Video Article

An Orthotopic Bladder Cancer Model for Gene Delivery Studies

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Abstract

Bladder cancer is the second most common cancer of the urogenital tract and novel therapeutic approaches that can reduce recurrence and progression are needed. The tumor microenvironment can significantly influence tumor development and therapy response. It is therefore often desirable to grow tumor cells in the organ from which they originated. This protocol describes an orthotopic model of bladder cancer, in which MB49 murine bladder carcinoma cells are instilled into the bladder via catheterization. Successful tumor cell implantation in this model requires disruption of the protective glycosaminoglycan layer, which can be accomplished by physical or chemical means. In our protocol the bladder is treated with trypsin prior to cell instillation. Catheterization of the bladder can also be used to deliver therapeutics once the tumors are established. This protocol describes the delivery of an adenoviral construct that expresses a luciferase reporter gene. While our protocol has been optimized for short-term studies and focuses on gene delivery, the methodology of mouse bladder catheterization has broad applications.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50181/>

Introduction

Bladder cancer is the second most common cancer of the urogenital tract with nearly 75,000 new cases and 15,000 deaths expected in 2012¹. High rates of recurrence require lifelong follow-up, which makes bladder cancer one of the costliest cancers to treat. Bladder cancer that has invaded the muscle layer may metastasize to liver, lung or bone via the lymphatic system. Multimodal therapy of advanced tumors results in only 20-40% survival after 5 years. Therefore, effective treatment strategies aimed at reducing the recurrence and progression of superficial bladder cancer as well as improving therapeutic outcome in patients with advanced disease are urgently needed.

Development of novel therapeutics requires preclinical models to evaluate efficacy following initial *in vitro* assessment. The tumor microenvironment can significantly influence cancer development and responsiveness, which highlights the need for preclinical models in which tumors arise or can be established in the organ of origin. One approach is the development of transgenic models in which tumors arise spontaneously or can be induced in an organ-specific manner. An excellent protocol of a transgenic bladder cancer model has recently been published². The drawback of transgenic models is that tumors tend to develop slowly and with less uniformity than desired. In addition, the cost of maintaining a breeding colony has to be considered. An alternative to transgenic models is orthotopic implantation of tumor cells, which has the benefit of short time frames for tumor establishment in commercially available mice. While some human bladder cancer cell lines can be grown orthotopically (we have successfully used UM-UC-3), it may be desirable to establish tumors in immunocompetent mice. Two murine bladder cancer cell lines, which grow orthotopically are MBT-2 and MB49³. Since MBT-2 cells are contaminated with replicating type C retrovirus⁴, we have chosen MB49 for our studies. It is important to note that MB49 cells were isolated from a male mouse and orthotopic implantations are for anatomical reasons performed in female mice. This has the benefit of easy identification of the implanted cells by markers of the Y chromosome, but the gender mismatch can be a drawback for immunological studies.

The bladder epithelium is lined by a glycosaminoglycan (GAG) layer, which functions as a barrier for infection by microorganisms. This barrier can also interfere with implantation of tumor cells and several methods have been developed to overcome this difficulty (Table 1). Electrocautery has been used extensively as a physical means to disrupt the GAG layer⁵⁻¹³ and a protocol demonstrating electrocautery has recently been published in JoVE¹⁴. However, should an electrocautery unit not be available, chemical means to destroy the GAG layer such as silver nitrate or poly-L-lysine can also be used¹⁵⁻²⁴. Tumors are established effectively by a brief exposure of the bladder to a small volume of silver nitrate (5-10 µl, 0.15-1.0 M, ~10 sec) or longer contact with poly-L-lysine (100 µl of 0.1 mg/ml for 20 min) (Table 1). Here we describe a method that uses trypsin to facilitate implantation of MB49 cells.

In an attempt to improve therapeutic approaches for bladder cancer, gene therapy has garnered significant attention. From a clinical point of view, bladder cancer is an ideal target for gene therapy due to easy accessibility of the organ and the ability to locally deliver the payload. Viral vectors that have been explored for bladder cancer gene therapy include an oncolytic herpes simplex virus²⁵, retrovirus²⁶, canarypox virus²⁷, vaccinia virus, AAV, and adenovirus²⁸. In the second part of our protocol, we describe a method for viral delivery that is virtually identical to instillation of the tumor cells. Of interest in our lab is the development of novel approaches to gene delivery, which we assess via

bioluminescence using an adenoviral vector that expresses a luciferase transgene. However, the methodology of bladder catheterization can be used for delivery of various agents and therefore has broad applicability.

Protocol

All procedures involving animals have been reviewed and were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. The protocol was approved under USDA category D for pain.

1. Cell Implantation

1. Two days before performing the procedure, plate 1×10^6 MB49 cells into T-25 flasks. Use high glucose DMEM supplemented with 10% FBS (and antibiotics, if desired). One flask is sufficient for each group of up to five mice that will be anesthetized and implanted in parallel.
2. On the day of the procedure, prepare trypsin for instillation by diluting sterile 0.25% tissue culture grade trypsin to 0.125% with sterile DMEM base medium (1:2 dilution). 1.2 ml is sufficient for 10 mice. Place in a 37 °C waterbath to equilibrate.
3. Prewarm a heating pad and position it under the nosecones of the anesthesia system that will be used to deliver isoflurane. Place a clean absorbent pad over the heating pad.
4. Place mice into the induction chamber of the anesthesia system and induce anesthesia with 3% isoflurane. When mice have lost consciousness, which is verified by loss of the toe-pinch reflex, transfer them to the nosecones. Make sure each animal is in a supine position and properly placed on the heating pad to maintain body temperature.
5. Reduce the isoflurane concentration to 2% to maintain anesthesia.
6. Apply ophthalmic ointment to both eyes to prevent drying.
7. Optional step: Remove abdominal hair. Hair on the lower abdomen must be removed if *in vivo* bioluminescent imaging will be performed.
 1. Apply a generous layer of depilatory cream, such as Veet, to the lowest 1 in square of fur on the abdomen, taking care not to contact the external genitalia. Wait 3 min.
 2. Remove hair by rubbing the area with a dry sponge, and then use a damp sponge to cleanse the skin. Repeat once, if needed.
8. Catheterize the bladder
 1. Lubricate a new, sterile 24 G pediatric venous catheter with a nonirritating lubricating gel such as K-Y. Remove and discard the needle. If leaking of fluid around the catheter is a common problem, *i.e.* it occurs more frequently than in one of ten mice, larger bore catheters (20 G) can be used. This may be an important consideration in mice older than 8 weeks.
 2. Holding the hub of the catheter, use the thumb and index finger of your opposite hand to spread the hind legs of the mouse and expose the urethral meatus. Gently insert the catheter into the urethra at a 45° angle, changing the angle to one parallel with the bench to insert it fully.
 3. After full insertion, lift the catheter gently, keeping it parallel to the bench, and visually confirm that it is placed in the urethra and not the vagina, which is below it. If correctly placed, the catheter will be able to be inserted fully since the vagina is shorter than the urethra.
 4. Reduce the isoflurane concentration to 1%.
9. Attach a tip to a P200 pipettor and remove urine from the bladder by applying suction to the external end of the catheter. Remove any remaining urine from the hub of the catheter. Discard urine and leave catheter in place.
10. Carefully pipette 80 μ l 0.125% trypsin solution into the hub of the catheter, avoiding air bubbles. Attach a 1 ml air-filled syringe to the catheter hub and slowly depress the plunger 0.1-0.2 ml to deliver the trypsin into the bladder.
11. Leave the syringe and catheter assembly in place for 15 min. Repeat steps 1.8-1.11 for any of the remaining anesthetized mice (up to 4). Proceed to the next step during the waiting period. HINT: Initially it may be helpful to have someone else prepare the cells (step 1.12).
12. Prepare MB49 cells for implantation. Cells were plated at 1×10^6 per T-25 flask 48 hr before implantation (step 1.1).
 1. Remove media and add 500 μ l of 0.25% trypsin to the flask. When cells detach add 5 ml of complete DMEM to resuspend cells. Remove a 50 μ l aliquot to count, and centrifuge the remainder at 1,000 rpm for 5 min. During the centrifugation step continue to steps 1.12.2 and 1.12.3.
 2. Count the cells using a hemocytometer. Calculate the cells/ml. Then calculate the volume needed to bring the cell pellet to 4×10^6 cells/ml (2×10^5 cells per 50 μ l).
 3. Pour off supernatant from centrifuged cells and resuspend the pellet in DMEM base medium using the volume required to suspend cells to 4×10^6 cells/ml (as calculated in step 1.12.2). Keep cells at room temperature.
13. When the 15 min time point for trypsin treatment of bladders is reached, detach the air-filled syringe from the catheter leaving the catheter in place. Spent trypsin will normally flow back out through the catheter. Remove any remaining trypsin from the bladder by suction with the P200 pipettor as in step 1.9 above and discard.
14. Immediately pipette 50 μ l of MB49 cell suspension into the hub of the catheter. Attach the 1 ml air-filled syringe to the hub of the catheter and deliver the cells to the bladder by slowly depressing the plunger 0.1-0.2 ml. Repeat this step up to four additional times to implant all five mice. Discard any unused cells.
15. Leaving the catheter-syringe assembly in place, allow the cells to dwell in the bladder for 50 min.
16. Gently withdraw the catheter-syringe assembly from the urethra. Turn the isoflurane concentration to zero and move each mouse an inch or two away from the nosecone to recover on the heating pad.
17. When a mouse has regained its righting reflex, return it to its cage.

2. Intravesical Delivery of Adenovirus (8 Days after Implantation of Cells)

CAUTION: This part of the protocol uses adenovirus, which is an infectious agent and should be handled under strict BSL2 guidelines (<http://oma.od.nih.gov/manualchapters/intramural/3035/>).

All procedures performed with infectious agents were approved by the Institutional Biosafety Committee of the Medical University of South Carolina.

1. Eight days after implanting mice with MB49 cells, check for hematuria. Pick up individual animals over a white piece of absorbent paper and gently press on the abdomen to blot drops of urine onto the paper. Urine that is pink to red indicates hematuria and is a sign that tumors have established. Tumor take rate is at least 80% and with excellent technique can be as high as 100%.
2. Thaw adenoviral stock on ice and dilute to 10^9 PFU/50 μ l (2×10^{10} PFU/ml) in sterile, room temperature PBS.
3. Anesthetize mice as described in steps 1.3-1.6 above.
4. Catheterize mice as described in steps 1.8 and remove urine as in step 1.9
5. After urine is removed, immediately pipette 50 μ l of virus suspension into the hub of the catheter. Attach the 1 ml air-filled syringe to the hub of the catheter and deliver the virus to the bladder by slowly depressing the plunger 0.1-0.2 ml.
6. Leaving the catheter-syringe assembly in place, allow the virus to dwell in the bladder for 40 min.
7. Gently withdraw the catheter-syringe assembly from the urethra, and discard into 10% bleach solution to inactivate any remaining virus.
8. Turn the isoflurane concentration to zero and move each mouse an inch or two away from the nosecone to recover on the heating pad.
9. When a mouse has regained its righting reflex, return it to its cage.
10. Mark cages to indicate that mice have been instilled with adenovirus. Virus will be shed into the cage bedding for several days and all cages and bedding must be handled and disinfected appropriately.

Representative Results

Hematuria is observed in nearly all mice within 8 days after implantation of 200,000 MB49 cells. As shown in **Figure 1**, bladder weight more than doubles from 34.7 ± 3.3 mg (range 31-37 mg, n=4) in nontumor bearing mice to 87.5 ± 19.2 mg (range 77-120 mg, n=10) in mice that have been implanted with MB49 cells. In terms of gene delivery, we found that imaging mice 24 hr after viral instillation yields a stronger signal than after 48 hr (**Figure 2**). Adenoviral delivery is highly variable between animals, which should be taken into account when planning group size for statistical purposes (**Figures 2 and 3**). If a small animal imaging system is not available, an alternative approach to measure expression of the luciferase transgene is to remove and homogenize the bladder for *in vitro* analysis. Comparison between *in vivo* analysis using the IVIS200 small animal system and *in vitro* analysis using the Steady-Glo kit, indicate excellent correlation between data sets (**Figure 3**).

Figure 1. Analysis of tumor burden. Weight of nontumor (n=4) and tumor-bearing (n=10) mouse bladders 9 days after instillation of 200,000 MB49 cells. Statistical significance was determined by the Student's t-test using the Graphpad software. *p= 0.0002. [Click here to view larger image.](#)

Figure 2. *In vivo* analysis of gene expression. Small animal imaging performed 24 and 48 hr after instillation of virus storage buffer (-) or 1×10^9 PFU AdCMV.Luc (+). To visualize luciferase, mice were injected intraperitoneally with 200 μ g luciferin/mouse. Expression of the luciferase transgene was measured *in vivo* using the IVIS200 small animal imaging system. [Click here to view larger image.](#)

Figure 3. Comparison of bioluminescence signals obtained by *in vivo* small animal imaging and an *in vitro* assay. Twenty-four hours after delivery of virus storage buffer (open circles) or 1×10^9 PFU AdCMV.Luc (closed circles), mice were imaged using the IVIS200 and then sacrificed. Bladders were removed and homogenized for *in vitro* analysis of the bioluminescent signal using the Steady-Glo kit. Luciferase signal obtained by each method is expressed in arbitrary units (AU). The coefficient of determination (R^2) was calculated in with the Data Analysis add-in of Microsoft Excel. [Click here to view larger image.](#)

Insult to GAG layer	Cells implanted	Retention time	Tumor detection and Development	reference
Electrocautery	0.01-1 x 10 ⁵ MB49		Hematuria: 1,000 cells: 0/0; 10,000 cells: 4/6; 100,000 cells: 6/6 Tumors: 1,000 cells: 0/6; 10,000 or more cells: 6/6, 6/6 (100%)	5
Electrocautery	5 x 10 ⁴ MB49-Luc		Tumor incidence: 90% (as in reference ¹¹)	6
Electrocautery	1 x 10 ⁵ MB49	~3 hr	Tumor incidence: 90% at 50 days	7
Electrocautery	1 x 10 ⁵ MB49-PSA		PSA: detected as early as 4 days after implantation	8
Electrocautery	1 x 10 ⁵ MB49		Tumor incidence: 90%	9
Electrocautery	5 x 10 ⁴ MB49	2 hr	Tumor incidence: 83.3% (10/12) on day 21	10
Electrocautery	2 x 10 ⁴ MB49	2 hr	Tumor incidence: 100% by day 28	11
Electrocautery	1-5 x 10 ⁴ MB49	3 hr	Hematuria: 100% by day 16; Tumor incidence: 100%	12
Electrocautery	1 x 10 ⁵ MB49	3 hr	Tumor incidence: 97.3% (73/75)	13
PLL (100ul 0.01% 20 min)	1 x 10 ⁵ MB49-PSA	2 hr	Tumor incidence: 100%	15
PLL (0.1 mg/ml)	1 x 10 ⁵ MB49-PSA	2 hr	Tumor incidence: 100%	16
PLL (100 µl 0.1 mg/ml 20 min)	1 x 10 ⁵ MB49	1 hr	Tumor incidence: 94% (15/16)	17
PLL (0.1 mg/ml)	1 x 10 ⁶ MB49		Hematuria at day 7: 50%, 100% at day 14	18
PLL (100 µl 0.1 µg/ml 20 min) or 22% ethanol	1 x 10 ⁵ MB49	1 hr	Tumor incidence: Unmodified bladder: 0%, PLL: 80-100%; Ethanol 40-80%	19
Silver nitrate (5 µl 0.2 M)	1 x 10 ⁶ MB49	1 hr	Tumor incidence: 100%	20
Silver nitrate (10 µl 0.15 M 10 sec)	5 x 10 ⁵ MB49		Tumor incidence: 92% (46/50)	21
Silver nitrate (8 µl 1 M 10 sec)	5 x 10 ⁵ MB49	2 hr	Hematuria: 100% at day 7; days hematuria 100%; Tumor incidence: 96.7% (29/30 mice) at day 15	22
Silver nitrate 5 µl 0.2 M	0.5-2 x 10 ⁶ MB49-PSA	1 hr	PSA detected	23
HCl (100 µl 0.1 M 15 sec)	1 x 10 ⁶ MB49	1 hr		24

Table 1. Tumor detection and development following orthotopic implantation of MB49 cells. Different physical and chemical insults have been used for disruption of the GAG layer to facilitate intravesical growth of MB49 cells. Experimental conditions and results from previous studies using the MB49 orthotopic model are summarized. When available, the information in parentheses in the first column includes volume, concentration, and contact time of agent used for chemical disruption of the GAG layer.

Discussion

The primary methodology described in this protocol is catheterization of mouse bladders, which has broad applications for instillation of cells or any agent intended for local delivery to the bladder epithelium. The specific protocol outlined above has been optimized for short-term studies (~10 days). Implanting the accurate number of cells is critical, since a higher cell number will result in more rapid tumor growth and possibly loss of animals due to large tumor burden. Using 200,000 MB49 cells for instillation may require euthanizing up to 25% of the animals by day 14 due to excessive tumor burden. In our experience, mice are not adversely affected by tumor load within 12 days. In addition to standard criteria such as lethargy, poor grooming, and/or loss of appetite excessive tumor burden in this model is evidenced by the inability to urinate.

An important consideration for this protocol is logistics. First, MB49 cells grow rapidly and plating one million cells two days before implantation will yield optimal cultures in log phase on the day of instillation. If the number of animals required for an experiment exceeds the number of animals that can be implanted in one day, MB49 cultures will have to be set up accordingly (on multiple days) to prevent overgrowth of cultures. Second, until the technique has been perfected, users should work with a small group of animals. Experienced users will be able to implant 6 groups of 5 mice each in one day without an assistant (1.5 hr/group including dwell times). However, initially it is helpful to have an assistant prepare and count the cells for instillation. Lastly, location of equipment is important. Ideally the biosafety cabinet and anesthesia equipment are located in the same room.

A potential limitation is that the anesthesia equipment has 5 nosecones, which are in use during dwell time. If large numbers of mice are routinely implanted, several such systems could be used in parallel. In theory, other methods of anesthesia could be used. However, a benefit of isoflurane inhalation anesthesia is that dwell time can be controlled.

Once the technique of catheterization is mastered, this protocol can be applied to a number of experimental conditions. New bladder cancer cell lines or cells derived from primary bladder tumors can be evaluated for their potential to grow orthotopically. Furthermore, it is possible to implant different numbers of cells to determine the threshold amount required for tumor take. Tumor growth rates can also be established. These variations may be particularly useful, if comparisons are made between genetically modified cells to determine the impact of the gene of interest on tumor establishment or growth rate. For monitoring of adhesion, growth rate, or tumor take, cells may be transfected with a reporter gene such as luciferase⁶. However, an important consideration is the impact of hypoxia and necrosis on the bioluminescence signal²⁹. Catheterization can also be used to deliver a therapeutic agent. We are currently using the orthotopic model to evaluate gene delivery strategies in an *in vivo* setting. This model will be useful to optimize delivery of therapeutics and to determine their impact on tumor regression and survival.

Disclosures

The authors declare that they have no competing financial interests.

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