Intravaginal and Subcutaneous Immunization Induced Vaccine Specific CD8 T Cells and Tumor Regression in the Bladder

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Abbreviations and Acronyms

BCG = bacillus Calmette-Guérin
CV = cervix-vagina
ELISPOT = enzyme linked immunosorbent spot
Foxp3 = forkhead/winged-helix transcription-factor
GM = genital mucosa
IFN-γ = interferon-γ
IN = intranasal
IVAG = intravaginal
IVES = intravesical
PE = phycoerythrin
SC = subcutaneous
TAA = tumor associated antigen
Treg = T-regulatory cell

Accepted for publication August 6, 2013.

Study received institutional animal care and use committee approval.

Supported by Swiss Cancer League KFS 2808-08-2011, Swiss National Science Foundation 31003A-135109 (DN-H) and Swiss National Science Foundation Sinergia Grant CRSII3 141879 (PR).

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Purpose: Vaccines targeting tumor associated antigens are in development for bladder cancer. Most of these cancers are nonmuscle invasive at diagnosis and confined in the mucosa and submucosa. However, to our knowledge how vaccination may induce the regression of tumors at such mucosal sites has not been examined previously. We compared different immunization routes for the ability to induce vaccine specific antitumor CD8 T cells in the bladder and bladder tumor regression in mice.

Materials and Methods: In the absence of a murine bladder tumor model expressing a tumor antigen relevant for human use we established an orthotopic model expressing the HPV-16 tumor antigen E7 as a model. We used an adjuvant E7 polypeptide to induce CD8 T cell mediated tumor regression.

Results: Subcutaneous and intravaginal but not intranasal vaccination induced a high number of $TetE7^+CD8^+$ T cells in the bladder as well as bladder tumor regression. The entry of vaccine specific T cells in the bladder was not the only key since persistent regression of established bladder tumors by intravaginal or subcutaneous immunization was associated with tumor infiltration of total CD4 and CD8 T cells. This resulted in an increase in $TetE7^+CD8^+$ T cells and a decrease in T regulatory cells, leading to an increased number of effector interferon-γ secreting vaccine specific CD8 T cells in the regressing bladder tumor.

Conclusions: These data show that immunization routes should be tailored to each mucosal tumor site. Subcutaneous or intravaginal vaccination may be of additional value to treat patients with bladder cancer.

Key Words: urinary bladder, urinary bladder neoplasms, immunotherapy, drug administration routes, CD8-positive T-lymphocytes

Bladder cancer is the second most common urological malignancy.[1] Of the tumors 70% to 80% are diagnosed as nonmuscle invasive and remain confined to the bladder mucosa. According to specific tumor stage and grade characteristics IVES immunotherapy with BCG partially limits the high propensity of these tumors to recur and progress after transurethral endoscopic resection.[2] However, repeat BCG instillations are associated with significant toxicity and treatment may fail in 30% to 40% of cases.[3] Thus, there is a need for alternative or complementary immunotherapy.

Bladder cancer cells express TAAs, which can be targeted by therapeutic vaccination.[4] Clinical trials of

0022-5347/14/1913-0814/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2013.08.009
Vol. 191, 814-822, March 2014
Printed in U.S.A.

systemic vaccination in patients with bladder cancer have been reported or are ongoing.[5–8] However, since lymphocytes primed by antigens in vivo are endowed with specialized homing programs that guide their migration to specific mucosal sites,[9] it appears crucial to characterize vaccination routes that can optimize this process.

In this regard the induction of immune responses in the bladder has been poorly investigated and remains limited to humoral responses and infectious disease. In humans uropathogenic Escherichia coli infection elicits IgG and SIgA responses in urine,[10] and IVAG immunization with heat killed bacteria decreases recurrent urinary infections.[11] In monkeys systemic vaccination induces protective antibody responses in the urinary tract.[12] In mice IVES instillation of live attenuated E. coli vaccine[13] or immunization by the intramuscular or IN route with a protein vaccine[14] provided protection against a bladder challenge.

In the absence of a murine bladder tumor model expressing a tumor antigen relevant for human use we established an orthotopic murine bladder tumor model that expresses the HPV tumor antigen E7. We then took advantage of a previously developed, adjuvanted E7-polypeptide vaccine that efficiently induces high E7 specific CD8 T cell responses after a single immunization by different routes, in addition to CD8 T cell mediated tumor regression.[15,16] In this study we assessed which immunization routes would efficiently induce vaccine specific CD8 T cells in the bladder as well as effective antitumor immunity resulting in bladder tumor regression.

MATERIALS AND METHODS

Mouse Immunization

Eight-week-old female C57BL/6 WT mice (Charles River, Chatillon-sur-Chalaronne, France) were used according to the ethical directives of the Swiss veterinary authorities. $E7_{34\text{-}98}$ and $E7_{49\text{-}57}$ peptides were chemically synthesized elsewhere. Deeply anesthetized mice were immunized by the SC, IVAG or IN route[16] with E7 vaccine[15] (50 μg $E7_{34\text{-}98}$ and 0.4 μg heat labile enterotoxin) and 10 μg CpG (Coley Pharmaceutical Group, Wellesley, Massachusetts), and 75 μg R-848 (Huang-Lisheng Pharmatec, Tianjin, People's Republic of China), the latter for IN immunization.

Murine Cell Preparation

Mice were sacrificed by CO_2 inhalation. The spleen, CV and bladder were harvested. Single cell suspensions were performed by mechanical dissociation, as previously described.[17] The CV and bladder were minced and digested stepwise with 0.5 mg/ml thermolysin and 1 mg/ml collagenase/dispase (Roche, Basel, Switzerland). Between 3×10^5 and 10^6 cells were obtained from 2 bladders or 1 CV. Bone marrow derived dendritic cells were isolated using bone marrow cells collected from the tibias and femurs of female C57BL/6 mice. Bone marrow was cultured in the presence of 150 U/ml recombinant granulocyte-macrophage stimulating factor (R&D Systems®).[16]

IFN-γ ELISPOT Assay

IFN-γ ELISPOT assays were performed as described[17] using MAHA S4510 Multiscreen-HA 96-well plates (Millipore, Darmstadt, Germany), antiIFN-γ monoclonal antibody (R4-6A2, 1:250), biotinylated antiIFN-γ monoclonal antibody (XMG1.2, 1:500, Becton Dickinson Pharmingen, Allschwil, Switzerland) and Streptavidin-AP (1:2,000, Roche). Bone marrow derived dendritic cells (30,000 per well) serving as antigen presenting cells were incubated for 1 hour in duplicate with 1 μg/ml H-2Db restricted $E7_{49\text{-}57}$ peptide or medium alone (the latter in control wells) before adding 100,000 bladder or CV cells for 16 to 24 hours. E7 specific responses were defined as the number of IFN-γ spots per 10^5 cells in E7 stimulated wells minus the number of IFN-γ spots per 10^5 cells in control wells (fewer than 3 spots per well).

Tetramer and T Cell Labeling

PE conjugated $E7_{49\text{-}57}$ and $L1_{165\text{-}173}$ (as a control) H-2D b restricted tetramers (TetE7 and TetL1, respectively, Tetramer Core Facility, Ludwig Institute for Cancer Research, Epalinges, Switzerland) and allophycocyanin labeled CD8a (clone 53-6.7, eBioscience, Vienna, Austria) staining was performed as previously described.[16] The percent of $TetE7^+CD8^+$ T in the total number of cells was calculated after subtracting the background, as measured with control TetL1 (fewer than 20 events per bladder). Cell acquisition and analysis were performed using a FACSCalibur™ and BD CellQuest™ Pro, version 4.0.1. The monoclonal antimouse antibodies used were allophycocyanin or PE-Cy7-antiCD8a (53-6.7). CD4 (antiCD4-fluorescein isothiocyanate, RM4-5) and Foxp3-PE-Cy5 (FJK-16s) staining was performed on permeabilized cells with the Mouse Regulatory T cell Staining Kit #3 (eBioscience) according to manufacturer instructions.

Orthotopic Tumor Bladder Model

TC-1 cells are primary mouse lung epithelial cells that were transduced with retroviral vectors expressing the oncogenes HaRas, HPV16 E6 and E7, as previously described. TC-1 cells expressing luciferase were then generated by lentiviral infection.[18] To establish bladder tumors deeply anesthetized mice were instilled with 250,000 TC-1-luc cells by urethral catheterization using a 24 gauge $^3/_4$-inch Introcan catheter (Braun, Melsungen, Germany). Tumor growth was monitored by bioluminescence 15 minutes after intraperitoneal injection of D-luciferin (150 μg/gm body weight, Promega) using a Xenogen imaging system (Caliper Life Science, Hopkinton, Massachusetts).

Statistical Analysis

Statistical analysis was done with Prism® 5.00 for Windows®. Multiple comparisons were performed using Kruskal-Wallis 1-way ANOVA and the Dunn multiple comparison test with other statistical tests applied as indicated.