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

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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 male-to-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.

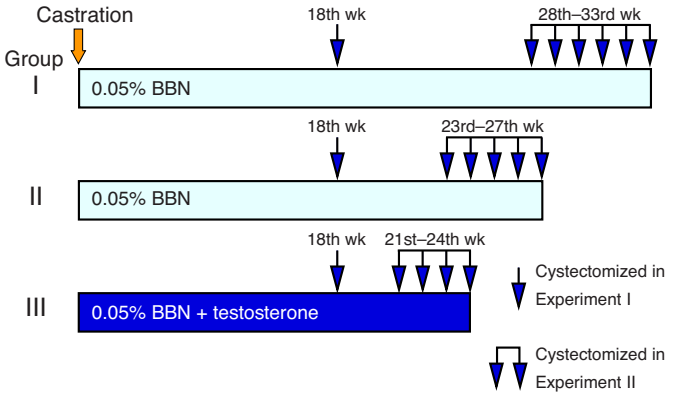


Fig. 1. Experimental protocol. In Group I, castration is carried out and 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) is administered. In Group II, 0.05% BBN is similarly administered. In Group III, 0.05% BBN is administered and 3 mg/kg of testosterone is injected intramuscularly weekly. Mice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

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 ± 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). 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-μm 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 digoxigenin-dUTP in a moist chamber for 90 min at 37˚C. Incubation with anti-digoxigenin-antibody-peroxidase 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, TUNEL-positive 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.

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

	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	$P < 0.001$‡

† 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†
Group I	1.1	6.7	17.1	$P < 0.001$
Group II	0.2	6.4	31.0	$P < 0.001$
Group III	1.1	13.2	42.7	$P < 0.001$
Statistical analysis	NS	NS	$P = 0.005$	

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

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

	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	$P = 0.012$	$P = 0.039$	$P = 0.004$	

†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

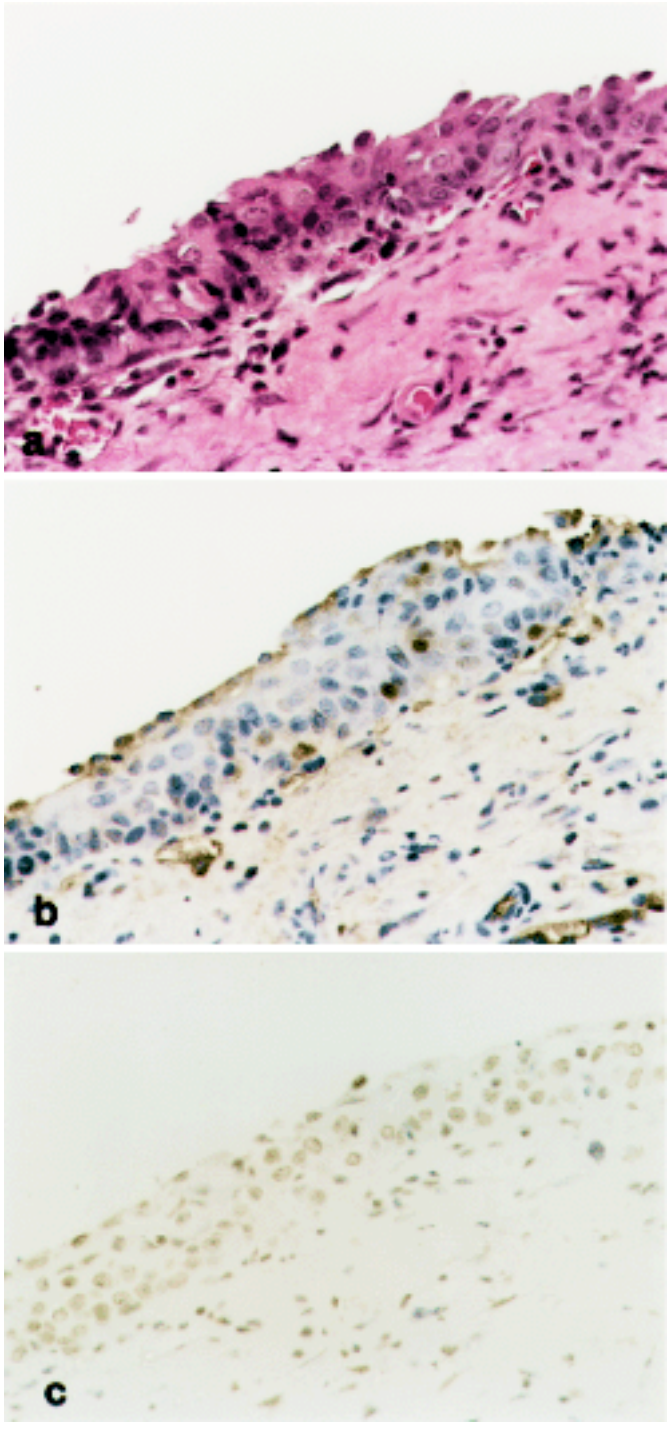


Fig. 2. A benign bladder lesion in Group II. **a:** Hematoxylin and eosin staining. **b:** A few proliferating cell nuclear antigen-positive cells shown by immunohistochemical staining. **c:** A number of apoptotic cells shown by terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end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

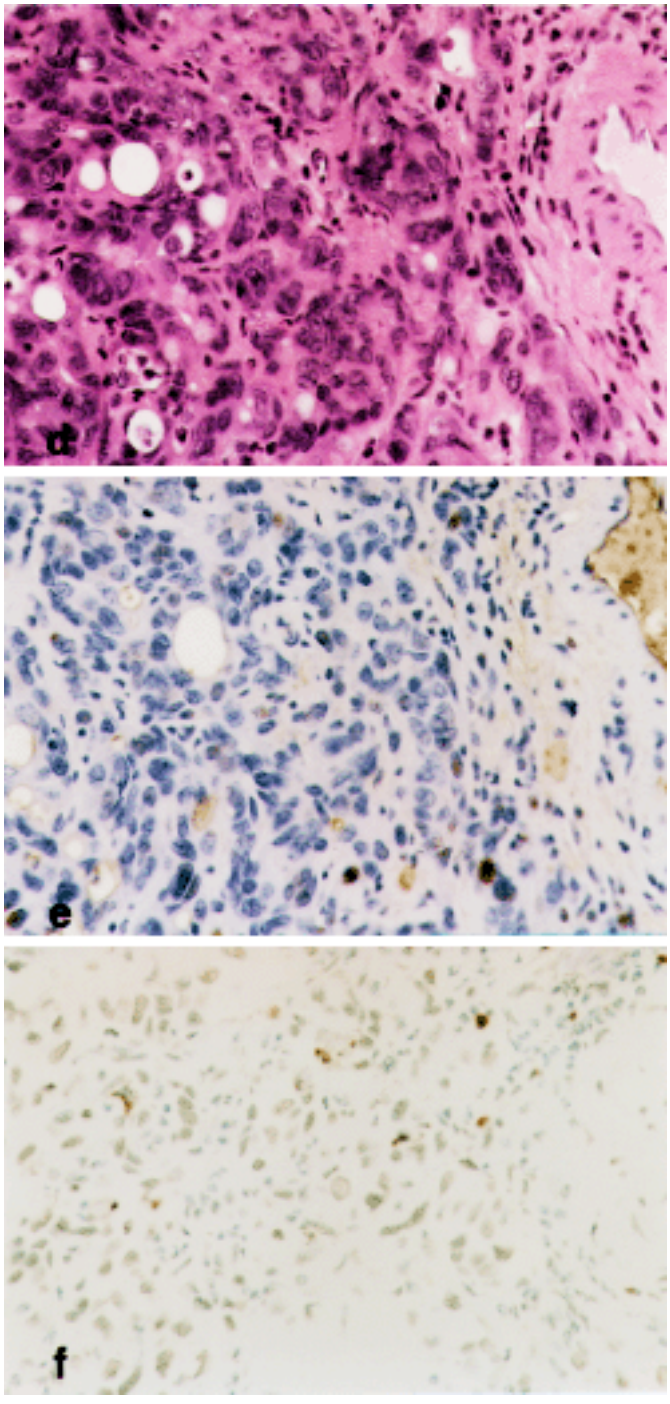
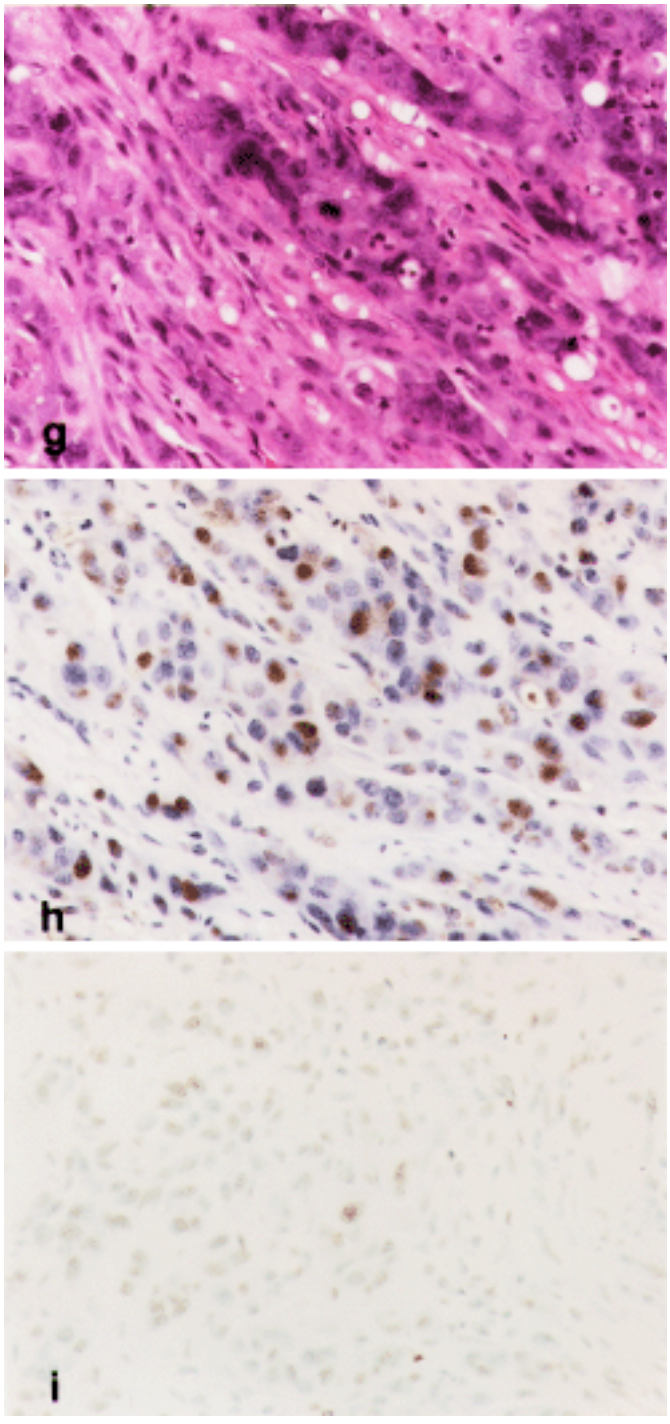


Fig. 3. A Grade-3 invasive bladder carcinoma in Group I. **d:** Hematoxylin and eosin staining. **e:** Proliferating cell nuclear antigen-positive cells shown by immunohistochemical staining. **f:** Some apoptotic cells shown by terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end labeling, × 200.

coagulation, but its prognosis is good. The latter frequently shows metastasis and has a very poor prognosis and many problems. Most of the previous studies reported the sex hormone influence on urinary bladder carcinogenesis in the rat model. In all BBN-treated rat strains, bladder carcinomas are usually papillary, multiple and superficial. Thus bladder carcinomas in rats are a satisfactory model for studying papillary superficial bladder carcinomas in humans (Ito et al., 1975). On the other hand, mice whose bladder epithelium has changed to dysplasia, carcinoma in situ and invasive tumor by BBN are good models for human invasive bladder carcinomas (Hirose et al., 1976). Thus, mice were selected in the present investigation as models for invasive bladder carcinomas in humans.

In Experiment I, the occurrence of carcinomas was significantly higher in Group III and significantly lower in Group I than in the other groups. In Experiment II, the tumor induction time was significantly shorter in Group III and significantly longer in Group I than in the other groups. A previous report showed that both surgical castration and luteinizing hormone-releasing hormone agonist treatment significantly reduced the occurrence of carcinomas as compared with controls; thus, surgery and ether anesthesia had little influence on carcinoma occurrences (Imada et al., 1997). Similarly, intramuscular injection was not found to affect induction of carcinogenesis (Kono et al., 1975). Our findings also showed that an overdose of testosterone promotes the occurrence of blad-



der carcinomas in BBN-induced mice and that suppression of testosterone decreases it.

Previously, the effects of testosterone on bladder carcinogenesis in animal models were considered to suppress the detoxication of BBN, induce a stimulation pathway for BBN-proximate carcinogen in the liver (Bertram and Craig, 1972) or increase cell growth in premalignant lesions of the bladder epithelium (Okajima et al., 1975). Recently, Imada et al. (1997) demonstrated the existence of androgen receptors on the bladder epithelium of mice and rats using an immunohistochemical staining. Noronha and Rao (1986) also reported that sex hormone receptors were identified in human advanced transitional cell carcinomas, and dihydrotestosterone and testosterone receptors were found more frequently than estrogen receptors. Although there is no clear evidence, testosterone may act on bladder mucosa and promote bladder carcinogenesis as it does in prostate cancer.

Previous studies have shown significant correlations between PCNA and histological stages or grades in human bladder cancer (Waldman et al., 1993; Skopeliou et al., 1997). PCNA expression in rats was also found to gradually increase as histological stages and grades advance (Yamashi, 1996). The present study yielded similar results, and our findings agreed significantly with those of the previous reports. In Group III,

Fig. 4. A Grade-3 invasive bladder carcinoma in Group III. **g:** Hematoxylin and eosin staining. **h:** Proliferating cell nuclear antigen-positive cells shown by immunohistochemical staining are significantly more stained than in Group I (Fig. 3e). **i:** Apoptotic cells are almost equal in quantity to in Group I, shown by terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP-biotin nick end labeling (Fig. 3f), × 200.

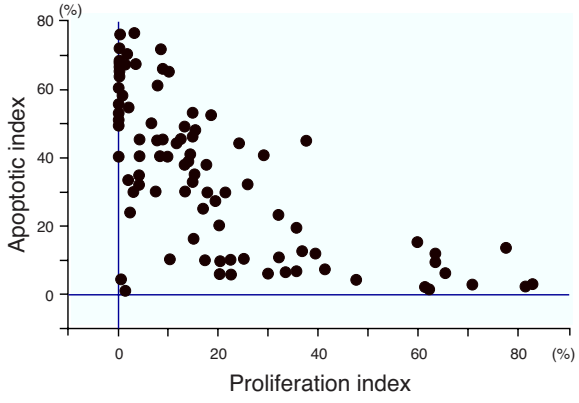


Fig. 5. Correlation between the proliferation index and the apoptotic index. A significant negative correlation is found ($r = -0.7$, $P < 0.001$, Pearson's test).

PCNA showed the highest expression, and in Group I, the lowest expression among the 3 groups. These findings support that testosterone promotes increased cell proliferation in BBN-induced mouse bladder carcinogenesis.

Apoptosis is one of the most important molecular mechanisms in the process of carcinogenesis. Originally, apoptosis is characterized by a marked reduction in cell volume and increase in density. Apoptotic bodies are histologically characterized by their small size, nuclear chromatin condensation, DNA fragmentation and compactness of cytoplasmic organelles (Kerr et al., 1972, 1994). TUNEL was introduced by Gavriel et al. (1992), and shown to be able to detect apoptosis cells in routine formalin-fixed, paraffin-embedded sections. The extent of apoptosis has been examined in several experimental tumor types (Aihara et al., 1994; Tatebe et al., 1996). The previous studies reported that the apoptotic index in bladder tumors, such as cell proliferation, indicates a positive correlation between histological stages and grades in humans (Koyuncuoglu et al., 1998) or a negative correlation in rats (Shirai et al., 1995). In the present study, the occurrence of apoptosis in a BBN-treated mouse showed a negative correlation with histological stage, grade or proliferation index. These different results may be influenced by differences in numerous oncogenes and tumor suppressor genes, including *bcl-2*, c-*myc*, *p53*, etc. among the various models.

This is the first study that indicates a correlation between the effects of testosterone in BBN-induced mouse bladder carcinogenesis and cell proliferation and apoptosis. Among the 3 groups, the proliferation index of invasive carcinomas was significantly higher in Group III and lower in Group I than in other groups; on the other hand, no significant difference was observed in apoptotic index. These results suggest that one of the effects of testosterone in BBN-induced mouse bladder carcinogenesis increases cell proliferation rather than decreasing apoptosis, and that this effect promotes mouse bladder carcinogenesis.

However, the exact mechanisms by which testosterone promotes bladder carcinogenesis are not known. Thus, it is necessary to investigate the relationship between testosterone and growth factor production in the process of bladder carcinogenesis. In the future, it might be possible to perform a chemoprevention trial on bladder cancer with hormonal therapy, provided that further investigations clarify the mechanisms related to the effects of testosterone on human bladder carcinogenesis.

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References

1 Aihara M, Truong LD, Dunn JK, Wheeler TM, Scardino PT, Thompson TC. Frequency of apoptotic bodies positively correlates with Gleason grade in prostate cancer. Hum Pathol 1994;25: 797–801.

2 Bertram JS, Craig AW. Specific Induction of bladder cancer in mice by butyl-(4-hydroxybutyl)-nitrosamine and the effects of hormonal modifications on the sex difference in response. Eur J Cancer 1972;8:587–594.

3 Druckrey H, Preussmann R, Ivankovic S, Schmidt CH, Mennel HD, Stahl KW. Selektive Erzeugung von Blasenkrebs an Ratten durch Dibityl- und N-Butyl-butanol(4)-nitrosamine. Z Krebsforsch 1964;66:280–290.
4 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragment. J Cell Biol 1992;119:493–501.
5 Hirose M, Fukushima S, Hananouchi M, Shirai T, Ogiiso T, Takashi M, et al. Different susceptibilities of the urinary bladder epithelium of animal species to three nitroso compounds. Gann 1976;67:175–189.
6 Imada S, Akaza H, Ami Y, Koiso K, Ideyama Y, Takenaka T. Promoting effects and mechanisms of action of androgen in bladder carcinogenesis in male rats. Eur Urol 1997;31:360–364.
7 Imada S, Akaza H, Ohtani M, Koiso K. Effects of androgen regulation system on bladder carcinogenesis in male mice. Nippon Hinyokika Gakkai Zasshi 1995;86:1666–1672 (in Japanese).
8 Ito N, Arai M, Sugimura S, Sugihara S, Hirao, K, Makiura S, et al. Experimental urinary bladder tumors induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine. Gann Monogr 1975;17:367–381.
9 Iriya K. The effects of sex hormones on development of urinary bladder tumors in rats induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine. Nara Igagu Zasshi 1979;30:69–82 (in Japanese).
10 Kakizoe T. Clinical aspects of bladder cancer from a perspective of carcinogenesis. Tokyo: Medical view; 1995 (in Japanese).
11 Kerr JFR, Winterford CM, Harmon BV. Apoptosis: its significance in cancer and cancer therapy. Cancer 1994;73:2013–2026.
12 Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972; 26:239–257.
13 Kono N, Sasaki N, Tanahashi T, Muraoka N, Azuma T. The effect of sex hormones on the incidence of experimental urinary bladder tumors in rats. Nippon Hinyokika Gakkai Zasshi 1975; 66:409–417 (in Japanese).
14 Koyuncuoglu M, Kargi A, Cingoz S, Kirkali Z. Investigation of *p53*, c-*erbB-2*, PCNA immunoreactivity, DNA content, AgNOR and apoptosis in bladder carcinoma as prognostic parameters. Cancer Lett 1998;126:143–148.
15 Laor E, Toria MB, Schiffman Z, Freed ZS, Reid ER. Sex-dependent variations in natural history of transitional cell carcinoma of bladder. Urology 1984;23:237–239.
16 Messing EM, Catalona W. Urothelial tumors of the urinary tract. In: Walsh PC, Retic AB, Vaughan ED, Wein AJ, eds. Campbell's urology. 7th ed. Philadelphia: Saunders; 1998. p. 2327–2410.
17 Noronha RFX, Rao BR. Sex hormone receptors in localized and advanced transitional cell carcinoma of urinary tract in humans. Urology 1986; 28:401–403.
18 Okajima E, Hiramatsu T, Iriya K, Matsushima S, Yamada K. Effects of sex hormones on development of urinary bladder tumors in rats induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine. Urol Res 1975;3:73–79.
19 Risch A, Wallace DMA, Sim E. Slow N-acetylation genotype is a susceptibility factor in occupational and smoking related bladder cancer. Human Mol Genet 1995;4:231–236.
20 Shirai T, Shibata M, Takahashi S, Tagawa Y, Imaida K, Hirose M. Difference in cell proliferation and apoptosis between reversible mucosal lesions associated with uracil-induced urolithiasis in N-butyl-N-(4-hydroxybutyl) nitrosamine-pretreated rats. Carcinogenesis 1995;16:501–505.
21 Skopelitou A, Hadjiyannakis M, Dimopoulos D, Kamina S, Krikoni O, Alexopoulou V, et al. *p53* and c-*jun* expression in urinary bladder transitional cell carcinoma: correlation with proliferating cell nuclear antigen (PCNA) histological grade and clinical stage. Eur Urol 1997;31:464–471.
22 Tatebe S, Ishida M, Kasagi N, Tsujitani S, Kaibara N, Ito H. Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal-deoxynucleotidyl-transferase-mediated dutp-biotin nick end labeling. Int J Cancer 1996;65:173–177.
23 Waldman FM, Carroll PR, Cohen MB, Kerschmann R, Chew K, Mayall BH. 5-Bromodeoxyuridine incorporation and PCNA expression as measures of cell proliferation in transitional cell carcinoma of the urinary bladder. Mod Pathol 1993;6:20–24.
24 Wingo PA, Tong T, Bolden S. Cancer statistics, 1995. Ca Cancer J Clin 1995;45:8–30.
25 Yamashi S. Immunohistochemical study of proliferating cell nuclear antigen on rat bladder cancer induced by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine. Ehime Igaku Zasshi 1996;15:569–575 (in Japanese).
26 Yamada T, Yokokawa M, Fukui I, Sekine H, Noro A, Ohshima H, et al. Statistical observation on our bladder tumor registry 3: sex and prognosis. Nippon Hinyokika Gakkai Zasshi 1986; 77:612–617 (in Japanese).

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