Effects of Testosterone on Cell Proliferation and Apoptosis in BBN-Induced Mouse Urinary Bladder Carcinogenesis

Hirofumi Ohno

Department of Urology, Faculty of Medicine, Tottori University, Yonago 683-0826 Japan

Human bladder cancer is nearly 3 times more common in men than in women. In general, the sex difference in incidence of human bladder carcinoma is considered to be due to industrial and environmental carcinogens or other factors, though there is no clear evidence supporting this. We suspected that the sex difference in incidence of bladder carcinoma might be due to the effects of testosterone. We investigated the effects of testosterone on mouse bladder carcinogenesis by using immunohistochemical staining and terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). A total of 94 four-week-old male mice were divided into 3 groups. In Group I, castration was carried out, then 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was administered until the end of the experiment. In Group II, 0.05% BBN was administered similarly, without castration. In Group III, 0.05% BBN was administered similarly, without castration, and 3 mg/kg of testosterone was injected intramuscularly weekly from the beginning of the experiment. All mice were cystectomized at the end of the experimental period to investigate the incidence of bladder cancer, and immunohistochemical staining and TUNEL were performed to examine the correlation between cell proliferation and apoptosis. Among the 3 groups, occurrence of bladder tumor was most frequent in Group III, and tumor induction time was the shortest. The proliferation index for tumors significantly increased as the stage and grade progressed. On the other hand, the apoptotic index for tumors significantly decreased. The proliferation index was the highest in Group III and the lowest in Group I. A significant difference in the proliferation index was observed among the 3 groups. No significant difference was observed in the apoptotic index among the 3 groups. Our results indicate that the effect of testosterone on mouse bladder carcinogenesis is more significantly related to cell proliferation than to suppression of apoptosis, with the result that testosterone promotes the occurrence of BBN-induced mouse bladder carcinomas.

Key words: apoptosis; BBN-induced mouse urinary bladder carcinogenesis; cell proliferation; testosterone

The fact that the incidence of human urinary bladder carcinoma is higher in males than in females has often been reported in the literature. It has been concretely established that the maleto-female ratio among patients is about 3:1 (Laor et al., 1984; Yamada et al., 1986). However, there is no clear evidence why gender results in different incidences of urinary bladder tumors. In general, the cause of sex difference in incidence of human bladder carcinogenesis is believed to be from industrial and environmental factors. Although over the past 30 years, women have joined the male-predominant workplace, changed their habits and been exposed to both industrial and environmental carcinogens, new cases of human bladder cancer in

BBN, a carcinogen of urinary bladder cancer in animal models

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine; TdT, terminal-deoxynucleotidyl-transferase; TUNEL, TdT-mediated dUTP-biotin nick end labeling; PCNA, proliferating cell nuclear antigen

United States still show no statistical change, and bladder cancer still remains about 3 times more common in men than in women (Messing and Catalona, 1998). We considered that sex difference in bladder carcinogenesis might be related to sex hormones, and specifically to testosterone, and decided to investigate the influence of testosterone on bladder carcinogenesis.

One of the most important molecular mechanisms in the process of carcinogenesis is apoptosis. Apoptosis is a morphologically and biochemically distinct form of cell death, and its major

mechanism is the maintenance of homeostasis and the balance of cell proliferation in normal and malignant cells (Kerr et al., 1972, 1994). Terminal-deoxynucleotidyl-transferase (TdT)mediated dUTP-biotin nick end labeling (TUNEL) has been successfully used to detect apoptotic cells in routine paraffin-embedded sections (Gavrieli et al., 1992).

Thus, we investigated whether testosterone influenced mouse bladder carcinogenesis and analyzed the relationship between cell proliferation and apoptosis by using immunohistochemical staining and TUNEL.

Materials and Methods

A total of 94 four-week-old male C57BL/6 mice (Shimizu, Kyoto, Japan) were housed 5 to 6 per plastic cage with hardwood chips for bedding, in an air-conditioned room at 23 ± 2 °C and $60 \pm$ 10% humidity with a 12-h light-dark cycle. They were divided at random into 3 groups of 30 to 33 mice each. *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) (Tokyo Kasei, Tokyo, Japan) was dissolved in tap water to concentrations of 0.05%, and was added to a few drops of Tween 80 (Wako, Osaka, Japan).



Fig. 1. Experimental protocol. In Group I, castration is carried out and 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) is administered. In Group II, 0.05% BBN is similarly administered. In Group III, 0.05% BBN is administered and 3 mg/kg of testosterone is injected intramuscularly weekly. Mice are cystectomized at the end of each experimental period. wk, weeks.

0.05% BBN was supplied to each group beginning at 5 weeks of age.

Experiment I

A total of 44 male mice were investigated in Experiment I. In Group I, castration was carried out under ether anesthesia at 4 weeks, and from 5 weeks of age, mice were administered 0.05% BBN orally for a period of 18 weeks. In Group II, mice were administered 0.05% BBN as described above, without castration, from 5 weeks of age, for a period of 18 weeks. In Group III, castration was not performed, and from 5 weeks of age, 0.05% BBN was given as described, and 3 mg/kg (body weight) of testosterone propionate (Enarmon-oil, Teikokuzoki, Tokyo) was injected intramuscularly once weekly for a period of 18 weeks. All mice were cystectomized on the 18th week (Fig. 1).

Experiment II

A total of 50 male mice were investigated in Experiment II. Mice were divided into 3 groups and housed as described in Experiment I. All mice were cystectomized at the end of the different experimental period because we believed that the difference in cystectomized period results in no significant difference in tumor stage and tumor grade among the 3 groups. Thus in Group I, mice were cystectomized in groups of 3 to 5 from the 28th to the 33rd week, in Group II, from the 23rd to the 27th week and in Group III, from the 21st to the 24th week (Fig. 1).

In Experiments I and II, all mice were cystectomized under ether anesthesia to investigate the incidence of bladder cancer. All tissue specimens were fixed by an injection of about 0.10 to 0.15 mL of 10% formalin into the lumen, and immersed in the same solution. After fixation, each specimen was cut into 4 sections and embedded in paraffin, and a series of 3- μ m thick sections was prepared for hematoxylin and eosin staining, immunohistochemical staining and TUNEL.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA)

Immunohistochemical staining was performed with a Histofine SAB-PO kit (Nichirei, Tokyo). Paraffin-embedded sections were placed on silane-coated glass slides and air-dried at room temperature. Deparaffinized and rehydrated sections were heated by microwave oven at 92°C for 10 min, and then cooled for 60 min at room temperature. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxidase in methanol for 20 min. After blocking nonspecific reactions with 10% normal rabbit serum, the sections were incubated with a monoclonal antibody against PCNA (DAKO, Glostrup, Denmark) with 1:200 dilution at 4°C overnight. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min, followed by incubation with streptavidin-peroxidase complex for 30 min and rinsed with several changes of phosphate-buffered saline between steps. The color was developed with diaminobenzidine solution. Finally, they were lightly counterstained with hematoxylin.

TUNEL

TUNEL was performed by using an Apop Tag Plus in situ apoptosis detection kit (Intergen, New York, NY). In brief, paraffin sections were de-waxed, rehydrated through a graded alcohol series and washed with distilled water. Subsequently, tissues were digested with 40 µL/mL proteinase K (Wako) at 37°C for 80 min, because proteinase K is known to enhance positive nuclear labeling in apoptotic cells. After prehybridization treatment, the sections were incubated with TdT and digoxigenindUTP in a moist chamber for 90 min at 37°C. Incubation with anti-digoxigenin-antibodyperoxidase for 30 min at room temperature was employed for detection of digoxigenin-dUTP labeling. The color was developed with diaminobenzidine solution and counterstained with hematoxylin or methyl green.

The proliferation and apoptotic indices

PCNA-positive cells were used to quantify the proliferation index (percentage of PCNA-positive cells in 1000 cells). Similarly, TUNELpositive cells were used to quantify the apoptotic index (percentage of TUNEL-positive cells in 1000 cells). In benign lesions and carcinomas in situ, the proliferation and apoptotic indices were measured in the mucosal layers, and in invasive carcinomas, counted at the infiltrative layers.

Statistics

The difference for occurrence of bladder tumor in Experiment I was analyzed with the Kruskal-Wallis rank test. The distribution of histological stages, tumor grades and tumor induction times in Experiment II was analyzed with the one-way analysis of variance. The relationship of the proliferation or apoptotic index to the stage and grade was examined with the Kruskal-Wallis rank test. The correlation between the proliferation index and the apoptotic index was analyzed by using a Pearson's correlation coefficient. A level of P < 0.05 was regarded as statistically significant.

	Group I	GroupII	Group III	Statistical analysis
Occurrence of bladder tumor (%)	13.3	64.3	80.0	P < 0.001†
Invasive tumor induction time (week)	30.3	25.4	22.8	$P < 0.001 \ddagger$

Table 1. Occurrence of bladder tumors in Experiment I and invasive tumor induction time in Experiment II

† Kruskal-Wallis rank test.

‡ One-way analysis of variance.

Results

Experiment I

A total of 44 mice were cystectomized in the 18th week. Occurrence of urinary bladder cancer was 13.3% (1 carcinoma in situ; 1 invasive carcinoma) in 15 mice from Group I, 64.3% (6 carcinomas in situ; 3 invasive carcinomas) in 14 mice from Group II and 80.0% (7 carcinomas in situ; 5 invasive carcinomas) in 15 mice from Group III. Statistically significant differences were found among the 3 groups (P < 0.001) (Table 1).

Experiment II

A cumulative total of 50 mice were cystectomized between the 28th and 33rd week in Group I, between the 23rd and 27th week in Group II and between the 21st and 24th week in Group III. Occurrence of invasive bladder cancer was 72.2% in Group I, 82.4% in Group II and 86.7% in Group III. No significant distribution of cases was observed according to the histological stage and grade of tumors in the 3 groups. The tumor induction time was a mean of 30.3 weeks in Group I, 25.4 weeks in Group II and 22.8 weeks in Group III. A significant difference in tumor induction time was found among the 3 groups (P < 0.001) (Table 1).

The proliferation and apoptotic indices

The relationship between the mean proliferation index and the stage of tumors is shown in Table 2. In each group, the proliferation index significantly increased as the tumor stage progressed. In invasive carcinomas, a significant difference in the proliferation index was observed among the 3 groups (P = 0.005). The relationship between the proliferation index and the grade of invasive carcinomas is shown in Table 3. In Groups I and III, the proliferation index significantly increased as the tumor grade increased. In Group II, no significant difference of the proliferation index was observed, though values tended to increase as mentioned above. In every grade of tumor, a significant difference in the proliferation index was found among the 3 groups. The relationships between the mean apoptotic index and the tumor stage or grade are shown in Tables 4 and 5, respectively. In each group, the apoptotic index significantly

Table 2.	Relationship between	the proliferation index and the stage of tumors	

Benign lesion (%)	Carcinoma in situ (%)	Invasive tumor (%)	Statistical analysis†
1.1	6.7	17.1	<i>P</i> < 0.001
0.2	6.4	31.0	P < 0.001
1.1	13.2	42.7	P < 0.001
NS	NS	P = 0.005	
	Benign lesion (%) 1.1 0.2 1.1 NS	Benign lesion (%) Carcinoma in situ (%) 1.1 6.7 0.2 6.4 1.1 13.2 NS NS	Benign lesion (%)Carcinoma in situ (%)Invasive tumor (%)1.16.717.10.26.431.01.113.242.7NSNS $P = 0.005$

†Kruskal-Wallis rank test.

NS, not significant.

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	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Statistical analysis†
Group I	9.9	16.1	25.3	P = 0.035
Group II	24.7	26.3	41.2	NS
Group III	14.0	33.0	71.4	P = 0.001
Statistical analysis	P = 0.012	P = 0.039	P = 0.004	

Table 3. Relationship between the proliferation index and the grade of tumors

†Kruskal-Wallis rank test.

NS, not significant.

decreased as the stage and grade progressed. No significant difference in apoptotic index was found among the 3 groups. Examples shown in Figs. 2, 3 and 4 are a benign lesion in Group II, a Grade-3 invasive bladder carcinoma in Group I and a Grade-3 invasive bladder carcinoma in Group III, respectively.

Correlation between the proliferation index and apoptotic index

The correlation between the proliferation index and apoptotic index is shown in Fig. 5. A negative significant correlation was observed (r = -0.7, P < 0.001).

Discussion

Druckrey et al. (1964) first reported that oral administration of BBN in rats induced bladder carcinoma only organotrophically. Since then, some investigators have reported on histogenetic studies of urinary bladder tumors in various animals induced by BBN (Kakizoe, 1995). In the 1970's, several reports indicated hormonal effects on the sex difference in the incidence of bladder carcinogenesis. Some of these studies showed that the occurrence of bladder carcinoma in animal models induced by BBN is higher in males than in females (Bertram and Craig, 1972; Iriya, 1979). Others have shown that testos-

Table 4. Relationship between the apoptotic index and the stage of tumors

	Benign lesion (%)	Carcinoma in situ (%)	Invasive tumor (%)	Statistical analysis†
Group I	54.0	51.3	26.7	P = 0.001
Group II	64.5	43.5	21.4	P < 0.001
Group III	69.0	36.3	18.8	P = 0.009
Statistical analysis	NS	NS	NS	

[†]Kruskal-Wallis rank test. NS, not significant.

Table 5. Relationship between the apoptotic index and the grade of tumors

	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Statistical analysis [†]
Group I	35.4	39.3	8.0	P = 0.010
Group II	28.5	28.7	8.2	P = 0.042
Group III	36.4	21.9	5.1	P = 0.006
Statistical analysis	NS	NS	NS	

†Kruskal-Wallis rank test.

NS, not significant.

terone promotes the occurrence of bladder carcinomas, and that diethylstilbestrol suppresses it in BBN-induced rats (Okajima et al., 1975; Kono et al., 1975). However, at present there is no clear evidence to explain these hormonal influences on urinary bladder carcinogenesis. In general, the effects of human bladder carcinogenesis are considered to be due to industrial and environmental carcinogens (Risch et al., 1995) such as aniline dye, cigarette smoking, etc., and hormonal influence is considered negligible. Recently, few reports have appeared on hormonal effects in bladder carcinogenesis. It was estimated that, in 1995, among 50,500 new cases of human bladder cancer diagnosed in the United States, the incidence was about 3 times more common in men than in women (Wingo et al., 1995). Over the past 30 years, women have joined the male-predominant workplace, changed their habits and have subsequently been exposed to both industrial and environmental carcinogens, but surprisingly, this sex difference in the number of new cases of human bladder cancer remains (Messing and Catalona, 1998). We considered that hormonal influence, especially of testosterone, might relate to this contradictory sex difference in incidence, and that the hormonal influence on bladder carcinogenesis should be investigated.

Human bladder carcinomas can be classified into papillary superficial bladder carcinoma and non-papillary invasive bladder carcinoma. The former frequently recurs after treatment such as transurethral resection or



Fig. 2. A benign bladder lesion in Group II. **a:** Hematoxylin and eosin staining. **b:** A few proliferating cell nuclear antigen-positive cells shown by immunohistochemical staining. **c:** A number of apoptotic cells shown by terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end labeling, \times 200.