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Irreversibility of Low-Grade Superficial Rat Bladder Carcinomas[1]

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ABSTRACT

The present investigation was conducted to determine: (*a*) whether the superficial papillary tumors developing in heterotopically transplanted bladders (HTBs) of rats after *N*-methyl-*N*-nitrosourea (MNU) initiation and subsequent weekly urine treatment would regress when placed in a urine-free environment; (*b*) whether tumors would develop in HTBs if MNU initiation is not followed by further manipulation, such as instillation of urine or 2.1% NaCl solution; and (*c*) whether tumors would develop in HTBs if urine instillation is delayed for as many as 25 weeks after MNU initiation. The results indicate: (*a*) that low-grade superficial tumors, once developed, do not appear to regress in a urine-free environment; (*b*) that tumors develop in MNU-initiated bladders even if they receive no further treatment; and (*c*) that late institution of urine instillation to HTBs still effectively enhances MNU-initiated tumorigenesis. If the current observation is extrapolated to the human situation, our data suggest that low-grade superficial tumors are indeed neoplastic, and spontaneous regression cannot be expected by urinary diversion. It, however, might be effective in controlling progression of at least some of the early neoplastic lesions to overt cancer.

INTRODUCTION

A great majority of bladder tumors are low-grade superficial carcinomas and, characteristically, are multiple in terms of time and space (6). They are usually readily controlled by endoscopic resections. Thus, a suggestion has been made that papillary tumors with minimal cytological atypia may not be neoplastic but represent hyperplastic reactions marking urothelium at increased risk of becoming neoplastic (2). This issue has been further complicated by the repeated observations that papillary lesions indistinguishable from the papillary urothelial tumors can be induced in animals by "noncarcinogenic" substances (2). It is quite possible that at least some of the low-grade superficial tumors may regress spontaneously under specific circumstances (*e.g.*, loss of contact with urine by permanent urinary diversion). Regression of bladder tumors following urinary diversion has been observed (1, 5).

The HTB[4] model developed in our laboratory (10, 11) may be suited to test reversibility of putative preneoplastic and neoplastic lesions. With this model, it is feasible to create a situation akin to clinical urinary diversion. In brief, the model consists of a whole rat bladder transplanted into the gluteal muscle and connected to a reservoir implanted s.c. at the back of a syngeneic recipient. Using this model, we have demonstrated a tumor-enhancing role of normal rat urine in urinary bladder carcinogenesis (8, 9, 12). The tumors induced are, in general, of low grade and low stage, and some of them may be diagnosed as papillomas. The relatively benign nature of these tumors may be attributable to the relatively small carcinogen dose used for initiation (12).

The present study was designed to determine: (*a*) whether the papillary tumors induced in HTBs by MNU initiation and subsequent weekly instillation of urine would regress when such bladders are placed in a urine-free environment or are left untouched without physical manipulation, such as weekly instillation of urine or 2.1% NaCl solution (saline); (*b*) whether tumors would develop in HTBs if MNU initiation is not followed by the physical manipulation described above, thereby leaving the HTBs untouched; and (*c*) whether tumors would develop in HTBs if weekly urine instillation is delayed for as many as 25 weeks after MNU initiation.

MATERIALS AND METHODS

Animals. A total of 550 male Fischer 344 rats (Charles River Breeding Laboratory, Wilmington, MA) weighing 175 to 225 g (average, 205 g) was used. They were fed *ad libitum* commerical stock diet (Purina 5012; Ralston Purina Co., St. Louis, MO) and were housed 5 to a plastic cage in an air-conditioned room at 22° with 70% humidity.

HTB System. One-half of the rats served as donors of bladders. They were aseptically transplanted into the gluteal muscle of recipients by the technique described previously (3, 8, 11).

Carcinogen and Preparation of Urine Samples. MNU was purchased from ICN Pharmaceuticals, Inc., Life Science Group, Plainview, NY, and was dissolved in saline immediately before use. Urine was collected from rats arbitrarily selected before bladder transplantation and processed by the technique described previously (8). It was finally adjusted to the pH of 6.8 to 7.0 and osmolality of 700 mOsmol. It was stored in 50-ml portions at −80° until use.

Experimental Design. Four weeks after transplantation, 261 recipients of bladders which were free from infection or evidence of ischemic contracture were divided into 9 groups (Chart 1). Each HTB received 0.25 mg of MNU dissolved in 0.5 ml of saline once a week for 3 consecutive weeks (Groups 1 to 5) or only once (Groups 6 to 9). One week after the last dose of MNU, the preexisting fluid was aspirated and replaced with either 0.5 ml of urine or 2.1% NaCl solution (700 mOsmol) once a week for 25 weeks, except for the HTBs in Group 3, which after MNU administration were left untouched for the rest of the experimental period. After 25 weeks of the first phase of study, animals of Groups 1 to 3 and 6 were killed. Animals of the remaining groups received during the next 22 weeks (the second phase) either 2.1% NaCl solution (Groups 4 and 7) or urine (Group 9) into their HTBs, while the HTBs of Groups 5 and 8 received no further treatment and were left untouched. One week before termination of the experiment, an aspirate from all HTBs was routinely cultured in BHI (Brain Heart Infusion) agar plates, excepting those left untouched.

Histological Examination. All rats were killed by an overdose of ether. HTBs and natural bladders were inflated *in situ* with 10% phosphate-buffered formalin solution. After an overnight fixation, each bladder was cut vertically into 12 to 16 sections, embedded in paraffin, and stained

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[4] The abbreviations used are: HTB, heterotopically transplanted bladder; MNU, *N*-methyl-*N*-nitrosourea; NPH, nodulopapillary hyperplasia.

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with hematoxylin-eosin. Each tissue block was cut at 2 different levels. The criteria for histological classification (10) are described below briefly; NPH was defined as exophytic (intraluminal) and/or endophytic (downward) growth with minimal atypia. Exophytic growth consisted of either a papillary projection of epithelial cell nests supported by nonbranching fibrovascular stroma or a nodular excrescence associated with endophytic epithelial growth. Since exophytic growth frequently coexisted with an endophytic growth pattern, they were referred to as NPH. These lesions are regarded as early neoplastic lesions, many of which would progress to low-grade tumors (7). As exophytic and endophytic growths continued, epithelial cell nests became larger and developed a branching pattern. At this time lesions were classified as carcinoma. Carcinomas were divided into 3 grades. Grade 1 tumor, either papillary or nodular, showed a minimal cytological atypia. Mitotic divisions were infrequent. The nuclei of Grade 2 carcinomas were larger and more pleomorphic than were those of Grade 1 carcinomas, and nucleoli were prominent. Mitotic divisions were readily detectable. Grade 3 carcinomas were characterized by marked cytological and architectural abnormality. Depending upon the depth of invasion, invasive tumors were divided into Stage 0 (confined to the epithelium or to the tumor stroma only), Stage A (extension to lamina propria), Stage B (tunica muscularis), or Stage C (tunica adventitia).

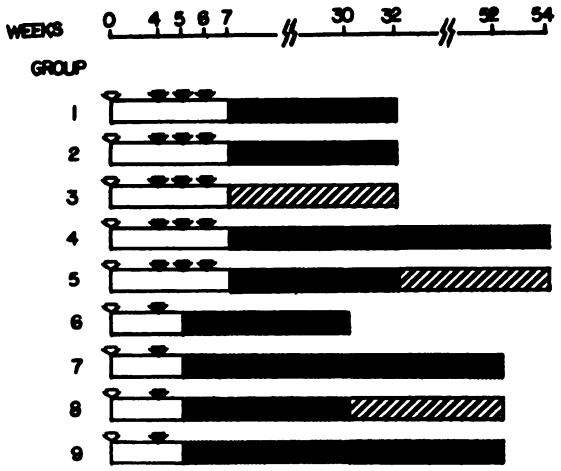

Chart 1. Experimental design. ⌔, bladder transplantation; ▾, instillation of 0.25 mg of MNU dissolved in 0.5 ml of 0.9% NaCl solution into HTB; □, instillation of 0.9% NaCl solution into HTB once a week; ▨, instillation of 2.1% NaCl solution into HTB once a week; ■, instillation of rat urine into HTB once a week; ▩, leaving HTB untouched instead of weekly instillation of urine or saline solution.

Statistical Analysis. Incidence of pathological lesions was analyzed by the χ^2 test.

RESULTS

All animals gained weight progressively with no significant difference in body weight among different groups. Six rats which died within 10 weeks after bladder transplantation and 3 rats which survived the experimental period but had infected HTBs were excluded from the results. Altogether, 255 rats survived the scheduled experimental period.

Bladder Lesions. Tumors in HTBs, when found, were randomly distributed throughout the mucosa and ranged in size from 1 to 10 mm and from one to 5 in number. A sessile polypoid lesion (inflammatory polyp) was frequently found in the mucosa around the tip of the intraluminal portion of the connector. Some carcinomas that developed at the sites inflammatory polyps were excluded from tabulation for the reasons discussed previously (12). No calculi were found in any bladder.

The incidences of NPH and carcinoma are shown in Table 1. The tumor enhancement by urine treatment as compared to saline treatment (3, 8, 12) was again demonstrated at 25 weeks (Group 1 *versus* Group 2, $p < 0.001$). Total avoidance of fluid injection leaving HTBs untouched (Group 3) did not prevent tumor development and resulted in an incidence equivalent to that of the saline group (Group 2) but lower than that of the urine group (Group 1 *versus* Group 3, $p < 0.05$). Saline treatment during the second phase of the study (Group 4) or leaving the HTBs untouched (Group 5) subsequent to the MNU-urine treatment did not decrease but tended to increase tumor incidence, although the increase was not statistically significant. There was, however, one exception; leaving HTBs untouched during the second phase after the initial MNU-saline treatment resulted in a significant increase in tumor incidence (Group 6 *versus* Group 8, $p < 0.025$). Delay in institution of urine treatment until the second phase of the study did not fail to exhibit tumor-enhancing effect (Group 7 *versus* Group 9, $p < 0.025$). A great majority of tumors were of Grade 1 and Stage 0 irrespective of difference in treatment. A few tumors were of higher grade and stage (data not shown). To determine if the second-phase treatment caused alteration in tumor size or number per bladder, tumors observed

Table 1

Histological changes in HTBs

Group	Treatment: Initial	Treatment: Subsequent, Phase 1	Treatment: Subsequent, Phase 2	No. of rats	NPH[a]	Carcinoma	% of rats with carcinoma
1	MNU,[b] 3 doses	Urine		28	16[c]	17[d]	61
2	MNU, 3 doses	Saline		28	1	3	11
3	MNU, 3 doses	Untouched		30	2	9[e]	30
4	MNU, 3 doses	Urine	Saline	26	10	22	85
5	MNU, 3 doses	Urine	Untouched	27	2[f]	22	82
6	MNU, 1 dose	Saline		28	3	1	4
7	MNU, 1 dose	Saline	Saline	28	0	7	25
8	MNU, 1 dose	Saline	Untouched	31	3	10[g]	32
9	MNU, 1 dose	Saline	Urine	26	7	15[h, i]	58

[a] Regarded as a precancerous proliferative change (7).
[b] MNU, 0.25 mg/administration; urine and saline (2.1% NaCl solution), 700 mOsmol.
[c] $p < 0.001$, Group 1 *versus* Group 5.
[d] $p < 0.001$, Group 1 *versus* Group 2.
[e] $p < 0.05$, Group 1 *versus* Group 3.
[f] $p < 0.025$, Group 4 *versus* Group 5.
[g] $p < 0.025$, Group 6 *versus* Group 8.
[h] $p < 0.001$, Group 6 *versus* Group 9.
[i] $p < 0.025$, Group 7 *versus* Group 9.

Table 2
Histological changes in HTBs left untouched

Group	Treatment: Initial	Treatment: Subsequent: Phase 1	Treatment: Subsequent: Phase 2	Total no. of rats	Capacity of HTBs[a]: <3 ml[b]: No. of rats	<3 ml[b]: NPH	<3 ml[b]: Carcinoma	>3 ml[b]: No. of rats	>3 ml[b]: NPH	>3 ml[b]: Carcinoma
3	MNU, 3 doses	Untouched		30	19	1	4	11	1	5
5	MNU, 3 doses	Urine	Untouched	27	12	1	12	15	1	10
8	MNU, 1 dose	Saline[c]	Untouched	31	20	1	5	11	2	5

[a] All numericals in the distended-bladder group are not significantly different statistically from those in the nondistended group.
[b] Bladder capacity at the end of the study.
[c] 2.1% NaCl solution.

in Groups 4 and 5 were compared with those in Group 1. There was no significant difference in either parameter (data not shown).

The incidence of NPH paralleled that of carcinoma as observed previously (3, 8, 12). The incidence of NPH in Group 5 (HTBs left untouched during the second phase) was lower than that of the corresponding saline group (Group 4, $p < 0.025$).

Approximately one-third of the HTBs, which had been left untouched during the first or second phase of the study, showed a marked increase in luminal capacity, ranging from 5 to 50 ml. This was in contrast to the average capacity in other groups being from 0.8 ± 0.3 (S.D.) ml (Group 6) to 1.7 ± 1.2 ml (Group 4). Apparently, enlargement resulted from a slow progressive increase over weeks, and its cause could not be determined. To determine if increase in size may have affected tumor incidence, these bladders were divided into 2 groups, those less than 3 ml and those over 3 ml in capacity. The incidences of NPH and tumors are shown in Table 2. There was no significant difference of either NPH or tumor between the groups. No abnormality was recognized in natural bladders on gross examination.

Other Significant Changes. Altogether, 8 malignant fibrous histiocytomas developed around the s.c. placed reservoir. All tumors occurred in rats involving the second-phase study, and their development was unrelated to the treatment.

DISCUSSION

Three significant observations were made. (*a*) The incidence of transitional cell carcinoma of low-grade cancer did not decrease but tended to increase even when contact with urine was discontinued (Group 1 *versus* Group 4 or 5). (*b*) Leaving the MNU-initiated bladders untouched did not prevent tumor development, and the incidence was similar to that of the bladders which were manipulated weekly by exchanging the luminal fluid with 2.1% NaCl solution (Group 2 *versus* 3). (*c*) Late institution of urine instillation to HTBs still effectively enhanced MNU-initiated tumorigenesis (Group 7 *versus* 9). Thus, the results suggest that tumors will develop in "dysfunctionalized" carcinogen-initiated bladders, although the incidence is lower than that of the bladders which maintain urine contact, and that tumors, once developed, do not appear to regress even if urine flow is diverted. This was in contrast to our previous observation in which progression of preneoplastic or early neoplastic lesions to overt carcinoma was inhibited when urine flow was diverted (13). If the current observation is extrapolated to the human situation, our data suggest that low-grade superficial tumors are indeed neoplastic, and spontaneous regression cannot be expected by urinary diversion. Urinary diversion, however, might be effective in controlling progression of at least some of the early neoplastic lesions (NPH) to overt cancer. Our results also suggest that the role of urine is consistent with that of the promoter in the classical 2-stage carcinogenesis model (4).

REFERENCES

1. Abeshouse, B. J., and Scherlis, I. Spontaneous disappearance or retrogression of bladder neoplasm; review of the literature and report of three cases. Urol. Cutan. Rev., *55:* 1–4, 1951.
2. Akaza, H., Murphy, W. M., and Soloway, M. S. Bladder cancer induced by noncarcinogenic substances. J. Urol., *131:* 152–155, 1984.
3. Babaya, K., Izumi, K., Ozono, S., Miyata, Y., Morikawa, A., Chmiel, J. S., and Oyasu, R. Capability of urinary components to enhance ornithine decarboxylase activity and promote urothelial tumorigenicity. Cancer Res., *43:* 1774–1782, 1983.
4. Boutwell, R. K. Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res., *4:* 207–250, 1964.
5. Davis, E. Disappearance of carcinomatous ulceration of bladder following ureterosigmoidostomy. J. Am. Med. Assoc., *137:* 450–453, 1948.
6. Friedell, G. H., Jacobs, J. B., Nagy, G. K., and Cohen, S. M. The pathogenesis of bladder cancer. Am. J. Pathol., *89:* 431–442, 1977.
7. Fukushima, S., Murasaki, G., Hirose, M., Nakanishi, K., Hasegawa, R., and Ito, N. Histopathological analysis of preneoplastic changes during *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in rats. Acta Pathol. Jpn., *32:* 243–250, 1982.
8. Miyata, Y., Babaya, K., and Oyasu, R. Carcinogenesis in heterotopically-transplanted urinary bladder by *N*-methyl-*N*-nitrosourea: a model for initiation and promotion. Carcinogenesis (Lond.), *2:* 495–497, 1981.
9. Oyasu, R., Hirao, Y., and Izumi, K. Enhancement by urine of urinary bladder carcinogenesis. Cancer Res., *41:* 478–481, 1981.
10. Oyasu, R., Iwasaki, T., Matsumoto, M., Hirao, Y., and Tabuchi, Y. Induction of tumors in heterotopic bladder by topical application of *N*-methyl-*N*-nitrosourea and *N*-butyl-*N*-(3-carboxypropyl)nitrosamine. Cancer Res., *38:* 3019–3025, 1978.
11. Oyasu, R., Manning, D. J., Matsumoto, M., and Hopp, M. L. Heterotopic urinary bladder with a communicating reservoir. Cancer Res., *36:* 2261–2267, 1976.
12. Ozono, S., Babaya, K., Morikawa, A., and Oyasu, R. A minimal dose of *N*-methyl-*N*-nitrosourea carcinogenic to heterotopically transplanted rat urinary bladder. Carcinogenesis (Lond.), *4:* 547–551, 1983.
13. Rowland, R. G., Henneberry, M. O., Oyasu, R., and Grayhack, J. T. Effects of urine and continued exposure to carcinogen on progression of early neoplastic urinary bladder lesions. Cancer Res., *40:* 4524–4527, 1980.