Oncogenic Activation of Pak1-Dependent Pathway of Macropinocytosis Determines BCG Entry into Bladder Cancer Cells

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Abstract

Bacille Calmette-Guerin (BCG) is an attenuated strain of Mycobacterium bovis that is used widely as a vaccine for tuberculosis and is used as an effective treatment for superficial bladder carcinoma. Despite being the most successful cancer biotherapy, its mechanism of action and response determinants remain obscure. Here, we establish a model system to analyze BCG interaction with bladder cancer cells, using it to show that these cells vary dramatically in their susceptibility to BCG infection. Unexpectedly, the uptake of BCG by bladder cancer cells occurs by macropinocytosis rather than phagocytosis. BCG entry into bladder cancer cells relied upon Rac1, Cdc42, and their effector kinase Pak1. The difference in susceptibility between BCG-permissive and -resistant bladder cancer cells was due to oncogenic activation of signaling pathways that activate macropinocytosis, with phosphoinositide 3-kinase inhibitor activation stimulating BCG uptake independently of Akt. Similarly, activated Ras strongly activated Pak1-dependent uptake of BCG. These results reveal that oncogenic activation of macropinocytosis determines BCG uptake by bladder cancer cells, implying that tumor responsiveness to BCG may be governed by the specific mutations present in the treated cancer cell. Cancer Res; 73(3): 1156–67. © 2013 AACR.

Introduction

Bladder cancer is among the most common tumors diagnosed in the United States, with an estimated annual incidence of 70,530 new cases and 14,680 deaths in 2010 (1). Approximately 70% of bladder tumors are classified as superficial (non–muscle-invasive). Treatment of superficial bladder cancer by transurethral resection alone is associated with a 40% to 80% risk of recurrence and a 10% to 27% chance of progressing to muscle-invasive, regional, or metastatic disease (2). Bacille Calmette-Guerin (BCG) is an attenuated strain of Mycobacterium bovis that was derived by prolonged in vitro passage of virulent M. bovis. Apart from its longstanding use as a vaccine against tuberculosis, BCG is also used as intravesical therapy for carcinoma in situ (CIS) of the bladder. BCG administration plays a central role in managing CIS as well as high-grade Ta (papillary) and T1 (lamina propria invasive) lesions after transurethral resection (3). However, up to 30% of treated patients experience recurrence or progression of disease (3).

Despite more than 30 years of clinical experience with intravesical BCG for bladder cancer, its mechanism of antitumor effect remains unknown and no markers exist to predict response. Direct and indirect immune mechanisms have been postulated to play a role (4), as have direct cytotoxic effects on the tumor cell (5). BCG attachment to tumor cells, leading to internalization and processing of the mycobacterium, likely plays a crucial role in activation of BCG-mediated antitumor activity (6–8).

The usual host cells of pathogenic mycobacteria, including M. tuberculosis and M. bovis, are macrophages. Macrophages internalize mycobacteria through phagocytosis, which is facilitated by attachment of the bacterium to receptors at the plasma membrane. However, M. tuberculosis is not known to actively promote its uptake to be internalized. In contrast, bacteria that invade nonphagocytic host cells often elaborate effector functions that induce their own uptake. One such strategy, which has been termed “zippering,” involves expression of invasion proteins on the bacterial surface that interact with cellular receptors, initiating signaling cascades that result in actin polymerization and membrane extensions with subsequent tight engulfment and internalization of the bacteria. This mechanism is used by bacteria such as Listeria monocytogenes and Yersinia pseudotuberculosis (9). An alternative strategy, termed “triggering,” uses a type III secretion system to inject bacterial proteins into the host cytoplasm that trigger actin polymerization and membrane ruffling, leading to co-uptake of bacteria and extracellular fluid in a pathway that mimics macropinocytosis. “Triggering” is used by Salmonella.
typhimurium and S. flexneri for cellular entry (10, 11). BCG is not known to use either of the above uptake mechanisms, and the pathway by which it enters bladder cancer cells, which are derived from nonphagocytic epithelial cells, is unknown. In this study, we investigated the mechanism and molecular requirements for BCG entry into bladder cancer cells.

Materials and Methods

Bladder cancer cell lines

Bladder cancer cell lines were a gift from Dr. Dan Theodeorescu. Cells were grown in Eagle Minimal Essential Medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine and 1% nonessential amino acids, and with 100 U/ml penicillin, and 100 μg/ml streptomycin (except where noted). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air. All cells were confirmed to be mycoplasma free by MycoAlert™ assay.

To ensure authenticity, all cell lines underwent mass spectrometry–based genetic fingerprinting using 42 highly variable single nucleotide polymorphisms across all chromosomes as previously described (12).

BCG, Mycobacterium smegmatis, and Escherichia coli

GFP-expressing BCG (BCG-GFP) was created by transforming BCG Pasteur strain with pYUB921 (episomal plasmid encoding GFP and conferring kanamycin resistance). mCherry-expressing BCG (BCG-mCherry) was created by transforming BCG Pasteur with pMSG432 (episomal plasmid encoding mCherry and conferring hygromycin resistance). GFP-expressing E. coli was created by transforming DH5α strain of E. coli with pGFP(ASV) vector (Clontech), which encodes GFP and confers ampicillin resistance. BCG strains were grown at 37°C in Middlebrook 7H9 media supplemented with 10% albumin/dextrose/saline, 0.5% glycerol, and 0.05% Tween 80, and in the presence of 20 μg/ml kanamycin (BCG-GFP) or 50 μg/ml hygromycin (BCG-mCherry). GFP-expressing M. smegmatis was created by transforming M. smegmatis with pYUB921. M. smegmatis was grown in LB media supplemented with 0.5% glycerol, 0.5% dextrose, and 0.05% Tween 80, in the presence of 20 μg/ml kanamycin. E. coli was grown in LB media in the presence of 100 μg/ml carbenicillin.

Pharmacologic inhibitors

Cells were pretreated with the inhibitors in serum-free media at the specified concentrations for one hour prior to infection with BCG, and kept in the media for the duration of infection. In all experiments using chemical inhibitors, the highest concentration of DMSO (0.1%) was used as vehicle control. The sources of the pharmacologic inhibitors used in this study are listed in the Supplementary Methods.

Plasmids and transfections

PLK0.1-PTEN and PLK0.1-SC were a gift from Dr. Xuejun Jiang. PLK0.1 shRNA constructs for knock-down of Pak1, Pak2, PDK1, and clathrin heavy chain (CHC) were purchased from the Memorial Sloan-Kettering High-Throughput Screening Core Facility. The scrambled shRNA lentivirus PLK0.1-SC was used as control for shRNA knockdown. Lentivirus for shRNA knockdown of PTEN, Pak1, or CHC was made by cotransfecting the respective plasmids with Mission Lentiviral Packaging Mix (Sigma) into 293T cells in 10 cm2 plates, using lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Lentivirus for overexpression of PTEN, Pak1, Cdc42, Rac1, K-ras, and H-ras was made by cotransfecting the respective constructs with the packaging plasmids VSV-G and pCPG into 293T cells in 10 cm2 plates, using lipofectamine 2000. After transfection, cells containing the lentiviral insert were selected using 1.5 μg/mL puromycin (Invitrogen) for 4 days. Please see Supplementary Methods for a complete list of shRNA sequences and sources of cDNAs.

BCG infection

Bladder cancer cells were plated a day prior to infection in antibiotic-free media to reach 50−80% confluence on the day of infection. Cells were washed and plated in serum and antibiotic-free media one hour prior to infection. BCG was thawed and diluted in serum and antibiotic-free media to achieve a multiplicity of infection of 10:1. Plates were incubated at 37°C for the indicated time period and then washed three times with PBS, three times with media containing 1% penicillin-streptomycin, rewarshed with PBS, detached using trypsin, and resuspended in PBS for analysis by flow-cytometry. Although absolute difference in BCG uptake between BCG-resistant and BCG-permissive cell lines is maximal after 24 hours, a 4-hour infection time point was chosen for experiments with chemical inhibitors to account for short half life of inhibitors.

Antibodies

Antibodies against pAkt (Ser473, #4060), Akt (#4691), S6K (#2012), p-S6K (Thr389, #9205), pPak1/pPak2 (pPak1 Ser144 / pPak2 Ser141 / #2606), Pak1 (#2602), Pak2 (#2608), Pak3 (#2609), PDK1 (#3062), β-actin (#3700), pMek1 (Ser298, #9128), Mek1 (#9124), pLIMK1 (Thr508, #3841), LIMK1 (#3842), CHC (#4796), and Myc-Tag (#2276) were purchased from Cell Signaling. pPAK1 antibody (Ser144, EP656Y, #3842), Pak3 (#2609), PDK1 (#3062), β-actin (#3700), pMek1 (Ser298, #9128), Mek1 (#9124), pLIMK1 (Thr508, #3841), LIMK1 (#3842), CHC (#4796), and Myc-Tag (#2276) were purchased from Cell Signaling. pPak1 antibody (Ser144, EP656Y, ab40795) was purchased from Abcam. PTEN antibody (clone 6H2.1) was purchased from Cascade BioScience.

Microscopy

Cells were plated on glass coverslips in 6-well plates and allowed to attach overnight. The following day the cells were infected with BCG as described above. Nuclei were stained using Hoechst (Invitrogen). Cells were then fixed with 4% PFA at room temperature and stained with Texas-Red Phalloidin (Invitrogen). Confocal images were obtained with a Leica inverted confocal SP2 microscope. Phase contrast microscopy was conducted using a Zeiss AxioVert 200M microscope with a CoolSnap ES camera. For live imaging using fluorescent dextran, cells were plated in glass-bottom 35-mm dishes (MatTek) and allowed to attach overnight. The following day the cells were infected with BCG as described above. Alexa Fluor 568–conjugated dextran MW...
10,000 (Invitrogen) at a concentration of 0.1 mg/ml was added to the media immediately following addition of BCG, and the cells were incubated at 37°C for the specified time period, and washed as described above. Live microscopy was performed on a Zeiss Axiovert 200M microscope, with a Yokogawa spinning disk (CSU-22) unit, and an incubation chamber set to 37°C with 5% CO₂ in air. Microscopy images were adjusted for contrast using Volocity software (PerkinElmer).

**Flow cytometry**

Cell suspensions were analyzed on an LSR II flow cytometer (BD Biosciences), using the FACS DiVa software (BD Biosciences) according to manufacturer’s instructions. Data analysis was performed with the Flowjo software package (Tree Star). GFP was detected on the FITC channel using a 488-nm laser. mCherry was detected on the PE-Texas Red channel using a 532-nm laser. As the cells had a high degree of auto-fluorescence, an empty channel (Pacific Blue) was used to optimize gating of GFP-positive or mCherry-positive events. Gating strategy is described in Supplementary Figure S1.

**Apoptosis and cell death assay**

Bladder cancer cells were infected with BCG-GFP for 4 or 24 hours, washed with PBS, detached with trypsin, and resuspended in PBS. In order to evaluate apoptosis, cells were stained using Pacific Blue Annexin V (Invitrogen) as per the manufacturer’s instructions. Proportion of apoptotic cells (positive for Annexin V fluorescence) was determined by flow cytometry. Unstained cells were used as gating controls.

**Transferrin uptake**

Cells were washed with serum-free media, and media was replaced with serum-free media one hour prior to addition of transferrin. Media was replaced with serum-free media containing 25 μg/mL Alexa-568–conjugated transferrin (Invitrogen) for 15 minutes at 37°C. Cells were chilled on ice and washed three times with ice-cold PBS. Cells were then washed with 0.1 mol/L glycine, 0.1 mol/L sodium chloride, and pH 3.0 to remove extracellular transferrin. Cells were detached using trypsin, resuspended in PBS, and analyzed by flow cytometry.

**Results**

**Bladder cancer cell lines derived from human tumors vary in their susceptibility to BCG infection**

To study the mechanism of BCG infection of bladder cancer cells, we established an *in vitro* infection system using a BCG strain expressing GFP and a panel of bladder cancer cell lines derived from human tumors. We first asked whether the cell lines differ in their susceptibility to BCG infection. We infected the cell lines J82, T24, UM-UC-3, MGH-U3, MGH-U4, and VMCUB-3 with BCG-GFP and determined uptake of BCG using flow cytometry (Figs. 1A and Supplementary Fig. S1). Cell lines could be categorized into 2 groups according to their susceptibility to BCG infection; 3 of the cell lines (UM-UC-3, T24, and J82) readily took up BCG, with up to 25% of the cells infected after 24 hours, whereas the others (MGH-U3, MGH-U4, and VMCUB-3) were resistant to BCG infection, with less than 2% of the cells infected after 24 hours (Fig. 1B). These findings were confirmed by confocal microscopy (Fig. 1C). To exclude the possibility that differences in BCG infection were due to apoptosis, we stained infected cells with Annexin V, an early marker of apoptosis. The proportion of apoptotic cells was similar in the BCG-resistant compared with the BCG-sensitive lines, suggesting that apoptosis did not account for the differences in BCG permissiveness (Fig. 1D).

**BCG uptake by bladder cancer cells is inhibited by cytochalasin D, EIPA, and staurosporine**

The data above indicate that a subset of bladder cancer cells efficiently take up BCG. This result is surprising insofar as bladder cells are nonphagocytic, and mycobacteria, in contrast to other bacterial pathogens such as *Salmonella* and *Listeria*, do not have pathogenic effector functions to invade epithelial cells. Thus, the mechanism of BCG uptake into bladder cancer cells susceptible to BCG infection is unclear. To determine the endocytic pathway mediating uptake of BCG in bladder cancer cells, we used a panel of small-molecule inhibitors, which are commonly used to investigate mechanisms of pathogen internalization (13), and assessed their effect on BCG uptake. We first tested the actin polymerization inhibitor cytochalasin D and found that it diminished uptake of BCG by 64% to 89% (Fig. 2). In addition, the Na⁺/H⁺ pump inhibitor ethylisopropyl amiloride (EIPA), which has been used as an inhibitor of macropinocytosis (14), inhibited uptake of BCG by 47% to 61%. The protein kinase inhibitor staurosporine inhibited uptake by 25% to 46%, and genistein (tyrosine kinase inhibitor) inhibited uptake in J82. BCG uptake was not inhibited by the non-muscle myosin inhibitor blebbistatin or G-6983 (a protein kinase C inhibitor). Taken together, these data suggest that uptake of BCG by bladder cancer cells is dependent on the actin cytoskeleton and on protein kinases. The inhibition by EIPA is suggestive of, albeit not specific for, uptake by macropinocytosis (14).

To verify that the action of the inhibitors was not mediated through a direct effect on BCG, we measured uptake of paraformaldehyde-fixed BCG-GFP by bladder cancer cells in the presence of the same panel of small-molecule inhibitors. The same effects seen with live BCG were seen with fixed BCG, confirming that the effect of the inhibitors was due to a bladder cell target and not through a direct effect on BCG such as compromising bacterial viability (Supplementary Fig. S2). Similar results were obtained with *M. smegmatis*, a nonpathogenic mycobacterium (Supplementary Fig. S3A) and *Escherichia coli* (Supplementary Fig. S3B). However, the efficiency of infection with *E. coli* was lower than seen with either BCG or *M. smegmatis*. Thus, the uptake of BCG by bladder cancer cells reflects activation of a generalized uptake pathway for bacterial particles.

**BCG uptake is dependent on Cdc42, Rac1, and Pak1**

Rho-family GTPases, including Rac1, Cdc42, and RhoA, are involved in actin cytoskeletal organization and in various pathways of endocytosis. Rac1 and Cdc42 control lamellipodia formation and membrane ruffling and are essential for
macropinocytosis and for Fc receptor–mediated phagocytosis (14–16), as is their downstream effector, p21-activated kinase 1 (Pak1; ref. 17). RhoA, through its downstream effector RhoA kinase (ROCK), is required for complement receptor–mediated phagocytosis (16). To determine the role of Rho-family GTPases in BCG uptake, we initially used 2 small-molecule inhibitors, Y-27632, an inhibitor of ROCK, and IPA-3, an inhibitor of group 1 PAK family kinases, including Pak1. Y-27632 did not have a significant effect on BCG uptake (Fig. 3A), but IPA-3 inhibited BCG uptake by 46% to 90% (Fig. 3A), an effect that was also observed with fixed BCG, confirming an effect on the bladder cell (Supplementary Fig. S2).

To further investigate the role of Rac1 and Cdc42, we stably transduced BCG-permissive cell lines with the dominant-negative Rac1(T17N) and Cdc42(T17N) (ref. 18) and measured BCG uptake. We observed that either construct inhibited BCG uptake: Cdc42(T17N) by 50% to 75% and Rac1(T17N) by 28% to 46% (Fig. 3B). To test whether Rac1 and/or Cdc42 are sufficient to activate BCG uptake in BCG-resistant cell lines, we stably transduced MGH-U3, MGH-U4, and VMU-B3 with Rac1(Q61L) and Cdc42(Q61L), activated forms of Rac1 and Cdc42, respectively (19). Either construct significantly increased BCG uptake: Rac1(Q61L) by 48% to 141% and Cdc42(Q61L) by 67% to 100% (Fig. 3C).
results indicate that Rac1 or Cdc42 are both necessary and sufficient to determine BCG uptake by bladder cancer cell lines.

We next investigated the role of Pak1, downstream effector of Rac1 and CDC42. We depleted Pak1 protein in the cell line UM-UC-3 by lentiviral delivery of 2 short hairpin RNA (shRNA) targeting Pak1 and verified depletion by Western blotting of whole-cell lysates with anti-Pak1 antibodies (Fig. 4A). Depletion of Pak1 by either shRNA resulted in striking inhibition of BCG uptake in presence of DMSO. BLEB, blebbistatin; CYTO, cytochalasin D; DMSO, dimethyl sulfoxide; GENI, genistein; STAU, staurosporine. *, P < 0.05; **, P < 0.005; ***, P < 0.0005.

These data suggested that activated Pak1 was the main group 1 PAK family member stimulating BCG uptake by bladder cancer cells. To confirm this result, we stably transduced the BCG-permissive cell lines with Pak1(K299R), a dominant-negative form of Pak1 (21). Consistent with our results using shRNA, DN-Pak1 decreased BCG uptake by 50% to 88% when compared with an empty construct or wild-type Pak1 (Fig. 4C). Furthermore, transduction of BCG-resistant cell lines with either of 2 activated forms of Pak1 (22) resulted in a 23% to 156% (for Pak1 T423E) and an 85% to 106% (for Pak1 L107F) increase in BCG uptake (Fig. 4D). Taken together, these data indicate that BCG entry into bladder cancer cells occurs through activation of a pathway that involves Rac1, Cdc42, and Pak1.

Uptake of BCG is independent of dynamin and clathrin

Receptor-mediated pathways for uptake of large particles, such as phagocytosis, or the "zippering"-type endocytosis used to internalize pathogens such as Listeria, are dependent on the GTPase dynamin (23, 24). To investigate the role of dynamin in BCG uptake, we transiently transfected the BCG-sensitive cells lines T24 and UM-UC-3 with wild-type dynamin2, or DN dynamin2(K44A) (25). As the C-terminus of dynamin in these constructs is fused to GFP, we used BCG-mCherry for these experiments. As shown in Supplementary Fig. S5A, transfection with either construct did not significantly alter BCG uptake. Similarly, neither construct had an appreciable impact on BCG uptake by MGH-U4 (Supplementary Fig. S4B). Conversely, transfection with dynamin2(K44A), but not wild-type dynamin2, significantly inhibited uptake of transferrin, a process that is known to be dynamin-dependent (ref. 26; Supplementary Fig. S5A). Clathrin has also been shown to be essential for the "zippering"-type endocytosis of pathogens such as Listeria (24). Despite effective knockdown of clathrin heavy chain (CHC) by 3 different shRNAs, no reduction in BCG uptake was observed (Supplementary Fig. S4C). Uptake of transferrin, a known clathrin-dependent process (27), was inhibited by CHC knockdown (Supplementary Fig. S5B).

Internalized BCG colocalizes with fluid phase fluorescent dextran

The molecular requirements for BCG uptake, namely, the involvement of Rac1/Cdc42/Pak1, the inhibition of uptake by EIPA, and the lack of dependence on dynamin or clathrin, suggest that uptake occurs through macropinocytosis. Particles internalized by macropinocytosis are taken up together with extracellular fluid, whereas particles internalized by receptor-mediated uptake pathways, such as phagocytosis or "zippering," exclude extracellular fluid (28). To determine whether extracellular fluid is being internalized with BCG, we infected the cell lines T24 and UM-UC-3 with BCG-GFP in the presence of red fluorescent dextran [molecular weight (MW) 10,000] in the medium. Red fluorescent dextran could be seen in the same vesicle as BCG-GFP (Fig. 5). We excluded the possibility that dextran was attaching to BCG before uptake by observing that fluorescent dextran did not colocalize with extracellular BCG (Fig. 5).

The PI3K/PTEN pathway determines BCG uptake by bladder cancer cells

The data presented above indicate the mechanism of entry of BCG into some bladder cancer cells is via macropinocytosis. However, some bladder cancer cells are resistant to BCG uptake, suggesting that they do not have an activated macropinocytosis pathway. We hypothesized that the pattern of mutations present in the BCG-resistant and BCG-sensitive cell lines may determine their permissiveness for BCG uptake. Of the cell lines used in this study, 2 BCG-permissive cell lines (J82 and UM-UC-3) are reported to have a deletion of PTEN (29) and 2 (T24 and UM-UC-3) have activating mutations in Ras (30). We investigated the causal relationship between these mutations and BCG susceptibility, focusing first on the PTEN/PI3K/Akt pathway.

J82 and UM-UC-3, the 2 cell lines with reported PTEN deletion, showed no detectable PTEN protein (Fig. 6A). All 3 sensitive cell lines (J82, T24, and UM-UC-3) had elevated levels of...
phospho-Pak1(Ser144) and pMEK1(Ser298), the latter being a direct phosphorylation target of Pak1 (31). We could not detect expression of pLimk1/pLimK2, also a downstream target of Pak1 phosphorylation (32), nor of Pak3 (data not shown).

Wortmannin (Fig. 6B) or BKM120 (a selective pan-PI3K inhibitor, Supplementary Fig. S6; ref. 33) inhibited BCG uptake by 33% to 50%. In contrast, inhibitors of Akt or mTOR did not decrease BCG uptake, despite clear inhibition of their downstream targets (Fig. 6B). We deduced that these inhibitors were not acting through a direct effect on BCG by showing the same effects on uptake of fixed BCG (Supplementary Fig. S2). To further determine whether the effect of phosphoinositide 3-kinase inhibitor (PI3K) on BCG uptake is mediated through the canonical PI3K/PDK1/Akt arm of the PI3K pathway, we depleted PDK1 using 2 different lentiviral-delivered shRNAs and confirmed depletion by Western blotting (Fig. 6C). PDK1 depletion did not inhibit BCG uptake (Fig. 6C), confirming that the effect of PI3K on BCG uptake is not mediated through the PDK1/Akt arm of the PI3K pathway.

To study the role of PTEN in BCG uptake, cDNAs encoding PTEN (wild-type) or PTEN (C124S), a PTEN protein deficient for lipid phosphatase activity (34), were stably transduced into the BCG-sensitive cell lines (Fig. 6D). Restoration of wild-type PTEN, but not PTEN(C124S), resulted in approximately 50% reduction in BCG uptake in the cell lines J82 and UM-UC-3 (Fig. 6D), but not in T24, which has a missense mutation of PTEN (29). Although expression of wild-type PTEN did not clearly decrease phosphorylation of Pak1-S144, we did observe a decrease in BCG uptake.

Figure 3. BCG uptake requires CDC42, Rac1, and Pak1. A, specified cell lines were pretreated with IPA-3 or Y-27632 and incubated with BCG-GFP for 4 hours in the presence of the inhibitors. BCG uptake was measured by flow cytometry. BCG uptake is shown as percentage of BCG uptake in presence of dimethyl sulfoxide (DMSO). B, specified cell lines were stably transduced with empty vector or with DN-Rac1(T17N) or DN-Cdc42(T17N) (both with N-terminal myc-tag). BCG uptake was measured by flow cytometry 4 hours after infection. Western blot analysis shows expression of myc-tagged Rac1(T17N) or Cdc42(T17N). C, specified cell lines were stably transduced with empty vector, Rac1(Q61L), or Cdc42(Q61L) (both with N-terminal myc-tag). BCG uptake was measured by flow cytometry 8 hours after infection. Expression of myc-tagged Rac1(Q61L) or Cdc42(Q61L) was shown by Western blotting.
decreased phosphorylation of Mek1 at S298 in the cells transduced with wild-type PTEN. Knockdown of PTEN in MGH-U3 resulted in approximately 2-fold increase in uptake of BCG compared with a nontargeting shRNA control, but the same phenomenon was not seen in VMCUB-3, despite PTEN depletion as evidenced by Western blotting (Fig. 6E).

To confirm that PTEN knockdown was activating the same pathway of BCG uptake observed in permissive cell lines, we tested whether the increase in BCG uptake following PTEN knockdown in the cell line MGH-U4 could be abrogated by inhibition of Pak1. IPA-3 completely abrogated the increase in BCG uptake stimulated by PTEN knockdown (Fig. 6F), indicating that hyperactivation of the PI3K pathway in a resistant cell line activates BCG uptake through the same pathway as in susceptible cell lines with loss of PTEN function. Overall, these data show that the PI3K/PTEN signaling pathway modulates BCG uptake by bladder cancer cells. Activation of the PI3K pathway activates macropinocytic uptake of BCG, but this effect is independent of the downstream kinases PDK1, Akt, and mTOR.

Activated Ras activates macropinocytotic BCG uptake

We next investigated the role of Ras in uptake of BCG by bladder cancer cells. We stably transduced BCG-resistant
cell lines with cDNAs encoding K-ras G12D and H-ras G12V, constitutively activated forms of K-ras and H-ras, respectively. Both activated forms of Ras caused a dramatic increase in BCG uptake, up to 7-fold higher than control cells (Fig. 7A). In 2 of 3 cell lines (MGH-U3 and MGH-U4), activated Ras-stimulated phosphorylation of Mek1 at the Pak1 target site serine 298, indicating activation of the Pak1 pathway. Fluorescence microscopy confirmed increased BCG uptake by Ras-transformed cell lines and revealed striking morphologic changes in these cells, including numerous cytoplasmic vacuoles (Fig. 7B). BCG-GFP localized in dextran-containing macropinosomes in cells expressing K-Ras G12D, but not control cells (Fig. 7C), indicating uptake by macropinocytosis. Activation of BCG uptake by K-Ras G12D could be abrogated by either IPA-3 or wortman- nin, suggesting that the action of Ras occurs upstream of PI3K and Pak1 (Fig. 7D).

Discussion

Our data show that bladder cancer cell lines vary considerably in their propensity to take up BCG. We found that this property is dependent on activation of several oncogenic signaling pathways, resulting in increased macropinocytosis and uptake of BCG. This is, to our knowledge, the first report showing that cancer cells may become susceptible to a microbial pathogen due to oncogenic mutations.

The pathways determining BCG uptake by bladder cancer cells, namely, PTEN/PI3K, Ras, and Cdc42-Rac1-Pak1, are known to be interconnected. The oncoprotein Ras can activate PI3K (35) and is also able to activate Rac1 through its action on the guanine nucleotide exchange factor (GEF) Tiam1 (36). Rac1 can also be activated by increased phosphatidylinositol (3,4,5)-triphosphate concentrations (37), which can occur through PI3K activation or through PTEN loss. Cdc42 can itself activate PI3K (38).

The same pathways determining BCG uptake are also involved in bladder cancer oncogenesis. Alterations in the PTEN/PI3K/Akt pathway are frequently present in human bladder cancers. These include decreased expression or deletion of the tumor suppressor PTEN, activating mutations of PI3K, and, rarely, activating mutations of Akt1 (39). A sizeable fraction of bladder cancers harbor activating mutations of Ras, most commonly H-ras mutations (40). Cdc42 also appears to have a role in bladder cancer; expression of Cdc42 has been shown to be higher in bladder cancer than in normal urothelial cells, and RNA interference of Cdc42 was found to suppress growth of bladder cancer cells (41, 42). Pak1 is overexpressed in a large proportion of bladder cancers (43) and may also be a marker of recurrence after transurethral resection of superficial bladder cancer (44).

Our findings could thus explain why treatment with BCG is successful in most, but not all, patients with bladder cancer. BCG therapy would be expected to provide the most benefit for those patients whose cancer contains mutations activating the pathways of BCG uptake, such as decreased PTEN expression, activating Ras mutations, or activated Pak1. Our findings could also provide a mechanism of specificity for BCG infection of tumor cell compared with the normal urothelium, which does not contain mutations activating BCG uptake.

Epithelial cells are not the usual target of mycobacteria; the main cell type involved in M. tuberculosis infection is the macrophage, and the receptors used by macrophages for phagocytosis of M. tuberculosis have been comprehensively described (45). We define a novel mechanism underlying BCG uptake within epithelial cells, which is dependent on the actin cytoskeleton, inhibited by EIPA, and controlled by Cdc42, Rac1, and Pak1, but independent of dynamin or clathrin. Perhaps most importantly, BCG was taken up with fluid phase markers. Overall, these features are most consistent with uptake by macropinocytosis.
Others have shown that BCG attachment and uptake by bladder cancer cells is facilitated through attachment of BCG fibronectin attachment protein (FAP) to fibronectin on bladder cancer cells (46). Receptor-mediated uptake of large particles, via phagocytosis, or by the clathrin-dependent pathway that is used for uptake of Listeria, is typically dependent on dynamin.
BCG uptake, however, was independent of dynamin. One explanation for this apparent discrepancy is that uptake of BCG does not occur through a classical receptor-mediated uptake pathway. Rather, BCG that is either adjacent to bladder cancer cells or attached to them through a receptor is internalized because of increased membrane ruffling that accompanies macropinocytosis. The presence of a receptor for BCG attachment, such as α5β1 integrin, would lead to more mycobacteria being in intimate contact with the cells and would thus promote this process, resulting in increased uptake of BCG. Although macropinocytosis has traditionally been described as receptor-independent (16), there have been some recent reports describing receptor-dependent pathways of macropinocytosis in the uptake of some viruses (47, 48).

If validated in clinical settings, our findings could have implications for the treatment of patients with bladder cancer.
On the basis of our results, Ras and PTEN aberrations may represent predictive biomarkers of BCG efficacy. Prospective genetic profiling of TUR specimens for mutations affecting these signaling pathways may allow clinicians to restrict BCG therapy to those patients most likely to respond. Furthermore, as novel therapies targeting oncogenic pathways are being developed for bladder cancer treatment, such as inhibitors of PI3K (49), receptor tyrosine kinase inhibitors (50), and RNA interference–mediated silencing of Cdc42 (42), care should be taken to consider possible effects of these treatment on BCG uptake and efficacy. Finally, BCG therapy could possibly be improved by local administration of activators of these pathways, thereby potentially rendering BCG-resistant cells sensitive. The findings presented here will catalyze a direct examination of the role of specific macropinocytosis activating mutations in clinical response to BCG.

In conclusion, we have shown that BCG uptake by bladder cancer cells is determined by some of the same pathways that lead to oncogenesis. Knowledge of the mechanism underlying responsiveness to BCG therapy will help tailor the treatment to individual patients based on their tumor genotype and will hopefully lead to the development of more effective treatment options for bladder cancer.

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

References

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# Oncogenic Activation of Pak1-Dependent Pathway of Macropinocytosis Determines BCG Entry into Bladder Cancer Cells

Gil Redelman-Sidi, Gopa Iyer, David B. Solit, et al.


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