Androgen Receptor Signaling Reduces the Efficacy of Bacillus Calmette-Guérin Therapy for Bladder Cancer via Modulating Rab27b-Induced Exocytosis

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ABSTRACT

Although intravesical bacillus Calmette-Guérin (BCG) immunotherapy has been the gold standard for nonsurgical management of non–muscle-invasive bladder cancer, a considerable number of patients exhibit resistance to the adjuvant treatment with unexplained mechanisms. This study aimed to investigate whether and how androgen receptor (AR) signals modulate BCG cytotoxicity in bladder cancer. AR knockdown or overexpression in bladder cancer lines resulted in induction or reduction, respectively, in intracellular BCG quantity and its cytotoxic activity. Microarray screening identified Rab27b, a small GTPase known to mediate bacterial exocytosis, which was upregulated in BCG-resistant cells and downregulated in AR-shRNA cells. Knockdown of Rab27b, or its effector SYTL3, or overexpression of Rab27b also induced or reduced, respectively, BCG quantity and cytotoxicity. In addition, treatment with GW4869, which was previously shown to inhibit Rab27b-dependent secretion, induced them and reduced Rab27b expression in bladder cancer cells. Meanwhile, AR expression was upregulated in BCG-resistant lines, compared with responsive controls. In a mouse orthotopic xenograft model, Rab27b/SYTL3 knockdown or GW4869 treatment enhanced the amount of BCG within tumors and its suppressive effect on tumor growth. Moreover, in non–muscle-invasive bladder cancer specimens from patients subsequently undergoing BCG therapy, positivity of AR/Rab27b expression was associated with significantly higher risks of tumor recurrence. AR activation thus correlates with resistance to BCG treatment, presumably via upregulating Rab27b expression. Mechanically, it is suggested that BCG elimination from urothelial cells is induced by Rab27b/SYTL3-mediated exocytosis. Accordingly, Rab27b inactivation, potentially via antiandrogenic drugs and/or exocytosis inhibition are anticipated to sensitize the efficacy of BCG therapy, especially in patients with BCG-refractory AR/Rab27b-positive bladder cancer.

Introduction

Urinary bladder cancer is one of the commonly diagnosed malignancies, especially in males, with an estimation of approximately 549,000 new cases and 200,000 deaths per year in the world (1). Of these new cases, approximately 70% to 75% is diagnosed in an early stage as non–muscle-invasive bladder cancer. Meanwhile, bacillus Calmette-Guérin (BCG), attenuated bacterial strains derived from Mycobacterium bovis, has been used as not only a vaccine primarily against tuberculosis but also immunotherapy of cancers for many years (2–4). Specifically, BCG has been administered intravesically for the treatment of urothelial carcinoma in situ or the prevention of disease recurrence in patients with non–muscle-invasive bladder cancer after transurethral surgery (4–6). Injected BCG into the bladder can be attached to the cell membrane and internalized by the cancer cells, followed by exhibiting anti-tumor activity directly by the bacteria as well as indirectly by host immune activation (6–8). However, a significant number of patients with non–muscle-invasive bladder cancer fail to have a successful response to intravesical BCG immunotherapy. Of note, several studies have demonstrated significantly lower response rates of BCG therapy in male patients than in females (9, 10), although controversial results exist. Moreover, little is known about the mechanisms for resistance to BCG therapy and molecular markers that predict its efficacy.

Androgen receptor (AR), a ligand-inducible transcription factor, belongs to the steroid hormone receptor superfamily that is known to possess a wide variety of functions, with cooperation of transcriptional coregulators, in nonneoplastic and neoplastic cells (11, 12). Importantly, AR signaling has been proved to contribute to urothelial cancer progression (reviewed in ref. 13), as well as chemoresistance (14, 15) and radioresistance (16) in bladder cancer. Thus, antiandrogens inhibit the growth of bladder cancer cells and enhance the cytotoxic effects of chemotherapeutic agents or irradiation. In addition, in a study by Shang and colleagues (17), AR antagonists were shown to increase the expression level of BCG-induced integrins (e.g., α5β1) and intake of BCG in bladder cancer cells, as well as recruitment of monocytes/macrophages, and treatment with one, together with BCG, could more strongly inhibit cell proliferation compared with that with
BCG alone. It has also been documented that dihydrotestosterone downregulates BCG-mediated IL6 expression in bladder cancer cells (18). These findings imply the involvement of AR signaling in modulating sensitivity to BCG therapy in bladder cancer cells. However, it has never been clearly demonstrated whether and how AR activity correlates with cytotoxicity of BCG in bladder cancer cells.

Rab27, a member of the small GTPase Rab family, is known to regulate various types of secretion (19) in not only traditional secretory cells, such as pituitary cells (20) and parotid acinar cells (21), but also others including Madin–Darby canine kidney cells (22) and cervical cancer HeLa cells (23). It has been documented in bladder urothelial cells that most of Escherichia coli (E. coli) are housed in vesicles enriched in an isoform of Rab27, Rab27b (24), which may result in enhancing the exocytosis of intracellular bacteria. Rab27b may thus play an important role in protecting urothelial cells from bacterial infection via mediating exocytosis. Rab27b has also been reported to be over-expressed in several types of malignancies, including bladder cancer where its overexpression is associated with poor patient outcomes (25). In addition, in nonurothelial cancer cells, Rab27b has been suggested to contribute to inducing tumor progression by exocytosis of vesicles containing growth regulators or other components necessary to cancer growth (26, 27).

The current study primarily aimed to investigate how bladder cancer cells show resistance to BCG therapy in relation to AR activity. We found that elimination of BCG form bladder cancer cells could be modulated via the AR-Rab27b pathway and that AR and Rab27b served as prognosticators by predicting response to intravesical BCG therapy.

Materials and Methods

Cell culture

Human bladder cancer cell lines, UMUC3, TCCSUP, and 5637, were originally obtained from the ATCC. All these lines were recently authenticated, using GenePrint 10 System (Promega). A murine bladder cancer cell line MB49 was a gift from Dr. Armine Smith (Johns Hopkins University School of Medicine, Baltimore, MD). Stable sublines, including UMUC3-control-short hairpin RNA (shRNA)/UMUC3-AR-shRNA and 5637-vector/5637-AR, were established in our previous study (28). Similarly, conditional or lentivirus generated from the transfected 293T cells as well as BCG by PureLink Genomic DNA Mini Kit (Invitrogen) were originally obtained from the ATCC. All these lines were recently authenticated, using GenePrint 10 System (Promega). A murine bladder cancer cell line MB49 was a gift from Dr. Armine Smith (Johns Hopkins University School of Medicine, Baltimore, MD). Stable sublines, including UMUC3-control-short hairpin RNA (shRNA)/UMUC3-AR-shRNA and 5637-vector/5637-AR, were established in our previous study (28). Similarly, conditional or lentivirus generated from the transfected 293T cells as well as BCG by PureLink Genomic DNA Mini Kit (Invitrogen) were originally obtained from the ATCC. All these lines were recently authenticated, using GenePrint 10 System (Promega). A murine bladder cancer cell line MB49 was a gift from Dr. Armine Smith (Johns Hopkins University School of Medicine, Baltimore, MD).

Chemicals and primary antibodies

BCG (TICE BCG; 1 × 10^6 CFU/50 mg) was obtained from Merck. Although the exact unit in each vial (considered as 5 × 10^6 CFU in this study) was thus not specified, we prepared aliquots of frozen stocks (50 mg of bacteria in a vial diluted in 5 mL of DMEM with 10% FBS and 20% glycerol as a final concentration of 1 × 10^6 CFU/mL) and used the same aliquot for a series of experiments. DHT, hydroxyflutamide (HF), and doxycycline were from Sigma-Aldrich, R1881 was from PerkinElmer, and GW4869 (hydrochloride hydrate; ref. 31) was from Cayman Chemical. Anti-AR (N-20), anti-GAPDH (6c5), anti-integrin-α5 (A-11), anti-integrin-β1 (IR1B), anti-Paki (A-6), anti-MEK1 (H-8), anti-phospho-MEK1 (p-MEK1; Ser298; B-4), and anti-Rab27a (E12A-1) antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Pak1 (p-Pak1; Ser144), anti-Rab27b (134121), and anti-SYT1 (PA5-24291) antibodies were purchased from Cell Signaling Technology, Thermo Fisher Scientific, and Proteintech, respectively.

Western blot analysis

Equal amounts of proteins (30 μg) obtained from cell extracts were subjected to electrophoresis with 10% SDS-PAGE, which was transferred to polyvinylidene difluoride membrane electrophoretically. After blocking with 0.03% Blotting-Grade Blocker (Bio-Rad), the membrane was incubated with a primary antibody at 4°C overnight, followed by 1-hour incubation with a HRP-conjugated secondary antibody (Cell Signaling Technology) at room temperature. Chemiluminescent signals were generated by Clarity western ECL substrate and detected by ChemiDOC MP (Bio-Rad).

BCG quantification by PCR

Cells (1 × 10^6) were subcultured in 24-well plate one day before BCG treatment, cocultured with BCG (1 × 10^6 CFU) for 5 hours at 37°C, and washed with PBS three times to remove BCG outside the cells or floating in media. Genomic DNA (10 ng) extracted from the cells as well as BCG by PureLink Genomic DNA Mini Kit (Invitrogen) was subjected to PCR, using OneTag DNA Polymerase (New England Biolabs), in T100 Thermal Cycler (Bio-Rad) at 95°C for 3 minutes, followed by 35 cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 68°C for 30 seconds. The primer sequences for BCG and GAPDH are given in Supplementary Table S1. PCR products were electrophoresed with 3% agarose in TBE containing 0.002% ethidium bromide, and signals were detected by ChemiDOC MP.

BCG quantification by immunofluorescence

BCG was labeled by FITC as described previously (32). Briefly, stocked BCG was diluted in 10 mL of DMEM and upper 6 mL of the dilution was taken after removing aggregated BCG by centrifuge at 12,000 × g for 10 minutes and incubated with 0.3 mg/mL FITC (Sigma–Aldrich) at room temperature for 30 minutes in the buffer (50 mmol/L Na2CO3 and 100 mmol/L NaCl at pH 9.2). To remove unconjugated FITC, the labeled BCG was washed with PBS and collected as pellet by centrifuge at 15,000 rpm for 10 minutes three times. Each cell line was then cocultured for 5 hours with the
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BCG suspended in DMEM at a concentration of 10 CFU/cell. The BCG-treated cells were washed with PBS three times and fixed with 10% formalin at room temperature for 8 minutes. For BCG quantification, the fixed slides were stained with 4',6-diamidino-2-phenylindole (DAPI) at 0.05 μg/mL in 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.1% (v/v) Tween-20 (TBST) at room temperature for one hour. The rate for BCG positivity was calculated as follows: FITC-labeled BCG-positive cell numbers were divided by total cell numbers from DAPI staining, followed by normalization by each control sample.

The fixed slides were permeabilized in TBST for 10 minutes and blocked with 0.3% BSA in TBST at room temperature for one hour, followed by incubation with a primary antibody in 0.3% BSA/TBST at 4°C overnight. Then, the slides were treated with a secondary antibody (Alexa 488 goat anti-rabbit Ig antibody) with 0.05 μg/mL DAPI in 0.3% BSA/TBST at room temperature for one hour. Each slide was embedded in Fluorescent Mounting Media (VWR) and observed under the immunofluorescence microscope (BX41, Olympus).

Cell proliferation

We used the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay to assess cell viability. Cells (3–4 × 10⁵/well) seeded in 96-well tissue culture plates were cocultured with or without BCG (10 CFU/cell) for 72 hours, washed with PBS, and then incubated in medium containing 0.5 mg/mL of MTT (Sigma-Aldrich) for 2 hours at 37°C. The media were replaced by DMSO, and the absorbance at 570 nm was measured.

DNA microarray

RNAs extracted from UMUC3, UMUC3-BCG-R, and UMUC3-AR-shRNA were subjected to microarray gene expression analysis at Johns Hopkins Microarray Core Facility, using GeneChip Human Gene 2.0 ST Array (Affymetrix). Scanned fluorescence signals were converted to continuous values by the Gene Expression Console software (Affymetrix).

Screening was performed to identify genes that meet the following criteria: (i) those with absolute high signals (i.e., top 20% of all probes); (ii) those upregulated (i.e., >2-fold increase) in UMUC3-BCG-R compared with UMUC3; and (iii) those downregulated (i.e., >70% decrease) in UMUC3-AR-shRNA compared with UMUC3.

Real-time PCR

Total RNA extracted from cultured cells, using TRIzol (Invitrogen), was reverse transcribed with Omniscript RT Kit (Qiagen) and Oligo- dT Primers (Qiagen). Real-time PCR was then performed, using IQ SYBR Green Supermix (Bio-Rad), in C1000 Thermal Cycler. The primer sequences are given in Supplementary Table S1.

Mouse orthotopic xenograft model

The animal protocol in accordance with the NIH Guidelines for the Care and Use of Experimental Animals was approved by the Institutional Animal Care and Use Committee.

MB49 cells (1 × 10⁶) suspended in 50 μL of DMEM containing 10% FBS were implanted into the bladder of 6- to 8-week-old female C57BL/6 mice (Charles River Laboratories) following a 15-minute intravesical pretreatment with 80 μL of 0.125% trypsin injected transurethrally via a 24-gauge catheter under anesthesia, as described previously (33). Then, 50 μL of DMEM with or without BCG (3 × 10⁶ CFU) was injected transurethrally into the bladder of the anesthetized mice. The animals were carefully monitored for health status and hematuria, and were euthanized for macroscopic/microscopic analyses of the bladder and other major organs when they lost >15% of body weight from the baseline, had severe gross hematuria, or showed difficulty in moving, eating, or drinking.

BCG quantification by acid-fast bacillus stain

MB49-control-shRNA, MB49-Rab27b-shRNA, or MB49-SYTL3-shRNA cells were injected into the mouse bladder, as described above. After 14 days, BCG (3 × 10⁶ CFU) in 50 μL of DMEM was injected to the bladder twice. The animals were euthanized to harvest the tumors 12 hours after initial BCG injection. These bladder tumor samples were fixed with 20% formalin and embedded in paraffin. The sections (4-μm thick) were stained with Ziehl–Neelsen carbol–fuchsin solution at 60°C for 1 hour. These samples were mounted with coverslips after washing out with 1% hydrochloric acid in 70% ethanol until turning light pink/stopping discoloration.

Immunohistochemistry (IHC) in surgical specimens

Upon appropriate approval from the Institutional Review Board (IRB), a total of 32 patients [26 males and 6 females; mean/median age 70.9/72 years (range: 47–81) who were histopathologically diagnosed as urothelial carcinoma in situ or high-grade urothelial carcinoma with or without lamina propria invasion in their bladder specimens obtained by transurethral surgery performed at Yokohama City University Medical Center (Yokohama, Japan) were subjected to assessment. The IRB also approved the request to waive the documentation of patient consent, and we were thus not required to obtain their signatures. In addition, this study was conducted in compliance with the Declaration of Helsinki and Belmont Report. All these patients without undergoing intravesical/systemic chemotherapy or radiotherapy prior to the collection of the tissues subsequently received 6 to 8 weekly intravesical instillations of BCG (Immunobacterium Intravesical 80 mg, Japan BCG Laboratory). The sections (4-μm thick) from the formalin-fixed paraffin-embedded tissues were probed with an anti-AR or anti-Rab27b antibody at 4°C overnight, followed by labeling with a secondary antibody and counterstaining, as we described previously (34). All stains were manually quantified by a single pathologist (H. Miyamoto) blinded to sample identity. AR or Rab27b expression was considered positive when weak or moderate/strong signals were seen in >10% or >1% of tumor cells, respectively.

Statistical analysis

Fisher exact test or χ² test was used to evaluate the associations between categorized variables. The numerical data were compared by Student t test. Survival rates in xenograft-bearing mice or patients with bladder cancer were calculated by the Kaplan–Meier method and statistically analyzed by the log-rank or Wilcoxon test. P values less than 0.05 were considered to be statistically significant.

Results

Reduction of cytotoxic activity of BCG by AR

To determine the impact of AR signaling on BCG cytotoxicity, we compared its effects between AR-positive and AR-negative/knockdown sublines (Fig. 1A). As BCG is internalized by bladder cancer cells to induce anticancer activity (6–8), we measured the amount of BCG by PCR (Fig. 1B) and the rate of BCG-positive cells by immunofluorescence (Fig. 1C; Supplementary Fig. S1A). AR knockdown or overexpression resulted in considerable increases or decreases, respectively, in the BCG amount/positive rate. MTT assay was then performed to assess direct cytotoxicity of BCG in AR-positive versus
AR-negative/knockdown cells (Fig. 1D) or AR-positive cells treated versus without a synthetic androgen R1881 (Supplementary Fig. S2). In this assay, the effects of AR expression or androgen treatment on cell viability, irrespective of BCG, were excluded by comparing with versus without BCG treatment in each subline/treatment. Correspondingly with intracellular BCG data, AR knockdown or AR overexpression/androgen treatment significantly enhanced or diminished, respectively, the cytotoxic effects of BCG. These findings confirmed that AR signaling did contribute to resistance to BCG therapy in bladder cancer cells.

Identification of Rab27b as a key molecule involving both BCG resistance and AR signaling

A BCG-resistant subline was established by coculturing with BCG over 6 months (Fig. 2A). Remarkably, AR expression was upregulated in UMUC3-BCG-R, compared with control cells (Fig. 2B). DNA microarray analysis in control UMUC3 versus UMUC3-BCG-R or UMUC3-AR-shRNA cells was then employed. Of those with absolute high signals, 20 genes met the criteria (i.e., upregulation in UMUC3-BCG-R and downregulation in UMUC3-AR-shRNA; Fig. 2C). A quantitative PCR was then performed to confirm the increase in the expression of candidate genes in UMUC3-BCG-R cells. Of 20 genes, the expression of six, including Rab27b showing the highest induction, was indeed upregulated (Fig. 2D). In Western blotting, the level of Rab27b protein expression was also considerably higher in UMUC3-BCG-R than in control UMUC3 where Rab27b was hardly detectable (Fig. 2B), as well as in AR-positive sublines than in AR-negative sublines (Fig. 2E). However, no upregulation of the expression of a Rab27b effector SYTL3 (19) in UMUC3-BCG-R, compared with control UMUC3, was observed (Fig. 2B). We thus decided to further investigate the role of Rab27b, as a potential AR-regulated molecule, in BCG resistance.
We next assessed the effect of androgens on the expression levels of Rab27b in bladder cancer cells. Consistent with Western blot analysis data, the basal level of Rab27b was significantly higher (approximately 5-fold) in UMUC3-BCG-R than in control UMUC3 (Fig. 2F). Moreover, DHT upregulated Rab27b expression in three AR-positive sublines. R1881 also induced the expression of Rab27b and AR proteins (Fig. 2G). In these experiments, androgen-induced upregulation of Rab27b mRNA/protein expression was antagonized by an antiandrogen HF. Thus, AR activity appeared to correlate with Rab27b expression in bladder cancer cells.

Reduction of cytotoxic activity of BCG by Rab27b
As described above (Fig. 2B), Rab27b expression was considerably upregulated in UMUC3-BCG-R cells (also see Fig. 3A), suggesting its involvement in BCG resistance. We therefore assessed the impact of Rab27b knockdown or overexpression in AR-positive bladder cancer cells on BCG cytotoxicity.

We first established sublines stably expressing Rab27b-shRNA (Fig. 3A; Supplementary Fig. S3A) and determined BCG quantity inside these cells via PCR and immunofluorescence. There was a decrease in the quantity in UMUC3-BCG-R, compared with control
cells, but it was at least partially restored (Fig. 3B and C) or otherwise increased (Supplementary Fig. S3B and S3C) in Rab27b knockdown cells. Correspondingly, Rab27b knockdown cells were more sensitive to BCG treatment in their viability (Fig. 3D; Supplementary Fig. S3D).

Again, the effects of Rab27b expression on cell viability, irrespective of BCG, were excluded by comparing with respective controls without BCG treatment, although Rab27b knockdown did not significantly change cell growth (Supplementary Fig. S4A). Conversely, when Rab27b was exogenously expressed in UMUC3 (Fig. 3E) or TCCSUP (Supplementary Fig. S3E), intracellular BCG (Fig. 3F and G; Supplementary Fig. S3F and S3G) or sensitivity to BCG treatment (Fig. 3H; Supplementary Fig. S3H) was reduced. In addition, when a

Figure 3. Rab27b and BCG cytotoxicity in bladder cancer cells. A, Western blot analysis of Rab27b (25 kDa) in control UMUC3, UMUC3-BCG-R, and UMUC3-BCG-R-Rab27b-shRNA. B, A semiquantitative PCR of BCG (334 bp) in control UMUC3, UMUC3-BCG-R, and UMUC3-BCG-R-Rab27b-shRNA sublines cocultured with BCG (10 CFU/cell) for 5 hours. C, Immunofluorescence of BCG in control UMUC3, UMUC3-BCG-R, and UMUC3-BCG-R-Rab27b-shRNA sublines cocultured with FITC-conjugated BCG (10 CFU/cell) for 5 hours. D, MTT assay in control UMUC3, UMUC3-BCG-R, and UMUC3-BCG-R-Rab27b-shRNA sublines cocultured with medium only (mock) or BCG (10 CFU/cell) for 72 hours. E, Western blot analysis of Rab27b (an additional higher molecular weight form for exogenous tagged protein) in UMUC3-vector and UMUC3-Rab27b. F, A semiquantitative PCR of BCG in UMUC3-vector and UMUC3-Rab27b sublines cocultured with BCG (10 CFU/cell) for 5 hours. G, Immunofluorescence of BCG in UMUC3-vector and UMUC3-Rab27b sublines cocultured with FITC-conjugated BCG (10 CFU/cell) for 5 hours. H, MTT assay in UMUC3-vector and UMUC3-Rab27b sublines cocultured with medium only (mock) or BCG (10 CFU/cell) for 72 hours. I, MB49-tetOn-Rab27b-shRNA cells were treated with BCG (10 CFU/cell) for 7 hours, washed with PBS three times, further cultured with/without doxycycline (Dox; 100 ng/mL) for 17 hours, and then subjected to Western blot analysis of Rab27b (J), PCR of BCG (K), and immunofluorescence of BCG (L). *, P < 0.05 (vs. control). In Western blot analysis/PCR, GAPDH (37 kDa/178bp) served as an internal control. In immunofluorescence, BCG positivity was estimated by counting at least 100 cells in each determinant. In MTT assay, cell viability is presented relative to that of each subline without BCG treatment. Each value represents the mean (±SD) from at least 3 determinants. *, P < 0.05 (vs. control).
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constitutive-negative mutant of Rab27b (Supplementary Fig. S5A) was expressed, no significant effects of the mutant on BCG quantity (Supplementary Fig. S5B and S5C) or BCG cytotoxicity (Supplementary Fig. S5D), compared with an empty vector, were seen. Rab27b overexpression (Supplementary Fig. S6A) or knockdown (Supplementary Fig. S6B) did not significantly affect the expression of AR, another Rab27 isoform Rab27a, or SYTL3. Meanwhile, in immunofluorescence experiments in UMUC3-derived sublines, it should be noted that the rate of BCG infection in UMUC3-control (for UMUC3-BCG-R subline; 22.2%; see Fig. 3C) was higher than those in other controls expressing control-shRNA, control-siRNA, or vector only (4.1%–11.3%).

We also determined the subcellular localization of BCG and Rab27b in bladder cancer cells, Rab27b is known to reside in intracellular vesicles related to molecular transportation and exocytosis (19, 23, 27). Immunofluorescence demonstrated colocalization of BCG with Rab27b (Supplementary Fig. S1B), suggesting that BCG is confined to Rab27b-positive vesicles in bladder cancer cells. To further elucidate whether Rab27b could enhance the elimination of BCG from urothelial cells following internalization, a doxycycline-inducible conditional knockout system was applied. MB49 cells stably expressing pLKO-tet-On-Rab27b-shRNA were cocultured with BCG, washed with PBS (to remove extracellular BCG), and then treated with doxycycline (to induce Rab27b silencing; Fig. 3J and J). The Rab27b knockdown subline had higher amounts of BCG (Fig. 3K) or comprised of higher numbers of BCG-positive cells (Fig. 3L), implying that Rab27b could induce exocytosis of BCG from bladder cancer cells.

We also assessed whether Rab27b could contribute to BCG attachment to bladder cancer cells and its internalization. Because activation of fibronectin/integrin and macropinocytosis was known to play a critical role in the attachment and internalization of BCG by urothelial cancer cells (6–8), we compared the expression levels of the fibronectin receptor, integrin α5β1, as well as macropinocytosis-related molecules, Pak1 and MEK1, and their phosphorylated forms. Western blot analysis showed no considerable changes in their expression between bladder cancer sublines with Rab27b overexpression/knockdown and respective controls (Supplementary Fig. S6C), implying an insignificant role of Rab27b in BCG attachment and internalization.

Reduction of cytotoxic activity of BCG by SYTL3

Rab27b has been shown to function as a membrane trafficking protein via interacting with specific effectors (19). Of eight major effectors for Rab27b, only SYTL3 was found to be expressed in all three bladder cancer lines examined, UMUC3, TCCSUP, and MB49. We therefore decided to further investigate the role of SYTL3 in BCG resistance.

A SYTL3-siRNA was transfected in cell lines with considerable (TCCSUP, UMUC3–BCG-R, UMUC3–Rab27b) or minimal (UMUC3) Rab27b expression (Fig. 4A). Immunofluorescence showed increases in the BCG amount in Rab27b-high cells, but not in Rab27b-low cells (Fig. 4B). Similar to Rab27b knockdown, SYTL3-siRNA transfection enhanced BCG cytotoxicity in Rab27b-high cells (Fig. 4C), while SYTL3 knockdown via its shRNA did not significantly change cell growth in the absence of BCG (Supplementary Fig. S4B). In addition, colocalization of BCG with SYTL3 was confirmed in Rab27b-positive cells (Supplementary Fig. S1C).

Induction of cytotoxic activity of BCG by GW4869

The findings described above suggested that exocytosis mediated by Rab27b/SYTL3 could reduce BCG cytotoxicity in bladder cancer cells. We next assessed the impact of treatment with GW4869, which is known to inhibit Rab27b-dependent secretion (35), on BCG effects. PCR (Fig. 5A) and immunofluorescence (Fig. 5B) showed higher quantity of BCG in GW4869-treated bladder cancer cells than in respective controls. Stronger inhibitory effects of BCG were also seen in the presence of GW4869 (Fig. 5C). The effects of GW4869 on cell viability, irrespective of BCG, although its treatment alone did not significantly inhibit cell growth (Supplementary Fig. S4C), were excluded by comparing with respective controls without BCG treatment. Meanwhile, GW4869 treatment in bladder cancer cells was associated with decreases in Rab27b protein expression (Fig. 5D).

Modulation of cytotoxic activity of BCG by Rab27b/SYTL3/GW4869 in a mouse orthotopic xenograft model

We used a mouse orthotopic xenograft model for bladder cancer to assess the impact of Rab27b/SYTL3 on tumor growth in vivo. A murine bladder cancer line MB49 (36) stably expressing Rab27b-shRNA or SYTL3-shRNA (Fig. 6A) was implanted into the bladder of immunocompetent mice through a urethral catheter to compare sensitivity to intravesical BCG therapy. Surrogate endpoints for survival included loss of >15% of body weight from the baseline, severe gross hematuria, and emergence of difficulty in moving, eating, or drinking. When euthanized, we confirmed extensive tumors in these animals (see Supplementary Fig. S7). In control xenograft-bearing mice, four injections of BCG did not significantly improve their survival (P = 0.547; Fig. 6B). Nonetheless, compared with control mice, BCG treatment resulted in significantly longer survival in the Rab27b-shRNA (P = 0.036; Fig. 6C), SYTL3-shRNA (P = 0.024; Fig. 6D), or GW4869-treated (P = 0.013; Fig. 6E) group. In addition, the amount of BCG in these xenograft tumors after two injections was separately compared. Acid-fast bacillus (AFB) staining showed significantly increased number of AFB-positive microorganisms in the Rab27b-shRNA (P = 0.012) or GW4869-treated (P = 0.016) mice, but not the SYTL3-shRNA (P = 0.167), compared with the control (Fig. 6F).

AR/Rab27b expression in surgical specimens and BCG efficacy

We immunohistochemically stained for AR and Rab27b in non–muscle-invasive bladder cancer specimens from patients who subsequently underwent intravesical BCG therapy and then compared the prognosis. Positive signals of AR (Fig. 7A) and Rab27b (Fig. 7B) were detected predominantly in the nucleus and cytoplasm, respectively, of tumor cells. Overall, AR was positive in 13 (40.6%) of 32 cases, including 6 (30.0%) of 20 without recurrence and 7 (58.3%) of 12 with recurrence (P = 0.057). Similarly, Rab27b was positive in 26 (81.3%) of 32 cases, including 14 (70.0%) of 20 without recurrence and 12 (100%) of 12 with recurrence (P = 0.018). Kaplan–Meier analysis coupled with the log-rank test revealed that patients with AR-positive (P = 0.024; Fig. 7C) or Rab27b-positive (P = 0.032; Fig. 7D) tumor had a significantly higher risk of tumor recurrence. When separately analyzed in male (n = 26) and female (n = 6) patients, there were statistically significant decreases in recurrence-free survival with AR (P = 0.044) or Rab27b (P = 0.035) positivity in males, but not in that with AR (P = 0.182) or Rab27b (NA; no Rab27b-negative cases) positivity in females.

Discussion

Bladder cancer has recently been considered to be an endocrine-related neoplasm where AR activation correlates with induction of its development and progression as well as resistance to chemotherapy and radiotherapy (13–16). In the current study, we have first found that...
AR-negative or AR-knockdown bladder cancer lines are considerably more sensitive to BCG treatment than those with exogenous or endogenous AR, respectively. We have then shown data indicating a new role of AR signaling in modulating sensitivity to BCG therapy via upregulating Rab27b expression. Previously, flutamide, an AR antagonist, was suggested to facilitate BCG attachment to bladder cancer cells presumably via inducing integrin \( \alpha_5/\beta_1 \) expression (17). We thus elucidated another AR-mediated mechanism for reducing the therapeutic effect of BCG immunotherapy via inducing the elimination of the bacteria from cancer cells mediated by Rab27b (and its effector SYTL3).

BCG is known to elicit not only direct cytotoxicity in bladder cancer cells but also indirect tumor-suppressing immune responses (6–8). In either case, BCG internalization by the cells is an initial step, which begins within a few hours after exposure and may then lead to suppressing their proliferation a few days later. Redelman-Sidi and colleagues further demonstrated that BCG uptake by bladder cancer cells could be mediated by macropinocytosis, rather than phagocytosis, in a Rac1/Cdc42/Pak1-dependent manner (8). PTEN loss or Ras activation was also shown to induce Rac1/Cdc42/Pak1 expression, as well as macropinocytosis, in bladder cancer cells. Indeed, we previously demonstrated an association between AR overexpression and reduced levels of PTEN expression in urothelial cells (37). We further found up-regulation of AR expression in BCG-resistant cells. However, Rab27b overexpression or silencing did not affect the expression of macropinocytosis- or BCG attachment–related molecules. These findings suggest that BCG internalization occurs in a Rab27b-independent manner.
Although Rab27a and Rab27b, two isoforms of Rab27 that are present in vertebrates, were originally thought to function redundantly, more recent evidence indicated that these could play different roles by interacting with specific domain on effectors, such as synaptotagmin-like protein (Slp; Slp1−5), Slp homolog lacking C2 domains (Slac2; Slac2-a−c), and unc-13 homolog D (also known as Munc13−4; ref. 19). In other words, these effectors specifically bind active Rab27 proteins and regulate their functions. For instance, Slp3/SYTL3 is one of the Rab27b effectors, and the Rab27b/SYTL3 complex has been shown to play an essential role in granule transportation and degranulation (38). We here found that AR activity was associated with the expression levels of Rab27b in bladder cancer cells. In addition, similar effects of Rab27b vs. SYTL3 knockdown on BCG amount in bladder cancer cells and its cytotoxicity were observed. Meanwhile, facilitators of exocytosis have been shown to preferentially extrude Rab27b-positive fusiform vesicles containing E. coli from bladder

Figure 5. Effects of GW4869 on BCG therapy in bladder cancer cells. A, A semiquantitative PCR of BCG (334 bp) in UMUC3-BCG-R, TCCSUP, and MB49 cocultured with BCG (10 CFU/cell) in the presence or absence of 10 μmol/L GW4869 for 5 hours. B, Immunofluorescence of BCG in UMUC3-BCG-R, TCCSUP, and MB49 cocultured with BCG (10 CFU/cell) in the presence or absence of 10 μmol/L GW4869 for 5 hours. BCG positivity was estimated by counting at least 50 cells in each determinant. C, MTT assay in UMUC3-BCG-R, TCCSUP, and MB49 and cocultured with or without BCG (10 CFU/cell) and/or 10 μmol/L GW4869 for 72 hours. Cell viability is presented relative to that of each line/treatment without BCG. Each value represents the mean (±SD) from at least 3 determinants. *, P < 0.05 (vs. mock treatment). D, Western blot analysis of Rab27b (25 kDa) in UMUC3-BCG-R, TCCSUP, and MB49 treated with or without 10 μmol/L GW4869 for 24 hours. GAPDH (37 kDa) served as a loading control.
These findings, along with our current data showing colocalization of BCG with Rab27b or SYTL3, as well as Rab27b/SYTL3–induced BCG extrusion following internalization, suggest Rab27b/SYTL3-dependent elimination of BCG from bladder cancer cells. Silencing or inactivation of Rab27b and/or SYTL3 is therefore anticipated to result in accumulation of BCG in bladder cancer cells and subsequent enhancement of its therapeutic effect. We additionally showed that the effects of treatment with GW4869, a neutral sphingomyelinase inhibitor which was known to suppress exosome release (39, 40), were analogous to those of Rab27b or SYTL3 knockdown. These observations further support that BCG can be eliminated from bladder cancer cells primarily by Rab27b/SYTL3–mediated exocytosis, while there is no direct evidence for Rab27b-mediated exocytosis of BCG.

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Figure 6. Effects of Rab27b/SYTL3 knockdown or exocytosis inhibitor treatment on tumor growth in a mouse model for bladder cancer. A, Western blot analysis of Rab27b (25 kDa) and SYTL3 (69 kDa) in MB49-control-shRNA, MB49-Rab27b-shRNA, and MB49-SYTL3-shRNA. GAPDH (37 kDa) served as a loading control. B–E, MB49-derived sublines were implanted into the bladder of female C57BL/6 mice. After 24 hours, DMEM (mock; n = 7–8/group) or BCG (3 × 10⁶ CFU; n = 10/group), with/without GW4869 (10 μmol/L), was injected transurethrally into the bladder twice a week for 2 weeks. Survival curves were estimated by Kaplan–Meier plots and compared statistically by the Wilcoxon test. F, MB49-derived sublines were implanted into the bladder of female C57BL/6 mice. After 14 days BCG (3 × 10⁶ CFU), with/without GW4869 (10 μmol/L), was injected transurethrally into the bladder twice in a 6-hour interval. The harvested bladder tumors (n = 3/group) were subjected to AFB staining. AFB-positive cells in six random high-power fields were counted.

Rab27b expression in several types of malignancies, such as breast cancer (41) and glioma (42), has been assessed, demonstrating that Rab27b overexpression in surgical specimens is associated with poorer patient outcomes. Conversely, in prostate cancer, patients with low Rab27b tumor had a higher risk of tumor recurrence following radical surgery, compared with those with high Rab27b tumor (43). More interestingly, Rab27b expression was positively correlated with AR expression in prostate cancer tissues (43). In addition, it was documented in two cohorts of bladder cancer cases that high Rab27b expression was associated with the risks of disease progression of non–muscle-invasive (Ta/T1) tumors and cancer-specific mortality in those with invasive (T1–4) tumor (25). Our earlier study also suggested that AR expression in non–muscle-invasive bladder cancers could predict the preventive effect of androgen deprivation therapy, primarily used...
for the treatment of concurrent prostate cancer, on bladder tumor recurrence (44). Our current IHC data in bladder cancer specimens further indicate that detection of AR or Rab27b expression is associated with resistance to BCG therapy, especially in male patients, and may thus serve as a predictor of BCG sensitivity. Previously, Rab27b knockdown in a bladder cancer line was shown to result in inhibition of cell invasion, but not cell proliferation, as well as vesicle secretion and exocytosis of miRNAs (25). No significant effects of Rab27b (or SYTL3) knockdown on the viability of urothelial cancer cells were confirmed in our assays (see Supplementary Fig. S4). In addition, our xenograft experiments without BCG treatment (see Fig. 6B) showing marginal improvement in survival in Rab27b-shRNA or SYTL3-shRNA tumor-bearing mice, compared with the controls, implied the promotion of cell invasion (and/or metastasis) by Rab27b and SYTL3, while the functional role of SYTL3 in cancer cell growth has not been established. Thus, in conjunction with other data in nonurothelial cells showing associations between Rab27b overexpression and induced cell invasion (and proliferation in some studies; refs. 26, 27, 41–43), Rab27b likely plays an important role in tumor growth via inducing exocytosis or other unknown mechanisms. Further studies are required to determine how Rab27b modulates cell proliferation and invasion.

In conclusion, we demonstrated that AR signaling could mediate sensitivity to BCG cytotoxicity in bladder cancer cells potentially via regulating Rab27b-induced exocytosis. Accordingly, concurrent androgen deprivation therapy or treatment with an exocytosis inhibitor may significantly enhance the efficacy of intravesical BCG immunotherapy in patients with bladder cancer. Nonetheless, further analyses in male xenograft models should be performed to confirm our findings, because we only used female mice in which tumor cells and BCG could be injected much more safely and reliably into the bladder. Meanwhile, IHC assessment of AR and/or Rab27b in transurethral resection specimens may be useful for predicting response to BCG therapy.

Disclosure of Potential Conflicts of Interest
H. Miyamoto reports grants from Ferring Research Institute outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Androgen and BCG Therapy in Bladder Cancer

References


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