Video Article

Induction of Invasive Transitional Cell Bladder Carcinoma in Immune Intact Human MUC1 Transgenic Mice: A Model for Immunotherapy Development

Daniel P. Vang¹, Gregory T. Wurz¹, Stephen M. Griffey², Chiao-Jung Kao¹, Audrey M. Gutierrez¹, Gregory K. Hanson¹, Michael Wolf³, Michael W. DeGregorio¹

¹Department of Internal Medicine, Division of Hematology and Oncology, University of California, Davis

²Comparative Pathology Laboratory, UC Davis School of Veterinary Medicine, University of California, Davis

³Merck Serono Research, Merck KGaA, Darmstadt, Germany

Correspondence to: Michael W. DeGregorio at mwdegregorio@ucdavis.edu

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Abstract

A preclinical model of invasive bladder cancer was developed in human mucin 1 (MUC1) transgenic (MUC1.Tg) mice for the purpose of evaluating immunotherapy and/or cytotoxic chemotherapy. To induce bladder cancer, C57BL/6 mice (MUC1.Tg and wild type) were treated orally with the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN) at 3.0 mg/day, 5 days/week for 12 weeks. To assess the effects of OH-BBN on serum cytokine profile during tumor development, whole blood was collected via submandibular bleeds prior to treatment and every four weeks. In addition, a MUC1-targeted peptide vaccine and placebo were administered to groups of mice weekly for eight weeks. Multiplex fluorometric microbead immunoanalyses of serum cytokines during tumor development and following vaccination were performed. At termination, interferon gamma (IFN- γ)/interleukin-4 (IL-4) ELISpot analysis for MUC1 specific T-cell immune response and histopathological evaluations of tumor type and grade were performed. The results showed that: (1) the incidence of bladder cancer in both MUC1.Tg and wild type mice was 67%; (2) transitional cell carcinomas (TCC) developed at a 2:1 ratio compared to squamous cell carcinomas (SCC); (3) inflammatory cytokines increased with time during tumor development; and (4) administration of the peptide vaccine induces a Th1-polarized serum cytokine profile and a MUC1 specific T-cell response. All tumors in MUC1.Tg mice were positive for MUC1 expression, and half of all tumors in MUC1.Tg and wild type mice were invasive. In conclusion, using a team approach through the coordination of the efforts of pharmacologists, immunologists, pathologists and molecular biologists, we have developed an immune intact transgenic mouse model of bladder cancer that expresses hMUC1.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50868/>

Introduction

Bladder cancer is the fourth most common form of cancer and the eighth leading cause of cancer deaths in American men. In the United States, an estimated 72,500 new cases and 15,000 deaths from bladder cancer are expected among men and women combined in 2013¹. The incidence of bladder cancer is approximately three times as high in men compared to women. In the United States, transitional cell carcinomas (TCC) account for over 90% of cases, while squamous cell carcinomas (SCC) have an incidence of less than 2%². The overall relative 5-year survival rate for papillary TCC is 91.5% compared to only 30.9% for SCC². Although noninvasive papillary TCCs account for approximately 75% of cases at the time of diagnosis, even with treatment more than 50% of patients will experience a recurrence within 5 years, with up to 30% of these patients progressing to muscle invasive disease^{3,4}. Typical treatment regimens for non-muscle invasive disease include transurethral resection (TUR) followed by intravesical chemotherapy. In patients with high-grade Ta or T1 tumors, a repeat TUR may be performed prior to chemotherapy^{3,4}. For those patients with low-grade Ta recurrences or high-grade Ta or T1 lesions, TUR followed by adjuvant chemotherapy or immunotherapy in the form of Bacillus Calmette-Guerin (BCG) may be used^{3,4}. Intravesical BCG has been shown to be superior to intravesical mitomycin C with respect to time to recurrence⁵. For T2 muscle invasive disease, radical cystectomy with or without neoadjuvant chemotherapy is the recommended course of treatment³. In patients with SCC, radical cystectomy appears to be the most effective treatment⁶. Given the very high rates of recurrence despite the best treatments available, there is clearly a need for new, more effective therapies for bladder cancer.

Expanding new immunotherapies for bladder cancer is one possible approach that may hold promise for extending disease-free survival. Historically, BCG has been the only effective immunotherapy for bladder cancer. Its mechanism of action is thought to involve the nonspecific induction of a T-helper 1 (Th1) type immune response via increasing levels of interleukin-2 (IL-2) and interferon gamma (IFN- γ)⁴. Cellular, or Th1 immunity, is critical in cancer immunotherapy as humoral, or Th2, immunity has never been shown to be effective against solid tumors, with the exception of antibodies directed against growth factor receptors⁷. In an attempt to improve upon the benefits of BCG monotherapy, IFN- α 2B/

BCG combination immunotherapy was evaluated in a phase II clinical trial with inconclusive results⁸. An alternative approach to immunotherapy for bladder cancer may be to target tumor-associated antigens (TAAs), the identification of which has made cancer immunotherapy more specific⁷.

One such TAA is mucin 1 (MUC1), which is a cell surface glycoprotein overexpressed in many epithelial cell cancers such as bladder, breast, lung, and pancreatic cancer^{9,10}. The expression and modification of MUC1 is also substantially altered during carcinogenesis, such that underglycosylation exposes antigenic sequences of amino acids known as variable number of tandem repeats (VNTR) on the peptide core. While MUC1 is a self-molecule, these immunodominant VNTR regions are not normally exposed due to extensive glycosylation, and thus they are seen by the immune system as foreign^{11,12}. Cytotoxic T-lymphocytes (CTLs) that specifically recognize MUC1 epitopes have been isolated from the tumor-draining lymph nodes of breast cancer patients¹³, as well as the blood and bone marrow of myeloma patients^{14,15}, making MUC1 a potential target for a cellular immune response. The immunodominant VNTRs of the underglycosylated form of MUC1 are recognized by CTLs, resulting in the destruction of tumor cells¹⁶⁻¹⁹. Native cellular and/or humoral immune responses to cancerous MUC1 are, however, not strong enough to eliminate tumors. To augment the already existing weak immune response to MUC1, synthetic immunodominant peptides can be introduced through vaccination to generate a CTL response strong enough to be of clinical benefit^{18,20}. A MUC1 liposomal vaccine has already been shown to increase survival in lung cancer patients^{21,22}, generate CTLs capable of killing MUC1-positive tumor cells, and produce a Th1-polarized cytokine response^{23,24}. With a high level of MUC1 expression^{9,11,25}, bladder cancer is a logical candidate for testing MUC1-directed immunotherapy^{26,27}. Furthermore, MUC1 has potential as a prognostic factor in bladder cancer²⁸, MUC1 expression in TCC is significantly associated with stage and grade, and metastatic TCC has been shown to continue to express MUC1²⁹.

In order to evaluate the potential utility of MUC1-directed immunotherapy in bladder cancer, we developed an immune intact human MUC1 (hMUC1)-expressing transgenic (MUC1.Tg) mouse model of bladder cancer congenic on the C57BL/6 background³⁰. Human MUC1 is expressed as a self-protein under the control of its own promoter, resulting in a tissue expression pattern consistent with that observed in humans^{30,31}. The mice were induced with the known bladder carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (OH-BBN)³², and then the resulting tumors were evaluated for hMUC1 expression and tumor type and grade. To assess the effect of the carcinogen on Th1/Th2 cytokine levels during tumor development, serum samples were collected periodically for multiplex analysis. Mice were then treated with a MUC1-targeted peptide vaccine, and the serum cytokine and immune responses were evaluated by multiplex fluorometric microbead immunoassay and ELISpot.

Protocol

All animal studies and experiments were conducted under a protocol approved by the University of California, Davis Institutional Animal Care and Use Administrative Advisory Committee.

1. MUC1.Tg Mouse Breeding and Propagation

1. The UC Davis Mouse Biology Program (MBP) breeds wild type C57BL/6 male mice with heterozygous MUC1.Tg C57BL/6 female mice to establish our breeding colony. MUC1.Tg offspring are delivered for studies as needed.
2. MBP personnel clip the toes of the offspring in a defined pattern (0-99) for mouse identification, and when applicable clip the tail. The toe or tail tissue is processed for genotyping using standard DNA extraction and Polymerase Chain Reaction (PCR) analysis.

2. Study Design

1. Study Group Assignments
 1. Weigh each mouse separately on a balance and record the weight in grams for each mouse.
 2. This part of the procedure involves the methodology and design of the study. For the first part of the study, assign age-matched MUC1.Tg and wild type mice to start bladder cancer induction with OH-BBN. Euthanize mice 8 weeks after the last OH-BBN dose to confirm the presence of bladder tumors by histology.
 3. For the second part of the study, randomize male MUC1.Tg mice into the appropriate number of treatment and control groups. These mice will be monitored for serum cytokines and T-cell immune responses during induction and tumor development, and then at termination of the study 8 weeks after the last OH-BBN dose.
2. Bladder Cancer Induction
 1. OH-BBN is a carcinogen and highly toxic. Please read and follow the guidelines of the material safety data sheet when handling and storing this chemical.
 2. Calculate the dosing solution concentration needed to deliver the appropriate dose (3 mg), in a volume of 100 μ l, to each mouse based on the average weights and number of mice in each study and group.
 3. Dilute the OH-BBN in 100% ethanol. Bring the final concentration to 30 mg/ml with sterile water. The final ethanol:water concentration should be 20:80, v/v.
 4. Administer the OH-BBN orally using stainless steel, 20 G gavage needles daily, 5 days/week for 12 weeks, based on the treatment group assignment starting at the age of 8 weeks.
3. Vaccination Treatments
 1. Reconstitute each vial of lyophilized peptide vaccine in 600 μ l of 0.9% sterile saline and thoroughly resuspend by drawing the solution through a 0.5 inch 27 G needle 6x. Using saline, adjust the concentration so that the desired dose is delivered in a volume of 100 μ l.
 2. Starting in Week 20, after the last dose of OH-BBN, administer the vaccine on a weekly basis for an eight-week cycle by subcutaneous injection of 100 μ l using a 25 G needle (week number corresponds to the age of the mice).
4. Monitoring and Sample Collection

1. Weigh all mice and palpate for the presence of new tumors once each week. Euthanize any mice that have lost $\geq 20\%$ of body weight or if there are palpable tumors, blood in the urine, and/or urinary retention.
2. Prior to the first OH-BBN dose and then at 4 week intervals thereafter, collect whole blood via submandibular bleeds. Collect the blood in serum clotting tubes (BD Microtainer), and allow 30 min for the blood to clot.
3. Centrifuge the blood samples in a microcentrifuge at $3,500 \times g$ for 10 min. Carefully transfer the serum to screw cap cryotubes using a pipette.
4. Flash freeze in liquid nitrogen, and store at -80°C until further analysis by multiplex.
5. Eight weeks after the last dose of OH-BBN, euthanize all mice by CO_2 asphyxiation.
6. Place each mouse on a dissection board and pin down by all four limbs.
7. Using forceps and scissors, make a horizontal incision in the upper abdominal region. Insert the scissors into the incision between the epidermal layer and abdominal wall and gently separate the skin from the underlying tissue with the help of forceps.
8. Make a vertical incision from the horizontal incision following the middle axis towards the anterior end of the mouse. Separate the skin from the rib cage, and using a 1 ml syringe and a 22 G needle, puncture the heart and collect blood with a smooth and steady draw.
9. Refer to step 2.4.3 and 2.4.4 for serum isolation and storage.
10. Using forceps and scissors, cut and peel back the rest of the epidermal layer. Cut through the abdominal wall and peritoneum and aseptically remove bladder tumor for Immunohistochemistry (IHC) and Western blot.
11. Collect spleen for cell viability analysis (Muse) and ELISpot. For IHC, place bladder tumor specimen in tissue cassette and fix in chilled formalin overnight at room temperature.
12. The following day, replace the formalin with 70% ethanol.
13. For Western blot analysis of tumor, homogenize the tumor, add protein extraction buffer plus Halt protease inhibitors and transfer to 1.5 ml microcentrifuge tubes.
14. Vortex for 30-60 sec and hold on ice for 5 min. Flash freeze in liquid nitrogen and thaw in ambient water. Repeat the process of vortexing, freezing and thawing twice.
15. Centrifuge the samples at $10,000 \times g$ for 10 min at 4°C and transfer the cellular extracts to new labeled tubes.
16. Quantify the concentration by performing a Bicinchoninic Acid Protein Assay (BCA) for protein measurements. Store samples at -80°C until ready for Western blot analysis.

3. Molecular Biology/Western

The following procedures were performed to verify the expression of MUC1 in mouse bladder tumor tissue using standard Western Blot protocol (data not shown).

1. Separate the protein extracts by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and then transfer onto PVDF membrane using semidry apparatus.
2. Block the protein transferred membrane with 5% nonfat milk in 0.1% Tween-20 in Phosphate Buffered Saline (PBS-T) pH 7.4 for 1 hr on an orbital shaker at room temperature.
3. Incubate the membrane for 1 hr at room temperature on a shaker with anti-MUC1 or anti- β -actin antibody in 0.1% PBS-T.
4. Wash the membrane by decanting the solution and adding 0.1% PBS-T. Swirl the membrane on the shaker for 5 min and decant. Repeat this step twice.
5. Incubate the membrane for 1 hr at room temperature with a horse radish peroxidase (HRP) conjugated secondary antibody.
6. Wash 3x (refer to step 3.4).
7. Follow the protocol for the Enhanced Chemiluminescence (ECL) kit to activate and read the fluorography.

4. Multiplex Fluorometric Microbead Immunoassay

1. Setup and Calculations
 1. Using a 96-well plate map, assign blanks, standards, controls, and unknowns to the wells. Calculate the number and volume of analytes, capture antibodies and Streptavidin-Phycoerythrin (SA-PE) needed for the assay.
 2. Allow all buffers and diluents to equilibrate to room temperature. Prepare the standards and sample dilutions using a blank 96-well plate.
 3. Reconstitute the serum matrix and lyophilized standard according to the manufacturer's protocol and make 1:5 serial dilutions of the standard with the appropriate diluent in the 96-well plate. For the blank wells, use the appropriate diluent.
 4. Dilute all controls and unknown samples 1:2 in the appropriate diluent in the rest of the 96-well plate.
 5. For the bead mix, pipette the required volume of the appropriate diluent into a 15 ml tube. Vortex each bead vial for 20 sec and pipette the required volume of each analyte into the 15 ml tube.
 6. Always protect the beads from light to avoid photobleaching. Do not place the 96-well plate on absorbent material to avoid loss of sample through wicking.
2. Assay Protocol
 1. Prewet the 96-well filter bottom plate with 200 μl of Assay Buffer and allow for complete soaking of the filter. Gently drain by using the 96-well plate vacuum apparatus and blot dry the bottom of the plate with a paper towel.
 2. Using a multichannel pipette, pipette 25 μl of serum matrix to the wells assigned for the blanks and standards and pipette 25 μl of Assay Buffer to the wells assigned for the controls and unknowns.
 3. Pipette 25 μl of the blank, standards, controls, and unknowns to the respective assigned wells. Vortex the bead mix for 20 sec and transfer the beads to a reservoir.
 4. Pipette 25 μl of the bead mix into each well. Cover the plate with aluminum foil or an opaque plate lid to protect from light.
 5. Shake the plate at 500 rpm for two hours at room temperature on a plate shaker. Drain and wash the plate with 200 μl of 0.1% Tween 20 in Phosphate Buffered Saline (PBS-T) twice. Drain and blot dry.

6. Prepare the capture antibody solution. Pipette the required amount of 0.1% PBS-T and capture antibody into a 15 ml tube and vortex for 10 sec. Transfer the capture antibody mix to a reservoir and pipette 25 μ l into each well.
7. Shake the plate at 500 rpm for one hour at room temperature on a plate shaker. Drain and wash the plate with 200 μ l of 0.1% PBS-T twice. Drain and blot dry.
8. Prepare the SA-PE solution. Pipette the required volume of 0.1% PBS-T and SA-PE into a 15 ml tube and vortex for 10 sec. Transfer the solution to a reservoir and pipette 25 μ l into each well.
9. Shake the plate at 500 rpm for 30 min at room temperature on a plate shaker. Drain and wash the plate with 200 μ l of 0.1% PBS-T twice. Drain and blot dry.
10. Pipette 100 μ l of 0.1% PBS-T and shake on a plate shaker at 500 rpm for at least two minutes to resuspend the beads. Read and analyze the plate on the Luminex Lx200 machine.

5. IFN- γ /IL-4 ELISpot Preparation and Analysis

1. In a biological safety cabinet, process the spleens through 100 μ m Nylon tissue sieves into 5 ml sterile Phosphate Buffered Saline (PBS) in sterile Petri dishes. Layer the splenocytes onto 3 ml of lymphocyte separation medium in sterile 15-ml tubes.
2. Centrifuge the tubes at 600 x g for 15 min to separate the lymphocytes from the red blood cells. Transfer the layered lymphocytes above the gradient to new sterile 15-ml tubes.
3. Adjust the volume to 10 ml with sterile PBS. Centrifuge the suspension at 600 x g for 10 min to pellet the cells.
4. Aspirate the supernatant and resuspend the cells in 1 ml of PBS for cell viability and count with the Muse analyzer. Follow the Muse Count & Viability Kit protocol.
5. Make serial dilutions of the lymphocytes in 1.5 ml screw cap centrifuge tubes at dilution factors of 1:10, 1:20, and 1:40 (or as necessary) in Count & Viability Reagent (minimum total volume 300 μ l). Pipette each dilution up and down several times to mix and analyze on the Muse.
6. Prepare a plate map of the samples and conditions for the ELISpot plate. Prepare the ELISpot plate according to the manufacturer's protocol and pipette 100 μ l of medium, peptide (10 μ g/ml), or scramble peptide (10 μ g/ml) to each well.
7. Pipette 100 μ l of cell suspension, delivering 1.0×10^6 cells to each well and incubate the plate at 37 °C overnight.
8. Follow the manufacturer's protocol for the ELISpot assay. Analyze the developed ELISpot plate using a dissection microscope.
9. Quantify the results by counting the number of colored spots corresponding to each analyte in each well. The spots correspond to the number of spot-forming cells (SFC) in each well.

6. Immunohistochemistry (IHC) and Hematoxylin & Eosin (H&E) Staining

1. Take bladder tumor tissue preserved as described above (Section 2.4.11), embed in paraffin and step-section at 4 μ m for immunohistochemical analysis.
2. Perform IHC using a MUC1 antibody that recognizes the tandem repeat region of MUC1. Use the Animal Research Kit peroxidase to minimize the reactivity of the secondary mouse antibody with endogenous immunoglobulin present in the tissue.
3. Perform H&E staining using standard protocols.

7. Statistical Methods

For the Multiplex Fluorometric Microbead Immunoassay, use a two-tailed Student's t-test to compare the average observed serum cytokine concentrations between the treatment and control groups. For ELISpot, use a one-way ANOVA to compare the spot forming colonies between the media control, scrambled peptide and peptide groups. Use Dunnett's Multiple Comparison Test to lessen the likelihood of a false positive result. A p-value of ≤ 0.05 is considered significantly different for all analyses.

Representative Results

The preclinical assessment of the effects of novel immunotherapies and combinations in bladder cancer requires the development of an appropriate animal model. In our transgenic mouse model, induction with the chemical carcinogen OH-BBN resulted in a high rate of bladder cancer incidence of predominantly TCC with some SCC, which is similar to bladder cancer in humans. To determine tumor histology, MUC1 expression status and the immune response to the peptide vaccine treatment, 21 MUC1.Tg and 18 wild type mice were euthanized for the collection of blood, bladders, and spleens (**Figure 1**) eight weeks following OH-BBN induction (Week 28). The bladder cancer incidence rate for both MUC1.Tg (14/21) and wild type (12/18) mice was 67%. Hematoxylin and Eosin (H&E) staining confirmed the presence of both TCC and SCC, with TCCs predominating at a 2:1 ratio. Among these, we observed a range of low and high-grade noninvasive to high-grade invasive tumors. All MUC1.Tg bladder cancer specimens were positive for MUC1 expression by IHC (**Figure 2**). It should be noted that the antibody used for MUC1 IHC recognizes both normal and cancerous human MUC1.

During model development, the serum levels of inflammatory cytokines were monitored serially between Weeks 8-28. We observed that inflammatory cytokine levels increased with time from induction through the end of the study (**Figure 3**). This cytokine pattern is very similar to what we observed previously in our lung cancer model³³, which strongly suggests that increasing inflammatory cytokine levels may correlate with tumor development.

To assess the Th1 serum cytokine response to the peptide vaccine, 15 vaccinated and 14 placebo-treated MUC1.Tg mice were euthanized and blood was collected at the end of the study in Week 28, 24 hr after the last vaccine treatment. Multiplex analysis (**Figure 4**) shows increased Th1 serum cytokine levels of TNF- α , IFN- γ , IL-2, IL-12 (p70), and IL-17 in the vaccine group compared to the placebo group. Levels of TNF- α , IFN- γ , and IL-17 were significantly higher ($p < 0.05$) in the vaccine-treated mice. These results suggest a Th1 polarized cytokine response to the peptide vaccine.

In order to evaluate the Th1/Th2 immune response to the peptide vaccine, splenocytes were assessed by IFN- γ /IL-4 ELISpot. Twenty-four hours after the last treatment, spleens were collected and processed to isolate lymphocytes for ELISpot analysis. Lymphocytes were counted and assessed for viability by Muse Analyzer (**Figure 5**). ELISpot plates were seeded with 1×10^6 viable cells per well and developed 48 hr later. Representative results (**Figure 6**) show a clear and specific IFN- γ response to the peptide, which confirms a Th1 immune response to the peptide vaccine.

Figure 1. Mouse Necropsy. Necropsy was performed at 28 weeks, 8 weeks after the end of OH-BBN induction. Liver, bladder tumor, and spleen are indicated. Asterisk (*) marks puncture point for blood collection. In this example, a high-grade, invasive SCC was observed. [Click here to view larger image.](#)

Figure 2. Representative bladder tissue sections stained with H&E (left) and human MUC1 IHC (right) of normal bladder, invasive squamous cell carcinoma, and invasive transitional cell carcinoma. (A) Normal urinary bladder with mucosa lined by transitional epithelium, which shows diffuse MUC1 reactivity. **(B)** Nests of invasive SCC (arrow) in submucosa. Organized keratin layers (asterisk) line the bladder mucosa. Diffuse MUC1 reactivity is seen in nests of SCC. **(C)** Mucosa contains TCC projecting into the lumen (left, at right). Transitional cell carcinoma is anaplastic with invasion into submucosa and muscle (arrow and inset). Mucosa and TCC projecting into lumen show diffuse MUC1 reactivity (right, at right), while invasive TCC has less prominent reactivity (right, at left). Bar= 200 μ m (main panel) and 50 μ m (inset). [Click here to view larger image.](#)

Figure 3. Inflammatory serum cytokines at different stages of tumor development. Serial serum specimens were collected by submandibular bleeds at baseline (8 weeks), then every 4 weeks thereafter until study termination. Blood was pooled (n=4), and the serum was isolated and analyzed for the presence of 20 cytokines. Concentrations represent the mean of pooled samples and bars represent the range. Arrows indicate the point at which OH-BBN dosing concluded. [Click here to view larger image.](#)

Figure 4. Th1 serum cytokines following peptide vaccine treatment. Serum samples were collected at study termination, 24 hr after the final dose of the vaccine (n=15) or placebo (n=14) and analyzed for the presence of 20 cytokines. Data is shown as mean cytokine concentrations and bars represent positive standard deviation. * $p < 0.05$ [Click here to view larger image.](#)

Figure 5. Representative mouse splenocyte histogram. Mouse splenocytes were isolated at study termination and assessed for count and viability using a Muse Analyzer. Left panel, cell viability based on cell size. Right panel, cell viability based on nucleated cells (live cells in green zone, dead cells in white zone). [Click here to view larger image.](#)

Figure 6. Splenocyte LISpot analysis at study termination. (A) Representative wells showing IFN- γ (red spots) and IL-4 (blue spots) production in response to media, scrambled peptide, and peptide. A clear IFN- γ , antigen-specific response was observed with peptide exposure. (B) Graphical representation of typical IFN- γ ELISpot data showing the mean ($\pm$ standard deviation) spot forming colonies in response to media, scrambled peptide and peptide. [Click here to view larger image.](#)

Discussion

The successful induction of invasive transitional and squamous cell bladder carcinoma in human MUC1.Tg mice offers a preclinical model for Immunotherapy development. Immunotherapeutic studies require the use of a spontaneous, immune intact model in order to evaluate the inflammatory response to tumor progression over time as well as the immune response to immunotherapy. In a spontaneous tumor development model, the tumor microenvironment remains intact and the tumors develop at a more representative growth rate that allows for the assessment of the antitumor effects of treatment. Moreover, the immune system can be measured and monitored through biomarkers, allowing for the evaluation of treatment efficacy.

Other tumor bearing mouse models described in the literature for testing immunotherapy include both xenograft models and transplant models. Although these models are convenient and have been extensively employed in cancer research, there are a number of important limitations to consider when conducting immunotherapeutic studies. Neither xenografts nor transplanted tumors develop spontaneously, and they proliferate in a microenvironment that is not representative of the tissue from which the tumors were originally derived. Furthermore, xenografts and

transplanted tumors grow more rapidly than spontaneous tumors, allowing less time to study the immune effects of therapy. Most importantly, these models require hosts with compromised immune systems.

In addition to our model, there are a number of other chemically induced bladder cancer models. For example, N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) and N-methyl-N-nitrosourea (MNU) have also been shown to induce bladder cancer in both rats and mice. However, these chemicals are slightly different with respect to the histology of the tumors they induce. FANFT primarily induces urothelial cell carcinoma (UCC) with some SCC, while MNU induces papillary carcinoma that eventually results in muscle-invasive tumors with a low incidence of metastasis³⁴. OH-BBN is commonly used for bladder cancer induction in rodent models because the TCCs that develop closely resemble high grade human TCCs³⁴. Urinary bladder cancer has also been induced in canines, rabbits and rats as well as in mice using OH-BBN. Although canines share similar metabolic processes with humans in respect to bioactivation of carcinogens³⁵, the latency period for bladder cancer development in beagles is 37 weeks³⁶, and experimentations with dogs have both financial and ethical considerations. Rabbits have even longer latency periods, and when added to the dosage period, a minimum of 21 months is required for TCC and SCC development³⁷. Similar to mice, rat models develop tumors that are histopathologically similar to humans, with short dosage periods of 8 weeks and latency periods of 5 weeks³⁸. However, a human MUC1 transgenic rat does not currently exist. Therefore, the transgenic mouse model described here is currently the only suitable animal model for studying human MUC1-directed immunotherapy.

Previously we developed two immune intact human MUC1-expressing spontaneous tumor models for both lung³³ and breast cancer³⁹. In order to evaluate immunotherapies in bladder cancer, we developed an OH-BBN-induced, spontaneous mouse model of bladder carcinoma. Similar to the previously described models^{33,39}, the tumors that develop in this model express the tumor-associated antigen MUC1 as a self-molecule, which is the target of the peptide vaccine. This model showed a 67% incidence of bladder tumors, all of which were positive for the expression of human MUC1. Histological assessments showed that TCCs predominated, which is consistent with what is observed in human bladder cancer. This model is ideal for studying carcinogenesis, prevention strategies and the treatment of localized and advanced bladder cancer in humans. In the future, we plan to pursue additional studies of immunotherapy in combination with chemotherapeutics and radiation therapy in the described bladder cancer model.

Disclosures

DPV, GTW, SMG, CJK, AMG, and GKH declare no competing interests. MWD is the Principal Investigator of a grant received from Merck KGaA, and MW is an employee of Merck KGaA.

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References

1. Siegel, R., Naishadham, D., *et al.* Cancer statistics, 2013. *CA Cancer J. Clin.* **63**, 11-30 (2013).
2. Lynch, C.F., Davila, J.A., *et al.* Cancer of the urinary bladder. In *SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program, 1988-2001, Patient and Tumor Characteristics*, Chapter 23, Ries, L.A.G., Young, J.L., *et al.* Bethesda, MD: National Cancer Institute, SEER Program, NIH Pub. No. 07-6215 (2007).
3. Jacobs, B.L., Lee, C.T., *et al.* Bladder cancer in 2010: how far have we come? *CA Cancer J. Clin.* **60**(4), 244-272 (2010).
4. Sexton, W.J., Wiegand, L.R., *et al.* Bladder cancer: a review of non-muscle invasive disease. *Cancer Control.* **17**(4), 256-268 (2010).
5. Bohle, A., Jocham, D., *et al.* Intravesical bacillus Calmette-Guerin versus mitomycin C for superficial bladder cancer: a formal meta-analysis of comparative studies on recurrence and toxicity. *J. Urol.* **169**(1), 90-95 (2003).
6. Shokeir, A.A. Squamous cell carcinoma of the bladder: pathology, diagnosis and treatment. *BJU Int.* **93**(2), 216-220 (2004).
7. Rosenberg, S.A. Progress in human tumour immunology and immunotherapy. *Nature.* **411**(6835), 380-384 (2001).
8. Joudi, F.N., Smith, B.J., *et al.* Final results from a national multicenter phase II trial of combination bacillus Calmette-Guerin plus interferon alpha-2B for reducing recurrence of superficial bladder cancer. *Urol. Oncol.* **24**(4), 344-348 (2006).
9. Lau, S.K., Weiss, L.M., *et al.* Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study. *Am. J. Clin. Pathol.* **122**(1), 61-69 (2004).
10. Hollingsworth, M.A. and Swanson, B.J. Mucins in cancer: protection and control of the cell surface. *Nat. Rev. Cancer.* **4**(1), 45-60 (2004).
11. Scholfield, D.P., Simms, M.S., *et al.* MUC1 mucin in urological malignancy. *BJU Int.* **91**(6), 560-566 (2003).
12. Devine, P.L. and McKenzie, I.F. Mucins: structure, function, and association with malignancy. *Bioessays.* **14**(9), 619-625 (1992).
13. Jerome, K.R., Barnd, D.L., *et al.* Cytotoxic T-lymphocytes derived from patients with breast adenocarcinomas recognize an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. *Cancer Res.* **51**(11), 2908-2916 (1991).
14. Takahashi, T., Makiguchi, Y., *et al.* Expression of MUC1 on myeloma cells and induction of HLA-unrestricted CTL against MUC1 from a multiple myeloma patient. *J. Immunol.* **153**(5), 2102-2109 (1994).
15. Choi, C., Witzens, M., *et al.* Enrichment of functional CD8 memory T cells specific for MUC1 in bone marrow of patients with multiple myeloma. *Blood.* **105**(5), 2132-2134 (2005).
16. Barnd, D.L., Lan, M.S., *et al.* Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T-cells. *Proc. Natl. Acad. Sci. U.S.A.* **86**(18), 7159-7163 (1989).
17. Ioannides, C.G., Fisk, B., *et al.* Cytotoxic T-cells from ovarian malignant tumors can recognize polymorphic epithelial mucin core peptides. *J. Immunol.* **151**(7), 3693-3703 (1993).
18. Tang, C.K., Katsara, M., *et al.* Strategies used for MUC1 immunotherapy: human clinical studies. *Expert Rev. Vaccines.* **7**(7), 963-975 (2008).

19. Mukherjee, P., Ginardi, A.R., *et al.* MUC1-specific cytotoxic T lymphocytes eradicate tumors when adoptively transferred *in vivo*. *Clin. Cancer Res.* **7**(3 suppl), 848s-855s (2001).
20. Acres, B. & Limacher, J.M. MUC1 as a target antigen for cancer immunotherapy. *Expert Rev. Vaccines.* **4**(4), 493-502 (2005).
21. Butts, C., Murray, N., *et al.* Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small-cell lung cancer. *J. Clin. Oncol.* **23**(27), 6674-6681 (2005).
22. Butts, C., Maksymiuk, A., *et al.* Updated survival analysis in patients with stage IIIB or IV non-small-cell lung cancer receiving BLP25 liposome vaccine (L-BLP25), phase IIB randomized, multicenter, open-label trial. *J. Cancer. Res. Clin. Oncol.* **137**(9), 1337-1342 (2011).
23. Agrawal, B., Krantz, M.J., *et al.* Rapid induction of primary human CD4+ and CD8+ T cell responses against cancer-associated MUC1 peptide epitopes. *Int. Immunol.* **10**(12), 1907-1916 (1998).
24. Palmer, M., Parker, J., *et al.* Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer. *Clin. Lung Cancer.* **3**(1), 49-57; discussion 58 (2001).
25. Walsh, M.D., Hohn, B.G., *et al.* Mucin expression by transitional cell carcinomas of the bladder. *Br. J. Urol.* **73**(3), 256-262 (1994).
26. Murray, A., Simms, M., *et al.* Production and characterization of rhenium-188-C595 antibody for radioimmunotherapy of transitional cell bladder cancer. *J. Nucl. Med.* **42**(5), 726-732 (2001).
27. Hughes, O., Bishop, M., *et al.* Targeting superficial bladder cancer by the intravesical administration of ⁶⁷copper-labelled anti-MUC1 mucin monoclonal antibody C595. *J. Clin. Oncol.* **18**(2), 363-370 (2000).
28. Conn, I.G., Crocker, J., *et al.* HMFG-2 as a prognostic indicator in superficial bladder cancer. *J. Clin. Pathol.* **41**(11), 1191-1195 (1988).
29. Hughes, O., Denley, H., *et al.* MUC1 mucin expression in transitional cell carcinoma of the bladder: a target for diagnosis and therapy with monoclonal antibody C595. *J. Urol. Pathol.* **12**, 185-197 (2000).
30. Rowse, G.J., Tempero, R.M., *et al.* Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res.* **58**(2), 315-321 (1998).
31. Mukherjee, P., Madsen, C.S., *et al.* Mucin-1 specific immunotherapy in a mouse model of spontaneous breast cancer. *J. Immunother.* **26**(1), 47-62 (2003).
32. McCormick, D.L., Ronan, S.S., *et al.* Influence of total dose and dose schedule on induction of urinary bladder cancer in the mouse by N-butyl-N-(4-hydroxy-butyl)nitrosamine. *Carcinogenesis.* **2**(3), 251-254 (1981).
33. Wurz, G.T., Gutierrez, A.M., *et al.* Antitumor effects of L-BLP25 antigen-specific tumor immunotherapy in a novel human MUC1 transgenic lung cancer mouse model. *J. Transl. Med.* **11**, 64, doi:10.1186/1479-5876-11-64 (2013).
34. Kunze, E., Schauer, A., *et al.* Stages of transformation in the development of N-butyl-N-(4hydroxybutyl)-nitrosamine-induced transitional cell carcinomas in the urinary bladder of rats. *Z Krebsforsch Klin. Onkol. Cancer Res. Clin. Oncol.* **87**(2), 139-60 (1976).
35. Crallan, R.A., Georgopoulos, N.T., *et al.* Experimental models of human bladder carcinogenesis. *Carcinogenesis.* **27**(3), 374-381 (2006).
36. Samma, S., Uemura, H., *et al.* Rapid induction of carcinoma *in situ* in dog urinary bladder by sequential treatment with N-methyl-N'-nitrosourea and N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Gann.* **75**(5), 385-387 (1984).
37. Bornhof, C., Wolfrath, G., *et al.* Induction of urinary bladder urothelial cancers in the rabbit by dibutyl nitrosamine with an artificial bladder calculus as cocarcinogen. *Urologe A.* **28**(6), 339-343 (1989).
38. Irving, C.C., Tice, A.J., *et al.* Inhibition of N-n-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder cancer in rats by administration of disulfiram in the diet. *Cancer Res.* **39**(8), 3040-3043 (1979).
39. Mehta, N.R., Wurz, G.T., *et al.* L-BLP25 vaccine plus letrozole induces a TH1 immune response and has additive antitumor activity in MUC1-expressing mammary tumors in mice. *Clin. Cancer Res.* **18**(10), 2861-2871 (2012).