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Modifying Factors in Urinary Bladder Carcinogenesis

by Nobuyuki Ito,* Shoji Fukushima,* Tomoyuki Shirai,* Keisuke Nakanishi,* Ryohei Hasegawa* and Katsumi Imaida*

N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) is a potent carcinogen in the urinary bladder of animals. The BBN model of bladder cancer is an excellent model of human urinary bladder cancer and has already led to a greater knowledge of its pathogenesis. In our studies, histogenesis and morphological characteristics of BBN urinary bladder cancer were analyzed in different animal species such as rats, mice, hamsters and guinea pigs and also in different rat strains. Papillary or nodular hyperplasia (PN hyperplasia) is found to be a preneoplastic lesion of the rat urinary bladder. Therefore, the promoting and inhibitory effects of various chemicals in two-stage urinary bladder carcinogenesis were judged by measuring PN hyperplasia in rats. Dose-dependent and organ-specific effects of the urinary bladder promoter, saccharin, in the induction of PN hyperplasia were shown in rats after initiation by BBN. The promoting effect of saccharin was seen more clearly in the urinary bladder of rats after potent initiation. A strain difference in susceptibility of the urinary bladder to the promoter was also shown. These results suggest that the above various factors may also have modifying activities on urinary bladder carcinogenesis in man.

Introduction

Animal models are very useful for studying the pathogenesis of human diseases. We have used rats given a carcinogen, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water as a model of urinary bladder cancer for more than ten years. This has proved to be a very good model of human bladder cancer and has provided much information on its pathogenesis. This paper describes the histogenesis and morphological characteristics of BBN urinary bladder cancer in rats and factors modifying BBN bladder carcinogenesis.

Urinary Bladder Carcinogenesis by BBN

BBN is a potent carcinogen in the urinary bladder of animals, and there are many reports on its carcinogenicity in rat urinary bladder (*1*). Histological lesions in the urinary bladder of rats induced by BBN were classified into four types: simple hyperplasia, papillary or nodular hyperplasia (PN hyperplasia), papilloma and cancer. PN hyperplasia of the urinary bladder in rats treated with 0.05% BBN developed before the induction of papilloma or cancer. The urinary bladders of rats treated with various doses of BBN for different periods were examined histologically to determine the incidence of PN hyperplasia and cancer. A high dose of BBN induced high incidences of PN hyperplasia and cancer in a short period. Dose-response relationships were observed for the induction of PN hyperplasia and cancer, the dose of BBN, and the period of BBN treatment. Moreover, a good correlation was found between the inductions of PN hyperplasia and cancer with different doses of BBN. Thus, it is clear that PN hyperplasia is a preneoplastic lesion of rat urinary bladder.

The histological types and grades of 611 urinary bladder cancers induced in rats by BBN were analyzed (*2*). Most of the cancers (95.4%) were transitional cell carcinomas, the remainder being squamous cell carcinomas (3.3%), undifferentiated carcinomas (1.0%) and carcinosarcomas (0.3%). Among the transitional cell carcinomas, 23.3% were Grade I anaplasias, 55.% Grade II and 21.5% Grade III. Some of the transitional cell carcinomas had areas

*Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan.

of squamous metaplasia and/or glandular metaplasia, and the incidence of metaplasia increased with the grade of anaplasia. There was a significant relation between the grade of transitional cell carcinomas and their extent of invasion into the bladder wall. More Grade I carcinomas were noninvasive, and one-third of Grade II were invasive. Of the transitional cell carcinomas of Grade III, 24.8% were noninvasive, 48.0% invaded the submucosa, and 13.6% invaded the muscle layer and through the serosa. Thus, there was a clear relation between the incidence of invasive transitional cell carcinomas induced by BBN and the grade. These results show that the morphological characteristics of bladder cancers in rats induced by BBN resemble those of human bladder cancer. BBN is also very useful in studies on factors modifying bladder carcinogenesis.

Synergistic and Additive Effects of BBN, 2-AAF, FANFT and 3,3′-DCB

The synergistic and additive effects of subcarcinogenic doses of chemicals may be important in human carcinogenesis, since several carcinogens are present in our environment at low concentrations. Experimentally, some chemical carcinogens have been shown to have synergistic and additive effects in chemical carcinogenesis when administered in combination or in sequence with other carcinogenic chemicals.

Whether BBN, 2-acetylaminofluorene (2-AAF), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) and 3, 3′-dichlorobenzidine(3,3′-DCB) have synergistic or additive effects on the urinary bladder when administered to rats at subcarcinogenic doses was also studied (*3*, *4*).

The incidence of PN hyperplasia of the bladder epithelium was significantly higher in groups treated with BBN plus FANFT, BBN plus 3,3′-DCB, BBN plus 2-AAF, and BBN plus 3,3′-DCB plus 2-AAF than in those treated with BBN, FANFT, 3,3′-DCB, or 2-AAF alone (Table 1). It was also higher in the group treated with 2-AAF plus FANFT than in that treated with 2-AAF alone. The incidence of papilloma was significantly higher in the group treated with BBN plus FANFT than in those treated with BBN or FANFT alone, and it was higher in the group treated with BBN plus 2-AAF than in those treated with BBN or 2-AAF alone. The incidence of transitional cell carcinoma was significantly higher in the group treated with BBN plus FANFT than in the group treated with BBN only. Thus synergistic effects on bladder tumorigenesis were observed on administration of BBN with FANFT, 2-AAF and/or 3,3′-DCB. The effects of BBN and FANFT were most synergistic.

The effects of sequential administrations of BBN, 2-AAF, FANFT and 3,3′-DCB at subcarcinogenic doses on urinary bladder carcinogenesis were examined in male Wistar rats (Table 2). Chemicals were each administered for 4 weeks in various sequences. No bladder carcinomas were present in rats given only BBN or any one of the other three chemicals. The incidences of bladder carcinomas were increased significantly by sequential administration of all four chemicals or the first three chemicals, without 3,3′-DCB. Bladder cancer also developed in rats administered FANFT, 2-AAF and 3,3′-DCB in se-

Table 1. Synergistic effects on urinary bladder carcinogenesis in rats of treatment with BBN, 2-AAF, FANFT and 3,3′-DCB for 40 weeks.

			Changes in urinary bladder, %				
					Cancer		
Group	Treatment[a]	Effective no. of rats	PN hyperplasia	Papilloma	No. of rats	With squamous metaplasia	With invasion
1	BBN	16	1 (6.3)	0 —	0 —	0 —	0 —
2	2-AAF	13	0 —	0 —	0 —	0 —	0 —
3	FANFT	11	1 (9.1)	1 (9.1)	0 —	0 —	0 —
4	3,3′-DCB	16	0 —	0 —	0 —	0 —	0 —
5	BBN + 2-AAF	11	9 (81.8)*	6 (54.5)*	1 (9.1)	0 —	0 —
6	BBN + FANFT	11	11 (100.0)*	10 (90.0)*	3 (27.3)**	2 (66.7)	1 (33.3)
7	BBN + 3,3′-DCB	7	5 (71.4)*	1 (14.3)	1 (14.3)	0 —	1 (100.0)
8	2-AAF + FANFT	14	6 (42.9)*	1 (7.1)	0 —	0 —	0 —
9	2-AAF + 3,3′-DCB	8	1 (12.5)	1 (12.5)	1 (12.5)	1 (100.0)	0 —
10	BBN + 3,3′-DCB + 2-AAF	10	8 (80.0)*	2 (20.0)	1 (10.0)	0 —	0 —
11	Control	12	0 —	0 —	0 —	0 —	0 —

[a]BBN: 0.001% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in drinking water; 2-AAF: 0.005% 2-acetylaminofluorene in diet; FANFT: 0.04% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide in diet; 3,3′-DCB: 0.03% 3,3′-dichlorobenzidine in diet.

*Significantly different from the groups treated with the respective carcinogens singly, at $p<0.01$.

**Significantly different from group 1 at $p<0.05$.

Table 2. Additive effects of BBN, FANFT, 2-AAF and/or 3,3'-DCB on urinary bladder carcinogenesis in rats.

			Changes in urinary bladder, %				
					Cancer		
Group	Treatment[a]	Effective no. of rats	PN hyperplasia	Papilloma	No. of rats	With squamous metaplasia	With invasion
1	BBN	20	8 (40.0)	5 (25.0)	0 —	0 —	0 —
2	BBN+FANFT	20	13 (65.0)	11 (55.0)	3 (15.0)	1 (33.3)	1 (33.3)
3	BBN+FANFT+2-AAF	19	11 (57.9)	7 (36.8)	5 (26.3)**	0 —	2 (40.0)
4	BBN+FANFT+2-AAF+3,3'-DCB	20	12 (60.0)	9 (45.0)	6 (30.0)*	1 (16.7)	2 (33.3)
5	FANFT	17	1 (5.9)	0 —	0 —	0 —	0 —
6	FANFT+2-AAF	19	2 (10.5)	0 —	0 —	0 —	0 —
7	FANFT+2-AAF+3,3'-DCB	20	4 (20.0)	1 (5.0)	1 (5.0)	0 —	0 —
8	2-AAF	19	0 —	0 —	0 —	0 —	0 —
9	2-AAF+3,3'-DCB	18	1 (5.6)	0 —	0 —	0 —	0 —
10	3,3'-DCB	19	0 —	0 —	0 —	0 —	0 —
11	Control	12	0 —	0 —	0 —	0 —	0 —

[a]BBN: 0.01% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in drinking water; FANFT: 0.15% *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide in diet; 2-AAF: 0.025% 2-acetylaminofluorene in diet; 3,3'-DCB: 0.03% 3,3'-dichlorobenzidine in diet.

*Significantly different from the incidence in group 1 ($p<0.02$).

**Significantly different from group 1 at ($p<0.01$).

quence. No antagonistic effects of the chemicals were observed.

Promoting Effects of Various Chemicals in Two-Stage Urinary Bladder Carcinogenesis

Saccharin and tryptophan are known to act as promoters after initiation of urinary bladder carcinogenesis with carcinogens (*5*). Various chemicals are present in the environment and many human cancers are attributable to environmental factors. Thus since some of the environmental chemicals to which humans are exposed may promote urinary bladder carcinogenesis, special consideration should be paid to evaluation of their promoting activities. Carcinogenesis of the urinary bladder was initiated in male 6-week-old F344 or Wistar rats by adding 0.01% BBN to their drinking water for 4 weeks, and then the animals were given 16 test chemicals to examine their respective promoting activities (*6*). The incidence and number of PN hyperplasias per 10 cm of basement membrane were measured with a color video image processor. Administration of 5.0% saccharin significantly increased the incidence and number of PN hyperplasias. This finding could be related to the induction of cancers in rat urinary bladder by high levels of saccharin. Sodium ascorbate, DL-tryptophan, allopurinol and diphenyl also increased the numbers of PN hyperplasias significantly, but other test chemicals, such as acetazolamide, quercetin, cyclamate, *o*-phenylphenol and caffeine, did not. The results suggest that sodium ascorbate, allopurinol and diphenyl have promoting activities in urinary bladder carcinogenesis in rats. The results indicating that sodium ascorbate is a promoter in urinary bladder carcinogenesis are consistent with other experiments.

In general, chemical carcinogens show dose-response relations for both tumor induction and the length of the induction period. Studies were made on the dose-response to saccharin as a promoter in induction of preneoplastic lesions of the urinary bladder (*7*). F344 rats of both sexes were initiated by adding 0.01% BBN to their drinking water for 4 weeks and were then given diets containing saccharin at various concentrations for 32 weeks and killed at the end of week 36. The incidences and average numbers of PN hyperplasia per 10 cm of basement membrane in males with a dose of 5.0% saccharin and in females with doses of 5.0% and 1.0% were significantly higher than those with BBN alone. Plots of the incidence of PN hyperplasia against the dose of saccharin gave parabolic curves in both sexes (Fig. 1); these dose-response curves showed enhanced hyperplastic responses in both sexes given 0.2 to 5.0% saccharin. The results suggest that if the experimental period had been longer, a dose-response relationship for the induction of papilloma and cancer might have been observed.

The organ specific effects of two different tumor promoters were investigated in a two-stage carcinogenesis experiment (*8*). In the initiation stage, the rats were given 0.02% 2-AAF in the diet for 4 weeks or 0.01% BBN in the drinking water for 4 weeks. Then, in the promotion stage, they were given 0.05% phenobarbital in the diet or 5.0% saccharin in the diet for 32 weeks. Phenobarbital greatly enhanced hepatocarcinogenesis and induced hepatocellular carcinoma after 2-AAF treatment, and it

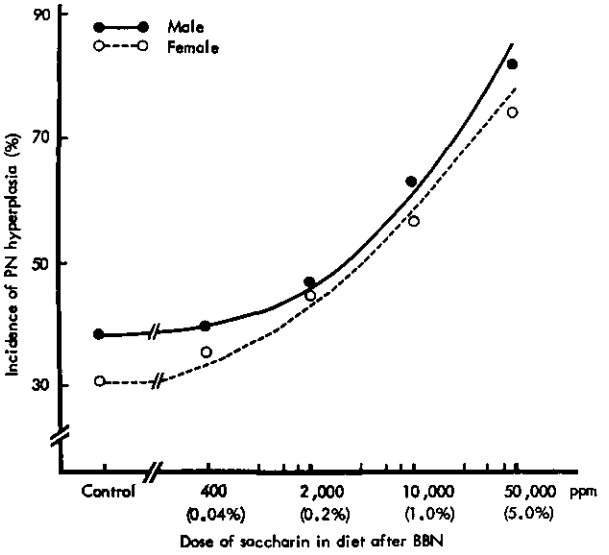

FIGURE 1. Dose-response curve for incidence of PN hyperplasia in rat urinary bladder.

significantly increased formation of hyperplastic liver nodules after BBN treatment. Saccharin significantly enhanced the induction of PN hyperplasias of the urinary bladder after BBN or 2-AAF treatment (Table 3). However, after 2-AAF or BBN treatment, there was no effect of phenobarbital on the urinary bladder or of saccharin on liver neoplasia induction. These data indicate that although 2-AAF and BBN have tumor-initiating effects in both liver and urinary bladder, the tumor-promoting effects of phenobarbital and saccharin are organ specific.

Strain difference is one of the factors that modify chemical carcinogenesis. Strain differences appear to modify promotion as well as initiation in two-stage carcinogenesis. Therefore, we tested whether there was any strain difference in the effect of saccharin as a promoter. Male ACI, Wistar, F344 and Sprague-Dawley (SD) rats were given a diet with 5.0% saccharin for 52 weeks (*9*). Histological examination showed that saccharin induced urinary bladder lesions in ACI rats, but no changes in Wistar, F344 or SD rats. PN hyperplasia and papilloma were increased significantly in the urinary bladder of ACI rats given saccharin. Scanning electron microscopy showed mucosal foci with slightly elevated cells, giving a cobblestone appearance, on the luminal surface of the urinary bladder. The superficial epithelial cells were covered with short, uniform microvilli and with ropy or leafy microridges. In addition, several superficial epithelial cells with pleomorphic microvilli on their luminal surface were found in these foci, as described previously. Among the groups given saccharin, the ACI rats had the greatest changes as shown by scanning electron microscopy. Slight formation of pleomorphic microvilli and short, uniform microvilli were seen in Wistar and F344 rats treated with saccharin, but not in SD rats. Thus, there was a clear strain difference in rats in susceptibility of the urinary bladder to saccharin. It is suggested that the promoting effect of saccharin in the urinary bladder was greatest in ACI rats.

Inhibitory Effects of Aromatic Retinoic Acid, OK-432 and DDPM on Urinary Bladder Carcinogenesis of Rats

There have been many reports that vitamin A and its analogs inhibit tumor induction in many or-

Table 3. Histopathologic changes of the urinary bladder in rats treated with 2-AAF or BBN followed by PB or SS.

				PN hyperplasia		Papilloma	
Group	Treatment[a]	No. of rats	Simple hyperplasia, incidence, %	Incidence, %	No./10 cm basement membrane[b]	Incidence, %	No./10 cm basement membrane[b]
1A	2-AAF + PB	24	0 —	0 —	0	0 —	0
1B	2-AAF + SS	29	6 (21)**	4 (14)**	0.3 ± 1.1	0 —	0
1C	2-AAF	28	0 —	0 —	0	0 —	0
2A	BBN + PB	30	19 (63)	14 (47)	0.6 ± 0.7	9 (30)	0.4 ± 0.8
2B	BBN + SS	29	27 (93)**	24 (83)**	2.6 ± 2.2*	6 (21)	0.2 ± 0.4
2C	BBN	28	19 (68)	11 (39)	0.7 ± 1.0	8 (29)	0.4 ± 0.7
3A	PB	29	0 —	0 —	0	0 —	0
3B	SS	28	0 —	0 —	0	0 —	0
3C	Control	29	0 —	0 —	0	0 —	0

[a]2-AAF: 0.02% 2-acetylaminofluorene in diet; BBN: 0.01% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in drinking water; PB: 0.05% phenobarbital in diet; SS: 5.0% sodium saccharin in diet.

[b]Values are means ± SD.

**$p<0.05$ compared with subgroup C (basal diet).

*$p<0.01$ compared with subgroup C (basal diet).

gans, such as the mammary gland, skin, urinary bladder, lung, bronchus, forestomach and colon. The inhibitory effect of an aromatic retinoic acid analog, ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, on bladder carcinogenesis in rats treated with BBN was evaluated (*10*). When given after BBN, 50 ppm of the aromatic retinoid greatly reduced the incidence and number of papillomas, and slightly inhibited the development of cancer. Administration of 100 ppm of the aromatic retinoid after BBN treatment also greatly reduced the incidences and numbers of papillomas and cancers. These results show that the aromatic retinoid inhibits bladder carcinogenesis of rats induced by BBN.

The relationship of immune status in patients with cancer of the urinary bladder at various stages of the disease has not been clearly defined, but it appears that there is a gradual decrease in the immune responsiveness of the patient as the disease progresses. OK-432 is a streptococcal preparation that enhances cellular immune and reticuloendothelial responsiveness to tumors. The effect of immunostimulation by OK-432 on bladder carcinogenesis in rats was evaluated by using the carcinogens (*11*). Short-term subcutaneous administration of OK-432 after BBN did not affect bladder carcinogenesis, but if OK-432 treatment was begun after BBN treatment and continued until the end of the experiment, the incidence of BBN-induced bladder tumor was significantly reduced. These results suggest that OK-432 may have a tumor inhibitory effect during bladder carcinogenesis but that its effect is reversible.

4,4′-Diaminodiphenylmethane (DDPM) is a potent, selective, aromatic hepatotoxic agent. It induces marked bile duct proliferation in the liver of rats like α-naphthylisothiocyanate (ANI). ANI reduced the incidence of bladder tumors when fed to rats either before or during the administration of bladder carcinogens (*12*). Thus, the hepatotoxic agent that produced bile duct proliferation had significant inhibitory effects on bladder tumorigenesis induced by chemical carcinogens. We examined the effects of administration of hepatotoxic agents after carcinogen treatment on the development of bladder tumors in rats (*13*). Rats were given drinking water containing 0.01% BBN for 4 weeks and then 0.1% DDPM in their diet for 34 weeks. DDPM reduced the induction of papillomas of the bladder as measured by the incidence and number/10 cm basement membrane, although it did not inhibit the induction of PN hyperplasia of the bladder. These results indicate that DDPM administration in the "post-initiation" stage inhibited bladder carcinogenesis in rats.

Conclusions

BBN-induced rat bladder cancers resemble human bladder cancers in morphological characteristics and there appear to be several close parallels between the human disease and rat models. 2-AAF is also a suitable bladder carcinogen to use experimentally, although it is less carcinogenic than BBN in the rat bladder. These carcinogens should be useful in experimental assessment of the etiology, prevention, and therapy of human bladder cancer.

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