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Timing Effects of Uracil-induced Urolithiasis on Amplification of Second-stage Promotion in Rat Bladder Carcinogenesis

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The post-initiation enhancing activities of the non-genotoxic agent $NaHCO_3$ and the genotoxic agent *N*-ethyl-*N*-(4-hydroxybutyl)nitrosamine (EHBN) in combination with uracil-induced urolithiasis were investigated in a rat bladder carcinogenesis model. Animals were treated with 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) for 4 weeks, and then 3% uracil was given for 3 weeks in the early (weeks 4–7), middle (weeks 8–11) or late (weeks 12–15) post-initiation phase. In addition, administration of 3% $NaHCO_3$, 20 ppm EHBN or no chemical supplement was performed for the 13 weeks when the rats were not receiving BBN or uracil. $NaHCO_3$ in sequential combination with early and middle stages uracil treatment strongly enhanced tumorigenesis in the urinary bladder, while EHBN treatment amplified lesion development at the middle stage only of uracil treatment. DNA synthesis and associated epithelial surface alterations observed by scanning electron microscopy tended to be increased in the $NaHCO_3$ and EHBN groups without BBN initiation, independently of uracil treatment timing. The present results demonstrated that uracil-induced urolithiasis during the middle post-initiation phase is highly active in enhancing bladder tumor development under the influence of a promoter or carcinogen.

Key words: Urinary bladder — Tumor promotion — Amplification — Uracil — Rat

Carcinogenesis in experimental systems can generally be divided into initiation, promotion and progression processes. During bladder carcinogenesis induced by various chemicals, epithelial lesions pass through several stages, i.e. beginning with simple hyperplasia at an early stage, developing to papillary or nodular hyperplasia in the middle stage and eventually progressing to papilloma and carcinoma at a late stage.[1–4]

It is becoming increasingly evident that an increment of DNA synthesis in target tissues can play a crucial role in the carcinogenic process; tissues are more susceptible to promoter or carcinogen treatment when they are stimulated to proliferate, as is, for example, the liver when subjected to partial hepatectomy.[5,6] The timing of the proliferative stimulus by partial hepatectomy is also important, as shown for hepatocellular preneoplastic lesion development.[7,8]

Previously, it was demonstrated that treatment with uracil in the diet results in urolithiasis[9] associated with papillomatosis in the urinary bladder of rats and mice.[10,11] While both calculi and bladder lesions disappear when the uracil administration is ceased,[10,12] it has been demonstrated that uracil treatment over only a relatively short time period can strongly enhance tumor development in the urinary bladder of rats pretreated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN[3]).[13] In addition, uracil administration in sequential combination with other agents during the post-BBN initiation stage results in enhanced activity, as documented for the promoter $NaHCO_3$ or the carcinogen *N*-ethyl-*N*-(4-hydroxybutyl)nitrosamine (EHBN) in a 20-week experimental model.[14] The purpose of the present study was to investigate the comparative influence of timing of uracil-induced urolithiasis on the enhancing activity of the non-mutagen $NaHCO_3$ and the mutagen EHBN.

MATERIALS AND METHODS

Animals A total of 135 male Fischer 344 rats, 5 weeks of age, were purchased from Charles River Japan, Inc., Atsugi, and housed 5 per plastic cage in an environment-controlled room maintained at 22±2°C and artificially illuminated for 12 h each day. The animals were quarantined for 1 week prior to the start of the experiment.

Chemicals BBN was obtained from Tokyo Kasei Kogyo Co., Tokyo; uracil was from Yamasa Shoyu Co., Choshi; $NaHCO_3$ was from Wako Pure Chemical Industries, Osaka. EHBN was provided by courtesy of Dr. M. Okada of the Tokyo Biochemical Research Institute, Tokyo.

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[3] Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; BrdU, 5-bromo-2′-deoxyuridine; EHBN, *N*-ethyl-*N*-(4-hydroxybutyl)nitrosamine; RBC, red blood cells; SEM, scanning electron microscopy; WBC, white blood cells.

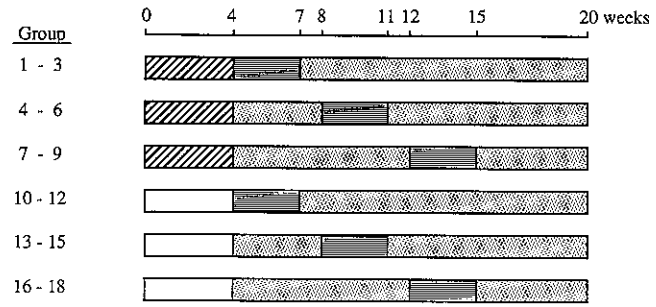

Fig. 1. Experimental protocol for evaluation of the enhancing effects of uracil on rat two-stage bladder carcinogenesis at different time points. Animals: 6-week-old, male F344 rats. ▨: 0.05% BBN in drinking water. ▤: 3% Uracil in diet. ▩: 3% $NaHCO_3$ in diet, 20 ppm EHBN in drinking water or no treatment.

Experimental procedure The experimental protocol followed is shown in Fig. 1. The animals were randomly divided into 18 groups of 5 or 10 rats each. Ten rats each in groups 1 to 9 were given drinking water containing 0.05% BBN for 4 weeks and then treated with 3% uracil in the diet (Oriental MF, Oriental Yeast Co., Tokyo) for 3 weeks during the early (weeks 4–7; groups 1–3), middle (weeks 8–11; groups 4–6) and late (weeks 12–15; groups 7–9) post-initiation phases, respectively. In addition, they received 3% $NaHCO_3$ in the diet (groups 1, 4 and 7), 20 ppm EHBN in the drinking water (groups 2, 5 and 8) or no chemical supplement (groups 3, 6 and 9) for the respective 13 weeks when they were not receiving BBN or uracil. Five rats each in groups 10 to 18 were given chemical supplements as in groups 1 to 9 without the initial BBN administration. The chemical doses chosen were the same as those used in earlier studies.[10–13] Animals were observed daily to assess general health and body weight, and levels of food and water consumption were measured weekly. Urinary cytology was performed to confirm the presence or absence of pathological changes in the urinary bladder at each stage of the post-initiation phase before and after uracil treatment. The experiment was terminated at week 20.

BBN-treated groups (groups 1–9): Histopathology of the bladder epithelium At week 20, the animals were killed by exsanguination under ether anesthesia and the urinary bladders were ligated at the neck, inflated by intraluminal injection of 10% phosphate-buffered formalin, and excised. After fixation, they were bisected sagittally and excess moisture was absorbed with filter paper. The bladders were weighed and cut into eight strips, which were embedded in paraffin, sectioned, and stained routinely with hematoxylin and eosin.

Non-BBN-treated groups (groups 10–18): Bladder epithelial morphology and DNA synthesis Circadian rhythms have been demonstrated in a number of different cell components.[15, 16] Therefore, in order to minimize variation between groups, single animal from each of the groups were sequentially injected i.p. with 100 mg/kg/body weight of 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co., USA), this being repeated until all had been processed. The animals were sequentially killed by exsanguination at 1 h after BrdU injection. The bladders were inflated with ice-cold 10% formalin in 0.1 *M* phosphate buffer (pH 7.4), removed quickly, and immersed in the fixative. At 2 h after fixation, the bladders were divided in half longitudinally. One half was processed for both histopathological examination and immunohistochemical BrdU staining[17] (Vectastain ABC Kit, Vector Laboratories, Inc., USA) to assess DNA synthesis: the numbers of cells incorporating BrdU into DNA per 1000 cells were counted. The other half was used for scanning electron microscopy (SEM) study (Hitachi S-450, Hitachi Co., Ltd., Tokyo).[18]

Statistical analyses The significance of differences between groups in body and bladder weights, levels of DNA synthesis and numbers of urinary bladder epithelial lesions was analyzed by using Student's *t* test. Insufficient homogeneity of variance was corrected with respect to the degrees of freedom according to the method of Welch. The significance of differences in tumor incidences in different groups was examined by using Fisher's exact probability test.

RESULTS

Clinical observation did not reveal any abnormalities or differences among the groups, and no deaths occurred during the treatment period. There were no differences in average intakes of BBN, uracil, $NaHCO_3$ and EHBN among the groups. Data on the body and urinary bladder weights are presented in Table I. There were no significant differences in the final body weights among the groups given uracil at any time point. Significant increase in bladder weight was observed for the $NaHCO_3$ and EHBN groups given uracil at all stages, with the exception of the EHBN group receiving uracil at the early stage, when compared to the corresponding control values.

Urinary cytology Urinary calculus formation was confirmed by palpation and forced micturition for urinalysis in rats after uracil ingestion for 3 weeks, small calculi being observed macroscopically in freshly voided urine. In the present study, the characteristics of the urinary sediment at each stage in the $NaHCO_3$ and EHBN groups were as follows. In the early stage, few exfoliated

Table I. Final Body and Urinary Bladder Weights in Rats Treated with BBN Followed by Uracil at Different Time Points[a)]

Group	Treatment		No. of rats	Final body weights (g)	Urinary bladder weights (g)
	BBN	Chemical			
Uracil treatment: early stage					
1	+	$NaHCO_3$	10	387±19	0.21±0.04**
2	+	EHBN	10	371±20	0.14±0.02
3	+	—	10	376±14	0.14±0.04
Uracil treatment: middle stage					
4	+	$NaHCO_3$	10	380±25	0.20±0.06*
5	+	EHBN	10	378±16	0.18±0.04*
6	+	—	10	366±10	0.15±0.03
Uracil treatment: late stage					
7	+	$NaHCO_3$	10	382±22	0.21±0.04**
8	+	EHBN	10	368±12	0.17±0.04*
9	+	—	10	375±12	0.15±0.02

a) Values are means±SD.
* $P<0.05$; ** $P<0.01$ compared with corresponding control values.

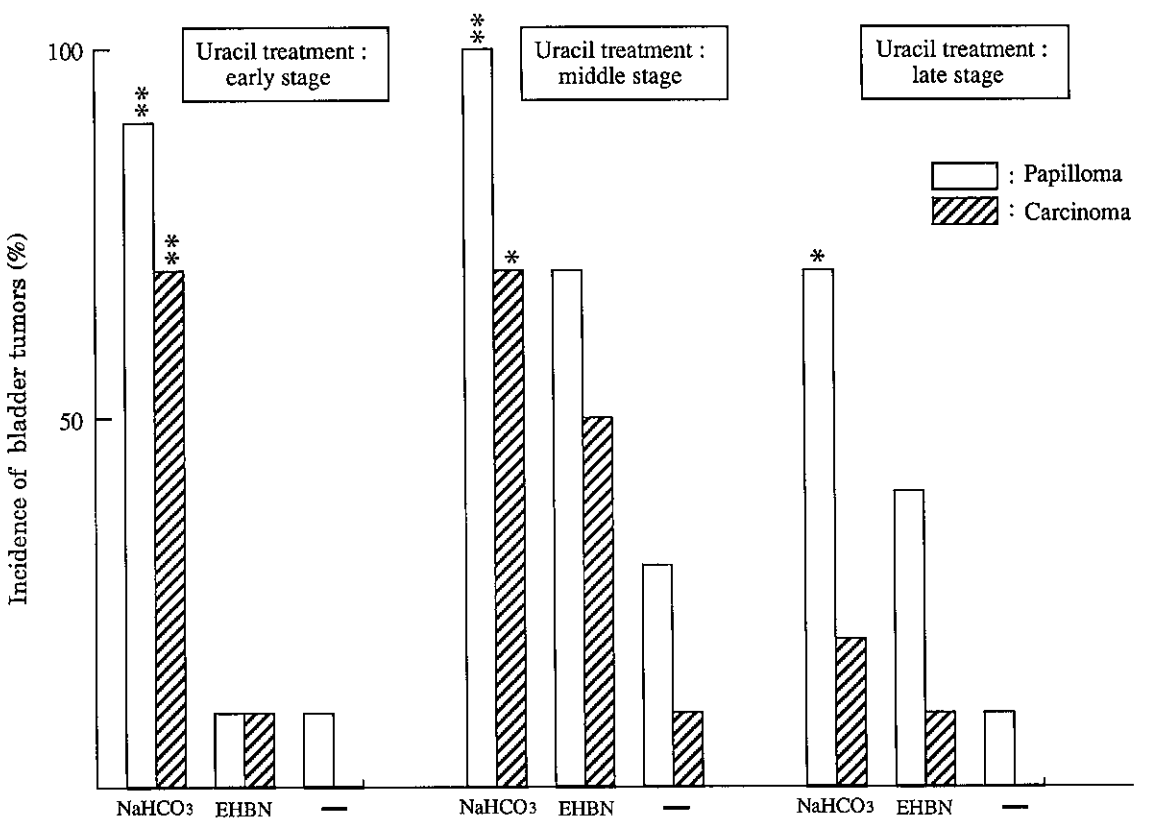

Fig. 2. Variation in tumor incidence with timing of uracil treatment in rat two-stage bladder carcinogenesis. Significantly different from corresponding control values (groups 3, 6 and 9) at $P<0.05$ (*) and $P<0.01$(**).

Table II. Numbers of Bladder Tumors in Rats Treated with BBN Followed by Uracil at Different Time Points

Group	Treatment		No. of rats	Numbers of bladder neoplasms		
	BBN	Chemical		Total	Average numbers of tumors per rat[a]	
					Papilloma	Carcinoma
Uracil treatment: early stage						
1	+	$NaHCO_3$	10	46	3.6 ± 2.2^{b}	$1.1\pm0.7^{c, e}$
2	+	EHBN	10	3	0.1 ± 0.3	0.2 ± 0.4
3	+	—	10	2	0.2 ± 0.4	0
Uracil treatment: middle stage						
4	+	$NaHCO_3$	10	38	2.8 ± 1.9^{c}	1.0 ± 0.8^{c}
5	+	EHBN	10	26	$1.3\pm1.2^{b, d}$	$1.1\pm1.1^{b, d, e}$
6	+	—	10	4	0.3 ± 0.5	0.1 ± 0.3
Uracil treatment: late stage						
7	+	$NaHCO_3$	10	33	2.7 ± 2.7^{b}	0.3 ± 0.7
8	+	EHBN	10	8	0.6 ± 0.8	0.2 ± 0.6
9	+	—	10	1	0.1 ± 0.3	0

a) Values represent means±SD.
b) $P<0.05$; *c*) $P<0.01$ compared with corresponding control values (groups 3, 6 and 9).
d) $P<0.05$ compared with the EHBN group given uracil at the early stage (group 2).
e) $P<0.05$ compared with the $NaHCO_3$ or EHBN groups given uracil at the late stage (groups 7 or 8).

cells, no red blood cells (RBC) and no white blood cells (WBC) were seen before uracil administration. In the literature, simple hyperplasia has been reported to occur in bladder epithelium after 0.05% BBN administration for 4 weeks.[19] The early stage in the present study represents this phase of simple hyperplasia development, this type of lesion sometimes but not always progressing to preneoplasia and neoplasia. After completion of the uracil treatment, the urinary sediment revealed high numbers of exfoliated cells with large nuclei, nucleoli and increased nuclear cytoplasmic ratio, as well as RBC and WBC. Since the exfoliated cells did not exhibit hyperchromasia or nuclear irregularities, this feature was considered to be due to mechanical stimulation by calculi, as shown for the human case.[20] In the middle stage, an increasing trend in urinary cells was seen, and small clusters of cells with slight atypia including hyperchromasia as well as RBC and WBC were occasionally observed even before uracil treatment ($NaHCO_3$ group, moderate; EHBN group, slight; control group, very slight). This was considered to suggest progression to the preneoplastic phase in both the $NaHCO_3$ and EHBN groups. Urinary cytology at the late stage revealed similar characteristics to those of the middle stage, but in addition, cell clumps with overlapping nuclei were also occasionally seen. This cytological finding indicated the beginning of neoplastic lesion development in both $NaHCO_3$ and EHBN groups. At 20 weeks, the calculi had completely disappeared in all groups after cessation of uracil treatment, as previously reported.[12]

BBN-treated groups: Histopathology of the bladder The incidence data for urinary bladder tumors are graphically illustrated in Fig. 2. Treatment with $NaHCO_3$ in sequential combination with uracil administration at the early and middle stages significantly increased the incidences of both papillomas and carcinomas, a similar tendency being observed with the late stage treatment, but only significantly for papillomas. Although the EHBN group given uracil at the middle stage showed a tendency for increase in papilloma and carcinoma incidences, treatment with EHBN and uracil at the other stages did not exert any marked influence. Quantitative data regarding numbers of bladder tumors are shown in Table II. Treatment with $NaHCO_3$ in combination with all stages of uracil treatment increased total numbers of bladder tumors (papillomas and carcinomas) and significantly elevated average numbers of papillomas or carcinomas per rat, with the exception of average numbers of carcinomas at the late stage of uracil treatment, when compared to the corresponding controls (groups 3, 6 and 9). Although average numbers of papillomas per rat in the $NaHCO_3$ groups were independent of timing of uracil treatment, average numbers of carcinomas per rat given uracil at the early or middle stages were higher than in the $NaHCO_3$ group given uracil at the late stage. EHBN treatment markedly increased total and average numbers of tumors (both papillomas and carcinomas) in the group given uracil at the middle stage. No uracil treatment-related erosion or necrosis was noted in any of the groups.

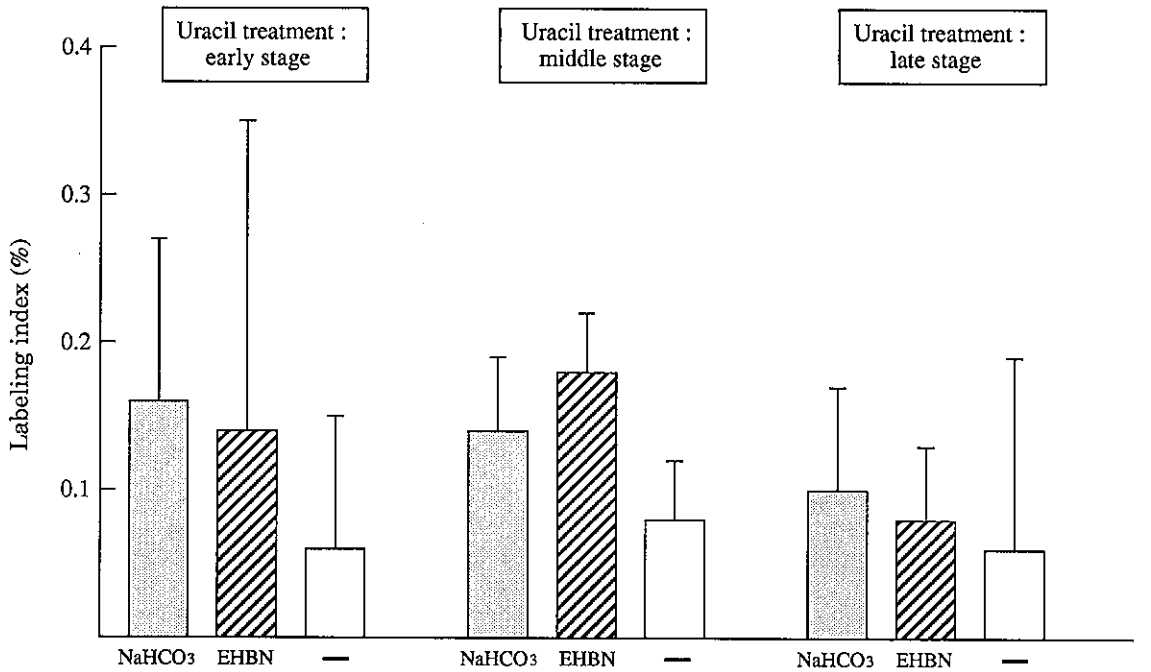

Fig. 3. DNA synthesis in bladder epithelium of rats treated with uracil at different time points without BBN initiation.

Non-BBN treated groups: Morphology and DNA synthesis of the bladder epithelium At 20 weeks, simple hyperplasia, consisting of diffuse thickening of the epithelium with approximately five layers of transitional epithelial cells, was observed in 4 or 5 of 5 rats in the $NaHCO_3$ and EHBN groups and in 2 or 3 of 5 rats in the group without any test chemical supplement. As shown in Fig. 3, DNA synthesis in bladder epithelium showed a tendency to increase in the $NaHCO_3$ and EHBN groups given uracil at all stages, but this was not significant. In addition, there were no differences in DNA synthesis between stages.

Under the SEM, normal bladder epithelium presents a flat appearance and is composed of large polygonal cells of relatively uniform size and shape whose luminal surfaces are covered with a complex network of microridges. Epithelial surface alterations of the bladder, such as pleomorphic microvilli, uniform or short microvilli and ropy or leafy microridges, which were observed by SEM were classified as described previously.[18, 21] Although epithelial surface alterations were noted in all groups, they were more severe in the $NaHCO_3$ and EHBN groups given uracil at any stage. However, no differences in bladder epithelial changes in the $NaHCO_3$ or EHBN groups between stages could be distinguished by SEM.

DISCUSSION

The results of the present investigation demonstrated that uracil-induced urolithiasis at different stages during the process of rat bladder carcinogenesis exerts different enhancing effects on promotion by the non-mutagen $NaHCO_3$ or the mutagen EHBN, in both cases being most effective when given at the middle stage.

Various treatments, such as partial cystectomy,[22, 23] unilateral ligation of the ureter,[24–26] *in situ* freezing [27] or uracil-induced urolithiasis,[12, 13] have been shown to increase DNA synthesis in the bladder epithelium and therefore to facilitate detection of promoting agents within a relatively short time period. Treatment with uracil for only 4 days induces particularly marked cell proliferation throughout the whole bladder epithelium without the necessity of any surgical manipulation, the calculi and papillomatosis also disappearing after cessation of treatment even after a 25-week administration.[12] Previously, it was reported that uracil treatment for 8 weeks strongly enhances bladder tumor development of rats initiated with BBN.[13] Indeed, long-term administration of uracil itself causes bladder cancer in rats and mice[28] but since uracil has no mutagenic properties,[29] it is thought to be an epigenetic carcinogen whose activity relies on chronic mechanical irritation by calculi. In fact,

many investigators have reported that calculi are associated with bladder cancer development.[30-32] In the present study, the process of bladder tumor development was divided into three phases, uracil treatment being separately conducted at each stage. The different responses to the enhancing effects of uracil treatment observed between the non-genotoxic $NaHCO_3$ and genotoxic EHBN groups were presumably due to the different natures of the chemicals. Non-genotoxic compounds induce only a transient early cell proliferation without DNA damage,[33-36] while genotoxic compounds cause genetic alterations and progressive cell proliferation, resulting in tumor development.[1-4, 37, 38]

Pleomorphic microvilli have been described on the cell surfaces of bladder cancer in both rats and man.[18, 39-41] It has further been shown that cytotoxic chemical or mechanical damage which induces cell proliferation of the epithelium leading to epithelial hyperplasia is associated with similar luminal surface structures.[42-44] Recent studies have demonstrated that bladder tumor promoters can commonly bring about cell surface alterations.[19, 36, 45] In the present study, DNA synthesis and epithelial surface alterations in the bladder tended to be increased in groups given $NaHCO_3$ or EHBN without BBN initiation, but the changes were independent of uracil treatment timing. With respect to uracil, treatment at the middle stage of the carcinogenic process most effectively enhanced tumor development by $NaHCO_3$ and EHBN in the present study. Further investigations are necessary to explore the mechanisms involved.

In conclusion, irrespective of the underlying mechanisms, uracil-induced urolithiasis during the middle post-initiation phase is most reliable for enhancing bladder tumor development by both tumor promoters and carcinogens, suggesting its use for testing of potentially active compounds.

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