A New in Vivo Model to Study Invasion and Metastasis of Human Bladder Carcinoma

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ABSTRACT

An animal model to investigate the invasive and metastatic properties of human bladder transitional cell carcinoma (HTCC) was established. Two long-term HTCC cell lines (RT4 and EJ) and one HTCC cell line derived in our laboratory (LD-71) were tumorigenic when injected s.c. into nude mice but had little potential to invade locally or metastasize before the animals succumbed to tumor burden. Experimental lung metastases were, however, observed in approximately 60% of animals given injections of RT4 or EJ cell lines in the tail vein. The cells were also implanted transurethrally into the urinary bladders of athymic mice. RT4 cells, which were originally isolated from a superficial papillary tumor, produced histologically noninvasive tumors after transurethral inoculation with no evidence of metastasis. In contrast EJ cells, which were originally isolated from a more aggressive tumor, produced invasive tumors in nude mouse bladders and metastasized to the lungs spontaneously. The invasive cell line LD-71 was weakly tumorigenic and was successfully implanted into the bladder on only one of five attempts. The human origin of the implanted tumors was documented by Southern blot analysis using human repetitive DNA as a probe. The results indicate that the site of injection strongly influences the behavior of the resulting tumors and that intravesical implantation of these HTCC cell lines produces pathological expression of invasiveness and metastatic potential.

INTRODUCTION

Transitional cell carcinoma of the human bladder is a good example of a neoplasm displaying a spectrum of invasive potentials. Generally, cytologically low-grade tumors tend to be multifocal with little potential to invade or metastasize. In distinction, more anaplastic bladder carcinomas tend to be unifocal with high potential to invade the bladder wall and metastasize. As a result, low-grade lesions have a much better prognosis than high-grade lesions (1–3).

The mechanisms involved in tumor cell invasion and metastasis are central to our understanding of the biological behavior of carcinoma. Much effort has been expended on the establishment of models to investigate tumor invasion and metastasis, but there has been less progress in establishing in vivo models to study human tumor invasion and metastasis particularly with epithelial tissues. The extent to which s.c. implanted human tumors will invade and metastasize in nude mice is presently not clear (4–8). Kyriazis et al. (6, 7) reported frequent invasion and metastasis of bladder cancer cells, whereas Russell et al. (8) observed no evidence for invasion and metastases of human bladder cancer xenografts. It is believed that invasive and metastatic potentials are influenced by both tumor cell properties and host factors (9, 10). Recently, attention has focused on the site of tumor implantation (4), and Giavazzi et al. (11) showed that human colorectal cancer cells implanted into the spleen were metastatic, whereas s.c. injected cells were not.

In the present study we confirmed that human transitional cell carcinoma cell lines implanted s.c. would grow locally but would not exhibit local invasive behavior or metastasize. Therefore, we modified a technique described by Soloway et al. (12) and implanted bladder cancer cell lines directly into the bladders of athymic mice. We hypothesized that tumors implanted directly into the bladders would display more typical pathological behavior. We implanted a low-grade, noninvasive HTCC cell line (RT4) (13, 14) into the bladder and found that it exhibited little or no potential to invade locally or metastasize, whereas a high-grade, invasive HTCC cell line (EJ) (15) exhibited aggressive behavior, and inoculated animals developed spontaneous metastases. Both cell lines showed similar incidences of lung metastases if they were injected i.v. A third cell line, LD-71, which was derived from an invasive human bladder tumor (16) was weakly tumorigenic and grew on just one occasion in the bladder. It produced a high-grade tumor that replaced large areas of the bladder wall. We have, therefore, established an in vivo model to study tumor invasion and metastasis of HTCC.

MATERIALS AND METHODS

Animals. Female athymic (nu/nu) nude mice 6 to 8 wk of age were obtained from Harlan, Sprague, Dawley Inc., Indianapolis, IN. The animals were maintained in pathogen-free, isolated barrier facilities provided with sterile food, water, bedding, and cages. Random animals tested negative for pneumonia virus of mice, murine hepatitis virus, Reo-3 virus, Sendai virus, murine encephalomyelitis virus, minute virus of mice, and Mycoplasma.

Tissue Culture Maintenance. The cell lines were maintained under sterile conditions utilizing standard tissue culture incubators (37°C, 5% CO2) and laminar flow hoods. The cells were maintained in Eagle's minimal essential medium (Gibco, Grand Island, NY) with 10% heat-inactivated fetal calf serum, 2% penicillin, and streptomycin and 2% tryptose phosphate broth (Difco, Detroit, MI). The cells were harvested utilizing 0.25% trypsin, pelleted, washed in medium, repelleted, and resuspended in PBS in preparation for implantation (concentration, 2 x 10^6 cells per 0.1 ml) into the bladder.

Cell Lines. A strain of human foreskin fibroblasts (T1) was used as our control cell line. RT4 (13, 14) is a cell line that was established in 1970 from a 63-yr-old patient who had a cystectomy because of recurrent multifocal superficial, low-grade papillary HTCC of the bladder. It was obtained from American Type Tissue Collection (ATCC HTB2). EJ (15) is a high-grade, invasive HTCC cell line that was established in 1970 from a patient undergoing cystectomy for high-grade invasive bladder cancer. The cell line was obtained from Dr. Eric J. Stanbridge, University of California, Irvine. LD-71 is a cell line derived under serum-free conditions in our laboratory (16) from a patient undergoing cystectomy for a high-grade invasive transitional cell carcinoma of the bladder.

Inoculation s.c. Mice were given injections in the anterior flank with 4 x 10^6 tumor cells suspended in 0.2 ml of PBS. The animals were sacrificed when the skin over the tumor became ischemic. If no tumor appeared the mice were observed for 3 mo. The tumor, lung, liver, and spleen were examined histologically.

Inoculation Intravesically. The inoculation technique was modified slightly from that described by Soloway et al. (12). Eight-wk-old female

Received 2/4/87; revised 9/10/87; accepted 9/22/87.

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This work was supported by Grant CA 40422 from the National Cancer Institute.

1 The abbreviations used are: HTCC, human transitional cell carcinoma; PBS, phosphate-buffered saline.

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athymic mice (nu/nu) were anesthetized with i.p. pentobarbital (5 mg/100 g) after being treated with p.o. tetracycline for 24 to 48 h before the procedure. A 22 gauge catheter (ANGIOCATH; Sandy, UT) was placed transurethrally into the bladder. If no cautery was used, $2 \times 10^6$ cells were injected through the catheter. If cautery pretreatment was used, the animal was placed on a moistened gauze pad on a grounding plate, and a 24 gauge surgical wire was passed through the catheter so that approximately 1 mm of wire was exposed to the bladder wall. Electrocautery (Model AM-5 Sensaur Microsurge; Coles Corp., Philadelphia, PA) was applied to the wire for 0.5 to 3 s with the machine set at 1-2. The wire was removed, and $2 \times 10^6$ cells were suspended in 0.1 ml of PBS injected through the catheter. The catheter was removed, and the animals remained anesthetized for approximately 20 to 30 min in order to prevent early expulsion of the cells by spontaneous voiding. The mice were examined daily and sacrificed at 8 to 12 wk postimplantation or earlier if they developed signs of distress. Autopsies were performed immediately to determine the cause of death and to evaluate for the presence and extent of tumor. The lungs, liver, spleen, pancreas, bladder, and kidneys were processed for histological examination. Because micrometastases may not be apparent on histological review, we routinely cultured the lungs, liver, and spleen in serum-free medium permissive for the growth of the tumor cells (16) in order to confirm the presence or absence of tumor cells. Other organs, such as the kidney or regional lymph nodes, were also cultured if tumor was suspected. The bladder tumors were examined histologically for extent of invasion into the bladder wall. The explanted tumor cells were analyzed for repetitive human DNA, using the Blur-8 probe (17), and labeled by random primer extension (18) with DNA extractions and Southern transfers as previously described (19).

Inoculation i.v. of Tumor Cells. Cells were resuspended in 0.1 ml of PBS, and $5 \times 10^6$ cells were injected into mouse tail veins using 25 gauge needles as described by Fidler (4).

Explant Culture Technique. Organs of interest were minced into small pieces of approximately 0.5-mm cubes with scalpel blades and placed on 60-mm tissue culture dishes coated with rat smooth muscle matrix containing 1 ml of defined medium (16) and incubated at 37°C for 24 h. An additional 3 ml of medium were then carefully added so as to not detach tissue pieces from the matrix. The medium was changed 3 times per wk, and outgrowths were normally visualized within 2 to 3 days if tumor cells were present.

RESULTS

Growth of HTCC Cell Lines after Injection s.c. The three tumor cell lines used in these experiments were all tumorigenic when implanted s.c. (Table 1). However, the LD-71 cell line was tumorigenic in only two of five attempts and grew more slowly than the RT4 and EJ cell lines. Grossly, none of the tumors was fixed to the abdominal wall or overlying skin, and they grew as pseudoencapsulated masses compressing the surrounding normal tissue. An example of a histological section of a tumor formed after injection of EJ cells is shown in Fig. 1. Minimal or no evidence of local invasion was seen, and no gross metastases were observed. One mouse was examined for micrometastasis by organ explant of the lungs, but no human cancers were recovered.

Intravesical Implantation of HTCC Cell Lines. Soloway et al. (12) showed that the successful implantation rate of transurethral injected murine bladder tumors (MBT-683) in syngeneic mice was significantly improved by electrocauterization. For our first attempts, we therefore pretreated bladders of the nude mice with a short burst of electrocautery (set at 2) for 1 to 3 s (Table 2). We found, however, that the implantation rate was not significantly improved by this treatment and that the procedure-related mortality, which was primarily related to bladder perforation, was high. In contrast the procedure-related mortality was 16% and was related primarily to anesthesia when no cautery was used.

Normal human fibroblast cells (T1) were not tumorigenic when the bladders were examined histologically or by explant culture (Table 2). The cell line LD-71 was weakly tumorigenic after intravesical implantation similar to s.c. injections (Table 1). RT4, the low-grade noninvasive HTCC cell line, had an approximately 50% successful implantation rate regardless of

<p>| Table 1  Incidence of tumor growth from HTCC cells implanted s.c. |</p>
<table>
<thead>
<tr>
<th>No. of animals with tumors/mice given injections</th>
<th>Time for appearance of tumors (wk)</th>
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<tbody>
<tr>
<td>LD-71</td>
<td>2/5</td>
</tr>
<tr>
<td>RT4</td>
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<tr>
<td>EJ</td>
<td>6/7</td>
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cautery pretreatment. The implantation rate for the EJ cell line was also approximately 50% and was not improved significantly by electrocautery (Table 2).

Table 2 also shows the abilities of the inoculated cells to establish metastases at distant organ sites following intravesical injection. No gross metastases to any of the organs examined were found in animals given injections of RT4 cells with or without cautery. Neither were any micrometastases observed when organ fragments were cultured. Four of the 18 animals given injections did, however, develop kidney tumors which were probably due to primary implantation because mice reflux urine up their ureters. The EJ cells, on the other hand, were metastatic, and micro- and/or macrometastases were observed in 8 of the 15 animals developing primary tumors after intravesical injection (Table 2). These metastases were mainly observed in the lungs, although a lymph node was also involved on one occasion. As with RT4 cells, cautery treatment had little effect on either primary or secondary take rates, although the lower number of metastases found in the cauterized group might have been due to the fact that the mice died too early from complications of their primary tumors for metastases to become apparent. Renal tumors were found in 6 of the animals receiving injections, probably due to urinary reflux (see above).

Inoculation i.v. of RT4 and EJ Cells. The results with intravesical inoculation of RT4 and EJ cells showed that the cells recapitulated their metastatic behavior in the patients from whom they were derived. We also injected the cells directly into the circulation of nude mice to determine whether the RT4 cells were inherently incapable of establishing colonies in the lungs. Five mice were each given injections of $2 \times 10^5$ RT4 or EJ cells in the tail vein and examined subsequently for the presence of lung tumors. Three mice in each group developed tumors in the lungs (see below), and thus this route of inoculation did not discriminate between the original metastatic behavior of the cells.

Histology of Implanted Tumors. The bladder tumors derived from the RT4 cell line were composed of small cells that were fairly uniform in size and shape and had prominent nucleoli and infrequent mitoses (Fig. 2). These changes indicate a lower degree of anaplasia (Grade 2 of 4) and in human patients are usually associated with a relatively good prognosis. The tumors consistently grew superficially in the bladder and showed no histological indication of bladder muscle invasion if no electrocauterization was done (Fig. 2A). However, tumor nests were seen deep in the bladder wall after cauterization (Fig. 2B). These tumor cells may have penetrated bladder tissue as a result of mechanical trauma induced by cautery and therefore may not represent true tumor invasion. The use of electrocautery may therefore cause artifacts and was subsequently avoided, since it did not improve tumor take (Table 2).

Four kidneys were found to have tumors growing in the renal pelvis and expanding into the medulla. In each case the tumor masses compressed the surrounding renal structure but were always sharply demarcated from the renal tissue and produced a noninvasive histological pattern regardless of whether electrocautery had been used (Fig. 2C).

The bladder tumors derived from the EJ cell lines consistently displayed a higher grade of anaplasia, with increased cellular pleomorphism and high mitotic activity (Fig. 3). These cytological features are usually associated with a more aggressive clinical course in humans. Histologically the tumors were implanted with RT4 cells. The large tumor mass is sharply demarcated from the surrounding renal tissue and compresses the adjacent renal structures but does not invade into the tissue.
EXPERIMENTAL BLADDER CANCER METASTASIS

Fig. 3. A, micrograph (x 25) of a representative bladder tumor from an uncauterized mouse implanted with the HTCC cell line EJ. A bulky tumor mass fills much of the bladder cavity. Tongues of tumor cells are also seen infiltrating deep into the bladder wall musculature. B, micrograph (x 100) from a kidney tumor from a mouse implanted with EJ cells. The tumor is anaplastic with obvious invasion into the renal parenchyma. Note remnants of renal tubules engulfed by the tumor (arrows).

clearly invasive, with tongues and sheets of neoplastic cells dispersed throughout the bladder muscle (Fig. 3A). In contrast to RT4, EJ cells that implanted in the renal pelvis were poorly demarcated from surrounding renal tissue and produced a clearly invasive histological pattern (Fig. 3B).

As mentioned earlier, EJ and RT4 cells had different metastatic abilities from the nude mouse bladder, and none of the mice given injections of RT4 cells had any gross or micrometastases at the time of sacrifice. In contrast, 8 of the mice given injections of EJ cells had either histologically visible lung metastases or micrometastases documented by recovering EJ cells from the lungs by tissue culture. In one instance, metastasis to a retroperitoneal lymph node was found (Fig. 4). Thus, the invasive abilities and metastatic potentials of EJ and RT4 cells injected in nude mouse bladder recapitulated those of their parent human tumors.

Fig. 5 shows tumors arising in the lungs of mice given injections i.v. of RT4 or EJ cells. A large number of tumor deposits arose in the lungs of animals given injections of either cell line, and it was difficult to assess the invasive activities of the cells in this organ.

Documentation of Human Origin of Implanted Tumors. When palpable tumors in the bladder or kidneys were identified at autopsy, a piece was also cultured in serum-free medium (16). Epithelial outgrowths were serially passaged until enough cells for DNA analysis were obtained. Additionally, the lungs from each animal were cultured to document the presence or absence of micrometastases. To document the human origin of the tumors, DNA from the cultured cells was extracted by routine procedures, cut with EcoRI, size separated by agarose gel elec-
EXPERIMENTAL BLADDER CANCER METASTASIS

Fig. 5. Micrographs of representative lung tumors from mice given injections i.v. of the HTCC cell lines RT4 and EJ. RT4 or EJ cells were injected into tail veins, and the mice were observed until they developed physical signs of tumor burden. The mouse given injection of RT4 (A) after 55 days, and the one given injection of EJ (B) after 88 days. In each case, tumor nests are seen implanted in otherwise relatively normal lung parenchyma.

trophoresis, blotted to nitrocellulose, and probed with a $^{32}$P-labeled human repetitive DNA probe (Fig. 6). No hybridization signal was detected with mouse DNA, but extensive hybridization occurred with human DNA and with all of the DNAs extracted from cells cultured from the mice. Thus the primary and metastatic tumors were of human origin.

DISCUSSION

Central to understanding the biological behavior of carcinoma is why and how malignant cells invade normal anatomical boundaries, disperse, and metastasize. Human bladder transitional cell carcinoma represents a good system to study these questions, because the clinical management of bladder cancer hinges on superficial versus invasive properties. Superficial bladder tumors are managed locally without removing the bladder. However, 10 to 20% of patients with superficial bladder tumors develop invasive tumors with time. In contrast, invasive bladder tumors with evidence of muscle invasion are usually managed by removal of the bladder and urinary diversion (1–3).

Our results substantiate earlier findings on the limited application of s.c. injection of human cancer cell lines into nude mice with regard to invasive and metastatic properties (4). All of the HTCC cell lines were tumorigenic s.c., but their invasive and metastatic properties were not manifested in contrast to the findings of Kyriazis et al. (6, 7). Our results were more similar to the results of Russell et al. (8), for bladder tumor xenografts and all three cell lines grew as freely mobile subcutaneous nodules and only became a burden to the mice because of local skin necrosis over the tumors. Our results with intravesical implantation of HTCC cell lines support a growing body of evidence that the site of injection in nude mice profoundly influences pathological behavior (4, 9, 10, 20).

In order to directly implant tumors into the bladders of nude mice we slightly modified a technique described by Soloway et al. (12) where they implanted MBT-683 cells, a high-grade invasive C3H/He mouse transitional cell carcinoma, into the bladders of C3H/He mice. Their data showed that implantation rates of 6% for normal untreated bladders could be significantly improved to 67% by electrocautery pretreatment. Under our conditions we did not observe such an effect of electrocautery, and because this treatment complicated the assessment of bladder tissue infiltration from histological sections and was associated with a high degree of procedure-related mortality, we suggest that human tumor cells should be inoculated directly without prior cauterization.

The superficial HTCC cell line (RT4) did not produce distant metastases even when the cells penetrated deep into bladder tissue after electrocautery. In the absence of cauterization the bladder tumors remained superficial, showing no histological evidence of invasion into the bladder wall musculature. EJ cells, which were originally isolated from an invasive and metastatic bladder tumor, invaded deeply into the nude mouse bladder wall with or without prior cauterization and were highly metastatic. The invasive behavior of RT4 and EJ cells was also
characteristics of the RT4 and EJ tumors in the mice were very similar to their human counterparts (Figs. 2 and 3). The pathophysiological behavior was also highly maintained as the tumors derived from the superficial tumor cell line produced no spontaneous metastases. In sharp contrast, the tumors derived from the invasive cell line produced spontaneous metastases.

The results of these experiments indicate that the pathophysiological expression of invasive and metastatic potential for HTCC cell lines can be investigated in an in vivo model that reproduces the human experience. We are currently exploring the reasons for the different biological behavior of the two cell lines and attempting to modify metastatic behavior by various treatments in vitro before intravesical implantation.

REFERENCES

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