Antiandrogen Therapy with Hydroxyflutamide or Androgen Receptor Degradation Enhancer ASC-J9 Enhances BCG Efficacy to Better Suppress Bladder Cancer Progression

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Abstract

Recent studies suggest that the androgen receptor (AR) might play important roles in influencing bladder cancer progression, yet its clinical application remains unclear. Here, we developed a new combined therapy with Bacillus Calmette–Guerin (BCG) and the AR degradation enhancer ASC-J9 or antiandrogen hydroxyflutamide (HF) to better suppress bladder cancer progression. Mechanism dissection revealed that ASC-J9 treatment enhanced BCG efficacy to suppress bladder cancer cell proliferation via increasing the recruitment of monocytes/macrophages that involved the promotion of BCG attachment/internalization to the bladder cancer cells through increased integrin-α5β1 expression and IL6 release. Such consequences might then enhance BCG-induced bladder cancer cell death via increased TNFα release. Interestingly, we also found that ASC-J9 treatment could directly promote BCG-induced HMGB1 release to enhance the BCG cytotoxic effects for suppression of bladder cancer cell growth. In vivo approaches also concluded that ASC-J9 could enhance the efficacy of BCG to better suppress bladder cancer progression in BBN-induced bladder cancer mouse models. Together, these results suggest that the newly developed therapy combining BCG plus ASC-J9 may become a novel therapy to better suppress bladder cancer progression. Mol Cancer Ther; 14(11): 1–9. ©2015 AACR.

Introduction

Bladder cancer has a high mortality with an estimated 74,690 new cases and 15,580 deaths in United States in 2014 (1). Most bladder cancer patients receive the complete transurethral resection of their tumor followed by intravesical instillation of anti-tumor agents, including Bacillus Calmette–Guerin (BCG) that represents a very successful adjuvant agent for treating noninvasive bladder cancer (2, 3). However, the BCG therapy still has its limitations: Approximately 30% of patients treated with intravesical BCG failed to respond, and for those patients with initial positive response, many suffered the high rate of recurrence (4). Recently, significant progress has been made toward understanding the mechanism of BCG antitumor response. With respect to the local response to BCG, accumulating data support a critical role for BCG–tumor interaction in initiating and directing the downstream antitumor response. By the combination of local effects and a systemic immune response, BCG therapy might have a remarkable antitumor effect.

Interestingly, a recent study suggested the potential linkage of androgen receptor (AR) with bladder cancer initiation and progression (5), and another study also indicated that androgens might be able to modulate IL6 to alter BCG interaction with bladder cancer cells (6).

Here, we found a new potential therapy with combination of BCG and antiandrogen hydroxyflutamide (HF) or AR degradation enhancer ASC-J9 better suppressed bladder cancer progression (7–9).

Materials and Methods

Reagents

The compound ASC-J9 was a gift from AndroScience Corp. BCG, a lyophilized preparation containing 10.5 ± 8.7 × 10⁸ colony-forming units (CFU), was resuspended in PBS. HF was obtained from Sigma. Polyclonal antibodies against F4/80 and integrin-α5β1 were from Abcam and bromodeoxyuridine (BrdUrd) was from BD Biosciences. The liquid DAB+ substrate chromogen system horseradish peroxidase used for immunocytochemistry was obtained from Dako Cytomation. Penicillin,
streptomycin, all media, and FBS were obtained from Invitrogen. Tris, glycite, NaCl, SDS, BSA, and monoclonal antibody against β-actin were obtained from Sigma.

Cell lines and culture conditions

The T24 cell line was a gift from Dr. Timothy Ratliff (Department of Urology, University of Iowa, Iowa City, IA) in 2009 and cultured in Hyclone McCoy’s 5A media supplemented with 10% FBS. The human bladder cancer cell line 253J was generously provided by Dr. Colin P.N. Dinney, The University of Texas (Houston, TX) in 2010 and cultured in T media supplemented with 10% FBS. The MB49 cell line was a gift from Dr. Ron Wood, University of Rochester Medical Center (Rochester, NY) in 2009. The human THP-1 cell line was obtained from the ATCC in September 10, 2009, and cultured in RPMI-1640 supplemented with 10% FBS, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin. All cell lines were authenticated and tested by PCR when we received them, and then were expanded and frozen down in several aliquots. Each aliquot was thawed and used for no more than 6 months.

Animals

FVB female mouse were obtained from The Jackson Laboratory. The animals were housed four per cage in a specific pathogenic-free animal facility and fed with regular chow diet with water ad libitum. Animal protocols/usage was approved by the University of Rochester Committee on Animal Resources, and the mice were kept in a specific pathogen-free environment at the animal facilities of the University of Rochester Medical Center.

DNA extraction, RNA extraction, RT-PCR, and quantitative real-time PCR

For RT-PCR, 1 μg of total RNAs was reverse-transcribed using the iScript Synthesis Kit (Bio-Rad Laboratories), according to the manufacturer’s protocol. The sequence of primers in the PCR RT-PCR and qPCR were as followed: Human β-actin: 5’-ATCTTGGCACCACTTCTTAT3’ (sense) and 5’-CGTATACCTCGTCTGC’-3’ (antisense); human GAPDH: 5’-GCTCTCACAGATCTATCC-3’ (sense) and 5’-TGGTACCACCCTTCTTG-3’ (antisense); human integrin-α5: 5’-CGCTTGGCCTCGTCTGATACTGC’-3’ (sense) and 5’-GTGGCCGGCCTTAATATGAC’-3’ (antisense); human integrin-β1: 5’-TTATGCTGCTGAGTACTGCTT3’ (sense) and 5’-CACCAGTCTGGCAGCACCCTCTAT3’ (antisense); human IL-6: 5’-AAAGCCAGCAGCTGAGATGTA3’ (sense) and 5’-TGGTCCCATCGACTGGTC-3’ (antisense).

MTT assay

The antiproliferative effects of HF and ASC-J9 on BCG against bladder cancer cell lines 253J and T24 were determined by the MTT dye uptake method.

BCG attachment and internalization assay

We used PCR to detect BCG internalization and attachment to bladder cancer cells. After incubation and washing out nonattached, excess BCG, the cells monolayer was washed twice with Hanks BSS (Gibco). Cells were then harvested using Cell Disassociation Solution (Sigma). Genomic DNA was extracted according to procedure of the Dneasy Blood and Tissue Kit (Qiagen). The bladder cancer cell lines were each plated at a density of 10^5 cells per well in 6-well plates (Costar) and pretreated with 5 μmol/L ASC-J9 or HF, then incubated at 37°C in complete culture media overnight. The following day 5 × 10^5 CFU BCG was added to each well, and placed at 37°C for 2 hours. At the time points indicated, the culture media with nonattached BCG was aspirated and the cell monolayer washed twice with Hanks BSS (Gibco). Cells were then harvested using Cell Disassociation Solution (Sigma). Parental 253J and T24 cells were incubated with a concentration of 50 CFU BCG per cell for 2 hours and compared with the negative threshold. Tests were performed in duplicate and repeated on a subsequent day.

Migration assay

A total of 5 × 10^4 bladder cancer cells were seeded into the bottom well (5-μm pore size Transwell; Corning Incorporated) treated with HF and ASC-J9 for 12 hours and treated with BCG for 2 hours. Then the excess BCG was removed and 4 × 10^3 THP-1 cells placed into the upper Transwell, incubated for 2 hours, then we collected the THP-1 cells migrated into the bottom wells for counting.

N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)–induced mouse bladder cancer model

Twelve-weeks-old FVB female mice (The Jackson Laboratory) were supplied ad libitum with sterile water containing 0.05% BBN (TCl America) in opaque bottles for 12 weeks, and thereafter with sterile water without BBN. The drinking water was prepared fresh twice a week, and consumption was recorded to estimate BBN intake. Then mice were divided into 4 groups (10 mice/group) as soon as urine blood tested positive, and treated with (i) ETOH-control, (ii) BCG (2 × 10^5 CFU/mouse, intravesical injection weekly) alone, (iii) ASC-J9 (75 mg/kg body weight, every other day, IP) alone, and (iv) BCG (2 × 10^5 CFU/mouse, intravesical injection weekly)+ASC-J9 (75 mg/kg body weight, every other day, IP). Mice were then sacrificed 48 hours after the fourth treatment and bladders were collected for further examination.

Immunohistochemical analysis for F4/80, and BrdUrd in tumor tissues

The expressions of F4/80 and BrdUrd were evaluated using an immunohistochemical method described previously (10). The antibodies used were anti-F4/80 (Abcam) and anti-BrdUrd (BD Biosciences). Results were expressed as the percentage ± SE of positive cells per ×200 magnification field. A total of 5 × 200 fields was examined and counted from each group.

Statistical analysis

Data are presented as mean ± SEM of at least three independent biologic replicates. The values were compared using a Student t test (two-tailed). We considered a P value inferior to 0.05 as significant.

Results

ASC-J9 and HF enhance BCG attachment/internalization to better suppress bladder cancer cells

Early reports suggested that BCG was able to function through attachment/internalization into bladder cancer urothelial cells to alter the immune responses to exert its chemotherapeutic effect (11–13). Other studies also indicated that AR might play important roles to influence bladder cancer progression (5). We
ASC-J9 and HF treatment increases IL6 expression to enhance the recruitment of monocytes/macrophages to bladder cancer cells

To study the consequences after enhanced BCG attachment/internalization to bladder cancer urothelial cells following treatment with HF or ASC-J9, we then applied the coculture system to examine whether ASC-J9 or HF could affect BCG-induced immune responses in bladder cancer cells as early reports suggested that BCG-suppressed bladder cancer progression was linked to the recruitment of immune cells, including monocytes/macrophages (15). We seeded T24 (or 253J) cells in the bottom chambers and monocytes/macrophage THP-1 cells on the top chambers (Fig. 2A), and cocultured cells were treated with BCG, or BCG with HF or ASC-J9 for 2 hours. As shown in Fig. 2B and C, addition of BCG increased THP-1 cells migration to bladder cancer cells, and addition of HF or ASC-J9 further increased significantly the THP-1 cells migration to bladder cancer cells.

To dissect the mechanisms at the molecular level by which HF or ASC-J9 could enhance BCG efficacy to promote monocytes/macrophages migration toward bladder cancer cells, we then examined the altered immune cytokines expression and found that the expression of IL6 increased after coculture of THP-1 cells with T24 or 253J cells, and the addition of HF or ASC-J9 further enhanced IL6 expression in both T24 and 253J cells (Fig. 2C–G). These results were in agreement with an early report showing that IL6 promotes monocytes/macrophages migration to bladder cancer cells (16).

Together, results from Fig. 2A–G proved that HF or ASC-J9 could enhance IL6 expression in the bladder cancer cells that
recruits more monocytes/macrophages to the bladder cancer cells.

More recruited monocytes/macrophages to bladder cancer cells leads to more TNFα secretion to kill more bladder cancer cells

We then asked what are the impacts of recruiting more monocytes/macrophages to bladder cancer cells. Early studies suggested that BCG might function through recruitment of macrophages to suppress bladder cancer that involved the release of soluble cytotoxic factors, including TNFα, IFNγ, and nitrous oxide (17). We first demonstrated that addition of BCG to the THP-1 cells released more TNFα compared with THP-1 only and importantly, adding ASC-J9 or HF released even more TNFα compared with BCG only (Fig. 3A). Importantly, an interruption approach with addition of anti-TNFα antibody also interrupted monocytes/macrophages/BCG/ASC-J9–induced bladder cancer cell viability (Fig. 3B and C).

Together, results from Fig. 3A–C suggest that HF- or ASC-J9–enhanced BCG efficacy to suppress more bladder cancer cells might function through recruiting more monocytes/macrophages that involve the increase of TNFα release.

**ASC-J9 or HF enhances BCG efficacy to suppress bladder cancer progression via high-mobility group box 1 release**

In addition to enhancing BCG efficacy via recruitment of more monocytes/macrophages to suppress bladder cancer cells, we also examined the potential BCG direct intracellular cytotoxic effect after attachment/internalization to bladder cancer urothelial cells. We first demonstrated that without coculture with THP-1 cells, BCG alone (for 2 hours in every 48 hours) could suppress bladder cancer cell proliferation in four different bladder cancer cell lines.
(3 human cell lines, TCCSUP, T24, and 253J and 1 mouse cell line, MB49), and that BCG plus 5 \( \text{mmol/L} \) HF or 5 \( \text{mmol/L} \) ASC-J9 treatment further suppressed cell proliferation significantly (Fig. 4A–D).

To dissect the potential mechanism, we examined high-mobility group box 1 (HMGB1) expression, as an early report suggested that HMGB1 release from bladder cancer could function as a paracrine factor to potentiate the direct cellular effects of BCG (18). Our results suggested that addition of BCG increased HMGB1 release from bladder cancer cells and addition of HF or ASC-J9 further enhanced BCG-induced HMGB1 release (Fig. 4E).

Together, results from Fig. 4A–E demonstrated that HF or ASC-J9 could also enhance the BCG intracellular efficacy to suppress bladder cancer cell proliferation that might involve the alteration of the HMGB1 release.

ASC-J9 enhances BCG efficacy to suppress bladder cancer development in BBN-induced mouse bladder cancer model

We then applied the bladder cancer mouse model to prove our above in vitro cell lines findings. Twelve-weeks-old FVB female mice were divided into 4 groups (10 mice/group), treated with BBN (0.05% in drinking water) for 12 weeks. Mice were then injected with (i) vehicle control, (ii) BCG alone (2 \( \times \) 10^6 CFU/mouse, intravesical injection weekly for 28 days), (iii) ASC-J9 alone (75 mg/kg body weight, every other day for 28 days, IP), or (iv) BCG (2 \( \times \) 10^6 CFU/mouse, intravesical injection weekly for 28 days)+ASC-J9 (75 mg/kg body weight, every other day for 28 days, IP). Mice were then sacrificed 24 hours after the fourth injection and bladders collected for further examination.

Using hematoxylin and eosin (H&E) staining (Fig. 5A), we found mice receiving vehicle injection developed bladder papilloma and carcinoma in situ, and BCG-treated mice developed less hyperplasia with little carcinoma. Importantly, mice injected with both BCG plus ASC-J9 developed little hyperplasia, suggesting that ASC-J9 indeed enhanced BCG efficacy to prevent bladder cancer development in the BBN-induced bladder cancer mouse model.

We also applied BrdUrd staining to assay the proliferation in these BBN-induced bladder cancer mouse models and found mice treated with BCG alone or ASC-J9 alone had less BrdUrd staining than those mice receiving vehicle control, and mice treated with both BCG and ASC-J9 had the least BrdUrd staining (Fig. 5B and D), which again confirmed the above findings showing ASC-J9 could enhance BCG efficacy to suppress bladder cancer cell proliferation in the BBN-induced bladder cancer mouse model.

Importantly, we examined the recruitment of monocytes/macrophages in this BBN-induced bladder cancer mouse model with antimacrophage F4/80 antibody and found that BCG alone helps to recruit more monocytes/macrophages to bladder cancer, and BCG plus ASC-J9 treatment recruited many more monocytes/macrophages to bladder cancer (Fig. 5C and E), which is in agreement with above in vitro coculture system (Fig. 2) showing ASC-J9 could
enhance BCG to recruit more monocytes/macrophages to bladder cancer cells.

Together, results from the BBN-induced bladder cancer mouse model (Fig. 5C–E) clearly demonstrated that ASC-J9 could enhance BCG efficacy to suppress bladder cancer development.

**Discussion**

BCG therapy has been used successfully to treat nonmuscle invasive bladder cancer since 1976 (2, 19, 20). However, even though BCG is quite effective, approximately 30% of bladder cancer patients treated with intravesical BCG fail to respond (6), and many patients have higher rates of recurrence and/or progression within 5 years (21, 22). How to improve the BCG efficacy to reduce the recurrence of bladder cancer is, therefore, clinically important and essential to extend the bladder cancer patients survival.

Understanding the mechanism of BCG function is critical for improving the efficacy of therapy. Presently, it is generally assumed that the BCG-induced antitumor activity is critically dominated by a local nonspecific immunologic reaction reflecting the activity of immunocompetent cells. After instillation, the BCG binds to fibronectin that is expressed on the urothelium, which could then be internalized by the urothelial cells, including cancer cells (23). Subsequently, BCG antigens can be presented at the cell surfaces of urothelial cells in the context of MHC class II, which may then stimulate the CD4+ T cells and induce a primary T helper type 1 immune response (24). This complex and robust immune reaction evoked by BCG is evidenced by a massive transient secretion of cytokines in voided urine, including IL1, IL2, IL5, IL6, IL8, IL10, IL12, IL15, and IL18, as well as interference of inducible protein (IP)-10, TNFα, granulocyte–monocyte colony-stimulating factor (GMCSF), and IFNγ (25). The final step in the eradication of bladder cancer cells is the activation of cytotoxic...
effector cells, such as the natural killer cells, monocyte/macrophage cells, and other innate immune cells (26, 27).

The linkage of androgen/AR signals to alter the BCG efficacy, however, remains unclear, and few studies suggest that targeting AR could be an alternative therapy to suppress bladder cancer progression. Using an ARKO mouse model, Miyamoto and colleagues (5) found that androgen/AR signals might play important roles to promote bladder cancer development/progression, and targeting AR with ASC-J9 suppressed bladder cancer development/progression. See and colleagues also reported that one of the cytokines elicited in response to BCG, specifically IL6, upregulates the cellular expression of integrin-α5β1, the receptor complex on which BCG adherence depends (4). They further confirmed that androgen could downregulate NF-κB–mediated IL6 expression by human TCC lines (6). These studies suggest that alteration of the autocrine IL6 response to BCG via pharmacologic manipulation of the androgen milieu may have therapeutic value for the BCG antitumor treatment efficacy for transitional carcinoma.

In this article, we used two methods to block androgen/AR signaling. One is the antiandrogen compound, HF, which is an active metabolite of flutamide approved by the FDA and inhibits androgen/AR signals by blocking androgen binding to AR. On the other hand, we used ASC-J9 to inhibit androgen/AR signaling by degrading AR in T24 and 253J cells (Supplementary Fig. S1A and S1B). On the basis of the clinical BCG intravesical therapy guidelines and a previously published article, we used BCG only or combined with ASC-J9 to treat bladder cancer cells or intravesical therapy in the BBN-induced bladder cancer mouse model both for 2 hours (28), and the results showed that the combination of BCG and HF or ASC-J9 better suppressed bladder cancer progression.

Importantly, we proved here that ASC-J9 and HF could also enhance the BCG therapeutic efficacy to suppress bladder cancer cell proliferation in the absence of infiltrating monocytes/macrophages (Fig. 4). Mechanism dissection suggested that BCG combined with HF or ASC-J9 induced more HMGB1 release from bladder cancer cells compared with BCG only. These results are in agreement with recent studies showing that HMGB1 release from bladder cancer after BCG treatment could be one of the direct cellular effects of BCG.

Interestingly, in addition to the direct effect, other indirect mechanisms also indicate that BCG could enter into bladder

**Figure 5.**

ASC-J9 potentiates the antitumor effects of BCG in BBN-induced mouse bladder cancer model. Twelve-week-old FVB female mice were divided into 4 groups (10 mice/group) and treated with 0.05% BBN in drinking water for 12 weeks. Mice were then injected with (i) vehicle control, (ii) BCG (2 × 10⁶ CFU/mouse, intravesical injection weekly) alone, (iii) ASC-J9 (75 mg/kg body weight, every other day, IP) alone, or (iv) BCG (2 × 10⁶ CFU/mouse, intravesical injection weekly) + ASC-J9 (75 mg/kg body weight, every other day, IP). Mice were then sacrificed 48 hours after the fourth injection and bladders collected for further examination, including H&E section (A) and BrdUrd stain (B) for the bladder cancer cell proliferation. C, we used monocytes/macrophages marker F4/80 antibody to assay monocyte/macrophage cells infiltration. D and E, the positive expression of BrdUrd and F4/80 were quantified in three random fields. Blue arrows, positive cells; *, P < 0.05 (Student t test).
cancer via adherence to the luminal surface of the bladder to promote immunologic responses that involve the recruitment of the infiltrating monocytes/macrophages to suppress bladder cancer (29). Importantly, using qPCR to assay the M1- versus M2-type of macrophage (30) during BCG treatment, we further found that BCG could enhance the recruitment of M1-type of macrophage that led to suppress bladder cancer cell proliferation (Supplementary Fig. S2.). This result is in agreement with early studies showing that infiltrated M1-type macrophages may suppress tumor cells (31, 32).

ASC-J9 could enhance BCG immunotherapeutic effects via alteration of those key factors, including IL6, integrin-α5β1, and TNFα, which is also in agreement in early studies showing ASC-J9 had high efficacy to suppress other AR-related tumors, including prostate, kidney, and liver (8, 33–37). We also found similar effects when we replaced ASC-J9 with HF. This result not only further supports the above findings showing that targeting androgen/AR signals can enhance BCG efficacy to suppress bladder cancer, but may also provide a much easier and quicker therapeutic approach to develop a new combined therapy of BCG with HF. In contrast, more time and effort will be needed in the future to see whether ASC-J9 can also pass human clinical trials to translate its in vitro and in vivo mouse effects into humans. In summary, our findings showing the combined therapy of BCG with HF or ASC-J9 can lead to better therapy than BCG alone to suppress bladder cancer progression may help patients to better battle bladder cancer in the near future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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ASC-J9 was patented by the University of Rochester, University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C.C. own royalties and equity in AndroScience.

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