Strong Promoting Activity of Reversible Uracil-induced Urolithiasis on Urinary Bladder Carcinogenesis in Rats Initiated with N-Butyl-N-(4-hydroxybutyl)nitrosamine

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ABSTRACT

The tumor-promoting effect of uracil-induced calculi on rat urinary bladder carcinogenesis was investigated in male F344 rats pretreated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). Since uracil-induced calculi and papillomatosis of the bladder are reversible, uracil was given for a limited period after the treatment with BBN. Animals were given 0.05% BBN in their drinking water for 4 wk and then treated with uracil as 3% of the diet for 8 or 16 wk. After the uracil treatment, rats were given basal diet without uracil until Wk 28 of the experiment. Animals were killed from each group at the end of either Wk 12, 20, or 28. The incidence of carcinoma of the bladder was 40% after only 8 wk of uracil treatment following BBN initiation and increased to 100% when uracil treatment was extended to 16 wk. After discontinuation of uracil treatment, the papillomatosis disappeared, but the incidence of carcinoma steadily increased with increasing time. In the control group given BBN alone, only 1 of 16 rats had carcinoma at Wk 28. The present findings clearly demonstrate that uracil-induced urolithiasis had a strong promoting activity on BBN bladder carcinogenesis.

INTRODUCTION

The coexistence of carcinoma and calculi in the urinary bladder of rats and mice has been observed in many studies. For example, two rat strains, the Brown Norway and DA/Han rats, have a high incidence of spontaneous bladder tumors that are often associated with the presence of calculi (1, 2). Insertion of foreign bodies, such as paraffin wax pellets (3–5), rough glass beads (6), chalk powder (7), wood or silastic pellets (8), into the bladder resulted in the development of bladder tumors. These results indicate that tumor formation may result from the proliferative stimulation by calculi or foreign bodies. When administration of chemicals to rodents has resulted in both tumor formation and calculi in the bladder, the question has arisen as to whether the chemical itself or the bladder calculi were responsible for tumor formation (8–10). Therefore, it is essential to clarify the role of bladder calculi in bladder carcinogenesis.

We recently found that all rats given a diet containing 3% uracil had urolithiasis accompanied by severe and extensive papillomatosis in the bladder, and the uracil-induced calculi and mucosal papillomatosis were reversible (11). It was shown that bladder calculi were induced in a short period of feeding of uracil, and soon after discontinuation of the feeding, the stones started to disappear.

In the bladder, the two stage system of initiation and promotion is well established, with several carcinogens acting as initiators, including N-methyl-N-nitrosourea (12), N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (13), and BBN (14). In our laboratory, using BBN initiation, many chemicals have been shown to exert tumor-promoting activity (15–17).

The present experiment was conducted to evaluate the tumor-promoting effect of uracil-induced calculi on rat urinary bladder carcinogenesis initiated with BBN. Utilizing the reversibility of uracil-induced calculi and papillomatosis, uracil was given for a limited period after the treatment with BBN, and the reversibility of bladder mucosal lesions was examined.

MATERIALS AND METHODS

Male F344 rats (purchased from Charles River Japan, Inc., Kanagawa, Japan) were 6 wk old and weighed about 124 g at the beginning of the experiment. Animals were housed 5 to a plastic cage with hardwood chips as bedding, in an air-conditioned room with a 12 h-12 h light-dark cycle, and given free access to water and food (Oriental MF; Oriental Yeast Co., Tokyo, Japan). BBN and uracil were purchased from Tokyo Kasei Co., Tokyo, Japan, and Wako Pure Chemical Co., Osaka, Japan, respectively.

The experimental protocol was shown in Fig. 1. Animals in Group 1 to 3 were given 0.05% BBN in their drinking water for 4 wk as initiation, and then 3 days later they were given diet containing uracil at a level of 3.0% by weight for 8 wk in Group 1 and for 16 wk in Group 2. After the treatment with uracil, rats were given basal diet without uracil until the end of the experiment. Rats in Group 3 were maintained with basal diet throughout the experiment as a control after the treatment with BBN. Groups 4 and 5 were given uracil for 8 and 16 wk, respectively, the same as Groups 1 and 2 but without BBN initiation. The experiment was ended 28 wk after the beginning of BBN administration. Animals in Group 1 were killed at the end of Wk 12 [after 8-wk feeding of uracil, Group 1(a)], Wk 20 [8 wk after the uracil feeding was completed, Group 1(b)], and Wk 28 [16 wk after uracil feeding was completed, Group 1(c)]. The rats in Group 2 were killed at the end of Wk 20 [after 16-wk feeding of uracil, Group 2(a) and Wk 28 [8 wk after 16-wk feeding of uracil was completed, Group 2(b)]. In control groups (Groups 3 to 5), rats were killed at the same times as the experimental groups.

The urinary bladder, ureter, and renal pelvis were inflated and fixed with 10% phosphate-buffered formalin. After fixation, the bladder was longitudinally cut into 6 to 8 slices, and each ureter was cut transversely into 3 pieces. They were embedded in paraffin for routine histological examination. The incidences of lesions were analyzed statistically by the 2 test.

RESULTS

Growth curves for all groups are shown in Fig. 2. Treatment with uracil suppressed the growth of the rats. The average body weights of rats 16 wk after starting the administration of uracil were 76% and 74% [Groups 2(a) and 5(a), respectively] of the control (BBN alone), while 4 wk after the discontinuation of uracil treatment the body weight recovered to 90% of the level of the control group. Rats that received uracil for 8 wk (Groups

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2 To whom requests for reprints should be addressed.
3 Unpublished data.

* The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; PN, papillary or nodular.

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PROMOTION OF BLADDER CARCINOGENESIS BY CALCULI

Experimental period (weeks)

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Fig. 1. Experimental design. ■, time of administration of 0.05% BBN in drinking water; □, time of administration of 3.0% uracil in diet; ▼, time of sacrifice of animals. Letters in parentheses, subgroup.

Fig. 2. Mean body weights of rats given BBN and/or uracil. O, Group 1; •, Group 2; △, Group 3; □, Group 4; ■, Group 5. —, period of administration of BBN; ---, period of administration of uracil; ——, period of no treatment.

1 and 4) showed a similar growth retardation and recovery rate as rats that received uracil for 16 wk.

Macroscopically, regardless of pretreatment with BBN, rats killed just after uracil treatment [Groups 1(a), 2(a), 4(a), and 5(a)] had numerous white calculi in the bladder, and the entire mucosa of the bladder was irregularly thickened (Table 1; Fig. 3). Microscopically the mucosa of these bladders showed diffuse finger-like epithelial projections, namely, extensive papillomatosis (Fig. 4), as described by us previously (11). In contrast to the rats given uracil without BBN treatment, many small tumor-like masses were noticed in the thickened mucosa of the bladders of rats given uracil after pretreatment with BBN. Histological sections of these bladders revealed many tumor growths among the papillomatosis (Fig. 5). When rats were killed after the interval of normal basal diet following uracil treatment [Groups 1(b), 1(c), 2(b), 4(b), 4(c), and 5(b)], all bladders were free of calculi. The thickness of the mucosa had returned nearly to normal, except for the development of multiple tumors in the bladder of rats that were pretreated with BBN (Fig. 6).

Besides the papillomatosis, bladder proliferative lesions were composed of simple hyperplasia, PN hyperplasia, dysplasia, and carcinomas. All but dysplasia have been described previously (15, 16). Dysplasia was seen as localized lesions with distinct cellular atypia in the epithelium of the papillomatosis (Figs. 7 and 8) or in the simply hyperplastic mucosa. This lesion was only found in rats given BBN followed by uracil. Since PN hyperplasia was sometimes indistinguishable from the lesions of papillomatosis, all rats with papillomatosis were included in the number of rats with PN hyperplasia. Although the actual distinction of papilloma from papillomatosis was sometimes difficult, polyplike structures composed of irregularly arranged transitional cells with connective tissue were observed among the papillomatosis, and these were referred to as papillomas.

Fifty to 60% of the rats treated with BBN only developed simple hyperplasia without an increase in incidence with increasing time. Only two rats treated with BBN alone and killed at Wk 28 had tumors, one a papilloma and the other a carcinoma. In contrast, many rats given BBN and then uracil developed high incidences of tumors regardless of the presence of any interval with basal diet before sacrifice (Figs. 5 and 6). The diagnosis of carcinoma was based on the loss of differentiation to the surface, presence of nuclear pleomorphism, and the presence of mitoses (Figs. 9 and 10). In Group 1, carcinoma

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<th>Papilloma</th>
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*Time of treatment (wk).

Papilloma was not counted in rats with papillomatosis.

This appeared in either tumorous or nontumorous regions.

Numbers in parentheses, percentage.
had already developed in 40% of rats as early as Wk 12, an incidence significantly different from that of Group 3(a) ($P < 0.05$). Even after discontinuation of uracil treatment, the incidence of carcinoma increased with increasing time [Groups 1(b) and 1(c)]. The effect of time on the incidence of carcinoma is shown graphically in Fig. 11. The incidence of papilloma also increased from 40% after Wk 20 to 73% after Wk 28 [Groups 1(b) and 1(c)]. When uracil treatment was extended from 8 to 16 wk [i.e., Group 1(a) versus Group 2(a)], the incidence of carcinoma significantly increased from 40% to 100% ($P < 0.01$). The yield of carcinoma was 100% even after 8 wk of basal diet [Group 2(b)]. In contrast, dysplasia, the incidence of which reached 100% by administration of uracil [Groups 1(a) and 2(a)], decreased after discontinuation of the uracil treatment [Groups 1(b), 1(c), and 2(b)].

Squamous metaplasia of the transitional epithelium of the bladder was frequently observed in the bladder of rats treated with BBN and uracil. It appeared mainly in the tumors, especially the papillomas and carcinomas, and appeared as a well-differentiated, keratinized epithelium (Fig. 12). The incidence of squamous metaplasia varied among Groups 1 to 5. No
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Fig. 8. A higher magnification of Fig. 6. The right half of the papillomatosis is composed of dysplastic epithelium. Note definite variability in cell and nuclear sizes and loss of nuclear polarity compared to the mucosa on the left side in which no irregularity of cells and nuclei is seen. H & E, x 400.

Fig. 9. Transitional cell carcinoma of the bladder induced by treatment with 4 wk of BBN and then 8 wk of uracil [Group 1(c)]. H & E, x 200.

Fig. 10. Transitional cell carcinoma of the bladder found in a rat given BBN and uracil [Group 2(b)]. H & E, x 200.

Fig. 11. Sequential changes in the incidences of bladder carcinoma of rats treated with BBN and/or uracil. Data are from those in Table 1. O, Group 1; C, Group 2; L, Group 3. □, time of administration of BBN; ■, time of administration of uracil; Δ, no treatment.

DISCUSSION

Production of bladder calculi in rats by p.o. administration of 3% uracil in the diet and their disappearance after stopping treatment (11) were confirmed in this study. The ability to reliably induce reversible calculi made possible an investigation of the effects of calculi on bladder carcinogenesis. The present data clearly demonstrate that uracil given after initiation with BBN strongly promoted the development of urinary bladder cancer. It is noteworthy that the incidence of carcinoma was 40% only after 8 wk of uracil treatment following BBN initiation and increased to 100% when uracil treatment was extended to 16 wk. Importantly, even after removal of uracil from the diet and replacement with normal basal diet, the incidence of carcinoma steadily increased [Groups 1(b) and 1(c)]. Promoting activity by uracil thus appears to be the most potent known bladder tumor promoter, compared to others such as sodium saccharin, sodium L-ascorbate, and butylated hydroxyanisole (14, 17), based on the duration of treatment and the resulting incidences of carcinoma. There is no doubt that the strong tumor promotion by uracil is associated with the induction of bladder calculi. We have shown that, soon after the starting of feeding of diet containing 3% uracil, numerous tiny calculi appear in the bladder, and at the same time, the entire bladder mucosa begins to proliferate and form papillary epithelial pro-

squamous metaplasia was found in rats given BBN or uracil alone [Groups 3(a) to 5(b)]. Some papillomas were squamous cell type, while all carcinomas were transitional cell type but with accompanying squamous metaplasia as described above. No metastasis of bladder cancer was found. The epithelium of the ureter also showed papillomatosis after treatment with uracil, although papillomatosis still remained after the disappearance of the stones. No tumors were found in the ureter of any of the rats in the present experiment.

Fig. 12. Squamous metaplasia in a papilloma [Group 1(a)]. Note keratinized well-differentiated squamous epithelium. H & E, x 100.
jection. Furthermore, labelling indices in the proliferating epithelium remain high until the end of the uracil treatment. These findings suggest that potent tumor promotion resulted from the continuous increased cell proliferation of the urinary tract induced by the irritant effect of the stones. No data have been obtained to indicate that uracil itself can act as a promoter. Additional studies using lower doses of uracil, doses at which no urinary calculi are formed, should provide important information as to the mechanism of uracil promotion.

Another striking finding observed in the present experiment was the appearance of many foci of dysplasia in the proliferating epithelium during uracil treatment after BBN initiation. These dysplastic epithelia were observed often as a part of papillomatosis and sometimes as small masses in the mucosa as shown in Figs. 7 and 8. These dysplastic foci have never been observed in the papillomatosis induced by uracil calculi without pretreatment with carcinogen. Therefore, it is suggested that dysplasia might represent an expression of carcinogen-initiated cells in response to a stimulus for abnormal proliferation. The mechanisms underlying enhancement of the appearance of dysplasia require further investigation.

Limited administration of uracil after BBN treatment revealed that, instead of the disappearance of papillomatosis, some or all of the coexistent dysplasias and carcinomas did not disappear and continued to develop and grow. This finding suggests that, although papillomatosis without atypia is a reversible benign lesion, papillomatosis with dysplasia may represent a true neoplastic alteration. The decrease in the incidence of dysplasia over time after discontinuing uracil treatment suggests that some of the dysplasias were reversible. However, the yield of carcinomas, increased in conjunction with the decrease in dysplasias, we believe provides some evidence that some dysplasias proceed to carcinomas. These points on histogenesis and reversibility require more quantitative analyses. Dysplasia as an early lesion is rarely found in the histogenesis of tumor development in the rat bladder induced by carcinogens alone or in combination with a weak promoter (18, 19). Therefore, the present method using controllable calculus production may provide a unique model to clarify early epithelial alterations and the relationship between dysplasia and carcinoma.

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