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ORIGINAL RESEARCH

Antitumor Efficacy of Intravesical BCG, Gemcitabine, Interferon-α and Interleukin-2 as Mono- or Combination-Therapy for Bladder Cancer in an Orthotopic Tumor Model

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Abstract

Objective: To reduce adverse effects and improve efficacy of intravesical BCG for bladder cancer, alternative treatment options were investigated in an orthotopic rat tumor model.

Methods: Superficial bladder cancer was established in syngeneic female rat bladders by instillation of AY-27 cells. Animals were randomly assigned to treatment groups including dose escalation of intravesical BCG with or without interferon-α (IFN-α) or interleukin-2 (IL-2); or graded doses of gemcitabine alone; or BCG plus gemcitabine. Treatments were given twice weekly for 3 weeks. Rats in control groups received saline instillations. Treatment response was monitored by animals' well-being, survival days, tumor growth inhibition, and histological examination at necropsy.

Results: Rats receiving monotherapy with intravesical BCG, gemcitabine, or IFN-α, attained significantly better survival and tumor reduction compared with control ($P = 0.002$; 0.001; 0.002, respectively, Log-rank Test). A dose-dependent treatment response was observed in animals with established bladder tumor receiving escalated BCG instillations. Only high-dose BCG significantly improved animal survival. Although high-dose BCG plus gemcitabine or IFN-α did not increase benefit over monotherapies, low-dose BCG plus IL-2 did show improved efficacy ($P = 0.01$).

Conclusion: Intravesical monotherapies with gemcitabine and IFN-α were as effective as BCG for treatment of early non-muscle-invasive urothelial bladder cancer in this immune competent rat model. Combining these agents with high-dose BCG did not further increase efficacy. However, combining low-dose BCG with IL-2 enhanced BCG effectiveness.

Keywords: BCG, bladder cancer, gemcitabine, interferon-α, intravesical therapy

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Introduction

Urothelial cancer of the bladder (UCB) is the fourth most common malignancy diagnosed in American men.[1] The majority of these cancers are non-muscle-invasive lesions at the time of diagnosis. Non-muscle-invasive urothelial cancer has a high incidence of recurrence (up to 75%) which requires close monitoring.[2,3] The main goal of intravesical therapy is to prevent tumor recurrence and progression following initial transurethral resection of bladder tumor (TURBT). Intravesical bacillus Calmette-Guérin (BCG) is so far the most effective and common form of adjuvant therapy for high risk bladder cancer.[4] Compared with controls, BCG immunotherapy has superior advantage in preventing tumor recurrence than intravesical chemotherapy.[5,6] In contrast to intravesical chemotherapy, BCG has also been shown to reduce the risk of tumor progression.[3] Despite its success, significant proportions (30%–40%) of patients do not respond to BCG therapy and 30% to 50% of initial responders have relapse within the first five years.[7] Furthermore, side-effects are common, which may be related to BCG dose, especially in highly sensitive patients.[8] BCG elicits a non-specific immune response within the bladder wall. This response is T-lymphocyte dependent and is mediated by both T-helper 1 (Th1) and T-helper 2 (Th2) cytokines.[9,10] $CD4^+$ Th1 cytokines [interleukin 2 (IL-2) and interferons (IFNs)] lead to activation of lymphokine activated killer cells, macrophages, NK cells, and apoptotic pathways.[11,12] The apoptotic pathways are thought to be related to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[13,14] TRAIL can trigger both the extrinsic and intrinsic apoptotic pathways.[15] Preliminary clinical studies of combination therapy with IFN-α plus low dose BCG demonstrate 42% to 53% tumor-free at 2 years in BCG refractory patients, for which effective alternative therapy remains very limited.[16,17] Also, combining low-dose BCG plus $CD4^+$ cytokines has been suggested to reduce BCG side effects.[18] In addition, IL-2 has been shown in some clinical studies to have a direct antitumor effect.[19] Furthermore, there is strong experimental evidence for maximally activating the apoptotic pathways with combination treatment of gemcitabine and TRAIL in urothelial cancer cells.[20]

Gemcitabine (2′-deoxy-2′, 2′-difluorocytidine, Gemzar®), is a nucleoside analogue with a molecular weight of 299.66 that is metabolized intracellularly by nucleoside kinases to the active diphosphate and triphosphate nucleosides. It primarily kills cells undergoing DNA synthesis (S-phase). Studies have shown it can also promote tumor cell apoptosis,[21] which may enhance the antitumor effect of BCG if used together as an intravesical agent.[22]

The ultimate goal of cancer therapy is to achieve maximal antitumor efficacy and minimal toxicity. To reach this goal, we investigated the antitumor efficacy of some alternative modalities including gemcitabine, IFN-α, IL-2, alone or in combination with BCG, which was compared to BCG monotherapy and saline control. Ideally new therapeutic strategies should be tested rigorously in relevant animal models. Thus, a previously well characterized orthotopic, immune competent rat bladder cancer model[23–25] was used in this study.

Materials and Methods

Tumor cells, BCG, and gemcitabine

The AY-27 cell line was originally induced in the bladders of Fischer F344 rats by using the FANFT carcinogen. The cells were cultured as monolayers in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum in 37 °C humidified 5% CO_2. These cells were passaged using standard trypsinization protocols and were previously characterized to confirm their urothelial cell origin by immunohistochemical analysis.[23]

Connaught strain BCG was initially provided by Aventis Pharmaceuticals (formerly Pasteur-Merieux Connaught, Montreal, QC, Canada). It was assayed for colony forming unit (cFU) quality by the manufacturer and our treatment aliquots were based on these functional calculations. This strain of BCG was used in the first part of the study. Our hospitals later switched over to use Tice strain BCG (OncoTICE®, Organon Canada Ltd., Toronto, ON) in patients. Accordingly, we used Tice strain BCG in the second part of the animal study. The medium BCG dose (2×10^6 cFU/ml) used for the animal studies was based on the clinically used dose ($1–8 \times 10^6$ cFU/ml). All lyophilized BCG aliquots were protected from light and stored at 4 °C. BCG was used in aliquots and never stored after being reconstituted in sterile saline (0.9% NaCl).

The lyophilized powder of gemcitabine (Eli Lilly Canada Inc., Toronto, ON) was first reconstituted with

saline, aliquoted and stored at –20 °C. These aliquots were further diluted to the desired concentrations with Dulbecco's phosphate buffered saline (PBS, pH 7.4) before each experiment.

Animals

All animal procedures were carried out in accordance with guidelines regulated by the Canadian Council on Animal Care and approved by the University of Alberta Institutional Animal Care Committee. Female Fischer F344 rats weighing ~150 gm were used for tumor implantation and randomly assigned to experimental groups (Tables 1 and 2). Animals were initially purchased from Charles River Laboratories (Quebec, Canada) and bred locally in the Cross Cancer Institute vivarium. Sterile technique was used for all procedures including catheterization of the animals.

Tumor cell implantation and intravesical therapy

Tumor cell implantation procedures were previously reported by us.[23,26] Briefly, animals were anesthetized with inhalation of 2% Isoflurane in oxygen. The bladder was catheterized with an 18 gauge angiocatheter (BD Insyte™, Utah, USA) and the mucosa preconditioned with 0.1 *M* HCl, neutralized with 0.1 *M* KOH, and then flushed with sterile PBS (pH 7.4) 3 times. Single cell suspensions of AY-27 cells (3×10^6) in 500 µl of serum-free medium were then instilled *via* the catheter and left indwelling for 1 h. The rats' position was changed from side to side to facilitate full bladder wall exposure. The catheter was removed after 1 h and the rats were allowed to void spontaneously. The well-being of the rats was monitored daily. With this instillation procedure, nearly 100% tumor engraftment has been achieved in syngeneic Fisher F344 rats if 2×10^6 or more tumor cells are inoculated.[23–25]

Based on our prior study using reovirus in the same tumor model,[26] treatments with dose escalation of intravesical BCG (Connaught strain) or BCG plus IL-2 (Invitrogen) commenced on day 10 after tumor cell inoculation (Table 1). BCG doses ranged from 5×10^5 cFU/ml (low-dose) to 5×10^7 cFU/ml (high-dose). Low-dose BCG plus IL-2 (5×10^5 Units) was used for combination treatment. Control animals received normal saline instillations. Treatments were administered twice weekly for 3 weeks. Instilled volume was 0.5 ml per treatment. To ensure accurate drug exposure a purse-string suture was placed in the skin around the urethral meatus to keep the solutions in the bladder while the animals were under anesthesia. The suture was removed 2 h later. After treatment the animals were monitored daily. Urine was collected for cytology at day 0, 10 and 60. In cases where urine could not be collected directly (ie, empty bladder) 0.5 ml of normal saline was flushed into the bladder and collected for cytology.

A 9.4T rodent magnetic resonance imaging (MRI, Magnex Scientific, Oxford, UK) was used to monitor bladder tumor growth. Although the 9.4T MRI had better resolution (0.5 mm) than the 1.5T clinical MRI we used previously,[23] it was still difficult to visualize tiny or flat tumors (Fig. 1). The MRI did detect papillary tumors and some were fairly large after 10 days post-inoculation (Fig. 1), suggesting that intravesical BCG should possibly be started earlier. Therefore in the second part of the study, treatments with gemcitabine commenced at day 6 post-implantation (Table 2). Graded doses of gemcitabine ranging from 0.5 mg/ml to 20 mg/ml, or BCG (2×10^7 cFU/ml), or recombinant rat IFN-α (Invitrogen, 18000 Units) were given intravesically twice weekly for 3 weeks. Combination

Table 1. Fisher rats receiving intravesical treatments starting 10 days after inoculation of 3-million AY-27 rat UCC cells.

Treatment groups	BCG† concentration	Treatment schedule	No. rats	No. LTS§	No. (LTS) tumor-free	*P* value vs. saline
Low BCG	5×10^5 cFU/ml	Twice/wk, 3 wks	10	2	0	0.115
Medium	5×10^6 cFU/ml	Twice/wk, 3 wks	10	4	3	0.05
High BCG	5×10^7 cFU/ml	Twice/wk, 3 wks	10	5	4	0.03
IL-2¶ + low BCG	5×10^5 cFU/ml	Twice/wk, 3 wks	10	5	3	0.01
0.9% NaCl	Not applicable	Twice/wk, 3 wks	10	1	1	Not applicable

Notes: †Connaught strain of BCG was used; instilled volume was 0.5 ml per treatment. ¶Recombinant IL-2; 5×10^5 units per treatment. §LTS means long-term survival, which is that rat survives greater than 90 days post implantation.

Table 2. Fisher rats receiving intravesical treatments starting 6 days after inoculation of 3-million AY-27 rat UCC cells.

Treatment groups	Drug concentration	Treatment schedule	No. rats	No. LTS	No. (LTS) tumor-free	*P* value vs. saline
Control	0.9% NaCl	Twice/wk, 3 wks	12	1	1	Not applicable
Gemzar A	>10 mg/ml	Not finished	6	0		
Gemzar B	2 mg/ml	Twice/wk, 3 wks	10	7	5	0.005
Gemzar C	1 mg/ml	Twice/wk, 3 wks	10	9	4	0.001
Gemzar D	0.5 mg/ml	Twice/wk, 3 wks	10	7	6	0.001
BCG (Tice®)	2×10^7 cFU/ml	Twice/wk, 3 wks	12	8	7	0.002
Gemzar/BCG	0.5 mg/ml 2×10^7 cFU/ml	Twice/wk, 3 wks	9	4	5	0.046
rIFN	18 000 IU	Twice/wk, 3 wks	12	8	7	0.002
rIFN/BCG	18 000 IU 2×10^7 cFU/ml	Twice/wk, 3 wks	11	6	6	0.005

Note: Instilled volume was 0.5 ml per treatment.

therapies of BCG plus gemcitabine (0.5 mg/ml) or IFN-α were administered. Once again, animals in a control group received saline instillations. Treatment procedures were identical to the first part of the study. However, urine cytology and MRI were abandoned due to their limitations in tumor detection.

Follow-up endpoint

Once the animals completed the treatment regimes, they were observed for more than 90 days for signs and symptoms of bladder cancer progression (body weight loss, hematuria, and urinary retention).

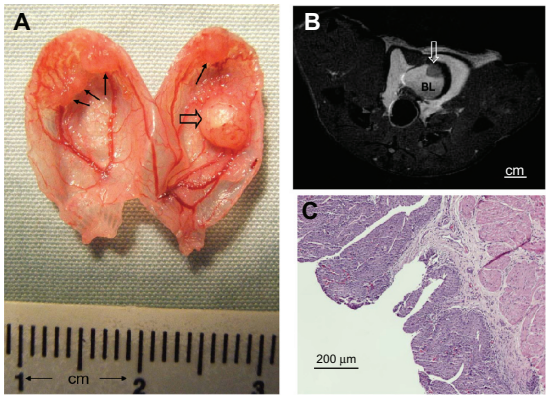

Figure 1. Orthotopic rat bladder tumors post-inoculation of 3×10^6 AY-27 UCC cells. (**A**), a gross photograph of a rat bladder, which is sagittally cut open from the anterior wall at 14 days post-implant, shows a solid tumor on the left side of the bladder cavity (open arrow). There are also multiple tiny 'satellite' tumors (small arrows) surrounding the solid tumor. (**B**), a transverse view of an MR image of the rat scanned with the 9.4T rodent MRI demonstrates a solid bladder tumor (open arrow) protruding to the bladder lumen (BL) from the bladder wall. With MRI, it is difficult to detect satellite tiny tumors shown in (**A**) and flat lesions. MR imaging was performed using T2-weighted scans (TR = 3000 ms, TE = 35 ms) with slice thickness of 1.0 mm. A total of 20 consecutive slices were scanned per bladder, which was filled with 0.5 ml of saline. (**C**), a bladder section (H&E staining) 7 days post-inoculation shows early stage (flat) UCB. Scale bar denotes 1 cm in 1B; or 200 μm in 1C.

Animals were then euthanized using pentobarbital (Euthanyl®, Bimeda-MTC Animal Health Inc.) overdose and subjected to necropsy. The bladders and kidneys were excised for gross and histological examination (standard formalin fixation, sectioning, and hematoxylin and eosin staining). Sections were microscopically reviewed by a pathologist. Tumor stage and grade, and extent of inflammatory cell infiltration were recorded. Other organs that appeared grossly abnormal were also excised and studied microscopically. If animals became severely distressed they were euthanized earlier and necropsy performed.

Statistic analysis

Survival curves from different groups of animals were plotted by the Kaplan-Meier method. Log-rank (Mantel-Cox) test (GraphPad Prism®, version 5.01, San Diego, USA) was used to compare survival time distributions. Individual groups were compared relative to control groups to calculate *P* values and a $P < 0.05$ was considered significant.

Results

Table 1 and Figure 2 summarize the data from tumor-bearing rats treated by graded doses of BCG alone or low-dose BCG plus IL-2 starting at 10 days post tumor cell inoculation. Animals surviving greater than 90 days post-implantation were considered long-term survivals (LTS). A dose-dependent tumor cure (tumor-free animals in LTS) and survival benefit were observed from intravesical BCG monotherapies. High-dose BCG attained significant survival benefit compared to control ($P = 0.03$), while low-dose BCG

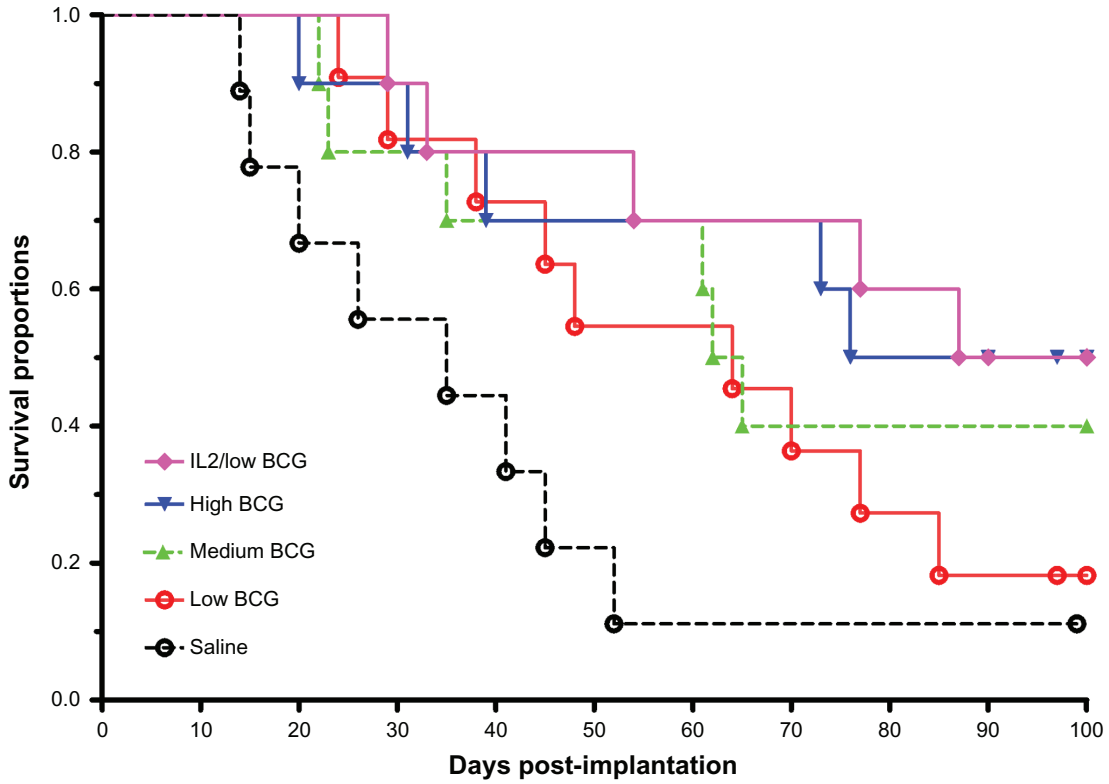

Figure 2. Kaplan Meier survival curves of rats bearing AY-27 bladder cancer and receiving BCG instillations commenced at day 10 post-implantation. A BCG dose-dependent survival is shown. Compared with saline control, high-dose BCG improved survival significantly ($P = 0.03$), while low-dose BCG appeared to be less effective ($P = 0.115$). IL-2 plus low-dose BCG enhanced efficacy of BCG monotherapy ($P = 0.01$).

did not ($P = 0.115$). Interestingly, when combining low-dose BCG with IL-2 similar efficacy to high-dose BCG was obtained ($P = 0.01$). Surprisingly, the benefit from medium-dose BCG (equivalent to dose used clinically) over saline instillations just reached significance ($P = 0.05$). The tumor burden at the time of treatment could have been a potential cause of failure, since MRI and histological examination at 10 days post tumor cell inoculation revealed some relatively 'large' papillary tumors with early invasion (Fig. 1). Clinically, intravesical BCG has been used mainly for treatment of carcinoma in situ or for minimal residual tumor following surgery to eliminate recurrence and progression.

Histological slides of bladder tissue did not detect marked difference of inflammatory cell infiltration between BCG and BCG plus IL-2 treated groups, suggesting that IL-2 may act directly on tumor cells. Urine cytology detected less than 50% of bladder tumors when compared with histological examination at necrospy. During and after BCG therapy, animals showed various degrees of distress (ocular porphyrin staining, hematuria and poor grooming). The main cause for animals to be terminated earlier was urinary retention (lower abdomen distention) resulting from bladder tumor and/or dystrophic stone obstruction of bladder outlet. Pyelonephritis was observed in 15% of animals, which might be related to tumor obstruction or reflux which is common in this model.

In the second part of the study, we investigated the efficacy of alternative agents in this tumor model using BCG as a standard treatment. In an attempt to treat animals with less tumor burden, intravesical treatments in this part of the study commenced at 6 days post-inoculation when mostly flat UCB was present (Fig. 1C). Data from the second part of the study are shown in Table 2 and Figure 3. Consistent to the first part, animals treated with BCG (Tice strain, 2×10^7 cFU/ml) instillations showed a significant survival advantage (66.7% LTS, $P = 0.002$) and tumor cure (tumor-free in LTS) compared with saline controls. Animals receiving gemcitabine instillations (0.5, 1, and 2 mg/ml) also attained 70–90% long-term survival, with 40–60% tumor cure, which were significantly different from animals receiving saline instillations

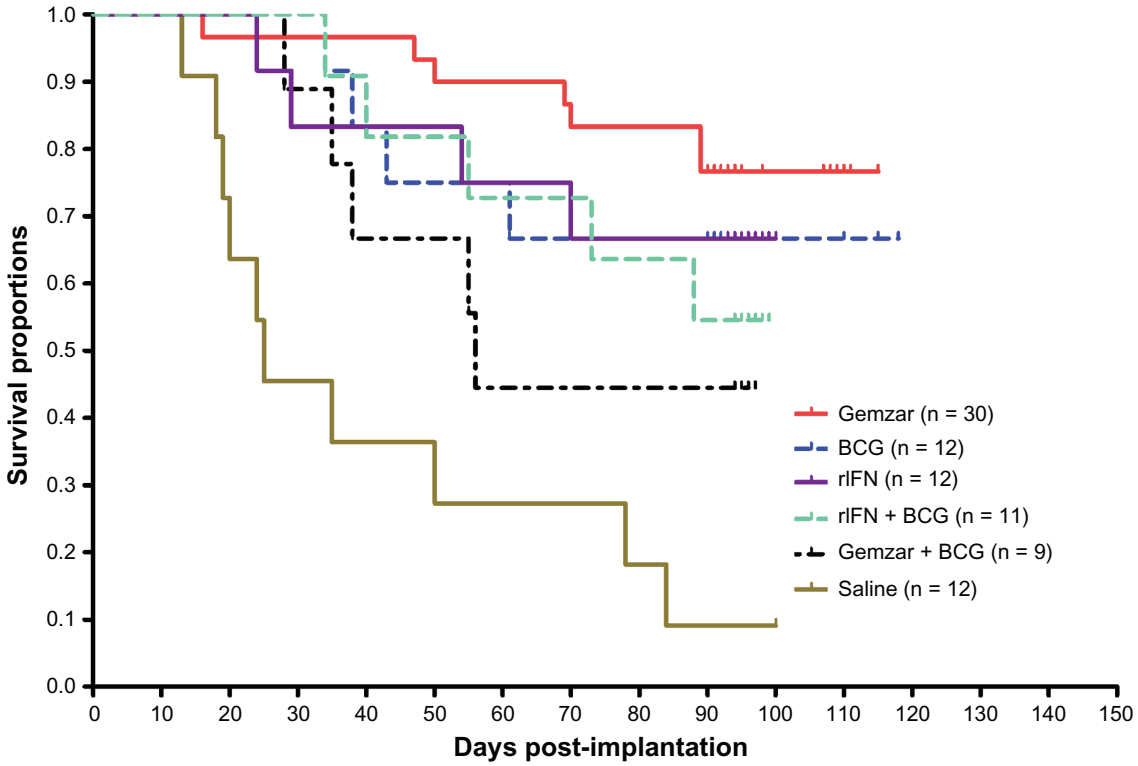

Figure 3. Kaplan Meier survival curves of rats bearing AY-27 bladder cancer and receiving instillations of gemcitabine, Tice strain BCG, IFN-α, or combination of these agents commenced at day 6 post-implantation. For clarity purpose, animals receiving lower doses of gemcitabine (0.5, 1.0, 2.0 mg/ml) were pooled together for survival analysis due to their similar survival data (see Table 2). Animals treated with gemcitabine (Gemzar), BCG, or IFN-α (rIFN) all attain significant survival benefits, compared with controls treated with saline ($P = 0.0001$). Combining BCG with gemcitabine or IFN has not further increased antitumor response.

($P = 0.001$, $= 0.001$, $= 0.005$, respectively). Since no dose-response was observed between the lower-dose gemcitabine groups, they were pooled into one group for survival analysis to generate a concise and more readable figure (Fig. 3). However, it should be noted that animals treated with higher doses of gemcitabine (10 and 20 mg/ml) died from toxicity after 2 instillations; as such, they were excluded from analysis as they did not finish the treatment schedule. Necropsy of these high-dose treated animals revealed hemorrhagic cystitis and gastrointestinal bleeding which appeared to be the etiology of death.

To test if gemcitabine could increase efficacy of BCG by targeting both the intrinsic and extrinsic apoptotic pathways, one group of animals received BCG (2×10^7 cFU/ml) plus gemcitabine (0.5 mg/ml) instillations. Unfortunately, this combination did not further increase effectiveness, but it slightly reduced the animals' tolerance to treatment (44% LTS). Surprisingly, rats receiving IFN-α alone instillations displayed similar effectiveness to BCG alone treatment. Interestingly, combining BCG (2×10^7 cFU/ml) with IFN-α did not enhance effectiveness over high-dose BCG monotherapy.

Discussion

Transurethral resection is the initial treatment for patients with non-muscle invasive UCB. Unfortunately, these tumors recur in 40% to 80% of patients following TURBT.[27] To prevent tumor recurrence and progression, intravesical instillation of BCG is the most commonly used adjuvant therapy. To date, there are limited proven effective alternative intravesical therapies for patients with BCG-refractory bladder cancers. Increasing the dose of BCG or enhancing the treatment schedules was suggested, but was associated with higher toxicities.[8,18] The doses of BCG used clinically are largely empirical and there is limited data in literature to show that BCG efficacy is dose-dependent. Studies in animals and preliminary clinical trials have shown that gemcitabine and IFN may be promising intravesical agents which could be used as monotherapy or in combination therapy with BCG.[12,17,18,21,28] However,

studies to date have been performed in various different clinical settings. To the best of our knowledge, there has been no side-by-side comparative studies performed using all these agents in a single preclinical animal model. In the present study, we used a well characterized syngeneic rat orthotopic bladder tumor model to examine gemcitabine as an alternative regional chemotherapeutic agent (which could be clinically administered earlier post-TURBT than the infectious BCG) and compared it with BCG. We also explored combination therapies of BCG plus gemcitabine, or T lymphocyte cytokines. The rationale for combining gemcitabine with BCG was to maximize apoptosis in UCC, as demonstrated in our previous in-vitro studies, where gemcitabine reduced Bcl-2 expression and enhanced TRAIL mediated apoptosis.[20]

We first performed a BCG dose escalation study because such a study is difficult to do in patients. A dose–dependent antitumor and survival effect was shown in animals receiving BCG instillations started 10 days post-inoculation of UCC cells (Table 1, Fig. 2). High-dose BCG treatments significantly improved survival benefit and tumor cure versus saline control. Low-dose BCG alone did not demonstrate a significant benefit over saline. The clinically equivalent medium-dose BCG treatment demonstrated a marginally significant antitumor and survival benefit ($P = 0.05$, Mantel-Cox test) over saline. Combination therapy of low-dose BCG with IL-2 significantly enhanced BCG's antitumor efficacy, which concurs with clinical results of combining low-dose BCG with INF-α.[16] Urinary IL-2 seems a positive predictor of response to BCG therapy.[9,10] Histological examination has not found noticeable difference of inflammatory cell infiltration between BCG and BCG plus IL-2 treated bladder tissues, which may suggest IL-2 (effector) has direct antitumor effect and enhances BCG's indirect (activator) activity.[9]

The reason for the lack of efficacy of low-dose BCG alone is not clear. We thought a heavier tumor burden at 10 days (rather than 6 days, Fig. 1) post-inoculation may be one of the obstacles for BCG therapy. This was based on the criteria established by Zbar, that successful BCG therapy requires close contact between BCG and tumor cells, a limited tumor burden, a host capable of mounting an immunological reaction to mycobacterial antigens and adequate numbers of viable BCG organisms.[4] Clinically, BCG is used to treat bladder flat lesions (CIS) or residual tumor cells.

We then examined the efficacy of gemcitabine and IFN-α, as well as in combination with high-dose BCG. Treatments were started earlier (6 days post tumor inoculation). The rationale of this was two-fold: 1, gemcitabine and INF-α are not infectious agents and can be used earlier than BCG in the clinic, and 2,the tumors tended to have early invasion (pT1 disease) beyond 6 days. Gemcitabine (low dose; 0.5–2 mg/ml), IFN-α, and BCG all demonstrated significantly superior antitumor responses (tumor-free long-term survival) when compared with saline controls (Table 2 and Fig. 3). Animals tolerated the treatments well. These data show that gemcitabine and INF-α may be promising alternative agents to BCG, especially in BCG-refractory patients. Also, we believe this is the first time to examine the two commonly used BCG strains in one study. Although the timing of BCG initiation was slightly different, both strains of high-dose BCG showed highly effective antitumor activity. Animals treated with high concentrations of gemcitabine (10–20 mg/ml) could not tolerate beyond 2 instillations and died from drug related toxicities. These toxicities may be attributable to an acidic chemical effect on the thin bladder wall at high drug concentration (pH of ~3.0) and systemic absorption of the drug. Absorption of gemcitabine (molecular weight, 299.66) may be facilitated by vesicoureteral reflux to kidneys in our rat model.[29] Systemic absorption in dogs after instillation has also been reported.[30] Fortunately, absorption of gemcitabine following instillation in humans appears to be limited,[28,31] which has allowed for dose escalation to facilitate a concentration gradient driven tissue penetration in human bladders. Contrary to our hypothesis, combining high-dose BCG with gemcitabine or IFN-α did not further increase their antitumor efficacy over monotherapy of either agent. It seems that a maximal antitumor activity of the monotherapy has been reached and there is limited room for further improvement from combining them in our model system. If this is the case, then rational combination therapy is more suitable for low-dose BCG

plus gemcitabine or Th1 cytokines in the treatment of early stage bladder cancer.

Conclusions

Based on the study in an orthotopic rat bladder tumor model, the antitumor efficacy of BCG is dose-dependent. IL-2 enhanced low-dose BCG's efficacy to the level of high-dose BCG treatment. Gemcitabine and IFN-α monotherapy showed similar efficacy to high-dose BCG and are promising intravesical agents for treatment of early stage UCB. These alternative and combinatory strategies warrant further exploration in patients with non-muscle invasive UCB, especially in those with BCG-refractory diseases.

Abbreviations

BCG, bacillus Calmette-Guérin; IFN-α, interferon-α; IL-2, interleukin-2; Gemzar, gemcitabine; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TURBT, transurethral resection of bladder tumor; UCB, urothelial cancer of the bladder; UCC, urothelial cell carcinoma.

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Disclosure

Author(s) have provided signed confirmations to the publisher of their compliance with all applicable legal and ethical obligations in respect to declaration of conflicts of interest, funding, authorship and contributorship, and compliance with ethical requirements in respect to treatment of human and animal test subjects. If this article contains identifiable human subject(s) author(s) were required to supply signed patient consent prior to publication. Author(s) have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have consent to reproduce any copyrighted material. The peer reviewers declared no conflicts of interest.

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