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Rat urinary bladder carcinomas induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine and *N*-methyl-*N*-nitrosourea

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Summary. The present investigation was conducted to examine the combination effect of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine(BBN) and *N*-methyl-*N*-nitrosourea(MNU) in rat urinary bladder carcinogenesis. Experiment 1 was performed in the groups treated by oral BBN administration in combination with MNU intraversical instillation and their control groups. In the groups given both BBN and MNU, the ratio of rats with the non-papillary type to carcinoma-bearing rats was significantly higher than in the controls. Since most of the carcinomas were non-invasive, the observation period was prolonged in the groups given both BBN and MNU in experiment 2. However, even after a longer observation period, no invasive carcinoma was observed. These results suggest that high-grade and invasive carcinomas similar to those induced by BBN and MNU in the heterotopically transplanted rat bladder cannot be induced in the natural bladder of the rat.

Key words: *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine – *N*-Methyl-*N*-nitrosourea – Rat bladder carcinogenesis – Papillary carcinoma – Non-papillary carcinoma

Human bladder carcinomas are classified into papillary non-invasive carcinoma and non-papillary invasive carcinoma. The establishment of an animal model of bladder carcinoma similar to human bladder carcinoma is important for analyzing various factors associated with the development and progression of bladder carcinoma and for obtaining basic data on the clinical diagnosis and treatment.

Animal models of carcinogenesis in the bladder have been reported for rats, mice and dogs. Rats treated by oral administration of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) develop multiple papillary peduncular carcinomas, which are generally low grade and non-invasive [3]. Mice given BBN orally develop highly malignant invasive bladder carcinoma [4]. We earlier established a dog model for bladder carcinogenesis using BBN and reported that BBN induce non-invasive bladder carcinoma after a long period of administration at low doses and non-papillary invasive bladder carcinoma after a short period at high doses [5]. In addition, we infused *N*-methyl-*N*-nitrosourea (MNU) into the bladder of dogs, administered BBN orally and observed development of carcinoma in situ (CIS) [9].

Thus, two types of bladder carcinoma similar to those in humans have been induced in dogs. However, in rats and mice, the induction of only one type or the other has been reported. The present study examined whether the two types of bladder carcinoma could be induced in rats in which only the development of papillary non-invasive carcinoma has been observed. Bladder carcinogenesis was evaluated following BBN and/or MNU administration.

Materials and methods

Animals

Two hundred forty-eight female Fischer 344 rats aged 8 weeks (Charles River, Kanagawa, Japan) were used. They were housed in plastic cages (5 animals per cage) in an air-conditioned room at 22 °C and a humidity of 50% under 12-h light/dark cycles with free access to water and chow diet (Charles River).

Chemicals

BBN (Iwai Chemical, Tokyo, Japan) was added to drinking water at a concentration of 0.05% and given ad libitum. MNU was purchased from Nakarai Chemical (Kyoto, Japan): 1.5 mg was dissolved in 0.5 ml of physiological saline and instilled into the bladder using a polyethylene tube (external diameter 0.8 mm) twice at a 2-week interval.

Experimental design

Experiment 1 was performed in eight groups (Fig. 1). Group 1 was treated by intravesical instillation of MNU followed by 12-week

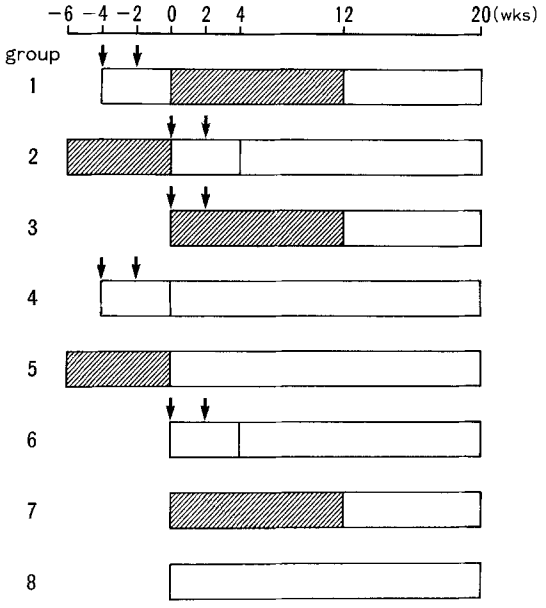

Fig. 1. Experimental design (experiment 1). ↓ = MNU 1.5 mg/0.5 ml instillation; ▨ = 0.05% BBN in drinking water

Table 1. Histopathological changes in the urinary bladder in rats (20 weeks)

Group	Treatment (weeks)	No. of rats	NPH[a] (%)	Carcinoma (%)
1	MNU → BBN (12)	22	22 (100)	13 (59)
2	BBN (6) → MNU	17	15 (88)	5 (29)
3	MNU + BBN (12)	23	23 (100)	11 (48)
4	MNU	13	1 (8)	0 (0)
5	BBN (6)	10	10 (100)	5 (50)
6	MNU	20	0 (0)	0 (0)
7	BBN (12)	19	19 (100)	16 (84)
8	Control	10	0 (0)	0 (0)

[a] Nodulopapillary hyperplasia

Table 2. Number of papillary or non-papillary neoplastic lesions

Group	No. of rats	Carcinoma*	Papillary type**	Non-papillary type***
1	22	13	11	8
2	17	5	3	3
3	23	11	5	9
5	10	5	5	0
7	19	16	15	3

* $p < 0.05$, group 3 versus group 7
** $p < 0.05$, group 3 versus group 7
*** $p < 0.05$, group 1 versus group 7; $p < 0.005$, group 3 versus group 7; $p < 0.05$, group 2 versus group 5

administration of BBN. Group 2 was treated by 6-week BBN administration, followed by intravesical MNU instillation. Group 3 received both drugs simultaneously. Groups 4–7 were control groups for groups 1–3 and given either BBN or MNU. Group 8 did not receive either drug.

In Experiment 2, rats groups in which both drugs were administered (Groups 1–3) were observed for 30 weeks + 40 weeks.

All animals were killed by overdoses of ether at the termination of each experiment. The urinary bladder was inflated in situ with 10% phosphate-buffered formalin solution. After overnight fixation, each bladder was cut vertically into 12–16 sections, embedded in paraffin, and stained with hematoxylin and eosin. Each tissue block was cut at two different levels. The criteria for histopathological classification of bladder lesions used were in accordance with those described previously [1, 3, 8]. Statistical analysis was performed by the χ^2 test.

Results

Experiment 1

Eleven animals died during the experiment, and most of the deaths were due to overdoses of the anesthetic used during instillation of MNU. In the other animals, normal weight gain was observed until the termination of the experiment. The histopathological changes in the bladder are shown in Table 1.

Nodulopapillary hyperplasia (NPH) was observed in most of the rats given BBN but in none of those treated with MNU alone. Carcinomas developed in the groups given both BBN and MNU and those given BBN alone, but not in the group treated with MNU alone. The incidence of carcinoma tended to be higher in the groups given BBN for 12 weeks than in those given BBN for 6 weeks. In group 3, which was given MNU and BBN simultaneously, carcinoma was induced in 11 of 23 rats (48%). However, this incidence was lower than that in group 7, which was given BBN alone (the control group for group 3).

All the carcinomas observed were non-invasive transitional cell carcinomas. Therefore, they were morphologically classified into the papillary type and non-papillary type (Table 2; Fig. 2A, B). The ratio of rats with papillary type carcinoma to all carcinoma-bearing rats was significantly higher in group 3 (5 of 11 rats) given BBN and MNU simultaneously than in group 7 (15/16), the control group for group 3 ($p < 0.05$). Conversely, the ratio of rats with non-papillary type carcinoma to all carcinoma-bearing rats was significantly higher in group 3 than in group 7 ($p < 0.005$). In group 1, the ratio of rats with the non-papillary type was significantly higher than in group 7 [the control group for group 1 ($p < 0.05$)] and also higher in group 2 than in group 5 ($p < 0.05$). Therefore, the ratio of rats with non-papillary type was significantly higher in the groups given both BBN and MNU (groups 1–3) than in their control groups given BBN alone.

Experiment 2

During the experimental period, 7 rats died due to overdoses of the anesthetic. The other rats grew normally until the end of the experiment.

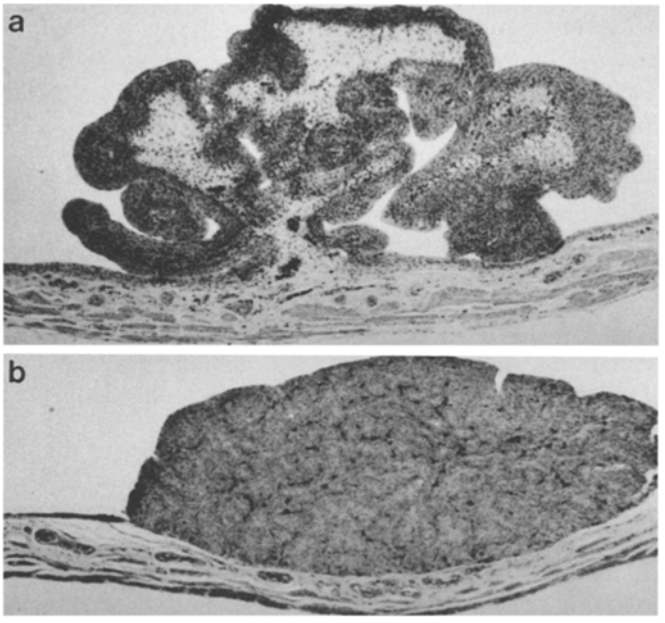

Fig. 2. **a** Papillary type of carcinoma (group 7 in experiment 1, H&E×40). **b** Non-papillary type of carcinoma (group 1 in experiment 1, H&E×40)

All rats except 1 in group 1 30-week developed carcinoma over the observation period. Most of the carcinomas were non-invasive transitional cell carcinomas. Morphologically, the percentages of the papillary type were significantly higher. With time, high-grade carcinomas increased (Table 3).

In Experiments 1 and 2, no stone formation was observed in the groups treated by intravesical instillation of MNU.

Discussion

Human bladder carcinomas can be classified into papillary non-invasive carcinoma and non-papillary invasive carcinoma. The former is treated primarily by transurethral resection with preservation of the bladder, but its recurrence rate is high. The latter often progresses rapidly and is not often curable even by radical surgery [10]. The natural histories of the two types of carcinoma, which occur in the same organ but show completely different growth and modes of spread, have not been adequately clarified. This makes the treatment of bladder carcinoma even more difficult.

Establishment of animal models of bladder carcinoma is important to clarify the natural history of this disease and obtain basic data on its clinical diagnosis and treatment. Many investigators have performed carcinogenesis experiments in the bladder using rodents. The development of papillary non-invasive carcinoma has been observed in rats [3] and that of non-papillary invasive carcinoma in mice [4]. In our dog model of BBN-induced bladder carcinoma, papillary non-invasiave carcinoma developed after administration for a longer period at low doses and non-papillarys invasive carcinoma after a short period at a high doses [5]. In addition, we infused MNU into the bladder of dogs and subsequently administered BBN and observed the development of CIS [9]. Thus, we have succeeded in inducing bladder carcinomas in dogs similar to those found in humans. The dog model is appropriate for the evaluation of the process of carcinogenesis and the development of treatment methods. Its disadvantages are that a long period is required for carcinoma induction and the number of animals was limited compared to a rat or mouse model. Therefore, it is important to induce the two types of bladder carcinogenesis models in the same rodent.

Recently, Oyasu et al. [7] induced highly malignant invasive bladder carcinoma in F344 rats. Heterotopically transplanted bladder (HTB) of donor rats to recipient rats, based on the hypothesis that urine not only transports carcinogens but also promotes growth of a carcinoma, was developed in his laboratory [6]. Using this HTB model system, the effects of urine as a promoter were evaluated using MNU as an initiator. Relatively low-grade papillary non-invasive carcinoma developed in a low-dose group (0.05 mg MNU) and high-grade invasive carcinoma in a high-dose group (0.5 mg), suggesting there is an association between the dose of MNU and the malignancy of the carcinoma that developed. In addition, they induced HTB using the bladder of donor rats pretreated

Table 3. Histopathological changes of the urinary bladder in rats (30 and 40 weeks)

Group	Termination (weeks)	No. of rats	Carcinoma	Type of carcinoma		
				Papillary/non-papillary	Superficial/invasive	Low grade/high grade
1	30	15	14	14/8	14/1	14/ 7
	40	14	14	14/5	14/1	14/11
2	30	15	15	14/3	15/0	15/ 7
	40	19	19	18/6	19/1	19/13
3	30	15	15	13/8	15/2	15/11
	40	18	18	18/5	18/1	18/17

Table 4. Number of nodular or papillary hyperplastic lesions/10 cm of the basement membrane

Group	No. of rats	NH		PH	
		Total no.	No./10 cm	Total no.	No./10 cm[a]*
1	22	21	3.84 ± 2.79[a]	22	3.66 ± 2.08
2	17	14	3.24 ± 2.70	9	1.47 ± 1.73
3	23	23	4.84 ± 3.29	23	4.57 ± 2.68
5	10	9	3.12 ± 2.09	10	4.5 ± 2.18
7	19	18	3.78 ± 1.71	19	17.23 ± 8.83

[a] Mean ± standard deviation
* $p < 0.001$, group 1 versus group 7; $p < 0.001$, group 3 versus group 7; $p < 0.05$, group 2 versus group 5

with BBN and observed higher-grade and higher-stage carcinoma following administration of MNU at a high dose. It is very interesting that papillary non-invasive carcinoma and non-papillary invasive carcinoma similar to those found in humans were induced in the same rodent.

The purpose of the present study was to determine whether high-grade and high-stage carcinomas similar to those observed in the HTB by Oyasu et al. can be induced in the rat natural bladder. Experiment 1 was performed in the groups treated by BBN administration in combination with MNU intravesical instillation and their control groups. In the groups given both BBN and MNU, the ratio of rats with the non-papillary type to carcinoma-bearing rats was significantly higher than in their controls. Since most of the carcinomas were non-invasive, the observation period was prolonged in the groups given both BBN and MNU (groups 1–3) in Experiment 2 for possible development of non-papillary invasive carcinoma. However, even after observation for 30 or 40 weeks, invasive carcinoma was observed in only a few animals. In all groups, the papillary type constituted significantly higher percentages than the non-papillary type. High-grade carcinomas increased with time in groups 1–3. Since non-papillary non-invasive carcinoma developed after 20 weeks, some additional treatment may be needed for its progression to invasive carcinoma. Even after a longer observation period such as in experiment 2, no invasive carcinoma was observed. The problem is that the invasive carcinoma that could be induced in HTB could not be produced in the natural bladder. What is the difference between the experimental design of Oyasu et al. and ours? The transplantation procedure to produce HTB in their experimental design may have had some effects on the outcome. The addition of a promoter to treatment with BBN and MNU may not only induce conventionally observed papillary non-invasive carcinoma but also non-papillary invasive carcinoma in rats.

In the present experiment, the carcinomas that developed were morphologically classified into the papillary type and non-papillary type. There are no reports on bladder carcinogenesis in which observations are done according to this classification. In experiment 1, we classified NPH, which is considered to be a preneoplastic lesion [2], into nodular hyperplasia (NH) and papillary hyperplasia (PH) and obtained their numbers per 10 cm basement membrane (Table 4). The incidence of NH did not differ significantly between groups 1–3 given both BBN and MNU and their controls (groups 5 and 7). However, PH was found significantly more frequently in the groups given BBN alone than in the groups given both BBN and MNU. Since this finding is consistent with the significantly higher ratio of non-papillary carcinoma in the groups given both drugs than in the groups given BBN alone, PH and NH seem to develop into papillary carcinoma and non-papillary carcinoma, respectively. Further clarification awaits future research on bladder carcinogenesis.

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