Bacillus Calmette-Guerin and BCG Cell Wall Skeleton Suppressed Viability of Bladder Cancer Cells In Vitro

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Abstract. Aim: Bacillus Calmette-Guerin (BCG) is one of therapeutic options for urothelial carcinoma (UC). The objectives of this study were to determine the direct effect of viable or heat-killed BCG and BCG cell wall skeleton (BCG-CWS) on UC cells in vitro. Materials and Methods: UC cell lines were co-cultured with viable or heat-killed BCG Immunobladder® (Tokyo 172 strain) and BCG-CWS. Viability of the cells, apoptosis and BrdU incorporation were estimated. Results: BCG induced cell growth retardation in highly malignant UC bearing integrin α5β1 (VLA5). VLA5-blocking antibody partially abrogated this effect. BCG treatment induced a modest increase in the sub-G1 fraction of cells and a decrease of BrdU incorporation. Cell growth retardation effect of viable BCG was reproduced by both heat-killed BCG and BCG-CWS. Conclusion: The results indicate that VLA5 may be a biomarker of UC with sensitivity to BCG. Moreover, BCG-CWS is a promising substance which might replace BCG, preventing life-threatening complications of viable BCG treatment.

In 2009, an estimated 70,980 adults (52,810 men and 18,170 women) were diagnosed with bladder cancer in the United States, with 14,330 deaths (10,180 men and 4,150 women) (Cancer.Net by ASCO, http://www.cancer.net). Seventy-four percent of bladder cancer patients are diagnosed with noninvasive/superficial urothelial carcinoma (UC). The five-year relative survival rate is 93% for those patients (Cancer.Net by ASCO, http://www.cancer.net). Although superficial bladder cancer has favourable prognosis, it poses a substantial treatment challenge. Progression occurs frequently, which is particularly important for grade 3 tumours, including carcinoma in situ (CIS). The broad involvement of CIS cannot be treated by transurethral resection (TUR) and usually progresses to invasive cancer (1). Bacillus Calmette-Guerin (BCG) is one therapeutic option for such patients. Several mechanisms of BCG action have been proposed. The most popular is an immune-mediating effect, however, a direct anti-tumour effect has also been discussed recently (2, 3).

It has been reported that BCG exerts its direct antitumour effect on cancer cells after binding to fibronectin, a high-molecular-weight glycoprotein of extracellular matrix (ECM) (4, 5). The integrin α5 subunit associates noncovalently with the integrin β1 subunit to form the α5β1 heterodimer very late antigen (VLA5) complex (4, 5). VLA5 is a fibronectin receptor that is expressed on the cell surface. A correlation between high expression of integrin subunits α5, β1 and the malignant potential of UC has previously been demonstrated (6). It is intriguing that BCG is prone to demonstrate a stronger effect on more malignant high-grade UC including carcinoma in situ (CIS) rather than grade 1 UC.

The SMP105 BCG cell wall skeleton (BCG-CWS) (7, 8) was developed as a substitute for viable BCG in cancer immunotherapy, and it is in current use as an immunomodulator in cancer patients (9).

There is much discrepancy among the published literature on the direct effect of BCG against UC cells. The precise mechanism of direct BCG action, internalisation and
induction of intracellular signaling is also controversial. Here the effect of treating three established UC cell lines with viable BCG, heat-killed BCG and BCG-CWS was studied.

Materials and Methods

Cell culture and reagents. Established bladder cancer cell cancer lines T24, HT1376, and RT4 were purchased from ATCC (Manassas, VA, USA). The cells were cultured as described previously (10) with the exception that antibiotics were not added to the culturing medium. BCG Immunobladder® (Tokyo 172 strain) was a kind gift of Japan BCG Laboratory (Tokyo, Japan). SMP105 BCG-CWS (7, 8) was a kind gift of Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan).

For blocking experiments, anti-α5β1 mAb (clone JBS5; Chemicon, Millipore Corporation, Billerica, MA, USA), which blocks VLA5 function was used. Fibronectin-coated plates were prepared according to a previously published method (11). BioCoat human fibronectin-coated plates (BD Biosciences, Bedford, MA, USA) were used. Fibronectin concentration in culturing medium was measured using Fibronectin EIA kit (Takara Bio Inc., Otsu, Japan).

Immunoblotting. Immunoblotting was performed as described previously (10). HRP-labelled second antibody was detected using a SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. The images were analysed using UN-SCAN-Itgel Automated Digitizing System software (version5.1 for Windows, Silk Scientific, Inc., Orem, Utah, USA). The following antibodies were used; anti-BCL-2 (clone 124, DAKO Japan, Tokyo, Japan), anti-pFAK (clone 18) from DAKO Japan (Tokyo, Japan), anti-XIAP (clone 28), anti-focal adhesion kinase (FAK) (clone 77/FAK), anti-pFAK (Y397) (clone 18) from BD Transduction Laboratories (Franklin Lakes, NJ, USA), and anti-β-actin from Abcam Inc. (Cambridge, MA, USA) as a control.

Flow cytometry of VLA5 (integrin α5β1) expression. For flow cytometry, cells were stained by the indirect immunofluorescence method as described previously (6). Tumour cells (1×10⁶) were reacted with anti-α5β1 mAb (clone JBS5; Chemicon, Millipore Corporation, Billerica, MA, USA), which blocks VLA5. Cells were stained by the indirect immunofluorescence method. Flow cytometry was performed as described previously (10). BCG size was much smaller than that of viable cancer cells and apoptotic cells in the sub-G₁ population and was gated-out.

Results

VLA5 (integrin α5β1) is expressed on high-grade but not low-grade bladder cancer cells. Previous experiments have shown that expression of VLA5 integrin subunits α5 and β1 correlated with bladder cancer of higher malignancy (6). Here, the expression of VLA5 integrin heterodimer molecule was detected on the surface of high-grade UC (T24, HT1376) but not low-grade (RT4) UC cells (Figure 1).

Both viable and heat-killed BCG, as well as SMP105 BCG CWS-induced cell growth retardation in UC cells with highly malignant phenotype. Culturing of UC cell lines with BCG resulted in a concentration-dependent decrease of cell viability (p<0.01) only in high-grade urothelial cancer (T24, HT1376) expressing VLA5, but not low-grade (RT4) urothelial cancer cells, which lack VLA5 on their surface (Figure 2A). Interestingly, there was no difference in viability between the cells cultured on uncoated and fibronectin-coated surfaces. Heat-killed BCG was also effective in retarding cell growth (p<0.01) (Figure 2B). As in the case of viable BCG, there was no significant difference between viability of the cells on uncoated and fibronectin-coated surfaces. This was in disagreement with the assumption that fibronectin-integrin complex is essential for direct effect of BCG on the cells. FCS-supplemented medium can contain fibronectin and cell lines can also shed fibronectin in the medium. Thus, the concentration of fibronectin in culturing medium was examined. It was clear that large amounts of fibronectin were present in culturing medium after culturing UC cells (Figure 3A) and these levels did not differ between cells cultured on uncoated and fibronectin-coated surface. Culturing medium supplemented with FCS contained only traces of fibronectin confirming...
that fibronectin was shed by UC cells. This finding explains why there was no difference in susceptibility to BCG between cells cultured on uncoated and fibronectin-coated surfaces. To test the effect of disrupting VLA5 and fibronectin interaction, T24 cells were cultured with VLA5-blocking antibody. VLA5-blocking antibody, but not irrelevant antibody, partially reversed cell growth retardation triggered by BCG ($p<0.01$) (Figure 3B). Since dead BCG was able to retard cell growth, the possibility that SMP105 BCG-CWS was sufficient to exert a similar effect was further explored. As shown in Figure 3C it significantly decreased cellular viability in both T24 and HT1376 cells ($p<0.01$).

*Increase in sub-G$_1$ population and suppression of BrdU incorporation in T24 cells by BCG.* A modest but consistent increase in the percentage of cells in the sub-G$_1$ population after co-culturing T24 cells with BCG was observed (Figure 4A). No significant changes in the cell cycle distribution (G1-S-G2 fractions) were observed. Co-culturing T24 cells with BCG resulted in decreased BrdU incorporation to 78% that of untreated cells ($p<0.01$) (Figure 4B).

*Expression of TLR2 and TLR4 in UC.* It has been reported that both viable tuberculosis bacillus and BCG-CWS confer TLR2- and TLR4-dependent signaling (13, 14). Both TLR2 (15, 16) and TLR4 (17) are known to trigger a protective immune response, as well as cellular apoptosis. In this light, the expression of TLR2 and TLR4 in UC cells was explored. Although both messengers were detected in T24 cells, they were undetectable in HT1376 cells by RT-PCR (Figure 4C). These results suggest that TLR2 and TLR4 signalling is not responsible for BCG effects, at least in HT1376 cells.

*Expression of antiapoptotic proteins and integrin downstream signaling mediator FAK in T24.* To seek evidence for fibronectin-itegrin engagement-induced intracellular signalling, FAK phosphorylation was examined. FAK is a non-receptor tyrosine kinase mediating the downstream signaling of VLA5 (integrin α5β1), a major fibronectin receptor (11). Co-culturing T24 cells with BCG promoted FAK phosphorylation (4.8- and 5-fold increase after 0.1 and 1 mg/ml BCG treatment respectively) (Figure 4D), which indicated activation of intracellular signaling downstream of VLA5. Despite the increase in the apoptotic sub-G$_1$ fraction (Figure 4A), there were no changes in anti-apoptotic proteins XIAP and BCL-2 (Figure 4D).

**Discussion**

BCG demonstrates high treatment efficacy (18) and recurrence prevention (19) in a fraction of superficial bladder cancer (Ta, T1 carcinoma and carcinoma in situ (CIS)) by intravesical instillation. However, about one
Figure 2. Relative viability of low-grade RT4 cells with low VLA5 expression and high-grade (T24, HT1376) TCC cells with high VLA5 expression cultured in the presence of BCG. The cells were cultured on uncoated (opened bars) or fibronectin-coated (closed bars) surface with the indicated amount of viable BCG (A) or heat-killed BCG (B) for 48 hours. Then relative cell numbers were estimated with crystal violet method. Bars indicate SD.
third of patients are primarily non-responsive to BCG and 15-25% have cancer recurrence after the treatment (18, 19). There is currently no good predictor for BCG response. Moreover, BCG therapy is associated with a high rate of complications. An 80% incidence of BCG-induced side-effects with 46% major complication rate has been reported (20). A relatively rare, but potentially fatal, complication is dissemination of BCG and sepsis (21, 22), which can occur several years after treatment (23). Thus, there is strong demand for a biological marker to predict response to BCG and therefore result in an increased BCG safety profile.
Figure 4. A: Analysis of cell cycle and sub-G1 population (apoptotic cells). B: BrdU incorporation assay. T24 cells cultured on fibronectin-coated surface with or without 1 mg/mL of BCG for 48 hours were stained with propidium iodide and subjected to FASCcan (A) or were analyzed for BrdU incorporation as described in the Materials and Methods section (B). C: Detection of toll-like receptor 2 and 4 (TLR2 and TLR4) mRNA in TCC cell lines by RT-PCR. RNA was extracted from untreated T24 and HT1376 cells; RT-PCR was performed using primers for TLR2 and TLR4 as described in the Materials and Methods. Actin was amplified as a control. N.C., template control. D: Western blot for fibronectin-signalling and anti-apoptotic proteins. Total protein was extracted from T24 cells cultured on fibronectin-coated surface with or without 0.1 or 1 mg/mL of BCG for 48 hours. After electrophoresis and Western blotting, the membranes were probed with antibodies against total FAK, Y397 phosphorylated FAK, XIAP and BCL-2. Actin was applied as control for loading.
BCG adherence to tumour cells is mediated by fibronectin (4). It has been demonstrated that direct antitumour effect of BCG is initiated via binding to VLA5, a cell surface receptor for fibronectin (5). Previously, a correlation between higher integrin expression and UC of higher malignancy was shown (6) which coincides with the antitumour profile of BCG. In this study, it was demonstrated that an antiproliferative effect of BCG was observed only in VLA5-positive T24 and HT1376 cells but not RT4 cells which lack VLA5 receptor on their surface. It was confirmed that a direct antitumour effect of BCG at least partially depended on VLA5 function (Figure 3B).

Both TLR2 and TLR4 have been shown to mediate Mycobacterium tuberculosis-induced intracellular signaling in vitro. Means et al. demonstrated that viable Mycobacterium tuberculosis bacilli activate cells via TLR2 and TLR4 (14). However, in the present study, a direct antitumour effect of BCG was observed on both TLR2- and TLR4-expressing T24 cells and non-expressing HT1376 cell line.

It was reported that BCG induced caspase-independent cell death (3) or non-apoptotic cell death (2, 3) in UC cells. Sasaki et al. reported an increase in apoptotic cells by flow cytometry. However, no TUNEL-positive apoptotic cells or DNA laddering were detected in T24 cells co-cultured with BCG (2). A modest increase in the sub-G1 apoptotic fraction from 0.79% to 4.02% (Figure 4A) without accumulation of typical apoptotic cells was observed by flow cytometry in this study. Thus, there is a possibility that BCG can trigger non-typical apoptosis or non-apoptotic cell death in UC.

A direct effect of BCG on UC cells in vivo has also been attributed to cell cycle arrest (24). Here, a retarded cell growth without prominent cell cycle arrest was detected (Figure 4A, B). This discrepancy can be explained by the use of a different BCG strain or different experimental conditions, including BCG dose.

As an approach to overcome potential complications of BCG treatment, heat-inactivation of BCG was used by Sasaki et al. without any obvious effect (2). Here a similar approach was applied, but a higher dose of BCG was used (1 mg/ml) which is closer to the concentration used in clinical routine (2 mg/ml). This study showed that heat-killed BCG inhibited T24 and HT1376 proliferative activity (Figure 2B).

BCG-CWS has been proposed for use as an immunomodulator in cancer immunotherapy (9). BCG-CWS interacts with TLR2 and TLR4, but its immunomodulatory profile is different from that of lipopolysaccharide (13). It was postulated that BCG-CWS binds multiple receptors. Here evidence is presented that BCG-CWS also exerts a direct antitumour effect (Figure 3C) irrespective of TLR2 and TLR4 expression. Thus, BCG-CWS may be potentially applied for intravesical instillation to control bladder cancer.

**Conclusion**

In conclusion, this study identifies VLA5 (integrin α5β1) as a potential biomarker of direct BCG effect on UC. It was also found that heat-killed BCG demonstrates antiproliferative activity on UC cells. Moreover, it was revealed that BCG-CWS was also effective in controlling UC cell proliferation, indicating a potential approach to increase the safety profile of bladder cancer immunotherapy.

**References**


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