Oncolytic Vesicular Stomatitis Viruses Are Potent Agents for Intravesical Treatment of High-Risk Bladder Cancer

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Abstract
Bladder cancer is the second most common genitourinary malignancy. At initial diagnosis, ~70% of cases are non–muscle-invasive; however, current treatment options for superficial disease are of limited efficacy because many patients will develop recurrent tumors. The purpose of this study was to examine two replication-competent oncolytic vesicular stomatitis virus (VSV) strains as intravesical agents in an orthotopic murine model of high-grade bladder cancer. Four human bladder cancer cell lines (RT4, MGH-U3, UM-UC3, and KU-7) were treated with either wild-type VSV or a mutant Δ51M variant (AV3) in vitro. Both wild-type VSV and AV3, which has an impaired ability to shutdown innate immunity, preferentially killed the more aggressive, IFN-nonresponsive cell lines UM-UC3 and KU-7, whereas IFN-responsive RT4 and MGH-U3 cells were less susceptible. In vivo, KU-7-luc bladder tumor cells, which stably express firefly luciferase, were inoculated into nude mice by intravesical instillation and tumor growth was quantified using bioluminescence imaging. Mice with established xenografts were administered VSV intravesically on days 4, 9, and 14, and necropsy was performed after 3 weeks. AV3 as well as wild-type VSV significantly inhibited KU-7-luc tumor growth by 90% (AV3) and 98% (wild-type), respectively, as compared with controls treated with UV-inactivated VSV. Despite using immunocompromised hosts, there was no evidence of toxicity in either group. In conclusion, VSV instillation therapy showed promising antitumor activity and safety in an orthotopic model of bladder cancer. These findings provide preclinical proof-of-principle for the intravesical use of VSV against non–muscle-invasive bladder cancer, especially in IFN-refractory patients. [Cancer Res 2008;68(12):4506–10]

Introduction
With more than 67,000 cases diagnosed in the United States in 2007, bladder cancer is both a significant clinical and economic problem (1). Approximately 70% of bladder cancers are non–muscle-invasive at initial diagnosis; yet, present treatment strategies for superficial disease following transurethral resection are not very effective. Despite intravesical chemotherapy and/or immunotherapy, up to 80% of patients with non–muscle-invasive bladder cancer develop recurrent tumors, of which 20% to 30% evolve into more aggressive, potentially lethal tumors. Treatment with Bacillus Calmette-Guérin (BCG) has shown significant benefits for tumor prophylaxis compared with current intravesical chemotherapeutics (2). Unfortunately, it is associated with frequent local or systemic adverse effects and yet >30% of patients at high-risk ultimately require cystectomy within 15 years. Therefore, novel intravesical therapies for early-stage high-risk bladder cancer are clearly required to prevent both recurrence and progression. Recently, several studies have shown that an intravesical combination therapy of BCG and IFN-α may be useful as salvage regimen in BCG failures (3). However, as tumors evolve, they acquire defects in their ability to respond to IFNs (4), and treatment effects in true BCG-refractory disease are limited (3). Similarly, it has been shown both in vitro and in vivo that various more aggressive bladder cancer cell lines are highly resistant to IFN administration (5, 6).

Oncolytic viruses have been selected or engineered to specifically target cancer cells by exploiting their genetic defects (4). Unlike conventional gene therapy that uses viral vectors for targeted gene delivery, oncolytic viruses are inherently fully replication-competent. They actively spread through neoplastic tissues and kill tumor cells by direct lysis and induction of apoptosis. One of these, vesicular stomatitis virus (VSV), is an enveloped, negative-sense RNA virus that selectively replicates in IFN-resistant cells, but is strongly suppressed in IFN-responsive normal tissues (7). IFN-resistance confers a growth advantage over normal tissues, but simultaneously compromises the antiviral response of cancerous lesions (7). This vulnerability of tumor cells has been found to be present in a wide variety of human malignancies. Experiments on members of the NCI-60 panel of cancer cell lines showed that 47 of 57 tested cell lines were highly sensitive to VSV oncolysis (8). Similarly, studies by Barber have indicated that VSV can infect many types of tumor cells (9). Importantly, the oncolytic potential of VSV did not seem to be restricted to tumor cells with specific genetic aberrancies. For example, VSV was able to destroy cells carrying defective Myc or p53 and cells with activated Ras. Finally, Wollmann and colleagues have recently compared VSV to eight other oncolytic viruses (10); in their studies, using seven in vitro tests and one in vivo trial, VSV showed excellent infectivity, high rates of replication, and strong cytolitic action. The tumor specificity of wild-type VSV has been further enhanced in an attenuated strain (AV3) which has a defect in its ability to short-circuit the antiviral activity of IFNs and instead induces an enhanced protective response in normal tissues (8). Because viral therapy with both VSV strains has been reportedly active against a variety of human tumor xenografts (4), we decided to examine the efficacy of intravesically instilled VSV using a recently validated orthotopic bladder cancer model (11). Intravesical therapy offers strong benefits over systemic administration as it avoids many

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barriers to virus delivery that normally compromise therapeutic efficacy such as neutralizations by antibodies and complement or scavenging in the liver. Our results reveal the promising antitumor efficacy of VSV as an intravesical agent and provide the basis for future clinical trials.

Materials and Methods

Cell lines, vectors, and viruses. Human bladder cancer cell lines RT4 and UM-UC3 were purchased from the American Type Culture Collection. Cells were maintained in McCoy's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS). MGH-U3 cells were obtained as a gift from Dr. Y. Fradet (L'Hôtel-Dieu de Québec, Quebec, Canada) and maintained in MEM supplemented with 10% FBS and 2 mmol/L of l-glutamine (Invitrogen). The KU-7 cell line was kindly provided by Dr. M. Tachibana (Keio University, Tokyo, Japan). These cells were grown in DMEM containing 5% FBS. For visualization purposes, KU-7-luc cells were infected with a lentiviral vector coding for firefly luciferase (11).

The Indiana serotype of VSV was used throughout this study. The δ51M recombinant IFN-inducing mutant of wild-type VSV (AV3) has been previously described (8). Both viruses encode green fluorescent protein (GFP) and were propagated in Vero cells (American Type Culture Collection). Virions were purified from supernatants bypass age through a 0.2-μm filter and by centrifugation at 30,000 × g before resuspension in PBS. Plaque-forming units (pfu) were used for calculating infectious titers and inactivated virus was produced by exposure to UV light for 45 min.

In vitro proliferation assay. Cells were grown in 96-well plates in 100 μL of medium at a density of 5,000 cells/well, and were infected with either virus strain with or without preincubation with universal type I IFN (PBL Biomedical Laboratories). Cell viability was determined 72 h postinfection using the CellTiter96 AQueous Nonradioactive Cell Proliferation Assay (Promega). Data was analyzed with Prism 4.03 (GraphPad) and normalized to controls infected with UV-inactivated virus. Each experiment was performed with three replicates and repeated thrice.

Flow cytometry. Cells were grown in six-well plates in 2 mL of medium at a density of 500,000 cells/well, and were infected with either virus strain at different multiplicities of infection. Four, 8, 12, and 24 h after infection, the amount of GFP was determined in paraformaldehyde-fixed cells on a FACS Canto II cytometer (BD Biosciences) using standard protocols and acquiring 10,000 cells per sample.

Orthotopic murine bladder cancer model. The orthotopic mouse model was recently described by our group (11). Briefly, 8-week-old female Hsd:athymic nude-Foxn1nw mice (Harlan) were anesthetized with isoflurane. A superficial 6–0 polypropylene purse-string suture was placed around the urethral meatus before a 24-gauge angiocatheter was passed through the urethra into the bladder. After a single irrigation with PBS, two million KU-7-luc cells were instilled as a single cell suspension in 50 μL and the purse-string suture was tied down for 2.5 h. To quantify in vivo tumor burden, animals were imaged in the supine position 15 min after i.p. injection of 150 mg/kg luciferin on days 4, 8, 12, 16, and 21 with an IVIS200 Imaging System (Xenogen). Data was acquired and analyzed using Living Image 2.50 (Xenogen). Necropsy was performed after 3 weeks. The whole bladders and appropriate tissues were removed and either snap-frozen in liquid nitrogen or fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were prepared and stained with H&E. All slides were reviewed by a pathologist (L. Fazli) and scanned on a BLISS workstation at ×20 magnification (Bacus Laboratories). Animal procedures were performed according to the guidelines of the Canadian Council on Animal Care.

In vivo treatment studies. Following quantitation of tumor burden on day 4 after the initial tumor inoculation, 26 mice were randomized for treatment with either wild-type VSV, AV3, or UV-inactivated wild-type VSV. Levels of bioluminescence were equivalent among the experimental groups.

Figure 1. Efficacy of wild-type (WT) VSV (A) and the mutant AV3 strain (B) against different bladder tumor cell lines. Cells were infected with either virus at incremental titers up to 20 pfu/cell. MTS assays were performed 72 h after infections. RT4 (C) and KU-7 (D) cells were incubated with AV3 at a multiplicity of infection of 0.1, and virus replication was assessed over time by flow cytometric analysis of GFP expression. Representative assays of three independent experiments.
Intravesical therapy was administered on days 4, 9, and 14 with an instillation volume of 100 mL per mouse and a dwell time of 2 h (7 × 10³ pfu/mL). An additional set of four mice was treated with either virus to monitor for GFP fluorescence inside the bladders 24 h after infection (8).

Titeration of VSV from mouse tissues. Tissue samples were homogenized in 0.5 mL of PBS and serial dilutions were applied to confluent Vero cells for 45 min. Subsequently, plates were overlaid with 0.5% agarose in medium and plaques were grown for 48 h. Plaques were counted by visual inspection (12).

Quantitative real-time PCR. Total RNA from mouse tissue was isolated using the Trizol method (Invitrogen), and RNA from serum was extracted using the QiAamp Viral RNA Mini Kit (Qiagen). Serial dilutions of RNA obtained from 1 × 10⁶ pfu of VSV were used as internal standards. RNA extracts were reverse-transcribed and amplified using primers for GFP (forward, 5'-CAAGGTAACCTCAAGATCC; reverse, 5'-CCACGAGACCATGTTAGTCG) on an Applied Biosystems 7900HT Fast Real-time PCR System following the SYBR Green PCR Master Mix protocol. Relative quantification of gene expression was performed using β-actin as a control. The lower detection limit for VSV was <10 pfu. 

Statistical analyses. All results were expressed as mean ± SE. Statistical tests were performed using InStat3.06 (GraphPad). P < 0.05 was considered statistically significant.

Results

Cytopathic effects of wild-type and mutant VSV. The effects of both wild-type VSV and the AV3 strain on bladder cancer cell growth were examined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assays 3 days after infection with various multiplicities of viral infection. Two representative low-grade (RT4 and MGH-U3) as well as two high-grade (UM-UC3 and KU-7) urothelial carcinoma cell lines were tested. Both viruses were found to be potent inhibitors of proliferation in the more aggressive UM-UC3 and KU-7 cell lines, whereas well-differentiated RT4 and MGH-U3 cells were less susceptible to oncolysis (Fig. 1A and B). Regarding virus replication, GFP expression was detected by flow cytometry 1 day postinfection with higher doses of wild-type VSV in all cell lines at high levels, demonstrating excellent infectivity (data not shown). However, using the attenuated AV3 virus, there was little virus replication in AV3-resistant cells versus abundant virus replication in AV3-sensitive cells. Representative data for RT4 and KU-7 cells are shown in Fig. 1C and D. As VSV is exquisitely sensitive to IFN, this behavior is most likely related to intact IFN pathways and greater responsiveness of RT4 and MGH-U3 cells to IFNs. In an intact system like RT4, which is the least malignant bladder tumor cell line available, IFN binds to its receptor, activating a signaling cascade that leads to growth inhibition (Fig. 2A) and a strong antiviral response at low IFN concentrations (Fig. 2B; ref. 13). Therefore, endogenous cytokine production that is triggered rather than disabled by AV3 might explain the diminished anticancer potency of the mutant virus as compared with wild-type VSV (Figs. 1A and 2B). The observed differences in IFN sensitivity are in agreement with previous results from other groups (5, 6), but protection of bladder cell lines from viral infection with IFN has not been previously reported.
Intravesical treatment of orthotopic xenografts in nude mice. In vivo studies were performed on a total of 31 athymic nude mice to evaluate the efficacy of wild-type VSV and the AV3 strain as intravesical agents. Using both histologic evaluation and magnetic resonance imaging, we have recently validated an orthotopic murine model of bladder cancer in which tumor burden is longitudinally quantified using bioluminescence imaging (11). The KU-7-luc tumor cells used in our model are of human origin and are the only cell line thus far that reproducibly provides reliable tumor take rates as orthotopic xenografts without the use of secondary agents such as trypsin or electrocautery that traumatizes the urothelium in order to promote tumor cell adhesion. The key characteristic of the model is a lack of deeper invasion at early time points, which makes these tumors amenable to intravesical therapy. Thirty out of 31 mice in which we transurethrally inoculated KU-7-luc cells developed bladder tumors with no procedure-related deaths. Initially, two mice were each treated with wild-type VSV or AV3 and sacrificed 24 hours later to confirm replication of virus in orthotopic tumors via GFP fluorescence (data not shown). Subsequently, three groups of mice were randomly selected to receive intravesical treatment with either virus strain or with UV-inactivated VSV as control. As early as day 8 post-tumor inoculation, the control mice progressed significantly faster than mice treated with wild-type VSV ($P < 0.05$; Fig. 3). At the end of the study, tumor growth in the treatment arms were reduced to 10% (AV3) and 2% (wild-type) compared with controls, respectively (Fig. 3A). In the wild-type VSV-treated cohort of nine mice, there were seven complete responses and one partial response, whereas out of the nine AV3-treated mice, three were cured and four were partial responders. Representative bioluminescence images of mice in each treatment group are shown in Fig. 4. Upon histopathology, KU-7-luc tumors exhibited an aggressive growth pattern with frequent multifocality, but after 3 weeks of growth, they were usually still confined to the lamina propria and correlated with high-grade T1 stage disease. Surprisingly, regardless of the virus strain administered, no significant changes in body weight or deaths of the immunocompromised animals were observed (Fig. 3B). Furthermore, no viral escape from the bladders was detected via plaque assay of serum, brain, lung, liver, spleen, and kidney tissues at the time of necropsy 1 week after the last treatment (viral titer was 0 in all specimens examined). Using quantitative reverse transcription-PCR, we detected trace amounts of virus in only a few serum samples, but not in other organs. These results are in agreement with studies using intravesical cytotoxics in the same murine model in which a systemic bioavailability of $<2.5\%$ of the total dose can be expected (14). To monitor long-term safety and efficacy, we chose to keep three mice of each group beyond 21 days. Although the control mice had to be euthanized at 7 weeks, the six virus-treated mice continued to behave normally and showed no evidence of systemic toxicity or residual disease on imaging 100 days post-tumor inoculation.

Discussion

BCG is the most effective regimen for bladder cancer prophylaxis (2), but causes irritative voiding symptoms, and occasionally, systemic adverse events including life-threatening BCG sepsis. Patients who do not tolerate or respond to BCG immunotherapy represent a significant therapeutic challenge. In an aging population, definitive surgical treatment for recurrent high-risk bladder cancer is often not feasible or desirable for the patient. Recently, the addition of IFN to BCG has evolved as a second-line treatment option (3). However, many high-grade tumors have been
shown to be IFN-non–responsive (5, 6). Such behavior confers a growth advantage over normal tissues, but concurrently compromises the antiviral response of these lesions (7). To exploit this specific vulnerability, we explored the therapeutic potential of two oncolytic VSV strains that selectively kill IFN-refractory tumors as a possible new treatment modality (8). Although oncolytic herpes, reoviruses, and adenoviruses have been previously tested in bladder cancer models (15–17), compared with these viruses, VSV’s distinct targeting approach combined with a high inherent infection efficiency and a proven track record of broad spectrum oncolytic activity without requiring the application of transduction enhancement agents holds great promise. The current study is the first to describe the use of VSV against bladder cancer and to determine its efficacy as an intravesical agent in vivo.

In vitro, both viral cytotoxicity and virus replication as well as IFN protection from oncolysis were dependent on the degree of differentiation of the cell lines studied (Figs. 1 and 2). The two high-grade tumor cell lines tested, KU-7 and UM-UC3, which are known to be nonresponsive to exogenous IFNs (5), showed excellent sensitivity to wild-type and mutant VSV. In vivo, wild-type VSV as well as the attenuated AV3 strain showed a profound response in our model system and significantly inhibited KU-7-luc tumor growth by 90% (AV3) and 98% (wild-type), respectively, compared with UV-inactivated virus control. Complete and durable responses were observed in both treatment arms. In our opinion, orthotopic implantation of human bladder cancer cells into immunodeficient mice remains one of the best models currently available to assess the effects of intravesical antitumor therapy in vivo. Transgenic models are also important, offering advantages such as an immune response of the host, but with shortcomings as well. Although they allow the evaluation of tumor growth after mutation of single genes, they are, as with syngeneic systems, murine instead of human tumors. VSV efficacy was not tested in immunocompetent syngeneic bladder tumor models because the two commonly used murine bladder cancer cell lines, MBT-2 and MB49, are responsive to exogenous IFN administration (18, 19). Thus, their tumors do not represent an adequate model to evaluate VSV which is selective for IFN-resistant cells (7). Because it has recently been published that VSV also induces antitumor immune responses (12), we anticipate that the oncolytic potential of intravesical VSV installation therapy may be even higher in the presence of an immune system. Moreover, VSV has previously been tested against a variety of syngeneic tumors in immunocompetent mice and rats and showed robust oncolytic antitumor activity (20).

The urothelium provides a natural barrier against systemic uptake, but a potential concern with VSV treatment is safety, especially because oncolytic viruses are replication-competent. Although there were only trace amounts of virus detected in the serum via reverse transcription-PCR and even wild-type–treated nude mice, which do not have the ability to eradicate viral infection, showed no evidence of toxicity, some systemic bioavailability of intravesical therapeutics has to be expected (14). BALB/c nude mice are more sensitive to toxicity caused by VSV infection than CD-1–derived nude mice (7). Thus, our Hsd athymic nude-Foxn1nu mice, which have a BALB/c background, represent a good model to monitor for adverse effects caused by intravesically administered VSV. Despite the absence of toxicity in our study, for a planned phase I clinical trial, we will focus on the attenuated AV3 strain instead of wild-type VSV because this strain has an impaired ability to shutdown innate immunity and thereby has an improved therapeutic index (8).

In conclusion, our studies support further evaluation of the use of VSV as adjuvant therapy against high-grade non–muscle-invasive urothelial carcinoma, especially in IFN-non–responsive patients with comorbidities in whom definitive surgical treatment is not possible. VSV’s distinctive mechanism of action, in addition to the safety of the attenuated AV3 strain, suggests a complementary response to existing therapies.

Disclosure of Potential Conflicts of Interest

J.C. Bell is cofounder of Jennerex Biotherapeutics, a company involved in the development of oncolytic virus therapeutics. The other authors disclosed no potential conflicts of interest.

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6. Qu XJ, Yang JL, Russell PJ, Goldstein D. Changes in differentiation of the cell lines studied (Figs. 1 and 2). The two high-grade tumor cell lines tested, KU-7 and UM-UC3, which are known to be nonresponsive to exogenous IFNs (5), showed excellent sensitivity to wild-type and mutant VSV. In vivo, wild-type VSV as well as the attenuated AV3 strain showed a profound response in our model system and significantly inhibited KU-7-luc tumor growth by 90% (AV3) and 98% (wild-type), respectively, compared with UV-inactivated virus control. Complete and durable responses were observed in both treatment arms. In our opinion, orthotopic implantation of human bladder cancer cells into immunodeficient mice remains one of the best models currently available to assess the effects of intravesical antitumor therapy in vivo. Transgenic models are also important, offering advantages such as an immune response of the host, but with shortcomings as well. Although they allow the evaluation of tumor growth after mutation of single genes, they are, as with syngeneic systems, murine instead of human tumors. VSV efficacy was not tested in immunocompetent syngeneic bladder tumor models because the two commonly used murine bladder cancer cell lines, MBT-2 and MB49, are responsive to exogenous IFN administration (18, 19). Thus, their tumors do not represent an adequate model to evaluate VSV which is selective for IFN-resistant cells (7). Because it has recently been published that VSV also induces antitumor immune responses (12), we anticipate that the oncolytic potential of intravesical VSV.
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