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 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 men have not shown a significant decrease. The fact that the incidence of bladder cancer is higher in men than in women has 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 men have not shown a significant decrease.

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 ± 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 weekly. Mice were cystectomized at the end of each experimental period. wk, weeks.

**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 experiment.

**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.
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 (\( P \leq 0.001 \)) (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 1. Occurrence of bladder tumors in Experiment I and invasive tumor induction time in Experiment II

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occurrence of bladder tumor (%)</td>
<td>13.3</td>
<td>64.3</td>
<td>80.0</td>
<td>( P &lt; 0.001 )†</td>
</tr>
<tr>
<td>Invasive tumor induction time (week)</td>
<td>30.3</td>
<td>25.4</td>
<td>22.8</td>
<td>( P &lt; 0.001 )‡</td>
</tr>
</tbody>
</table>

† Kruskal-Wallis rank test.
‡ One-way analysis of variance.

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

<table>
<thead>
<tr>
<th></th>
<th>Benign lesion (%)</th>
<th>Carcinoma in situ (%)</th>
<th>Invasive tumor (%)</th>
<th>Statistical analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>1.1</td>
<td>6.7</td>
<td>17.1</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Group II</td>
<td>0.2</td>
<td>6.4</td>
<td>31.0</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Group III</td>
<td>1.1</td>
<td>13.2</td>
<td>42.7</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>NS</td>
<td>NS</td>
<td>( P = 0.005 )</td>
<td></td>
</tr>
</tbody>
</table>

† Kruskal-Wallis rank test.
NS, not significant.
Table 3. Relationship between the proliferation index and the grade of tumors

<table>
<thead>
<tr>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>Grade 3 (%)</th>
<th>Statistical analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>9.9</td>
<td>16.1</td>
<td>25.3</td>
</tr>
<tr>
<td>Group II</td>
<td>24.7</td>
<td>26.3</td>
<td>41.2</td>
</tr>
<tr>
<td>Group III</td>
<td>14.0</td>
<td>33.0</td>
<td>71.4</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>P = 0.012</td>
<td>P = 0.039</td>
<td>P = 0.004</td>
</tr>
</tbody>
</table>

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

developed 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

<table>
<thead>
<tr>
<th>Benign lesion (%)</th>
<th>Carcinoma in situ (%)</th>
<th>Invasive tumor (%)</th>
<th>Statistical analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>54.0</td>
<td>51.3</td>
<td>26.7</td>
</tr>
<tr>
<td>Group II</td>
<td>64.5</td>
<td>43.5</td>
<td>21.4</td>
</tr>
<tr>
<td>Group III</td>
<td>69.0</td>
<td>36.3</td>
<td>18.8</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>Grade 3 (%)</th>
<th>Statistical analysis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>35.4</td>
<td>39.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Group II</td>
<td>28.5</td>
<td>28.7</td>
<td>8.2</td>
</tr>
<tr>
<td>Group III</td>
<td>36.4</td>
<td>21.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

†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 environ-
mental carcinogens (Risch et al., 1995) such as aniline dye, ciga-
rette smoking, etc., and hormonal influence is considered neglig-
ible. 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 wo-
men (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 envi-
ronmental 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 in-
fluence, especially of testosterone, might relate to this contra-
dictory sex difference in incidence, and that the hormonal in-
fluence on bladder carcinogenesis should be investigated.

Human bladder carcinomas can be classified into papillary
superficial bladder carcinoma and non-papillary invasive blad-
ner 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.