Exogenous Epidermal Growth Factor Exerts Promoting Action during the Early Phase of Rat Urinary Bladder Carcinogenesis

Kazunori Hattori, Kiyohide Fujimoto, Tetsuya Tamatani, Alfred Rademaker1 and Ryoichi Oyasu2

Department of Pathology, Northwestern University Medical School and 1Northwestern University Cancer Center Biometry Section, 303E. Chicago Ave., Chicago, IL 60611-3008

Using the heterotopically transplanted rat urinary bladder (HTB) model that was developed in our laboratory, we examined the relationship between the duration of epidermal growth factor (EGF) treatment and acquisition of EGF-independence of urinary bladder tumors that were induced by EGF stimulation. After treatment with N-methyl-N-nitrosourea (MNU) (0.25 mg/0.5 ml of 0.9% NaCl once a week for 3 consecutive weeks), animals at week 3 received EGF [250 ng/0.5 ml phosphate-buffered saline (PBS)] into the HTBs once a week for 20, 28, or 36 weeks. For examination of the effect of EGF withdrawal, one half of the rats received the vehicle (PBS) only beginning at week 23 or week 31 for 8 weeks. When animals were examined at week 23, the incidence and the mean number of tumors per bladder were low, irrespective of EGF treatment. In the bladders that had been exposed to EGF during the first 20 weeks after MNU administration, however, both the incidence and the mean number of tumors per bladder had increased significantly at week 31, regardless of whether or not EGF treatment was continued beyond week 23. Between weeks 31 and 39, EGF treatment demonstrated no effect; both the incidence of tumors and the mean number of tumors were the same as those at week 31. These results suggest that EGF exerts its promoting effect only during the early phase of MNU-initiated bladder carcinogenesis, but that its effect becomes manifest during the subsequent 8 weeks. EGF independence may be due to establishment of an autocrine growth-stimulatory mechanism in bladder tumors.

Key words: Rat bladder cancer — N-Methyl-N-nitrosourea — Epidermal growth factor

MATERIALS AND METHODS

Animals A total of 420 young male Fisher 344 rats (130 to 160 g body weight, Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used, 210 as donors and 210 as recipients of urinary bladders. They were housed 4 or 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 a chow diet (Purina 5012; Ralston Purina Co., St. Louis, MO) and tap water throughout the experiment.

HTB system We used the HTB system that was developed in our laboratory for the investigation of the role of urine in bladder carcinogenesis. The HTB is a rat bladder transplanted aseptically into the gluteal muscle of a syngeneic host. It is attached by a connector to a reservoir placed subcutaneously. Test material is administered to the HTB system by percutaneous puncture of the membrane of the reservoir port.

Experimental design The experimental design is illustrated in Fig. 1. A total of 210 male Fisher 344 rats with a surgically placed HTB system received 0.25 mg of N-methyl-N-nitrosourea (MNU) (Sigma Chemical Co., St.
Louis, MO), dissolved freshly in 0.9% NaCl, once a week for 3 consecutive weeks. They were then divided into 7 groups of approximately 30 rats each, assigned randomly. One week after the last MNU administration (at week 3), EGF (250 ng/0.5 ml phosphate-buffered 2.1% NaCl solution (PBS), a concentration comparable to that in normal rat urine; Harlan Bioproducts for Science, Indianapolis, IN) was administered once a week for 20 weeks (groups 1 and 3), for 28 weeks (groups 4 and 6), and for 36 weeks (group 7). Rats of group 3 and group 6 then received PBS for 8 weeks before being killed at weeks 31 and 39, respectively. Rats of groups 2 and 5 received PBS after MNU throughout the experiment. At autopsy, HTBs were dissected out. After fixation in 10% neutral buffered formalin, bladders were cut, and the lumens were inspected for grossly visible tumors. If found, they were measured under a dissecting microscope, and their distribution was mapped. Tumor volume was calculated on the assumption that the tumor was an elliptical mass. Bladders were cut into multiple strips in such a way that the tumors could be oriented correctly in microscopic sections. The grade and stage of tumors and the type of epithelial differentiation were established according to criteria described previously.6)

Statistical analysis

The incidence of tumors (the percentage of rats with tumors) was compared between groups with Fisher’s exact test. The mean number of tumors per HTB was compared between groups by use of the unpaired t test with adjustment for unequal variance. The tumors were divided into 2 categories according to their volume. Any tumors less than 2 mm in their maximal dimension were classified as “small” (≤4.2 mm3), and those of more than 4.2 mm3 were classified as “large” tumors. The volumes of tumors were compared between groups with Fisher’s exact test. The criterion of statistical significance was a P value of less than 0.05. All calculations were done with the use of SAS statistical software (SAS Institute Inc., Cary, NC).

RESULTS

There was no difference in body weight among groups. One rat that died before the scheduled termination of the experiment was excluded because of extensive post-mortem autolysis. An additional 12 rats that developed malfunctioning of their HTB-reservoir system were also removed from consideration because of inadequate delivery of test substance to the HTB system. Thus, the results were evaluated based on 197 rats.

Incidence, mean number per bladder, and volume of tumors

When the bladders were examined at week 23 from the start of MNU administration, there was no difference in the incidence or the mean number of tumors per bladder whether or not EGF was administered (groups 1 and 2) (Table I). However, EGF treatment (group 4) significantly enhanced carcinogenesis when bladders were examined at week 31, with an increase in the mean number of tumors per bladder as compared to the control group (group 4 vs. group 5, \( P = 0.02 \)). The incidence of tumors at week 31 tended to be higher in the group receiving EGF than in the group receiving PBS (group 4 vs. group 5). When the HTBs received EGF for 20 weeks, the incidence and the mean number of tumors continued to increase between weeks 23 and 31 irrespective of EGF treatment during that period (groups 1 and 3 vs. group 4; and \( P = 0.03 \), group 1 vs. group 3; and \( P = 0.01 \), group 4 vs. group 5). When the HTBs received EGF for 20 weeks, the incidence and the mean number of tumors continued to increase between weeks 23 and 31 irrespective of EGF treatment during that period (groups 1 and 3 vs. group 4; and \( P = 0.03 \), group 1 vs. group 3; and \( P = 0.01 \), group 4 vs. group 5). Switching treatment from EGF to PBS between weeks 23 and 31 (group 3) resulted in an apparent decrease in the mean number of tumors as compared to the group which continued to receive EGF (group 4) (\( P = 0.19 \)). The tumor incidence in group 3 was comparable to that of group 4. From week 31 to week 39, there was no change in the incidence, the mean number of tumors or the size of tumors, when EGF treatment was continued (groups 4 vs. groups 6 or 7).

The tumors were divided into 2 groups according to their volume. Any tumors less than 2 mm in their maximal dimension were classified as “small” (≤4.2 mm3), and those of more than 4.2 mm3 were classified as “large” tumors. The latter ranged in volume from 5.2 to 4189 mm3. Of the total of 101 tumors, 37 were “small,” and none of them was invasive. Two “large” tumors were invasive. There was a tumor of an exceptionally large volume (4189 mm3) in group 1, accounting for the large
mean tumor volume and standard error of the mean of group 1. Because of the wide variation in tumor volume among the groups, there was no significant difference in the mean volume of tumors or in the proportion of large tumors to total tumors (data not shown).

Of the total of 101 tumors, the great majority (87) were classified as grade 1 transitional cell carcinoma (TCC), 12 tumors as grade 2 TCC, and one tumor as grade 3 TCC. There were 2 invasive carcinomas; one invaded the submucosa, and the other invaded the muscularis propria. These tumors were in group 7 and group 4, respectively.

DISCUSSION

We performed the present investigation to examine the relationship between the duration of EGF treatment and acquisition of EGF-independence of bladder tumors that were induced by EGF stimulation. When bladders were examined at week 23 after 20 weeks of EGF treatment, there was no effect of EGF on the incidence and the mean number of tumors per bladder. However, at week 31, both the incidence of tumors and the mean number of tumors per bladder increased significantly in the bladders that had been exposed to EGF during the first 20 weeks after MNU administration. Continuous EGF treatment from week 23 to week 31 had no enhancing influence on the same parameters. EGF treatment from week 31 to week 39 had no effect whatsoever: both the incidence of tumors and the mean number of tumors per bladder were exactly the same as those at week 31. Thus, the first 20 weeks of EGF treatment were the only time during which EGF demonstrated tumor-promoting action. Yet it took an additional 8 weeks before an EGF effect became visible. Contrary to our anticipation, withholding EGF after the first 20 weeks of treatment did not affect tumorigenesis at week 31. Furthermore, EGF had no effect on tumor volume.

Our data support the suggestion that exogenous EGF exerts its tumor-enhancing effect only during the early phase of tumorigenesis by acting on dormant tumor cells, and that its tumor-promoting action requires more than 20 weeks before potential tumor cells reach the stage of visible tumors. Our data further suggest that, once tumors have reached a certain stage, they no longer respond to exogenous EGF and continue to grow without EGF.

Several possible mechanisms can explain the EGF-independence. First, it is well known that tumor cells can establish an autocrine growth mechanism. In a study similar to the present one,5 we showed by using immunohistochemical methods that growth of tumors was closely associated with the expression of EGF receptor (EGF-R) and of transforming growth factor-α (TGF-α). Second, a paracrine growth mechanism and angiogenesis may have been established in EGF-independent tumors and may support further growth. Third, growth factors supplied from the bloodstream may support continuous tumor growth. It is now widely accepted that angiogenesis is an essential step for solid tumor growth.9 We have shown immunohistochemically that endothelial cells of tumor stroma express EGF-R.10 TGF-α is a potent angiogenic factor itself.11 Thus, TGF-α produced by tumor cells might be the ligand for EGF-R expressed in endothelial cells, upregulating neovascularization of tumors. Furthermore, we have shown that bladder carcinoma cells contain immunohistochemically demonstrable basic fibroblast growth factor,12 which is also a potent angiogenic factor. In fact, the tumors that had reached a certain size always had ingrowing tumor vessels around them. Yoshiji et al. reported that vascular endothelial growth factor is essential during the initial stages of s.c. growth of human breast carcinoma cells in nude mice but was not needed for continued growth after the tumors had reached a certain size.13 Further investigation is needed to clarify the mechanism(s) for the EGF-independence.

Table I. Incidence, Mean Number of Tumors per Bladder, and Volume of Tumors after EGF Treatment of MNU-initiated Rat Urinary Bladder

| Group | Treatment (weeks) | n  | No. of rats with tumor (%) | No. of tumors per HTB (mean±SE) | Tumor vol. (mm³) (mean±SE) | Tumor size large/total (%) |
|-------|------------------|----|---------------------------|---------------------------------|---------------------------|--------------------------|
| 1     | EGF (20)         | 28 | 6 (21)                    | 0.21±0.08                       | 715.5±694.7               | 6/6 (100)                |
| 2     | PBS (20)         | 30 | 5 (17)                    | 0.20±0.09                       | 8.3±3.5                   | 3/6 (50)                 |
| 3     | EGF (20)→PBS (8) | 27 | 13 (48)a                  | 0.52±0.11b                      | 66.9±30.8                 | 10/14 (71)               |
| 4     | EGF (28)         | 27 | 14 (52)b                  | 0.81±0.19c                      | 12.5±3.9                  | 11/22 (50)               |
| 5     | PBS (28)         | 31 | 9 (29)                    | 0.29±0.08                       | 37.5±18.9                 | 6/9 (67)                 |
| 6     | EGF (28)→PBS (8) | 27 | 15 (56)                   | 0.81±0.21                       | 14.1±2.7                  | 16/22 (73)               |
| 7     | EGF (36)         | 27 | 16 (59)                   | 0.81±0.15                       | 33.4±23.5                 | 12/22 (55)               |

a) P=0.05 compared with group 1.
b) P=0.03 compared with group 1.
c) P=0.007 compared with group 1.
d) P=0.02 compared with group 5.
In conclusion, the present investigation supports the notion that exogenous EGF (derived from urine) is critically involved in the early phase of MNU-initiated rat urinary bladder neoplasia until autocrine and/or paracrine mechanisms take over to support the continuous growth of tumors.

ACKNOWLEDGMENTS

This investigation was supported by NIH grant CA14649 and the Joseph L. Mayberry Sr. Research Foundation Fund.

(Received May 19, 1998/Revised July 6, 1998/Accepted July 14, 1998)

REFERENCES

1) Greene, L. F., Hanash, K. A. and Farrow, G. M. Benign papilloma or papillary carcinoma of the bladder? J. Urol., 110, 205–207 (1973).

2) Kaye, K. W. and Lange, P. H. Mode of presentation of invasive bladder cancer: reassessment of the problem. J. Urol., 128, 31–33 (1982).

3) Gregory, H., Holmes, J. E. and Willshire, I. R. Urogastrolone levels in the urine of normal adult humans. J. Clin. Endocrinol. Metab., 45, 668–679 (1979).

4) Oyasu, R., Manning, D., Matsumoto, M. and Hopp, M. L. Heterotopic urinary bladder with communicating reservoir. Cancer Res., 36, 2261–2267 (1976).

5) Fujimoto, K., Tanaka, Y., Rademaker, A. and Oyasu, R. Epidermal growth factor-responsive and -refractory carcinomas initiated with N-methyl-N-nitrosourea in rat urinary bladder. Cancer Res., 56, 2666–2670 (1996).

6) Oyasu, R., Samma, S., Ozono, S., Bauer, K., Wallemark, C.-B. and Homma, Y. Induction of high-grade, high-stage carcinomas in the rat urinary bladder. Cancer (Phila.), 59, 451–458 (1987).

7) Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., 1, 27–31 (1995).

8) Schreiber, A. B., Winkler, M. E. and Derynck, R. Transforming growth factor: a more potent angiogenic mediator than epidermal growth factor. Science, 232, 1250–1253 (1986).

9) Yoshiji, H., Harris, S. R. and Thorgeirsson, U. P. Vascular endothelial growth factor is essential for initial but not continued in vivo growth of human breast carcinoma cells. Cancer Res., 57, 3924–3928 (1997).