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Methods to assess anticancer immune responses in orthotopic bladder carcinomas

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Abstract

Urothelial bladder cancer is the most common malignancy of the urinary tract resulting in over 165,000 deaths worldwide. Immunotherapies targeting the programmed cell death protein-1 (PD-1) checkpoint pathway were recently approved for the treatment of bladder cancer, but favorable responses to this treatment are still limited to a minority of patients. This resistance to therapy has driven a need to optimize syngeneic models of bladder cancer that enable evaluation of the tumor immune microenvironment under varying conditions. Several models have been in place for many years, and we discuss in this chapter the optimization of an orthotopic model of bladder cancer that can be employed to study the anti-tumor immune response.

Keywords

Bladder cancer; Immunotherapy; Mouse models; Orthotopic; Urothelial cancer; Syngeneic

1. Introduction

Urothelial bladder cancer is the most common malignancy of the urinary tract with over 400,000 diagnoses worldwide annually resulting in over 165,000 deaths. Immunotherapies have been in use for bladder cancer for many decades beginning with the use of a tuberculosis vaccine. More recently, antibodies targeting the PD-1 checkpoint pathway were recently approved for the treatment of bladder cancer (Apolo et al., 2017; Balar, Castellano, et al., 2017; Balar, Galsky, et al., 2017; Bellmunt et al., 2017; Massard et al., 2016; Rosenberg et al., 2016; Sharma et al., 2017). However, most patients do not respond and clinical responses to immune checkpoint therapy requires a tumor microenvironment with a pre-existing endogenous anti-tumor immune cell response (Ayers et al., 2017). Because of the resurgence of cancer immunotherapy in the context of bladder cancer, there is a growing

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need to understand mechanisms of resistance to bladder cancer. To work toward that goal optimization of existing pre-clinical models is needed as is the development of novel methods for immune monitoring in pre-clinical bladder cancer models.

The study of the native anti-tumor immune response under varying conditions is best accomplished through the use of syngeneic model systems that avoid the use of immunosuppressive conditions needed for establishment of tumors derived from other hosts (i.e., human tumor xenografts implanted into mice). To this end the MB49 murine bladder cancer cell line was established 40 years ago and remains in use until today (Summerhayes & Franks, 1979). Importantly, this model allows the orthotopic implantation of tumors directly into the tissue of origin, the bladder. However, historical methods have had some challenges including low throughput, use of invasive methods or treatment with irritants to enable bladder tumor formation, and difficulty in assessing tumor growth kinetics in real time. In this chapter, we review this model and provide an optimization of its use in the study of bladder cancer tumor immunology.

2. Essential requirements for assessing the anti-tumor immune responses in bladder cancer

Optimal model systems for evaluating the anti-tumor immune response in bladder cancer consists of several required features. The most essential feature is the presence of an intact immune system with a shared genetic background relative to the tumor. This varies from models used predominantly for investigating therapeutic targets with direct cytotoxicity. In that case the activity of a drug, or effect from a change in conditions, can be assessed with xenograft models in immunocompromised hosts. These models have been the workhorse for oncology research for many decades. In the current era of immunotherapy, however, a major limitation of these models is the requirement for immunocompromised mice (i.e., *Rag*-deficient mice). Thus, there has been an increased focus on optimizing or developing better syngeneic transplantable tumor models, or genetically engineered models of cancers, to enable the evaluation of an anti-tumor immune response and allows for local and systemic immune monitoring during experiments.

Additional essential features of an optimal model are latency to develop invasive cancer, throughput, and reproducibility. With most transplantable tumor models including MB49, latency is less of a concern as tumors typically develop rapidly (i.e., 1–3 weeks). In the orthotopic context tumors can develop even faster (<1 week). With carcinogen-induced models, tumor formation is frequently delayed to months, as is the case with BBN induced bladder carcinomas. Genetically engineered mouse models can also take months to develop. When several conditions need to be evaluated simultaneously, throughput is another important feature to consider with model systems. In general models with more complex manipulations (i.e., survival surgery) produce additional strain on laboratory and animal facility resources. An optimal model system would allow 20 more mice to be inoculated with bladder carcinomas in one instance with a short time interval (i.e., <2h). Finally, reproducibility is an important factor to consider especially when using transplantable cell lines that can vary with passage number or lab source. Larger projects should use cell lines

from similar passage numbers and cells should only be maintained in culture for 6–8 weeks before regenerating from a frozen stock of similar passage number.

3. Development of orthotopic bladder carcinoma models

Orthotopic models for bladder cancer is optimal for assessing the immune response compared with urothelial carcinoma cell lines implanted in subcutaneous tissues. Bladder-implanted tumors more closely re-capitulate the tumor microenvironment of the cancer in its native context. For many tumors such as prostate cancer or renal cell carcinoma, orthotopic models can be cumbersome and involve surgical procedures. Bladder cancer is highly amenable to orthotopic cancer model development, since the tissue is readily accessible via catheterization and without the need for survival surgery. Additionally, the ease of access to the bladder not only enables the implantation of tumors in a transplantable fashion, but also facilitates the use of genetic modification in a localized fashion in the context of appropriate genetically engineered mice. For instance, the use of bladder-instilled adenoviral *Cre* recombinase or incorporating uroplakin-driven *Cre* (Mo, Cheng, Lee, Sun, & Wu, 2005). The use of carcinogen-induced models is another method to enable the formation of bladder tumors that result in syngeneic bladder tumors. There are advantages and disadvantages to this approach as discussed in Sections 3.1–3.3.

3.1 Transplantable tumor models

In orthotopic tumor models, tumor cells are either implanted or injected into the equivalent organ that the tumor is derived from. The bladder lends itself to this approach by virtue of its accessibility via the urethra by catheterization. In this sense, the use of the C57BL/6-mouse derived MB49 bladder cancer cell line has been commonly employed for over 40 years (Summerhayes & Franks, 1979). This cell line is derived from male mouse bladder epithelial cells transformed with the chemical carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA). Numerous technical methods have been developed for implantation of tumors into the bladder to generate tumors. This process typically involves some disruption of the glycosaminoglycan layer of the bladder inner surface followed by implantation of tumor cells with a fixed dwell time (Kasman & Voelkel-Johnson, 2013). The GAG layer is an important layer of protection in a normal bladder, and disruption of it can be accomplished by varying methods including administration of poly-L-lysine, silver nitrate, or even electrocautery. Surgical approaches can also be used to implant tumors. In this method, survival surgery is employed to make a small incision is made into the bladder and tumor cells implanted prior to suturing it closed (Dinney et al., 1995). Recently, gene expression profiling has indicated that the MB49 cell may not faithfully reflect human urothelial carcinomas. This model system can, however, be adapted to generate tumors using other cell lines. The MBT-2 cell line is an bladder cancer cell line derived from C3H/He mice (Jiang & Zhou, 1997). This cell line can similarly be used to generate a syngeneic model in that strain for assessment of immune response.

3.2 Carcinogen-induced models

One of the most common bladder cancer models is the *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) model. In this model the chemical carcinogen BBN, which is similar to

carcinogens linked with tobacco use in humans, is instilled in the mice drinking water, typically at a dose of 0.05% (Fantini et al., 2018). BBN is given systemically, but has a preferential mutagenic effect on the bladder urothelium. Mice develop similar stages of tumor as humans including hyperplasia, carcinoma-in-situ (CIS), invasive bladder tumors. Additionally, they are associated with a similar mutational profile to human bladder cancers and their neoantigen burden may be advantageous when investigating the immune responses to cancer. Onset of tumors can take 20 weeks, which is a limitation of this approach. This latency has been improved with the use of combination genetically engineered mouse models as noted in Section 3.3.

3.3 Genetically engineered mouse models

Genetically engineered autochthonous mouse models have long been employed to generate bladder cancers (Table 1). These models can involve genetic induction by though uroplakin-driven *Cre* recombinase, intravesicular instillation of adenoviral *Cre* recombinase, or exposure to BBN. The latter approach effectively creates hybrid GEM/carcinogen-induced models that have faster development of tumors. Many combinations of oncogene drivers and tumor suppressor knockout genes have been explored, and many do not produce invasive tumors. For instance, combining urothelium-specific PTEN knock out with the Kras activation mutation G12D or the FGFR activating mutation K644E both lead only to hyperplasia or non-invasive papillary tumors (Ahmad, Iwata, & Leung, 2012). Successful genetic combinations include double loss of tumor suppressor genes such as PTEN and p53 as noted in Table 1. Other limitations to genetically engineered mouse models include the requirement for management of GEM colonies, the delay in tumor formation compared with transplantable tumors (even with the hybrid GEM/carcinogen-induced models), and lower overall throughput. Nonetheless these models are very important especially in the context of investigating the effects of specific genetic alterations on histology or immune responses. They also enable the generation of molecular subtype-specific bladder cancers (i.e., luminal vs basal). GEM models are also especially important in the context of studying the anti-tumor immune response, since GEM tumors are not impacted by artificial inflammatory effects that may occur with transplantable tumor models as a result of mechanical or chemical factors used to implant those tumors.

4. Monitoring tumor growth kinetics and experimental end points

With orthotopic models the tumors cannot be measured by examination of mice until tumors become large enough for palpation. At this point mice become at risk for mortality given the urinary obstruction that occurs. Survival in a transplantable MB49 model is typically shorter than 4 weeks without treatment. Studies investigating late-time point events such as antigen-specific T cell dysfunction can incorporate a fixed endpoint at 2–3 weeks. Early events such as T cell priming can be studied by ending the experiment at fixed early time point such as 5 days. Monitoring of tumor size in real time, however, requires the use of intravital imaging. This has been successfully employed using luciferase transduction of implanted tumor cells and injection of luciferin prior to measurement on an optical imaging system. In this way, bladder tumors may be detected as early as day 3 and monitored as frequently as needed for each experiment. An example of this is shown in Fig. 1. Occasionally tumor size assessment

by this method can be impacted by therapies that impact angiogenesis, which limits the ability of the injected luciferin to reach the tumor. This point must be considered when designing experiments with agents known to have this effect.

5. Protocol: MB49 orthotopic bladder tumors

The following protocol has been optimized after adaption from prior published techniques (Kasman & Voelkel-Johnson, 2013; Summerhayes & Franks, 1979). Of note, C57/B6 female mice purchased from Jackson Laboratories or Taconic Farms are used for this technique. Though more technically challenging, recent methods have been reported for the catheterization of male mice, which could also be adapted to rule out sex-specific immune effects (Zychlinsky Scharff et al., 2019). First, MB49 cells are maintained in DMEM, with added glutamate, pyruvate (110nM), 10% fetal bovine serum with 1% penicillin/streptomycin. Cells are maintained in culture at 37°C with 5% CO₂. On the day of implantation cells are trypsinized, washed with phosphate-buffered saline (PBS), suspended in PBS, and counted. A solution is made in PBS at the desired concentration, which is typically between 1 and 2×10⁶ cells per mL and placed on ice for subsequent implantation.

On the first day of the experiment, mice are anesthetized in an induction chamber with isoflurane. Once mice have lost consciousness, as verified by loss of the toe-pinch reflex, they are transferred to nosecones and maintained at 1%. Multiple animals can be treated concurrently through the use of a multi-animal breather system that includes six mouse nose cones. A warming block is used for maintenance of body heat. Mice are maintained in a supine position and properly placed on the heating pad to maintain body temperature. Ophthalmic lubrication should be used to prevent corneal drying. Bladder catheterization is performed using a sterile 24G angiocatheter with non-irritating lubricating gel. The technique begins by using a thumb and forefinger to gently abduct the hind legs of the mouse to expose the urethral meatus. Then, the catheter is gently inserted at a 45-degree angle initially, followed by changing the angle to one parallel with the bench to insert fully. Once in place, urine should be allowed to drain out with gentle external pressure on the mouse bladder. Next, 100µL of poly-L-lysine at a concentration of 0.1mg/mL is instilled into the bladder using a 1.0cc syringe, with care being taken to remove all air bubbles. It is expected that 50µL may be wasted in the catheter space and thus only 50µL is actually instilled in the bladder. This assumption applies to all other instillations as well. The catheter is then capped and allowed to remain in place for 10min. Poly-L-lysine is a polycation and disrupts the glycosaminoglycan layer of the bladder to facilitate tumor implantation. After 10min, the catheters are uncapped and removed from the bladders to facilitate drainage of poly-L-lysine.

Instillation of tumor cells is then performed using the exact catheterization technique as above, except the intravesicular dwell time is 20min instead of 10min. The amount of MB49 bladder carcinoma cells used can vary depending on the expected duration of the experiment (higher numbers result in shorter time course before experimental endpoint). Typically, 1–2×10⁵ cells in 100µL of phosphate-buffered saline. The optimal cell count within this range should be determined with pilot studies. Once implantation of tumor cells is complete. Catheters are uncapped and removed. Then the isoflurane concentration is reduced to zero

and mice are allowed to recover. When mice have regained their righting reflex, they are returned to their cages.

Using this method, tumors can be grown, but in addition, the same catheterization technique can be employed for intravesical instillation of local therapies as well. Drugs for bladder instillation can be diluted in 50–100µL of an inert solvent such as PBS and administered with varying dwell times by capping of the catheter. Tumor growth kinetics can be monitored as described in Section 4. At experimental endpoint, mice can be euthanized and the bladder and draining pelvic or retroperitoneal lymph nodes can be extracted to assess local immune response. Splenocytes can be harvested to assess systemic immune responses. For the bladder tumor, once the bladder is dissected away from the pelvis, a single cell disruption can be generated by manual manipulation in PBS through a 70-micron strainer. Given that the bladder wall is typically resistant to tearing, an incision may be required at the superficial edge of the tumor on the bladder wall to enable disrupting of the tumor into suspension. This suspension can be used for single-cell techniques for analysis including CyTOF, FACS, or RNA seq. Systemic immune responses can be monitored if the MB49 cell line is transduced to express a model antigen such as ovalbumin (SIINFEKL) with a matched pentamer for monitoring. It is worth noting that the MB49 cell line was derived from a male mouse, however, cytogenetic characterization has revealed a complete loss of Y chromosome. Thus, when implanting MB49 cells into female mice, the intensity of an endogenous anti-tumor immune response should not be impacted.

6. Summary and future methods to assess anti-tumor immune responses

The surge in cancer immunotherapies impacting the clinical arena has led to redoubled efforts to develop modern pre-clinical murine models to evaluate human-derived tumors in the context of an intact immune response. To this effect, the orthotopic models such as the MB49 cell line model have been successfully used to track immune responses under varying conditions and at different time points. This remains one of the most common techniques in place to evaluate immune responses to bladder cancer, but this system and others are limited by many factors. Importantly, they are restricted to murine models that may not fully recapitulate human bladder tumors. Additionally, the heterogeneity in human bladder cancers are not reflected in a single model system. Because of these limitations, newer approaches are also under development. Human transgene knock-in mice models have enabled, to an extent, the engraftment of human tumor xenografts without rejection (De La Rochere et al., 2018). With these humanized mouse models, special attention must be taken to ensure the appropriate cytokine interactions given the lack of inter-species reactivity of many cytokines. Engraftment with human hematopoietic system after irradiation of mice is another approach to generate a humanized model of bladder cancer. This can be used to effectively generate an avatar of both the human tumor and the patient's immune system. Organoid models are also emerging as tools in the lab to study tumors with three-dimensional structure and may be derived from either mouse or human bladder cancers. Combining organoid models with humanized mice systems may offer important new methods to in the near future. For the past 30 years bladder, pre-clinical bladder cancer model development has been slow compared to other cancers. Thus, new approaches to

study immune responses in bladder cancer are in need and will likely be complimentary to the approach described in this protocol.

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Table 1

Selected examples of genetically engineered autochthonous mouse models.

Inducer	Genetic background	Histology	Latency time	References
BBN	Unmodified O57/B6	Basal-squamous	20 weeks	Fantini et al. (2018)
BBN	P53 ^{-/+}	Basal-squamous	16 weeks	Van Batavia et al. (2014)
BBN	UPII-Cre Rb ^{flox/flox} p53 ^{flox/flox}	Basal-squamous	10 weeks	He et al. (2009)
None	UPIIIa-Cre PTEN ^{flox/flox} p53 ^{flox/flox}	Luminal	10 weeks	Saito et al. (2018)
Adeno-Ore	PTEN ^{flox/flox} p53 ^{flox/flox}	Basal-squamous	10 weeks	Puzio-Kuter et al. (2009)