Orthotopic Implantation of Primary N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide-induced Bladder Cancer in Bladder Submucosa: An Animal Model for Bladder Cancer Study

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ABSTRACT

Primary bladder tumors induced in Fischer 344 inbred rats by N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide were transplanted in syngeneic rats by the intravesical, s.c., i.v., and orthotopic routes. Attempts were made to establish bladder cancer cell lines in vitro. No success was achieved in transplantation by either the s.c., i.v., or intravesical routes when primary tumor cells were transplanted as cell suspensions. Cell suspensions of primary tumors also failed to grow in culture. However, orthotopic implantation into the bladder submucosa gave 45% success. Tumor fragments obtained from either the primary tumor or its lung metastases resulted in 10.6 and 36% tumor takes, respectively, when implanted s.c. However, after one orthotopic passage in the bladder submucosa, the tumor cells injected as cell suspension grew s.c. in 14% and orthotopically in 79% of the animals. Tumor fragments obtained from orthotopic tumors and implanted s.c. resulted in 15% tumor takes. After the second orthotopic passage, tumor cells could be grown in cultures and orthotopically in 100% of animals. The technique of orthotopic implantation as well as the usefulness of this tumor model for bladder cancer studies are described.

INTRODUCTION

Animal models of transplantable bladder tumors in rodents are rare in spite of the fact that dyestuff intermediates are known to be bladder carcinogens in humans. Although β-naphthylamine is a known human carcinogen and 2-acetylaminofluorene, 2-methoxy-3-amino dibenzofuran, and tryptophan are suspected in the etiology of bladder cancer in animals and humans, few transplantable bladder tumor lines were available from these studies (2-4, 9, 10, 12, 13, 18, 19). It was only in recent years that FANFT1 was tested and shown to be a relatively specific bladder carcinogen in rats, hamsters, and mice (1, 5, 6, 7, 8, 11, 17). Soloway (15) was successful after several attempts to transplant FANFT-induced rodent bladder tumors. He showed that by traumatization of the bladder mucosa by MNU, the bladder tumor cells could be seeded in the bladder epithelium by intravesical tumor inoculation (15). The major disadvantage of this animal model, especially for the study of the intravesical immunotherapy, was that MNU caused local massive inflammation of the bladder mucosa and submucosa and infiltration of the bladder wall with acute inflammatory cells and exudate. Furthermore, our attempts to use this technique in the case of FANFT-induced rat bladder cancer in the syngeneic hosts were unsuccessful. We did not try instillation of tumor cells into bladder after its cauterization, a technique introduced by Soloway and Masters (16) that gave 54% success in tumor implantation.

In this paper, we shall describe our attempts to implant FANFT-induced tumors into appropriate recipients by several routes. Orthotopic implantation into bladder submucosa was the most convenient and dependable method for bladder cancer maintenance. In addition, the development of the transplanted tumor at the orthotopic site followed a course reminiscent of primary animal and human bladder cancer development. Our investigations have indeed yielded a bladder tumor model system ideal for the study of bladder tumor progression and testing of treatment regimens for application to human bladder cancer.

MATERIALS AND METHODS

Animals

Two hundred sixty-six inbred Fischer rats, bred in our laboratory (3 to 8 weeks old), were used as tumor recipients.

Tumors

They were FANFT-induced bladder tumors in female Fischer rats. The rats were fed FANFT for 14 months. A majority of the tumors were well-differentiated transitional-cell carcinomas. In some experiments, lung metastases of the primary bladder tumors were used. In the control group of 17 animals that received no therapy during or after FANFT feeding for 14 months, there were 13 animals with lung metastasis and 4 with suprarenal metastasis.

Routes Utilized for Transplantation of the Tumors

All the experiments described herein were carried out under aseptic conditions. The routes and modes of transplantation were as follows.

Implantation s.c. of Tumor Fragments

Tumor pieces, 8 to 15 cm in size, cut from the primary nonnecrotic tumor or its lung metastases, were inserted under the skin of syngeneic rats and then sutured.

Implantation of Single-Cell Suspensions

Tumor cell suspensions were prepared by mechanical tumor fragmentation with scissors in PBS. The suspended cells were removed and allowed to settle under gravity. They were washed 3 times with

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PBS and were tested for viability by trypan blue exclusion. The cell suspensions were inoculated by the following routes.

**Subcutaneous.** Five to ten million malignant cells in 0.2 ml PBS were injected s.c. in the right flank region.

**Intravesical Instillation.** The bladder mucosa was traumatized by MNU under Nembutal anesthesia (30 mg/kg) 48 hr before the instillation of 2 x 10^6 malignant cells/rat in 0.2 ml PBS. The tumor cell suspension was injected via a Teflon polyethylene sterile urethral catheter (19 gauge) inserted in the urethra. The fluid was retained in the bladder for 1 hr by a purse string suture around the external urethral meatus.

**Injection i.v. (Transcaval).** Inferior vena cava was utilized, as a vascular access, for injecting 10 x 10^6 tumor cells in 0.2 ml PBS per anesthetized rat. The time required for the injection was approximately 3 min.

**Orthotopic Injection into the Bladder Submucosa.** The bladder was evacuated of its urine content, and the tumor cell suspension was injected into the submucosa of a localized area of the anterior wall of the anesthetized rat with a 26 gauge 0.5-inch needle. To achieve this, the bladder was exposed and pulled upwards, gently, with a smooth forceps. The needle was then inserted obliquely for a few mm between layers of the bladder wall to deposit the cells in a submucosal location (Fig. 1). After the injection of 0.1 to 0.2 ml of tumor cell suspension, a bleb formed at the site of injection. In no instance was there breakage of the bleb and spilling of the tumor cells into the lumen. Injection of the suspension into a rat usually took about 2 to 3 min. Follow-up was done at 0.5-, 1-, and 2-month intervals to evaluate tumor growth, using a transillumination technique that permitted detection of tumors as small as 1 mm in diameter and of the development of the vascular architecture.

Orthotopic injection of tumor cells, cells that had migrated from the explants into the tissue culture dishes were trypsinized, and a specimen of the cells was seeded onto microscopic coverslips in plastic Petri dishes. The cells were allowed to attach to the coverslips and form confluent monolayers during 1 to 3 days. They were then washed with PBS, fixed with methanol, and stained with May-Grunwald-Giemsa for morphological examination.

The cytological examination of solid tumors was carried out by formalin fixation of the tissue, embedding fixed tissue in paraffin, and the preparation of tissue sections (5 µm thick). The sections were fixed to glass slides and stained with hematoxylin-phloxin and periodic acid-Schiff reagents.

### RESULTS

Table 1 summarizes the rate of our success in primary bladder cancer transplantation when different techniques of tumor implantation were used. The s.c. implant of a small piece of the tumor was more successful when it was obtained from the pulmonary metastases rather than from the primary tumor. Primary s.c. tumor takes were detected in 5 of 47 rats (10.6%) during the first transplantation passage and in 3 of 20 (18%) for the second passage. On the other hand, the successful tumor takes from pulmonary metastases were 5 of 14 rats (36%). They were not tried in the second passage.

A single-cell suspension from the primary tumor resulted in only one successful transplantation of 7 rats tried by the s.c. route.

No tumors developed when tumor cells were transplanted by either the intravesical instillation (with or without MNU traumatization) or the i.v. routes.

The orthotopic injection technique yielded a higher percent-
age of tumor takes. During the first passage, the bladder tumor takes were obtained in 10 of 22 rats (45%) tried, compared to 0% takes when tumor cells were injected s.c. Using the transillumination technique, the normal bladder appears semitransparent with fine vascularity (Fig. 2). The very early changes 2 weeks after orthotopic tumor implantation were in the form of neovascularity exhibited by the development of well-formed tortuous large blood vessels going toward the tumor site (Fig. 3). These vessels get larger at 4 weeks as the tumor grows (Fig. 4). When the tumors growing successfully by the orthotopic route in their first passage were converted into cell suspension and reinjected into the bladder submucosa of 14 rats, the tumor takes were obtained in 11 rats (79%).

The third orthotopic passage of the tumor in the bladder resulted in tumor takes in 100% of the rats that received the following tumor cell doses: 2 x 10^6; 1 x 10^6; 5 x 10^5; and 25 x 10^4 tumor cells/rat. It was 80% in animals that received 5 x 10^4 tumor cells. Using 25 x 10^5 cells from animals bearing orthotopic tumor in its second passage, an additional 50 rats were inoculated, and we observed 100% tumor takes for the third passage.

Using the third orthotopic passage tumor cells inoculated s.c. in a number of animals, there were 10 animals that failed to develop the tumor by the s.c. transplantation route. However, the same animals developed tumors when given injections orthotopically in the bladder (1 x 10^6 tumor cells/rat).

The natural history of orthotopic bladder tumor (third passage) was studied in 10 rats. The tumor developed in all the animals, reaching as big as 5 to 7 cm in diameter. No necrotic changes were found in these tumors, in spite of their large size. The tumors invaded the intestine and peritoneum locally, and bladder perforation was detected in 2 rats. Lymph node metastases were detected in all the rats. The tumor sometimes caused obstruction of the ureters with subsequent hydronephrosis (Fig. 5). Lung metastases were found in all the 10 rats that survived for more than 4 months (Fig. 6). The animals, if left, died from their tumors and its distant metastases. The orthotopically injected bladder tumor cells developed into papillary transitional-cell carcinoma (Fig. 7).

**DISCUSSION**

Transplantable animal tumors have been a cornerstone of experimental cancer research and its subsequent application to human cancer for more than 30 years. For reasons of easy access, s.c., i.d., and i.p. sites have been preferred for tumor implantation. Although these sites are appropriate for tumor maintenance, it is questionable if the development of the tumor at these sites parallels the development of the tumor in the organ of its origin. Few experiments have been conducted on transplantation of tumors into various organs to determine favorable sites of implantation or to mimic growth of primary cancer by implantation of tumor into the tissue of its origin.

Although it was our aim to have a transplantable bladder tumor in the rat, we were not oblivious to the fact that transplantable bladder tumor growing as a primary bladder cancer would provide a more useful animal model for the study of bladder cancer therapy. Hence, intravesical and orthotopic routes for tumor implantation in the bladder were attempted along with conventional implantation routes. As described in "Results," the orthotopic route proved to be the most favorable site for transplantation of these tumors.

Our inability to grow primary tumors by the s.c. and i.v. routes is not unique. Chemically induced tumors generally require several attempts before successful transplants in syngeneic hosts are achieved. The relative ease with which the orthotopic transplant was accepted by the recipients was surprising. It is possible that implantation in the bladder submucosa and subsequent selective growth of certain primary tumor clones probably overpowered the more antigenic slow-growing tumor cells, so that future transplants were 100% acceptable orthotopically and, to a limited extent, s.c.

Histologically, the orthotopically growing tumors were transitional-cell carcinomas. From the first-passage orthotopic tumors, we were also able to obtain a cell line that grew s.c. (Table 1) when inoculated as cell suspension. This tumor proved to be squamous-cell carcinoma of the bladder. It is apparent that FANFT induces tumors of both cell types and that the s.c. milieu may be more favorable for the growth of squamous-cell carcinoma.

The technique of orthotopic implantation into the bladder is relatively simple and can be performed after opening of the abdomen as easily as a s.c. transplantation. Under aseptic conditions, mortality rate is nil. The procedure can be completed in about 3 min. Growth of the tumor can also be judged at various intervals by transillumination of the bladder without sacrifice of the animal. We believe that this model will be exceptionally suitable to study the effects of new forms of intravesical chemotherapy and immunotherapy or their combination before the application of these treatment regimens in bladder cancer patients. Partial cystectomy of the bladder to remove the tumor may also show if tumor spill into the bladder lumen during surgery results in reseeding and future recurrence of the tumor at other sites in the bladder.

**REFERENCES**

1977.


Orthotopic Implantation of Bladder Tumor

Fig. 1. Technique of orthotopic injection of bladder tumor cells into the bladder submucosa.

Fig. 2. Transilluminated photo of normal rat bladder showing fine capillaries in a semitransparent organ.

Fig. 3. Transillumination of rat bladder 2 weeks following orthotopic transplantation of bladder tumor cells. It shows the early vascular changes, which include the thickening of tortuous blood vessels growing towards the implanted tumor at the dome of the bladder.

Fig. 4. Same as Fig. 3 except that the rat bladder transillumination was carried out 4 weeks after orthotopic inoculation of tumor cells. The vascular changes have become more prominent as the tumor increased in size (see shadow).
Fig. 5. A massive orthotopic bladder tumor 6 months after orthotopic inoculation of bladder tumor cells. A, bilateral hydronephrosis; B, lymph node metastasis.

Fig. 6. Lung metastasis from an orthotopically implanted bladder tumor.

Fig. 7. Histological appearance of tumor in the rat bladder 10 days following orthotopic injection of $25 \times 10^4$ tumor cells. The muscle layer is free of tumor at this time, and the tumor is localized. Hematoxylin-phloxine, $\times$ 400.
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