Tumor-promoting Effect of Urinary Epidermal Growth Factor in Rat Urinary Bladder Carcinogenesis

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ABSTRACT

We previously demonstrated that the specific component of rat urine designated as Fraction I (Fr.I), which has been known to enhance carcinogenesis in the rat urinary bladder, contains epidermal growth factor (EGF) and transferrin (TF). The present study was designed to determine whether EGF or TF is responsible for the tumor-enhancing effect of Fr.I. The heterotopically transplanted rat urinary bladder (HTB), which has been developed in our laboratory, was used for the study. Fr.I was prepared from normal rat urine by a method published previously. Fr.I deficient in EGF or TF was prepared by passing this fraction through an Affi-Gel HZ column coupled with anti-rat EGF or TF antibodies, respectively. EGF and TF eluted from the column (designated as eluted EGF and eluted TF) were also tested for tumor-enhancing activity. Fr.I passed through the column coupled with non-immune rabbit IgG served as control (Fr.I column control). After initiation of carcinogenesis in HTBs by instillation of a single dose of 0.25 mg of N-methyl-N-nitrosourea, test materials were administered into these HTBs once a week for 30 weeks. The results showed that removal of EGF significantly reduced the tumor-enhancing effect of Fr.I (P < 0.001 as compared to that of the Fr.I column control) and that eluted EGF by itself significantly enhanced the carcinogenesis as compared to that of the vehicle control (P < 0.006). Removal of TF from Fr.I also reduced the tumor-enhancing effect of Fr.I (P < 0.01). However, removal of both EGF and TF from Fr.I did not enhance the inhibitory effect demonstrated by the Fr.I which was deficient in EGF. Likewise, combined use of TF and EGF did not exceed the tumor-promoting effect of EGF. The results indicate that EGF in Fr.I may play a significant role in the promotion of bladder carcinogenesis by urine.

INTRODUCTION

A majority of urinary bladder cancers are noninvasive and of low grade, but new tumors frequently develop (so-called recurrence) after endoscopic resection. To test the hypothesis that the high rate of "recurrence" is due to the proliferation of dormant neoplastic cells that are stimulated by growth factors contained in urine, we developed an animal model designated as the HTB system. This model has been proved to be quite useful for studies on initiation and promotion of urinary bladder carcinogenesis (1). With this system, normal rat urine was shown to have a potent tumor-enhancing effect (2, 3). Subsequent studies with gel-filtration chromatography demonstrated that the tumor-enhancing effect of rat urine was associated with two urinary fractions designated as Fr.I and Fr.II. Both of these fractions also stimulated ODC activity in bladder carcinoma cells (4). Because the tumor-enhancing effect was more striking with the high-molecular-weight Fr.I than with the low-molecular-weight Fr.II, we focused our attention on the analysis of the biological activity of Fr.I. By further chromatographic analysis, two distinct components of Fr.I with different biological activities were isolated. The first was capable of stimulating DNA synthesis in cells of a rat bladder carcinoma cell line, 804G, but did not induce ODC; this was identified as TF (5). The second component, which stimulated ODC activity, was only weakly mitogenic in 804G cells; this was identified as EGF (6).

We conducted the present study to investigate whether EGF is the tumor-enhancing component of Fr.I and to determine whether TF has any additive effect on EGF activity. Effects of EGF and TF on DNA synthesis in proliferating urothelial cells were also examined.

MATERIALS AND METHODS

Isolation of Fr.I from Normal Rat Urine. Urine was collected from young male Fischer 344 rats (Hilar Sprague-Dawley, Inc., Indianapolis, IN) maintained on a commercial diet (Purina Chow 5012;Ralston Purina Co., St. Louis, MO). The urine was fractionated on a Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) column (2.6 x 90 cm; Bio-Rad Laboratories, Richmond, CA), as described previously (6). Each fraction was tested for its capacity to compete with mouse EGF for receptor (6). As was demonstrated in our previous study (6), the peak of mouse EGF-competing activity resided at about M, 54,000 and corresponded to that of the ODC-inducible fraction designated as Fr.I. Therefore, aliquots forming the peak of mouse EGF-competing activity (Fr.I) were pooled, dialyzed against distilled water (cutoff level, M, 2,000) at 4°C for 4 days, and lyophilized.

Removal of EGF and TF from Fr.I. EGF and TF were removed from Fr.I by affinity column chromatography. Anti-rat EGF rabbit IgG, prepared by a method described previously (6), was coupled to Affi-Gel HZ (Bio-Rad) according to the manufacturer's instructions. Fr.I (200 mg) dissolved in 10 ml of 25 mM Tris-HCl buffer, pH 7.5, was applied to a column (1.5 x 12 cm; bed volume, 25 ml) packed with the gel. The column was washed with 250 ml of the buffer, and the eluate was reapplied to the column for removal of EGF as completely as possible. The final eluate was dialyzed against distilled water (cutoff level, M, 2,000) and lyophilized (designated as Fr.I - EGF). Subsequently, EGF eluted from the column with 55 ml of 0.2 N acetic acid was dialyzed and lyophilized (designated as eluted EGF). The eluted EGF consisted of EGF in a high-molecular weight (54,000) and a low-molecular-weight (6,000) form (6).

TF was similarly removed from Fr.I by passage of Fr.I through an Affi-Gel HZ column coupled with anti-rat TF rabbit IgG (Organon Teknika-Cappel, West Chester, PA). The material obtained was designated as Fr.I - TF. TF was eluted with 55 ml of 0.2 N acetic acid to the column; this eluate was dialyzed and lyophilized. TF from Fr.I - EGF was removed by applying it to an anti-rat TF rabbit IgG column. The material obtained by this procedure was designated as Fr.I - EGF - TF. Separately, Fr.I was passed through an Affi-Gel HZ column coupled with non-immune rabbit IgG to yield Fr.I for procedural control (designated as Fr.I column control). To estimate the efficiency of EGF removal and EGF elution, we measured the amount of EGF in Fr.I, Fr.I - EGF, and eluted EGF by the radioreceptor assay described.

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2 To whom reprint requests should be addressed, at Department of Pathology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611.
3 The abbreviations used are: HTB, heterotopically transplanted rat urinary bladder; ODC, ornithine decarboxylase; Fr.I, fraction I; Fr.II, fraction II; TF, transferrin; EGF, epidermal growth factor; MNU, N-methyl-N-nitrosourea; PBS, phosphate-buffered 2.1% NaCl solution.

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described previously (6). The efficiency was calculated as follows:

Efficiency of removal (%)  
\[= \frac{1 - \text{Amount of EGF in Fr.I - EGF}}{\text{Amount of EGF in Fr.I}} \times 100\]

Efficiency of elution (%)  
\[= \frac{\text{Amount of EGF in eluted EGF}}{\text{Amount of EGF in Fr.I}} \times 100\]

Similarly, the amount of TF in Fr.I, Fr.I - TF, and eluted TF was measured by an enzyme-linked immunosorbent assay, described previously (5). The efficiency of removal was 93.4% for EGF and 92.0% for TF. The efficiency of elution was 78.5% for EGF and 90.5% for TF.

Experimental Animal Model. The HTB system, which was developed in our laboratory for investigation of the role of urine in bladder carcinogenesis (1-4), was used. The system consists of a urinary bladder from a donor rat transplanted into the gluteal muscle of a recipient rat with an attached s.c. placed reservoir. Male Fischer 344 rats, weighing 180 to 200 g (Harlan), were used as both donors and recipients. They were housed five/cage in an air-conditioned room at 22°C and 50% humidity under 12-h light/dark cycles and were given a chow diet (Purina 5012) and tap water ad libitum throughout the experiment.

Experimental Design. Rats with HTB were divided into 8 groups, each group consisting of 30 animals. Four weeks after urinary bladder transplantation (Fig. 1), each HTB received a single dose of 0.25 mg of MNU (ICN Pharmaceuticals, Plainview, NJ), which was recrystallized before use and dissolved immediately before use in 0.5 ml of 0.9% NaCl solution. Weekly injection of the test materials was begun 1 week later and continued until the experiment was terminated at 31 weeks after MNU injection.

The test samples were Fr.I (Group 1), Fr.I - EGF (Group 2), Fr.I - TF (Group 3), Fr.I - EGF + TF (Group 4), Fr.I - EGF - TF (Group 5), eluted EGF (Group 6), eluted EGF + TF (Group 7), and eluted EGF + eluted TF (Group 8). Each test material was dissolved in PBS (700 mOsm, pH 7.0). Another group received the buffer solution alone as a vehicle control group (Group 8). The weekly dose of Fr.I (Group 1) was 1.5 mg/rat. This dose was approximately 5 times the amount contained in 0.5 ml of rat urine when its osmolality was adjusted to 700 mOsm (7). This schedule results in a tumor incidence of approximately 70% (7). Other test materials were administered in amounts that are contained in this dose of Fr.I.

Pathological Examination. Rats were sacrificed 1 week after the final (30th) instillation of test materials. After euthanasia with CO2, HTBs were removed, opened longitudinally, and stretched over a piece of cardboard. The mucosa was inspected for gross tumors. The number, size, and location of tumors in the bladder were recorded. Tumor volume was calculated from measurement of the longest and shortest axes, with the assumption that a tumor was an elliptical mass. After overnight fixation in Bouin solution (Columbia Diagnostics, Inc., Springfield, VA), the bladders were cut into at least eight strips in such a way that each grossly visible tumor could be represented in histological sections. After embedding in paraffin, 4-μm-thick sections were stained with hematoxylin and eosin for light microscopic examination.

Urothelial lesions were classified as simple hyperplasia (four or more cells in epithelial cell layers), nodulopapillary hyperplasia (localized nodular and/or papillary proliferation of the urothelium), or carcinoma Grade 1, 2, or 3 according to criteria reported previously (8). The mean number of carcinomas and the mean total tumor volume per HTB were calculated for each group by assigning 0 to bladders without tumors.

\[^{3}H\]Thymidine Labeling Indices of Urothelial Cells. To evaluate as to whether once a week instillation of eluted EGF and TF affects urothelial cell proliferation, we investigated the acute effects of EGF and TF on urothelial \[^{3}H\]thymidine labeling indices of cells in rats of Group 1. This group was used because we expected it to have the highest tumor incidence. Twenty-four h before the scheduled termination of the experiment at 31 weeks, 18 rats in this group were divided randomly into 3 subgroups consisting of 6 rats each. One group received a single dose of EGF prepared from rat submandibular glands by a method described previously (6) (0.7 μg dissolved in 0.5 ml of PBS); another was given rat TF (Sigma Chemical Co., St. Louis, MO; 15 μg dissolved in 0.5 ml of PBS); and the third group received 0.5 ml of PBS into the HTB. These doses were equivalent to approximately 5 times the amount contained in 0.5 ml of normal rat urine (5, 6). Rats were killed between 10:00 and 10:30 a.m., preceded 1 h earlier by i.p. injection of \[^{3}H\]thymidine (1 μCi/g body weight; specific activity, 43 Ci/mmol; American Corp., Arlington Heights, IL). Histological sections were processed for autoradiography as described previously (9). The results were expressed as the mean number of cells labeled per 1000 cells ± SD.

Statistical Analysis. Tumor incidence was compared pairwise across groups using the \(x^2\) test with correction for continuity (10). Tumor multiplicity, total tumor volume, and labeling indices were compared pairwise across groups using the two-tailed unpaired \(t\) test with adjustment for unequal variance (10). To account for multiple statistical testing, \(P\) values less than or equal to 0.01 rather than 0.05 were considered statistically significant.

RESULTS

General. Animals of all groups gained weight progressively, and there was no significant difference in the final body weight among groups. Altogether, 231 rats were available for the final evaluation. Of the remaining 9 rats, 6 were excluded because of the malfunction of the HTB system (inability to deliver test material to the bladder because of closure of the connecting tubing) in 3 rats and bacterial infection of the HTB in 3 rats. One rat of Group 3 and 2 rats of Group 4 were found dead of unknown causes during the 8th, 22nd, and 25th weeks of the experiment, respectively.

Incidence, Multiplicity, Volume, and Histology of Urothelial Carcinomas. The results and data analysis are shown in Tables 1 to 3. Group 1 (Fr.I) and Group 2 (Fr.I column control) showed a high tumor incidence (93 and 86%, respectively), and no significant difference was observed between them (Table 1). Similarly, there was no significant difference between these two groups in the number of tumors per bladder (Table 2) or the total tumor volume per bladder (Table 3). When comparing these three parameters to Group 2, those of Group 3 (Fr.I - EGF) and Group 5 (Fr.I - EGF - TF) were significantly lower. Groups 3 and 5 were not significantly different from the vehicle control group on any of the three parameters. Group 4 (Fr.I - TF) was significantly different from Group 2 on tumor multiplicity and total tumor volume, but not on tumor incidence. Group 4 differed from the vehicle control group on tumor incidence and tumor multiplicity but not on total tumor volume. There was no difference among Groups 3, 4, and 5 on any of the three parameters. Group 6 (eluted EGF) showed a significantly higher tumor incidence and a larger number of tumors per bladder than did the vehicle control group (Group 8). No difference was observed between Groups 6 (eluted EGF) and 7 (eluted EGF + TF) in any parameter.

Altogether, 220 tumors were classified histologically according to the previously described criteria (8); 212 tumors (96%) were of the transitional and 8 (4%) were of the squamous cell
PROMOTING EFFECT OF URINARY EGF IN BLADDER CARCINOGENESIS

Table 1 Tumor incidence and data analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats examined</th>
<th>No. of rats with tumor (%)</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fr.I</td>
<td>29</td>
<td>27 (93)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fr.I CC</td>
<td>29</td>
<td>25 (86)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fr.I - EGF</td>
<td>30</td>
<td>10 (33)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fr.I - TF</td>
<td>28</td>
<td>16 (57)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fr.I - EGF - TF</td>
<td>28</td>
<td>9 (32)</td>
<td>NS</td>
</tr>
<tr>
<td>6</td>
<td>eEGF</td>
<td>29</td>
<td>12 (41)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>eEGF + TF</td>
<td>29</td>
<td>14 (48)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Vehicle</td>
<td>29</td>
<td>2 (7)</td>
<td></td>
</tr>
</tbody>
</table>

* P values are reported from the corrected chi^2 test.

Table 2 Tumor multiplicity and data analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of tumors/HTB (mean ± SD)</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fr.I</td>
<td>2.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fr.I CC</td>
<td>2.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fr.I - EGF</td>
<td>0.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fr.I - TF</td>
<td>0.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fr.I - EGF - TF</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>6</td>
<td>eEGF</td>
<td>0.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>eEGF + TF</td>
<td>0.6 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Vehicle</td>
<td>0.1 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Two-tailed P values are reported from Student’s t test.

Table 3 Total tumor volume per HTB and data analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Total tumor volume/HTB (mm^3) (mean ± SD)</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fr.I</td>
<td>14.9 ± 15.7*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fr.I CC</td>
<td>16.5 ± 18.6*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fr.I - EGF</td>
<td>2.7 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fr.I - TF</td>
<td>5.1 ± 11.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fr.I - EGF - TF</td>
<td>2.3</td>
<td>5.5</td>
</tr>
<tr>
<td>6</td>
<td>eEGF</td>
<td>4.4 ± 16.3*</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>eEGF + TF</td>
<td>6.0 ± 11.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Vehicle</td>
<td>0.4 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

* Two-tailed P values are reported from Student’s t test.

Type of the 220 tumors, 204 (93%) were classified as Grade 1, 16 (7%) as Grade 2, and none as Grade 3. All tumors were noninvasive. There was no significant relationship between the test materials injected and the grade or histological type of tumors.

Effects of EGF and TF on \[^3\text{H}\]Thymidine Incorporation into Urothelial Cells. The acute response to instillation of EGF and TF was evaluated at three different urothelial foci, including an apparently normal urothelium, simple hyperplasia (layer of four or more cells), and tumors. Foci of nodulopapillary hyperplasia were excluded because they were too few and too small to provide a sufficient number of cells for comparison. One rat each from the TF group and the control group was excluded because of failure to label cells. Labeled cells per 1000 cells were counted, and the total number of cells counted per lesion per bladder ranged from 2000 to 7000. As shown in Table 4, there was no significant difference in the labeling indices of "normal" (nonhyperplastic) urothelium among the three groups. However, the labeling index in hyperplastic urothelium was significantly higher in the rats that received EGF than in those of the other two groups (P < 0.01 for each comparison). There was no significant difference in the labeling index of the hyperplastic lesions between the TF and control groups.

For evaluating the labeling index in tumor cells, we chose only tumors of Grade 1 transitional cell type in which more than 1000 cells were countable. Cell counting was done as many times as possible in nonoverlapping fields of 1000 cells within a tumor. As shown in Table 4, the number of tumors examined was 8 for the EGF group, 11 for the TF group, and 6 for the control group. The mean number of fields counted per tumor was 2.6 for the EGF group, 3.1 for the TF group, and 3.0 for the control group. The mean labeling index of the EGF group was significantly higher than that of the TF group and that of the control group (P < 0.01 for each comparison). The TF
group also showed a significantly higher labeling index than did the control group (P < 0.01).

DISCUSSION

Our primary objective in this study was to investigate the effect of EGF on urinary bladder carcinogenesis. EGF is a major component of Fr.I, which has been shown to be a potent promoter of carcinogenesis in the rat urinary bladder (4). The results showed that EGF effect was quite clear; removal of EGF from Fr.I resulted in significant reduction in all three parameters (incidence, multiplicity, and total volume of tumors). This reduction was of such a magnitude that there was no longer significant difference between the vehicle control group and the groups that had received EGF-deficient Fr.I (Groups 3 and 5). Furthermore, weekly administration of urinary EGF enhanced bladder carcinogenesis significantly as compared to the effect of vehicle administration. In the thymidine incorporation assay, EGF significantly increased the labeling indices in both hyperplastic urothelium and tumors but did not do so in nonhyperplastic (normal) portions of the urothelial lining. These results indicate that urinary EGF is a potent promoter of bladder carcinogenesis and that it acts selectively on proliferative foci, including the foci of simple hyperplasia and carcinoma.

With regard to the role of TF in bladder carcinogenesis, our data suggest that TF did exert some effect on tumorigenesis. First, removal of TF from Fr.I reduced the number of tumors per bladder (P < 0.001) and the total tumor volume per bladder (P < 0.001). Second, in the thymidine incorporation study (Table 4), instillation of TF into the bladder lumen increased the labeling index in tumors but not in normal or hyperplastic foci. These data suggest that TF exhibits its effect only on tumors. This assumption may explain why HTBs initiated by MNU failed to develop tumors after weekly administration of TF in our previous study (7). This finding is not surprising, because TF is not a growth factor in the true sense. It merely serves as a transporter of iron, which actively proliferating cells require for DNA synthesis (11). The lack of an additive effect of TF in the group receiving eluted EGF (Group 7) may be due to the low tumor yield induced by eluted EGF that was shown in Group 6. If the experiment had been extended for a longer period, a TF effect might have become apparent.

The tumor-promoting effect in the eluted EGF groups (Groups 6 and 7) was significantly lower than that of the Fr.I column control group. To account for this difference, several possibilities are to be considered. First, the reduced effect may be due to incomplete removal and elution of EGF by affinity chromatography. Second, it may be due to spontaneous (or enzymatic) cleavage to smaller fragments of the high-molecular-weight EGF of Fr.I (6). Previously, we have demonstrated that Fr.I is more effective in enhancing bladder cancer than Fr.II, which consists of the native form of EGF (4, 6). The high-molecular-weight EGF may be retained for a longer time in the lumen of the HTB because of its slower permeability and therefore its effect may last longer. Finally, it is possible that Fr.I contains one or more tumor-enhancing factors. Additional studies are needed for clarification of these possibilities.

All of the tumors observed in the present investigation were noninvasive and of low grade. This is due to the small dose of MNU used; we demonstrated previously that the biological aggressiveness of MNU-induced tumors depends upon the dose of carcinogen (12, 13). It is quite possible that low-grade, noninvasive tumors depend on urinary EGF for growth. We believe that this report confirms that EGF is likely to play a significant role in the promotion of bladder carcinogenesis in the rat.

REFERENCES

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