Marked Enhancement of Rat Urinary Bladder Carcinogenesis by Heat-killed *Escherichia coli*

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ABSTRACT

Chronic urinary tract infection is an important risk factor for the development of carcinoma in the human urinary bladder. To test the effect of chronic persistent inflammation on bladder carcinogenesis, we instilled heat-killed *Escherichia coli* (1 × 10⁶ cells suspended in 0.5 ml of phosphate-buffered 2.1% NaCl solution) twice a week into the heterotopically transplanted rat urinary bladders in which carcinogenesis was initiated by a single dose (0.25 mg) of N-methyl-N-nitrosourea. When compared with the control animals, the rats treated with killed *E. coli* showed significantly enhanced bladder tumor progression, as reflected by an increase in the incidence of tumor (P = 0.05) and a 6- to 40-fold increase in the number of tumors per bladder (P < 0.0001). The tumors were characterized by intracellular clusters of neutrophils and by chronic inflammation and marked capillary proliferation in the tumor stroma. All of these features were rare in tumors in the control groups. The accelerated cell proliferation induced by killed *E. coli* treatment appears to play a significant role in the enhancement of tumorigenesis.

INTRODUCTION

The association of chronic inflammation with a variety of epithelial malignant tumors has been recognized for many years. For example, squamous carcinoma may develop along the draining sinus in chronic osteomyelitis (1), and development of adenocarcinoma is a significant risk in patients with chronic inflammatory bowel disease (2, 3). Several epidemiological and experimental animal studies have suggested that infection of the urinary tract is a significant risk factor for the development of bladder cancer (4–6). The risk is associated primarily with chronic urinary tract infection.

Based on the cause, human bladder cancers may be divided into 2 major groups. The first type occurs in close association with exposure to a variety of chemicals, either as an occupational hazard or as a result of tobacco smoking. This type of cancer typifies the bladder cancers seen in the Western world, the majority of which are transitional cell carcinoma with a variable biological potential. The second type of bladder cancer occurs as a complication of long standing (i.e., chronic) urinary tract infection. The majority of these cancers are deeply invasive squamous cell carcinomas that frequently have a lethal outcome despite aggressive surgical and chemotherapeutic intervention. In the Western world, this type of cancer typically occurs in patients with spinal cord injury because such patients invariably develop chronic bacterial cystitis (7–9). In Northern Africa and the Middle East, where infection with *Schistosoma hematobium*, a water-borne parasite, is endemic, chronic (bacterial) infection of the urinary tract in association with schistosomiasis is an important cause of bladder cancer; usually this is a deeply invasive squamous cell carcinoma. It is the leading cause of cancer deaths in Egypt (10).

Several recent reports indicate that urinary tract infection promotes carcinogenesis in the urinary tract of the rat (11–13). We were interested in elucidating the mechanism involved in infection-associated enhancement of urinary bladder cancer. We conducted a short-term (4 weeks) preliminary experiment by using the HTB system (vide infra) in an attempt to develop a reproducible animal model of bacterial cystitis-associated urothelial hyperplasia (14). Instillation into the HTB of live *Escherichia coli* resulted in persistent infection and acute and chronic inflammation, and also in diffuse urothelial hyperplasia. Instillation of KEC also induced hyperplasia, which regressed after withdrawal of KEC treatment. Furthermore, urothelial hyperplasia was induced by repeated instillation of protein-rich lipopolysaccharide, the endotoxin derived from a cell wall component of bacteria. A finding common to bladders showing hyperplasia was the infiltration of neutrophils into the intercellular space of the urothelium.

The present investigation was conducted to test the hypothesis that persistent hyperplasia induced by the cell wall component of *E. coli* (14) promotes MNU-initiated rat bladder carcinogenesis. The results indicate that repeated administration of heat-killed *E. coli* to the HTB markedly increases the incidence of tumors and the number of tumors per bladder.

MATERIALS AND METHODS

Animals. Male Fisher 344 rats weighing 130 to 160 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were housed 4 to 5 per cage in an air-conditioned room at 22°C with 50% humidity under 12-h light/dark cycles. They had free access to chow diet (Purina 5012; Ralston Purina Co., St. Louis, MO) and tap water.

HTB System. We used the HTB system, which we developed in our laboratory for investigating tumor promotion by urine components. The system consists of a rat urinary bladder transplanted aseptically into the gluteal muscle of a recipient rat (15). The system has advantages over others using natural bladders (11–13) in that contamination into the gluteal muscle of the recipient rat. In our system, KEC served as the source of the cell wall component. Our previous study showed that KEC could induce urothelial hyperplasia as effectively as can live *E. coli* (14). *E. coli* grown overnight in BHI broth was heat-killed in boiling water for 30 min; the suspension was centrifuged, and the sediment was suspended in PBS or normal rat urine (pH 7.0 and 700 milliosmotes) at a density of 2 × 10⁶ cells/ml and stored in 30-ml portions at −20°C until used. Once the suspension was thawed, any portion remaining after use was discarded.

Experimental Design. Four weeks after the establishment of the HTB system, a single dose of MNU (0.25 mg) dissolved in 0.9% NaCl was given to rats. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: HTB, heterotopically transplanted rat urinary bladder; KEC, killed *E. coli*; MNU, N-nitrosomethyl-N-nitrosourea; PBS, phosphate-buffered 2.1% NaCl solution.
solution (or vehicle only) was instilled into the transplant lumen via an attached reservoir (Fig. 1). This dose of MNU was expected to induce low-grade, noninvasive transitional cell carcinoma in approximately 10% of animals when the MNU treatment was followed by weekly instillation of 0.5 ml of 2.1% NaCl solution (17). Each experimental group consisted of 20 to 25 rats. One week after MNU (or vehicle) treatment, weekly injection of KEC, 1 × 10⁶ cells suspended in 0.5 ml of urine (groups 5 and 7), or PBS (groups 4 and 6) was begun and continued for 30 weeks. We added groups receiving latex beads (1–2 µm in diameter; Polyscience Inc., Warrington, PA) to rule out the possible effect of KEC as particulate foreign bodies. Test samples were instilled twice a week, each time after complete aspiration of the preexisting luminal content and washing with 2 changes of 0.5 ml PBS. Aspirates from all animals were routinely cultured once every 4 to 6 weeks to ensure their sterility.

Rats were sacrificed 4 days after the last regularly scheduled instillation of test material except for 5 rats randomly selected from each group. These rats received an additional dose of the same test sample and were killed 24 h later, between 11 a.m. and 1 p.m., preceded 1 h earlier by i.p. injection of [3H]thymidine (1 µCi/g body weight, specific activity 45 Ci/mmol; Amersham Corp., Arlington Heights, IL). This was done to estimate the effects of the test samples on urethral proliferation.

Gross and Microscopic Examination. At autopsy, HTBs were opened by a longitudinal incision, stretched over a piece of cardboard, and fixed in 10% neutral formalin or STF (a new fixative containing a purine derivative; Strech, Omaha, NE), and stored overnight in a refrigerator. After fixation, the mucosa was inspected for tumors; all neoplastic lesions at least 0.5 mm in the maximum dimension were mapped, and their diameters were recorded to estimate their volume. Any lesion less than 1.5 mm in diameter was classified as tumor size less than 2 mm³. Bladders were cut into several strips so that all tumors could be evaluated histologically. Bladder lesions were classified as simple hyperplasia, nodulopapillary hyperplasia, and carcinoma; grade 1, 2, or 3; stage P0, P1, P2, or P3, according to the criteria reported previously (18). Tumors were classified as transitional when the component cells were round to polygonal, had pale eosinophilic cytoplasm, and lacked squamous differentiation. When tumor cells exhibited eosinophilic cytoplasm and were tightly cohesive, but without clear-cut squamous differentiation, they were classified as squamoid because these lesions demonstrated differentiation toward the squamous type. Squamous metaplasia or carcinoma was so defined if unequivocal squamous differentiation was demonstrated. If more than one feature was found, the lesion was classified as mixed, for example, transitional-squamoid or transitional-squamous.

If the inflammatory response was dominated by neutrophils, it was classified as acute inflammation. When lymphocytes, mast cells, macrophages, and plasma cells were predominant, the lesion was defined as chronic inflammation.

DNA synthesis in a bladder lesion was estimated by autoradiography of [3H]thymidine. The number of labeled cells per 1000 cells was counted. The results were expressed as the mean number of labeled cells per 1000 cells per group.

Statistical Analysis. Tumor incidence, tumor size, and the number of tumors per bladder were compared between groups. Tumor incidence was defined as the percentage of rats with tumors, and was compared between groups by Fisher's exact test (19). The mean number of tumors per bladder was compared by the unpaired t test, accounting for unequal variance (20). We analyzed tumor size categorizing tumors as less or more than 2 mm³. The percentage of tumors that were larger than or equal to 2 mm³ was compared between the groups by Fisher's exact test. In the rats with tumors, the number of tumors was compared between groups with the use of the one sample binomial test for proportions. Labeling indices were compared by either 2-way or nested analysis of variance (20).

RESULTS

There was no difference in body weight among groups. Eighteen rats were removed from the study; in 5 rats, the communication between the transplant and the connecting reservoir was lost, which made delivery of test material impossible. An additional 13 rats were excluded because cultures of aspirate yielded growth to microorganisms, indicating bacterial contamination of the HTB system. Thus the analysis was based on 217 rats.

As was demonstrated previously (17, 21), weekly instillation of urine promoted the incidence of carcinoma significantly as compared to the PBS treatment (P < 0.0001, group 3 versus 4) (Table 1).

When the tumor incidence and tumor size in group 6 were compared to those in group 4, the KEC treatment in the absence of urine enhanced the incidence of tumor (P < 0.0001) and the number of tumors per bladder (P < 0.0001) (Table 1). Comparison of the data for groups 3 and 5 indicates that KEC treatment together with urine increased the tumor incidence significantly (P = 0.05). The KEC treatment resulted in a significant increase in the mean number of tumors per bladder (P < 0.0001, group 3 versus 5 and group 4 versus 6).

Latex bead treatment (groups 9 and 10) showed no adverse effect on tumorigenicity. This finding suggested that the KEC action was not due to its effect as foreign particles.

Microscopic examination (Fig. 2) showed several striking differences between KEC-treated and untreated groups in the histology of the tumors. First, the tumors observed in group 4 (MNU + PBS) were all small (<2 mm³) and consisted of grade 1 transitional cell carcinoma with little stroma between epithelial cell nests (Fig. 2, A and B). The tumors in group 3 (MNU + urine) were generally larger (Table 1) and contained more stroma, which was rich in dilated capillaries (Fig. 2, C and D). There were a small number of mononuclear cells (lymphocytes and macrophages) in the stroma.

The tumors in groups 5 and 6 (MNU + KEC + urine or PBS) were quite different: (a) Markedly dilated capillaries were abundant and were in close contact with tumor cells. (b) Aggregates...
of polymorphonuclear neutrophils were frequently found within the slit-like space created by opposing epithelial cell fronds and within neoplastic epithelium, frequently associated with focal necrosis of epithelial cells (Fig. 2, E and F). In addition, scattered single neutrophils were also found within the intercellular space of tumor epithelium. A varying number of polymorphonuclear neutrophils were frequently found in KEC-treated animals. This was in contrast to 1 of 51 tumors observed in groups 3 and 4 that showed squamoid change (P = 0.027). (e) KEC treatment itself induced urothelial hyperplasia, and a low incidence of carcinoma occurred even without MNU initiation. The present investigation has demonstrated that KEC, when instilled at a regular interval, markedly enhances tumorigenesis initiated by MNU in the bladder mucosa. Because lipopolysaccharide is resistant to boiling, we believe that the action of KEC is due to its cell wall component, lipopolysaccharide. The enhancing effect of KEC was evident in an increase in tumor incidence and in the number of tumors per bladder. Despite an active DNA synthesis in tumors, as demonstrated by enhanced thymidine incorporation, KEC treatment did not increase the labeling index significantly higher than that for animals of group 4 (P < 0.03).

### DISCUSSION

The present investigation has demonstrated that KEC, when instilled at a regular interval, markedly enhances tumorigenesis initiated by MNU in the bladder mucosa. Because lipopolysaccharide is resistant to boiling, we believe that the action of KEC is due to its cell wall component, lipopolysaccharide. The enhancing effect of KEC was evident in an increase in tumor incidence and in the number of tumors per bladder. Despite an active DNA synthesis in tumors, as demonstrated by enhanced thymidine incorporation, KEC treatment did not increase the labeling index (mean ± SEM) of tumors.

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**Table 1** Tumor incidence, mean number of tumors per bladder, and tumor numbers by volume

<table>
<thead>
<tr>
<th>Group</th>
<th>MNU</th>
<th>KEC</th>
<th>Urine or PBS</th>
<th>Tumor bearers/total rats</th>
<th>Mean tumor number/bladder (mean ± SEM)</th>
<th>Tumor no. by vol (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0/19</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>—</td>
<td>PBS</td>
<td>0/20</td>
<td>0</td>
<td>&lt;2</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>—</td>
<td>Urine</td>
<td>20/24[^a^]</td>
<td>1.88 ± 0.39</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>—</td>
<td>PBS</td>
<td>4/25</td>
<td>0.28 ± 0.15</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>Urine</td>
<td>25/25[^a^]</td>
<td>10.64 ± 0.95[^b^]</td>
<td>124[^c^]</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>PBS</td>
<td>24/24[^a^]</td>
<td>11.37 ± 0.93[^b^]</td>
<td>176[^c^]</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>—</td>
<td>Urine</td>
<td>2/21</td>
<td>0.19 ± 0.15</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>—</td>
<td>PBS</td>
<td>3/24</td>
<td>0.13 ± 0.07</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>+</td>
<td>Urine + LB[^d^]</td>
<td>16/21</td>
<td>1.81 ± 0.38</td>
<td>21</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>—</td>
<td>Urine + LB[^d^]</td>
<td>0/14</td>
<td>0</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

[^a^] Tumor incidence, group 3 versus group 4 and group 6 versus group 4, P < 0.0001; group 5 versus group 3, P = 0.05.
[^b^] Mean number of tumors per bladder, group 5 versus group 3 and group 6 versus group 4, P < 0.0001.
[^c^] Number of tumors by volume <2 mm³, group 5 versus group 3 and group 6 versus 4, P < 0.0001; ≥2 mm³, group 5 versus 3 and group 6 versus 4, P < 0.0001.
[^d^] LB, latex beads.

**Table 2** [3H]Thymidine labeling indices

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Group</th>
<th>Rat (no.)</th>
<th>Total measures[^a^]</th>
<th>Labeling index (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3</td>
<td>5</td>
<td>18</td>
<td>0.5 ± 0.2[^b^]</td>
</tr>
<tr>
<td>Simple hyperplasia</td>
<td>3</td>
<td>5</td>
<td>15</td>
<td>6.3 ± 0.7[^b^]</td>
</tr>
<tr>
<td>Tumors</td>
<td>3</td>
<td>4</td>
<td>12</td>
<td>9.5 ± 1.1[^c^]</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>56</td>
<td>52.3 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4</td>
<td>14</td>
<td>22.2 ± 2.6</td>
</tr>
</tbody>
</table>

[^a^] Each measure consists of 1000 cells counted.
[^b^] Normal versus simple hyperplasia, P < 0.0001 (group 3) and P < 0.03 (group 4).
[^c^] Tumors in group 3 versus group 5, P < 0.0006; group 3 versus group 9, P < 0.017; group 5 versus group 9, P < 0.004.

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* KILLED E. COLI PROMOTION OF RAT BLADDER CARCINOGENESIS

* Urine + LB, latex beads.
A fourth possibility is that the very small tumors, at least a small number of them, might be autotransplants derived from large tumors (24–26). This possibility cannot be neglected, because the HTB is a closed system. Any tumor fragments may start to grow on normal mucosa if the mucosal surface is in favor of cell attachment. The urothelial hyperplasia induced by the KEC treatment could provide a "fertile soil" for tumor growth. This, if substantiated, is a serious concern to urologists in their management of patients with bladder tumor. We have an experiment in progress to test this possibility.

A fifth possibility is that cytokines released from stromal macrophages and/or from tumor cells themselves may be involved in tumor enhancement. The 2 striking features associated with bladder tumors in KEC-treated animals were marked...
vascular proliferation in the tumor stroma and aggregations of neutrophils within the tumor parenchyma. The latter finding could be released and stimulate angiogenesis (29) and neutrophil emigration (30, 31). In addition, a significant number of mast cells were present in the tumor stroma. Histamine released by these cells could allow margination of neutrophils. It is also possible that tumor cells release angiogenesis factors (32, 33) in response to KEC treatment, and that bladder tumors secrete a chemotactic cytokine in response to interleukin 1 and tumor necrosis factor. The latter possibility is supported by a recent study in which interleukin 8, a neutrophil chemotactic cytokine, was released by a human transitional cell carcinoma (34).

Chronic inflammation has been recognized to be a risk factor for the development of carcinoma in several organ systems (2, 3, 35). The present experimental system provides an excellent model for investigations designed to elucidate the epithelial-stromal interactions during inflammation-associated tumorigenesis.

REFERENCES

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