early changes caused by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the bladder epithelium of different animal species. *Cancer Res.*, **36**, 2528–2531 (1976).
5) Mori, S., Takeuchi, Y., Toyama, M., Makino, S., Harauchi, T., Kurata, Y. and Fukushima, S. Assessment of L-ascorbic acid requirement for prolonged survival in ODS rats and their susceptibility to urinary bladder carcinogenesis by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Cancer Lett.*, **38**, 275–282 (1988).
6) Nakanowatari, J., Fukushima, S., Imaida, K., Ito, N. and Nagase, S. Strain differences in N-butyl-N-(4-hydroxybutyl)nitrosamine bladder carcinogenesis in rats. *Jpn. J. Cancer Res. (Gann)*, **79**, 453–459 (1988).
7) Fukushima, S., Arai, M., Nakanowatari, J., Hibino, T., Okuda, M. and Ito, N. Differences in susceptibility to sodium saccharin among various strains of rats and other animal species. *Gann*, **74**, 8–20 (1983).
8) Takahashi, M., Shumiya, S., Maekawa, A., Hayashi, Y. and Nagase, S. High susceptibility of an analbuminemic congenic strain of rats with an F344 genetic background to induced bladder cancer and its possible mechanism. *Jpn. J. Cancer Res. (Gann)*, **79**, 705–709 (1988).
9) Suzuki, E., Anjo, T., Aoki, J. and Okada, M. Species variations in the metabolism of N-butyl-N-(4-hydroxybutyl)nitrosamine and related compounds in relation to urinary bladder carcinogenesis. *Gann*, **74**, 60–68 (1983).
10) Garland, E. M., St. John, M., Asamoto, M., Eklund, S. H., Mattson, B. J., Johnson, L. S., Cano, M. and Cohen, S. M. A comparison of the effects of sodium saccharin in NBR rats and in intact and castrated male F344 rats. *Cancer Lett.*, **78**, 99–107 (1994).
11) Chappel, C. I. A review and biological risk assessment of sodium saccharin. *Regul. Toxicol. Pharmacol.*, **15**, 253–270 (1992).
12) Hicks, R. M. Multistage carcinogenesis in the urinary bladder. *Br. Med. Bull.*, **36**, 39–46 (1980).
13) Ito, N. and Fukushima, S. Promotion of urinary bladder carcinogenesis in experimental animals. *Exp. Pathol.*, **36**, 1–15 (1989).
14) Mori, S., Murai, T., Takeuchi, Y., Hosono, M., Ohhara, T., Makino, S., Hayashi,Y., Shibata, M.-A., Kurata, Y., Hagiwara, A. and Fukushima, S. No promotion of urinary bladder carcinogenesis by sodium L-ascorbate in male ODS/Shi-od/od rats lacking L-ascorbic acid-synthesizing ability. *Carcinogenesis*, **12**, 1869–1873 (1991).
15) Horio, F., Ozaki, K., Yoshida, A., Makino, S. and Hayashi, Y. Ascorbic acid requirement in a rat mutant unable to synthesize ascorbic acid. *J. Nutr.*, **115**, 1630–1640 (1985).
16) Horio, F., Ozaki, K., Kohmura, M., Yoshida, A., Makino, S. and Hayashi, Y. Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. *J. Nutr.*, **116**, 2278–2289 (1986).
17) Makino, S., Yamashita, H., Harada, M., Konishi, T. and Hayashi, Y. Genetic monitoring of the ODS rat. *In* "Vitamin C and the Scurvy-Prone ODS Rat," ed. T. Fujita, M. Fukase and T. Konishi, pp. 29–34 (1982). Elsevier, Amsterdam.

18) Mori, S., Kurata, Y., Takeuchi, Y., Toyama, M., Makino, S. and Fukushima, S. Influences of strain and diet on the promoting effects of sodium L-ascorbate in two-stage urinary bladder carcinogenesis in rats. *Cancer Res.*, **47**, 3492–3495 (1987).
19) Cohen, S. M., Cano, M., Earl, R. A., Carson, S. D. and Garland, E. M. A proposed role for silicates and protein in the proliferative effects of saccharin on the male rat urothelium. *Carcinogenesis*, **12**, 1551–1555 (1991).
20) Hard, G. C. Species comparison of the content and composition of urinary proteins. *Food Chem. Toxicol.*, **33**, 731–746 (1995).
21) Uwagawa, S., Saito, K., Okuno, Y., Kawasaki, H., Yoshitake, A., Yamada, H. and Fukushima, S. Lack of induction of epithelial cell proliferation by sodium saccharin and sodium L-ascorbate in the urinary bladder of NCI-Black-Reiter (NBR) male rats. *Toxicol. Appl. Pharmacol.*, **127**, 182–186 (1994).
22) Saito, K., Uwagawa, S., Kaneko, H. and Yoshitake, A. Behavior of alpha-2u-globulin accumulation in kidneys of male rats treated with *d*-limonene: kidney-type alpha-2u-globulin in the urine as a marker of *d*-limonene nephropathy. *Toxicology*, **79**, 173–183 (1991).
23) Roe, J. H. and Kuether, C. A. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.*, **147**, 399–407 (1943).
24) Fukushima, S., Murasaki, G., Hirose, M., Nakanishi, K., Hasegawa, R. and Ito, N. Histopathological analysis of preneoplastic changes during *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. *Acta Pathol. Jpn.*, **32**, 243–250 (1982).
25) Rao, G. N., Edmondson, J. and Elwell, M. R. Influence of dietary protein concentration on severity of nephropathy in Fischer-344 (F-344/N) rats. *Toxicol. Pathol.*, **21**, 353–361 (1993).
26) Matsuda, T., Imaoka, S., Funae, Y., Otori, K. and Fukushima, S. Induction of CYP isoenzymes in various organs of rats by 3-methylcholanthrene or $\beta$-naphthoflavone. *Cancer Lett.*, **97**, 137–143 (1995).
27) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
28) Scheffe, H. A. "The Analysis of Variance" (1959), Wiley, New York.
29) Dunn, O. J. Multiple comparisons among means. *J. Am. Stat. Assoc.*, **56**, 52–64 (1961).
30) Asamoto, M., Tsuda, H., Kagawa, M., de Camargo, J. L. V., Ito, N. and Nagase, S. Strain differences in susceptibility to 2-acetylaminofluorene and phenobarbital promotion of rat hepatocarcinogenesis in a medium-term assay system: quantitation of glutathione *S*-transferase P-positive foci development. *Jpn. J. Cancer Res.*, **80**, 939–944 (1989).
31) Asamoto, M., Tsuda, H., Kato, T., Ito, N., Masuko, T., Hashimoto, Y. and Nagase, S. Strain differences in susceptibility to 2-acetylaminofluorene and phenobarbital promotion of hepatocarcinogenesis: immunohistochemical analysis of cytochrome P-450 isozyme induction by 2-acetylaminofluorene and phenobarbital. *Jpn. J. Cancer Res.*, **80**, 1041–1046 (1989).
32) Fukushima, S., Shibata, M.-A., Shirai, T., Tamano, S. and Ito, N. Role of urinary sodium ion concentration and pH in promotion by ascorbic acid of urinary bladder carcinogenesis in rats. *Cancer Res.*, **46**, 1623–1626 (1986).
33) Otoshi, T., Iwata, H., Yamamoto, S., Murai, T., Yamaguchi, S., Matsui-Yuasa, I., Otani, S. and Fukushima, S. Severity of promotion by sodium salts of succinic acid in rat urinary bladder carcinogenesis correlates with sodium ion concentration under conditions of equal urinary pH. *Carcinogenesis*, **14**, 2277–2281 (1993).
34) Cohen, S. M., Arai, M., Jacobs, J. B. and Friedell, G. H. Promoting effect of saccharin and DL-tryptophan in urinary bladder carcinogenesis. *Cancer Res.*, **39**, 1207–1217 (1979).
35) Fukushima, S., Kurata, Y., Shibata, M.-A., Ikawa, E. and Ito, N. Promoting effect of sodium *O*-phenylphenate and *O*-phenylphenol on two-stage urinary bladder carcinogenesis in rats. *Gann*, **74**, 625–632 (1983).
36) Fukushima, S., Imaida, K., Sakata, T., Okamura, T., Shibata, M.-A. and Ito, N. Promoting effects of sodium L-ascorbate on two-stage urinary bladder carcinogenesis in rats. *Cancer Res.*, **43**, 4454–4457 (1983).
37) Fukushima, S., Kurata, Y., Shibata, M.-A., Ikawa, E. and Ito, N. Promotion by ascorbic acid, sodium erythorbate and ethoxyquin of neoplastic lesions in rats initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Cancer Lett.*, **23**, 29–37 (1984).
38) Fukushima, S., Thamavit, W., Kurata, Y. and Ito, N. Sodium citrate: a promoter of bladder carcinogenesis. *Jpn. J. Cancer Res. (Gann)*, **77**, 1–4 (1986).
39) Moolenaar, W. H., Tsien, R. Y., van Der Saag, P. T. and de Laat, S. W. $Na^+/H^+$ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature*, **304**, 645–648 (1983).
40) L'Allemain, G., Paris, S. and Pouyssegur, J. Growth factor action and intracellular pH regulation in fibroblasts. *J. Biol. Chem.*, **259**, 5809–5815 (1984).
41) Lewis, S. A., Eaton, D. C. and Diamond, J. M. The mechanism of $Na^+$ transport by rabbit urinary bladder. *J. Membr. Biol.*, **28**, 41–70 (1976).
42) Hotta, K., Yamamoto, Y., Imaida, K., Oshima, M., Fukushima, S. and Ito, N. Membrane potential of rat urinary epithelial cells. *Jpn. J. Physiol.*, **33**, 139–143 (1983).