Conversion from Low Grade to High Grade of Rat Urinary Bladder Carcinomas

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ABSTRACT

The present study was conducted to test if low-grade carcinomas induced by a single dose of N-methyl-N-nitrosourea (MNU) can be converted to high-grade carcinomas by a second identical dose of the carcinogen. The heterotopically transplanted rat urinary bladder system was used. Four wk after heterotopic bladder transplantation, the recipient male Fischer 344 rats were divided into 2 groups. The first group received 0.25 mg of MNU into heterotopically transplanted rat urinary bladder; the second group (controls) received 0.9% NaCl solution. At week 29 of the experiment, 1/3 of the animals from each group were killed for histological examination of the heterotopically transplanted rat urinary bladders. The remaining animals from each group were divided into 2 subgroups, the first receiving 0.25 mg MNU and the second, 0.9% NaCl solution. All animals were killed at 50 wk of the experiment. MNU-induced carcinomas at week 29 were all of low histological grade and were noninvasive. Longer follow-up without a second carcinogen administration resulted in both an increase in tumor incidence (P < 0.005) and more tumors per bladder (P < 0.001), but high-grade invasive carcinomas were rare. The second dose of MNU administered at the stage when low-grade carcinomas were prevalent (week 29) resulted in a significant increase in invasive high-grade carcinomas (P < 0.01).

Our data are consistent with the view that the second carcinogen administration induces a new mutation(s) within low-grade carcinomas which leads to invasive carcinomas.

INTRODUCTION

Bladder cancers may be divided into 2 histological types which correlate with their biological behavior. The more common type of tumor is of low histological grade (grades I and II); they grow slowly and frequently “recur” after excision of tumor. The second type consists of tumors of high histological grade, most of which grow rapidly, are already deeply invasive at the time of discovery, and are lethal. Recent increases in the latter type of carcinomas have become a serious clinical concern (1, 2). The foregoing suggest either a new carcinogenic hazard for the urinary bladder in the environment or additional noncarcinogenic factors which enhance progression of the carcinogenesis.

To test the hypothesis that the degree of malignancy of urothelial carcinomas is determined by the dose of carcinogen(s), we recently conducted a series of experiments using the HTB system (3). HTBs which were exposed to a low dose of MNU (0.05 mg of MNU, weekly, for 6 doses) developed only low-grade, low-stage carcinomas, whereas bladders exposed to a higher dose of carcinogen (0.5 mg of MNU for 6 doses) or those which were additionally treated with another carcinogen, N-butyln-N-(4-hydroxybutyl)nitrosamine, demonstrated frequent development of high-grade, high-stage carcinomas together with low-grade carcinomas within the same bladders.

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2 To whom requests for reprints should be addressed, at Department of Pathology.

3 The abbreviations used are: HTB, heterotopically transplanted rat urinary bladder; MNU, N-methyl-N-nitrosourea.

Microscopic examination demonstrated that high-grade carcinomas developed in 2 ways: the first involved more anaplastic clones within a preexisting low-grade carcinoma, and the second appears to be the direct induction of invasive high-grade carcinomas from the severely dysplastic urothelium or carcinoma in situ. Urine, which in our previous studies using the HTB system appeared to be a promoter (3–7), did not seem to play a significant role in the conversion of tumor grade. Emergence of a new population of higher histological grade within a low-grade carcinoma suggests to us that additional genetic changes occur following repeated exposure to MNU. This mechanism of malignant conversion has also been suggested in studies of mouse skin carcinogenesis (8, 9). In the study by Hennings et al. (8), 12-O-tetradecanoylphorbol-13-acetate, a potent promoter, was ineffective in converting 7,12-dimethylbenz(a)anthracene-initiated papillomas in mice to carcinomas whereas a second carcinogen when applied as a single dose was effective. It is, therefore, likely that a similar mechanism may operate in malignant conversion of low-grade urothelial carcinoma. In our earlier study cited above (3) carcinogen was administered in multiple doses during the early phase of study, and we were not able to delineate the action of each dose of carcinogen. In the present study, a relatively low dose of MNU (0.25 mg) was used as an initiator (5) and induced low-grade superficial carcinomas at 29 wk which were converted to higher grade following the administration of a similar dose of MNU at 29 wk.

MATERIALS AND METHODS

Animals and Chemicals. Young male Fischer 344 rats weighing 175 to 200 g (Charles River Breeding Laboratory, Wilmington, MA) were housed 4 to 5 per cage in an air-conditioned room at 22°C with 50% humidity and a 12-h light-dark cycle. They had free access to water and a chow diet (Purina 5012; Ralston Purina, St. Louis, MO). MNU was purchased from ICN Pharmaceutical, Plainview, NY, and was recrystallized before use.

Collection of Urine. Urine was collected from normal male Fischer 344 rats, adjusted to pH 7.0 with 0.5 n NaOH and to 700 mOsmol with distilled water, and then stored in 50-ml portions at −80°C until use (10).

Experimental Animal Model. The HTB system was used. This model, which was developed in our laboratory to investigate the role of urine in bladder carcinogenesis (4–7, 11), consists of a urinary bladder from a male Fischer 344 rat transplanted into the gluteal muscle of a syngeneic recipient with an attached s.c. placed reservoir. Our previous studies demonstrated that MNU when instilled percutaneously into the reservoir, induced transitional and squamous carcinomas in the HTB and the tumor incidence was in proportion to carcinogen dose (4, 5, 7).

Experimental Plan. Four wk after bladder transplantation (Fig. 1), recipient animals were arbitrarily divided into 2 groups. The first group received instillation into HTB of 0.25 mg of MNU freshly dissolved in 0.5 ml of 0.9% NaCl solution. The second group received 0.5 ml of 0.9% NaCl solution. One wk later, all rats received instillation into HTBs of 0.5 ml of normal rat urine once a week during the subsequent 24 wk. At week 29 of the experiment, 1/3 of the rats of each group were chosen randomly using random numbers and sacrificed for microscopic examination of the HTBs (groups A and F). The remaining rats of each group were further divided randomly into 2 subgroups. The first group...
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Fig. 1. Experimental design. Four wk after heterotopic transplantation (1) of urinary bladder, rats received into their transplanted bladders a single dose (0.25 mg) of MNU (7) or 0.9% NaCl solution (7) at Week 4 or 29. Subsequently the bladders received 0.5 ml of urine (a) once a week until the end of the experiment. S, weekly instillation of 0.9% NaCl solution.

RESULTS

There was no significant difference in body weights among comparable groups of animals that were killed at either week 29 or 50.

The results of microscopic examination of bladders are shown in Table 1. Treatment with a single dose of MNU yielded a tumor incidence of 60% (18 of 30 rats) at week 29 (group A), which was comparable to the incidence observed previously after an identical treatment (5). When the observation period was extended to 50 wk (group B), the carcinoma incidence rose to 93% (27 of 29 rats) (P < 0.005). This incidence was similar to that of the group which received the carcinogen for the second time at week 29 (97%; 30 of 31; group C). The group which received carcinogen for the first time at week 29 (group D) showed an incidence of 39% (12 of 31). The observation period following carcinogen administration, however, was shorter (21 versus 25 wk). In the 2 control groups which received no MNU, 3 tumors in group E and 1 in group F were found. This phenomenon had been observed in our previous studies (5, 7) and suggested that HTB mucosa is sensitive for tumorigenesis.

Foci of severe dysplasia, which has been now recognized as carcinoma in situ (13) and therefore was classified as grade III lesion in the present study, were seen in 2 rats of group B (first MNU dose only) and 6 of group C (first and second MNU doses).

The total number of tumors per group and the number of tumors per bladder are shown in Table 2. The numbers of tumors were dependent upon 2 factors: (a) observation period following MNU administration and (b) MNU dose. Comparison of the data of groups A, B, and C suggested that the majority of the tumors observed in group B had developed between weeks 29 and 50, and that the second MNU further increased tumor numbers, although not significantly (P < 0.001, group A versus B; P < 0.1, group B versus C; Student's t test).

The rate of tumor growth was evaluated by measuring total tumor volume per bladder and mean tumor volume per bladder. They were, respectively, 178 ± 307 mm³ (mean ± SD) and 21 ± 31 mm³ in group B. The respective values for group C were 582 ± 896 and 171 ± 369 mm³ and were higher than those in group B (P < 0.05, Student's t test). Thus the data suggested that the accelerated growth rate in group C was associated with the second MNU administration.

Development of more aggressive forms of carcinomas (invasiveness or grade III nuclear morphology; see "Materials and Methods" for detail) was seen in groups B and C only (Table 1). The overall frequency was 14% (4 of 29 rats) in group B and 45% (14 of 31) in group C (group B versus C, P < 0.01, x² analysis). The second dose of MNU significantly increased the incidence of invasive carcinomas (3 rats in group B and 10 in group C, P < 0.05) as well as the frequency of grade III
Table 1 Pathological changes induced by a single or double dose of MNU

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total no. of rats</th>
<th>Nodular papillary hyperplasia</th>
<th>Dysplasia (severe)</th>
<th>Carcinoma incidence (%)</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Incidence</td>
</tr>
<tr>
<td>A</td>
<td>M—</td>
<td>30</td>
<td>17</td>
<td>1 (0)</td>
<td>18 (60)</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>MS</td>
<td>29</td>
<td>27</td>
<td>9 (2)</td>
<td>27% (93)</td>
<td>4% (14)</td>
</tr>
<tr>
<td>C</td>
<td>MM</td>
<td>31</td>
<td>27</td>
<td>15 (6)</td>
<td>30 (97)</td>
<td>14% (45)</td>
</tr>
<tr>
<td>D</td>
<td>SM</td>
<td>31</td>
<td>9</td>
<td>0</td>
<td>12 (39)</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>SS</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>3 (10)</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>S—</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>1 (3)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Instillation into HTB of 0.25 mg of MNU (M) or 0.9% NaCl solution (S) at week 4 or 29. —, sacrifice at week 29.

a Significantly different from group A (P < 0.005).

Time rat with diffuse mucosal change with severe dysplasia (carcinoma in situ) is included.

b Significantly different from group B (P < 0.01).

c One rat with diffuse mucosal change (carcinoma in situ) with microinvasion is included.

d Significantly different from group B (P < 0.05).

Table 2 Incidence and histological classification of bladder tumors by grade, stage, and cell type

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total no. of rats</th>
<th>Total no. of tumors</th>
<th>Grade (%)</th>
<th>Stage (%)</th>
<th>Cell type (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I II III P0 &lt;P1 T SdSq</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>M—</td>
<td>30</td>
<td>18</td>
<td>64 (2.1 ± 2.9)</td>
<td>55 (86) 9 (14)</td>
<td>64 (69)</td>
</tr>
<tr>
<td>B</td>
<td>MS</td>
<td>29</td>
<td>27</td>
<td>18% (6.3 ± 4.4)</td>
<td>111 (61) 70 (39)</td>
<td>179 (98)</td>
</tr>
<tr>
<td>C</td>
<td>MM</td>
<td>31</td>
<td>30</td>
<td>25% (8.2 ± 7.1)</td>
<td>115 (45) 124 (49)</td>
<td>239 (94)</td>
</tr>
<tr>
<td>D</td>
<td>SM</td>
<td>31</td>
<td>12</td>
<td>25% (0.8 ± 1.8)</td>
<td>19 (76) 6 (24)</td>
<td>25 (0)</td>
</tr>
<tr>
<td>E</td>
<td>SS</td>
<td>29</td>
<td>3</td>
<td>3 (67)</td>
<td>1 (33)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>F</td>
<td>S—</td>
<td>29</td>
<td>1</td>
<td>1 (0)</td>
<td>0 (0)</td>
<td>1 (0)</td>
</tr>
</tbody>
</table>

* M, MNU; S, 0.9% NaCl solution; —, sacrifice at week 29.

a Tumors with combined grades are classified by the highest grade.

b Tumors with combined cell types are classified by the following order of dominance; squamous (Sq) > squamoid (Sd) > transitional (T).

c Diffuse papillomatosis is not counted (1 in group B and 11 in group C).

d Significantly different from group A (P < 0.001).

e Significantly different from group B (P < 0.005).

When all individual tumors were classified according to their grade, stage, and type of cell differentiation, their incidences are shown in Table 2. All tumors induced by a single dose of MNU (groups A and D) were confined to the mucosa (P0) and the majority of them remained as grade I and transitional cell type. A longer follow-up (from 29 to 50 wk) without additional carcinogen treatment (group B) resulted in more carcinomas with squamoid and squamous differentiation and development of 3 invasive carcinomas (P1, invasion of the lamina propria).

In group C which received MNU for the second time at week 29, this tendency became more obvious; carcinomas with grade III increased in number (P < 0.005) and the majority of them acquired squamous morphology (P < 0.001, group B versus C). Among the 15 invasive carcinomas in group C, 12 were in P1, 2 in P2 (invasion of the tunica muscularis), and 1 in P3 (invasion of the tunica serosa). Invasive carcinomas were, without exception, of either grade II or III nuclear morphology. Of the 9 grade III carcinomas, 8 showed coexisting foci of grade II nuclear morphology (data not shown). In all of these 8 carcinomas, the invasive front consisted of grade III carcinomas, either of squamoid or squamous type (Figs. 2 and 3).
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Diffuse papillomatosis was seen in 1 rat of group B and 11 of group C. These lesions were considered to evolve from diffuse mucosal proliferation. Among them, 10 in group C had a sign of the "conversion," suggesting that there is a close relationship between the development of papillomatosis and the high-grade conversion of tumors.

DISCUSSION

Although high-grade carcinomas of the urinary bladder are known to develop in animals following continuous administration of carcinogen (14, 15) there have been no studies of which we are aware which clearly focus on the possible mechanism(s) of their development. Based on the work we recently reported (3) it is quite likely that carcinogen administration each time induces random mutation in both normal and already neoplastic urothelial cells. This is also the mechanism of carcinogen action proposed for the mouse skin model (8, 9, 16).

In the present study, most of the tumors developing after a single dose of MNU remained as low-grade non-invasive carcinomas. A longer follow-up without additional carcinogen showed an increase in tumor incidence and in the total number of tumors. Malignant conversion was rare. In contrast, when MNU was administered for the second time at the stage when low-grade superficial carcinomas were prevalent (60% at Week 29), there was a significant increase in the incidence of invasive carcinomas ($P < 0.05$), carcinomas with grade III nuclear morphology ($P < 0.005$), tumors with squamous cell differentiation ($P < 0.001$), and in the tumor volume in both the median tumor volume per bladder as well as the total tumor volume per bladder. Thus the second MNU dose stimulated the growth rate of tumors in conjunction with increase in grade II and III carcinomas.

To determine whether the development of more aggressive forms of carcinomas seen in group C was indeed due to the second dose of MNU, tumors observed in this study were divided into 2 types, those developing after a short (less than 25 wk) and a longer (more than 25 wk) induction time (Fig. 4). All tumors which appeared within 25 wk were of low-grade carcinomas (LGCA) (those seen in group A in Fig. 1 and category I in Fig. 4). Even if the study period was extended to 50 wk, almost all tumors were low-grade carcinomas (II, Fig. 4). "Spontaneous" conversion to high-grade carcinomas (HGCA) were rare (group B, III in Fig. 4). Tumors which developed after 25 wk can be classified into the following 3 types: those induced by the first MNU, by the second MNU, and by the combination. Careful microscopic observation showed that only 1 of the 10 invasive carcinomas in group C was a pure grade III carcinoma and the remaining 9 demonstrated grade II and III morphology, and, in all, the invasive front was made up of grade III carcinoma. This strongly suggests that malignant conversion is more likely to take place within a low-grade carcinoma. In fact this was the common mechanism encountered in the previous similar study (3). We suggest that most high-grade carcinomas arise as a new mutation within a low-grade carcinoma by a second dose of MNU (group C, IV, Fig. 4). Rapid expansion of such a clone could be expected to replace a low-grade carcinoma. Thus, it is our current view that the biological aggressiveness of a given tumor is determined by the level of genetic alterations (e.g., oncogene activation) induced by carcinogen. This view is consonant with that expressed by other investigators (17). It is unknown whether malignant conversion takes place at only specific stages of multistage carcinogenesis. Our data suggest otherwise. Oncogene activation (Ha-ras) has been demonstrated in human urothelial tumors as well as rat bladder tumors induced by chemical carcinogens (18–20). In the study reported here, it is not known whether specific oncogenes are involved in urothelial neoplasia and its subsequent malignant conversion.

REFERENCES


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