Kaohsiung Journal of Medical Sciences (2015) 31, 391–397

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ORIGINAL ARTICLE

Bacille Calmette-Guerin can induce cellular apoptosis of urothelial cancer directly through toll-like receptor 7 activation

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Received 27 October 2014; accepted 5 January 2015

KEYWORDS
Apoptosis;
Bacillus Calmette-Guerin;
Toll-like receptors;
Urothelial cancer

Abstract Immunotherapy using bacille Calmette-Guerin (BCG) instillation is the mainstay treatment modality for superficial urothelial cancer (UC) through toll-like receptor (TLR) activation of cognitive immune response. We investigated the roles of TLR7 in the activation of apoptosis in UC cells after BCG treatment. The *in vitro* cytotoxicity effect of BCG on UC cells was measured by a modified 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium assay. Expressions of TLR7 mRNA and protein in native UC cells prior to and after BCG treatment were analyzed using real-time quantitative polymerase chain reaction and western blot methods. Phagocytotic processes after BCG treatment in UC cells were observed microscopically using a specific immunostain, subsequent cellular apoptosis-related signals induced by TLR7 were analyzed by western blot. Low-grade UC cells, TSGH8301, showed significant cellular death (4.23-fold higher than the high-grade UC cells T24 and J82) when treated with BCG and the BCG cytotoxicity was displayed in a dose–time-dependent manner. TSGH8301 cells had the highest content of TLR7 mRNA, 7.2- and 4.5-fold higher than that of T24 and J82 cells, respectively. TLR7 protein expression was also significantly increased in TSGH8301 cells. Phagocytosis-related markers, including beclin 1, ATG2, and LC3, were increased when TSGH8301 cells were treated by BCG. Interleukin-1 receptor-associated kinases 2 and 4 were also increased markedly in TSGH8301 cells. On the contrary, cellular apoptosis of TSGH8301 cells decreased by 34% when TLR7 activation was suppressed by the TLR antagonist IRS661 after BCG treatment. Our findings suggest that well differentiated TCC cells have higher

Conflicts of interest: All authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.kjms.2015.05.005

expression of TLR7 and BCG can drive cellular death of TCC cells directly via TLR7 activation and related apoptotic pathway.

Introduction

Urothelial cancer (UC) of the bladder accounts for about 4% of all cancer-related deaths. A large majority of tumors (80%) are superficial at diagnosis, and have a high rate of local recurrence (70%) and progression (30%) after local surgical interventions. Bacille Calmette-Guerin (BCG) has been shown to eliminate tumors and prevent recurrences of bladder UC with routine complete response rates of 60—70% [1,2]. Despite its well-recognized clinical efficacy, many questions about its precise mechanism of action remain unanswered.

The primary mechanism of the antitumor effect of BCG was that it induced an immune response, preferentially a Th1-polarized response characterized, by the production of elevated levels of interleukin-12 and interferon -γ [3,4]. Recent studies have demonstrated activation of toll-like receptors (TLRs) in immune responsive cells after BCG instillation. Downstream substrates of TLRs recruit other immune cells, suggesting that TLRs play a key role in the antitumor response to BCG therapy [5,6]. They provide essential requirements for initiating T-cell immunity: antigen uptake, processing and presentation by dendritic cells and other antigen-presenting cells, dendritic cell maturation, and -cell activation [7].

Directly killing of UC cells with BCG may be attributed to differential expression of the receptors of cytotoxic effector molecules, including TLRs, on target cells or adhesion molecules on both effector and target cells. These possibilities need to be investigated. Therefore, to determine the relationship between TLR expression and the direct killing effect of BCG on UC cells, we investigated TLR7 expression in BCG-treated UC cells and demonstrated that TLR7 activation can directly enhance BCG-induced apoptosis in UC cells.

Materials and methods

Cell culture

High-grade UC cell lines, T24 and J82, were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and low-grade UC cell lines, TCC9202 and TSGH8301, established at our laboratory were cultured in an RPMI-1640 medium (Thermo Scientific HyClone, Logan, UT, USA), and supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2 at 37°C.

Chemicals and antibodies

BCG (ImmuCyst 81 mg/vial) and IRS661 (a TLR inhibitor) were purchased from Sanofi Pasteur Limited (West Toronto, Ontario, Canada) and Sigma-Aldrich (St Louis, MO, USA), respectively. Antibodies of TLR7, IRAK2/4, caspase 8, beclin 1, ATG2, LC3, and antirabbit IgG horseradish peroxidase were obtained from Genetex (Irvine, CA, USA).

Measurement of cytotoxicity of BCG to UC cells

UC cells were exposed to the BCG reagent at concentrations of 0.05 mg/mL, 0.5 mg/mL, and 5 mg/mL. Cellular chemosensitivity was assayed using a modified 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium (MTT; Sigma-Aldrich) assay to determine cell viability *in vitro* [8]. In brief, cells (4000/well) in 100 μL culture medium were seeded into 96-well microplates and incubated at 37°C for 24 hours before drug exposure. The numbers of plated cells were calculated to keep control of the growth of cells in the exponential phase throughout the 72-hour incubation period. At one point, 50 μL of MTT was added to each well and allowed to react for 3 hours. The blue formazan crystals formed were dissolved in 150 μL of dimethyl sulfoxide. The optical density was determined by absorbance spectrometry at 492 nm using a microplate reader (MRX-2; Dynex Technologies, Inc., Chantilly, VA, USA). Three separate experiments, each run in triplicate, were performed to obtain the mean cell viability.

Quantitative reverse transcription polymerase chain reaction assay of TLR7 mRNA

Total RNA was extracted from cultured cells using a commercially available RNA extraction kit (Trizol; Gibco, Carlsbad, CA, USA), and cDNA was synthesized with a reverse transcription kit (Reverse Transcriptase Master Mix; Roche Diagnostics, Germany). The cDNA was amplified by quantitative reverse transcription-polymerase chain reaction (PCR) using 2 μL of the LightCycler PCR Master Mix. The amplicon was detected by fluorescence using a specific pair of TLR7 primers: 5′-CCTTGAGGCCAACAACATCT for sense and 5′-ACAGTAGGGACGGCTGTGAC for antisense. The mRNA of glyceraldehyde 3-phosphate dehydrogenase with specific pair primers (5′-ACCCACTCCTCCACCTTTGAC for sense and 5′-CATACCAGGAAATGAGCTTGACAA for antisense) was processed as a housekeeping gene. The fluorescence emitted after hybridization to the template DNA was measured by the LightCycler 480 Instrument. All PCR reactions were performed for 40 cycles of 60 seconds at 95°C, 10 seconds at 60°C, and 5 seconds at 72°C.

Western blotting analysis of apoptotic signaling

Protein expressions of TLR7, IRAK2/4, caspase 8, beclin 1, ATG2, and LC3 were measured by the western blot method.

Briefly, 30 μg of protein extracted from TSGH8301 cells after BCG treatment (0.05 mg/mL and 0.5 mg/mL) were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 8—12% resolution and 4% stacking gel. The SDS-PAGE was supplied with a voltage of 90 V for 45 minutes and 120 V for 90 minutes in running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, and 0.025—0.1% SDS) supplied with a voltage of 100 V at 4°C for 1 hour. The membrane was rinsed and shaken on a 40 rpm shaker in 5% milk in 0.1% Tris-buffered saline and Tween-20 at room temperature for 1 hour. Then the membrane was rinsed in specific antibodies at room temperature for 1 hour. After washing, the membrane was rinsed in horseradish peroxidase-conjugated secondary antibodies for 1 hour. Enhanced chemiluminescence signals were collected and analyzed using the UVP Biospectrum (USA).

Phagocytic assay of BCG in UC cells

Cellular BCG particles were studied by a fluorescence microscope. Cells were harvested and washed three times with phosphate-buffered saline after being incubated with BCG (5 mg/mL) for 24 hours, and stained with 100 μg/mL DAPI (AO; Sigma-Aldrich) for 10 minutes. β-actin stains were used to margin the cell bundles. The color distribution and structure of the cells were then observed under a fluorescence microscope (Olympus). The phagocytic index was calculated as the number of ingested BCG per cell [9].

Statistical analysis

Data were presented as mean ± the standard error of at least three independent experiments. For comparisons between three or more groups, two-way analysis of variance was used, followed by the Student method for multiple pairwise comparisons. Differences were considered statistically significant at $p < 0.05$.

Results

Dose-dependent cytotoxicity of BCG to UC cells

Various UC cells were treated with different dosages of BCG (0.05 mg/mL, 0.5 mg/mL, and 5 mg/mL) for 72 hours. The results showed that BCG directly inhibited cell growth in a dose-dependent manner, accounting for 3—5%, 4—7%, 2—25%, and 5—37% inhibition in T24, J82, TCC9202, and TSGH8301 cells, respectively. TSGH8301 cells showed significantly higher BCG cytotoxic sensitivity ($p = 0.003$) than T24 cells under the same dosage condition (Figure 1A). BCG treatment (0.05—5 mg/mL) also showed a cytotoxic effect on TSGH8301 cells in a time- and dose-dependent manner, which reduced cell viability by 12%, 25%, and 25—40% at 12 hours, 24 hours, and 48 hours, respectively (Figure 1B).

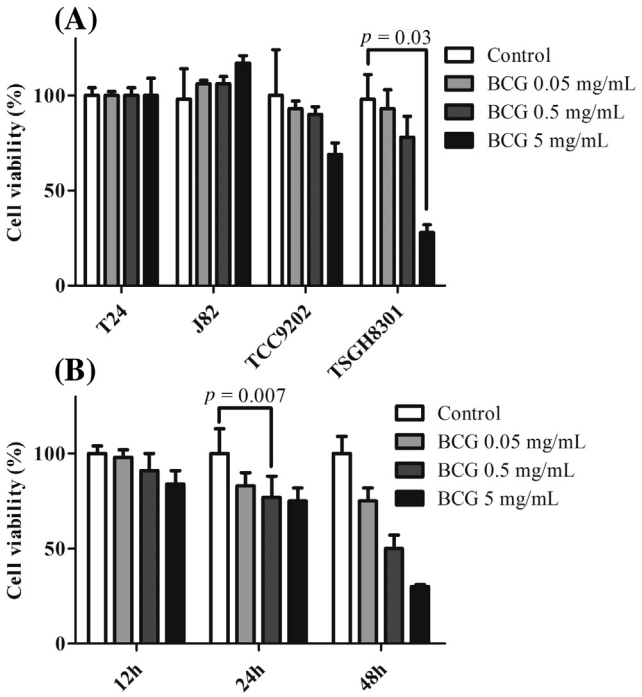

Figure 1. Cell viability of (A) T24, J82, TCC9202, and TSGH8301 cells when treated by BCG (0.05—5 mg/mL) for 72 hours and (B) TSGH8301 cells when treated with BCG at 0.05 mg/mL, 0.5 mg/mL, and 5 mg/mL dosages for 12 hours, 24 hours, and 48 hours, respectively. BCG = Bacille Calmette-Guerin.

Higher TLR7 expression in well-differentiated UC cells

Low-grade UC cells present a higher expression of TLR7 than high-grade UC cells. The increased expression of TLR7 was demonstrated in either the mRNA or protein level, as shown in Figures 2A and 2B. TSGH8301 cells had a significantly higher level of TLR7, 2.5- and 13.2-fold higher compared with T24 and J82 UC cells ($p = 0.003$ and 0.014). Furthermore, BCG treatment for 48 hours could enhance the expression of TLR7 in UC cells even when the BCG dosage was as low as 0.5 mg/mL, and TSGH8301 cells had the highest expression level of TLR 7, which was 4.1-fold higher than that of other UC cells ($p = 0.005$) (Figure 2C).

Phagocytotic activities increased in UC cells through TLR7 activation after BCG treatment

TSGH8301 cells were selected for further analysis since they had a higher expression of TLR7. The phagocytic activity of BCG-stimulated TSGH8301 cells increased markedly from 6 hours to 24 hours after treatment ($p = 0.05$) (Figure 3A). As shown in Figures 3B—E, phagocytosis-related factors, including beclin 1, ATG2, and LC3, were all upregulated in TSGH8301 cells after BCG treatment. Beclin 1 displayed a 3.9-fold increase compared with the control group. It also significantly increased (1.2-fold) 24 hours after stimulation with a lower dosage of BCG (0.05 mg/mL), but a reverse change was seen in the case of a higher-

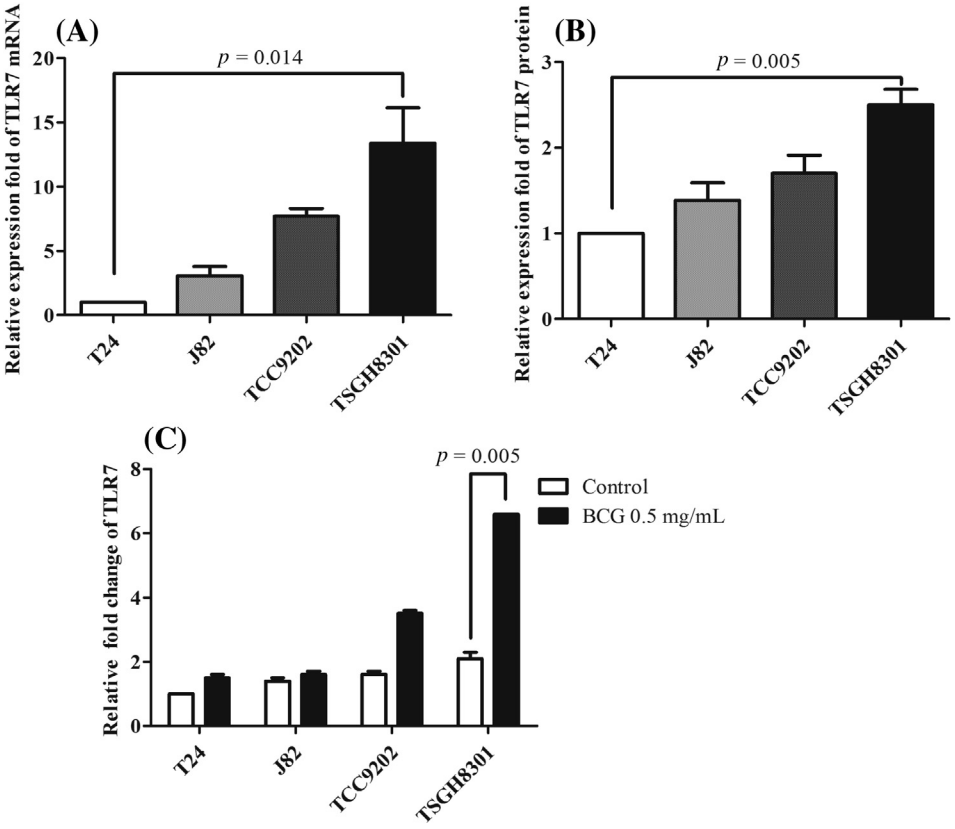

Figure 2. Relative expression of (A) mRNA and (B) protein fold change of TLR7 in T24, J82, TCC9202, and TSGH8301 cells. (C) Changes of TLR7 in T24, J82, TCC9202, and TSGH8301 cells after BCG stimulation (0.5 mg/mL) at 48 hours. All experiments were repeated three times. BCG = Bacille Calmette-Guerin; TLR = toll-like receptor.

dosage of BCG (0.5 mg/mL) treatment (Figure 3B). Both ATG2 and LC3 showed similar increasing results to beclin 1 with BCG treatment regardless of the dosage used (Figure 3B), ATG2 increased 1.3-fold compared with the control group at 24 hours, and LC3 increased significantly (2.6-fold) at 12 hours when stimulated by a higher dosage of BCG ($p = 0.03$) (Figures 3C and 3D). IRAK2/4, TLR7 downstream regulators, were significantly increased (2.5- and 4.2-fold, respectively; $p = 0.001$) after treatment with a higher dosage of BCG for 12 hours. The same finding was noted with caspase 8 when a significant maximum increase was observed at 12 hours ($p = 0.006$) (Figures 3E and 3F).

Changes of TLR7 in UC cells induced by BCG treatment was inhibited by IRS661 TLR7 antagonist

Increased TLR7 in TSGH8301 cells after 24 hours of BCG treatment was significantly suppressed to the basic level, as the cells were controlled by IRS661 treatment ($p = 0.001$) (Figure 4).

Discussion

Cell response to mycobacteria has been shown to depend on TLR signaling. BCG can stimulate various types of TLRs by different mycobacterial components with secondary inflammatory reaction, such as lipopolysaccharides (LPS) can stimulate TLR1 and TLR4 [10], and BCG DNA or RNA strains can stimulate TLR7 and TLR9 expression [11]. TLR4 is also involved in the response to mycobacteria, and an *in vitro* study on human epithelial cells showed that BCG induced activation of the ERK1/2 MAPK pathway and secretion of interleukin-8 via both TLR2 and TLR4 signaling [12]. TLR4 signaling is required for the development of a Th1 response that can increase lymphocyte-mediated cytotoxicity against tumor cells [13].

TLRs are poised to have a dual role in tumor biology [14,15]. In one way, they are potent activators of the nuclear factor (NF)-κB pathway [16]. NF-κB regulates the transcription of a number of antiapoptotic genes such as bcl-2, iNOS, c-FLIP, inhibitor of apoptosis, and TRAF molecules [17]. Therefore, they can promote growth of tumors such as lung cancers [18], colorectal cancers [19], ovarian cancers [20], renal cell carcinomas [21], and melanomas [22]. In another way, their role in the regulation of the innate and adaptive immune responses enables them to contribute to therapy for cancers, such as bladder cancer. Many studies have shown direct cytotoxicity of TLR agonists against bladder cancer cell lines and an antitumor effect *in vivo*. Ayari et al [23] reported the expression of many TLRs in normal urothelium and maintenance with reduced amounts in nonmuscle-invasive bladder tumors Normal urothelium expressed TLR5 weakly; TLR2, TLR3, and TLR7 moderately; and TLR4 and TLR9 strongly.

We performed a series of preliminary studies on BCG treatment of UC cells and showed that TLR2, TLR3, TLR5,

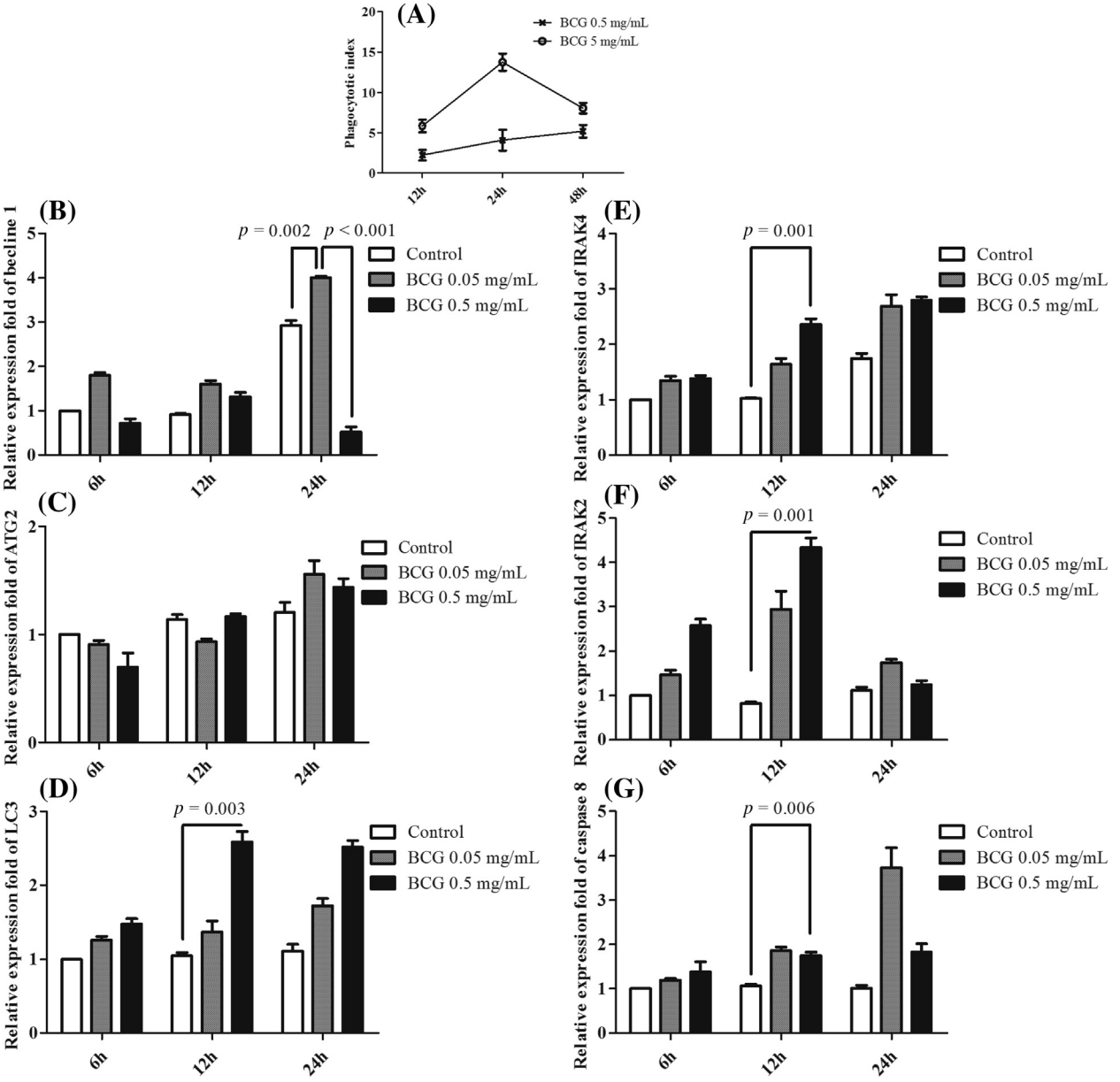

Figure 3. (A) Phagocytic index of TSGH8301 cells after being stimulated by 0.5—5 mg/mL BCG for 12 hours, 24 hours, and 48 hours ($p = 0.05$). (B—D) Changes of beclin 1, ATG2, and LC3 activities involved in phagocytosis after BCG (0.05 mg/mL and 0.5 mg/mL for 24 hours) treatment of TSGH8301 cells. (E—G) Activation of IRAK2, IRAK4, and caspase 8 after BCG (0.05 mg/mL and 0.5 mg/mL for 24 hours) treatment in TSGH8301 cells. BCG = Bacille Calmette-Guerin.

TLR8, TLR9, and TLR11 were not overexpressed, but TLR4 and TLR7 increased in UC cells after BCG stimulation. In a further study, we demonstrated that BCG can directly kill UC cells *in vitro* with activation of TLR4, TLR7, and TLR9 proteins through the caspase 8 signaling pathway (data not shown). In this study, we found that BCG can kill UC cells in a dose-dependent manner and assumed that an alternative specific pathway may be involved in the cytotoxicity of UC cells caused by BCG, in addition to its direct killing effect. Therefore, we speculated that TLRs, especially TLR7, may play an important role in BCG cytotoxicity. Ayari et al [23] reported that normal urothelium expressed TLR7 moderately; in the current study, we noted that overexpression or secondary activation of TLR7 in UC cells by BCG was closely related to its cytotoxicity. In addition to the higher expression of TLR7 intrinsically in low-grade UC cells, such as TSGH8301 and TCC9202, either in mRNA or in protein levels, TLR7 overexpression after BCG treatment in UC cells was confirmed in our study. We found that well-differentiated UC cells had a higher content of TLR7, making them more sensitive to TLR agonist—BCG treatment. Hence, we hypothesize that BCG can also kill UC cells directly through the TLR7-activated pathway, in addition to its direct cytotoxic effect.

We next investigated the mechanism of cell death via TLR7 activation by BCG treatment in TSGH8301 cells. BCG

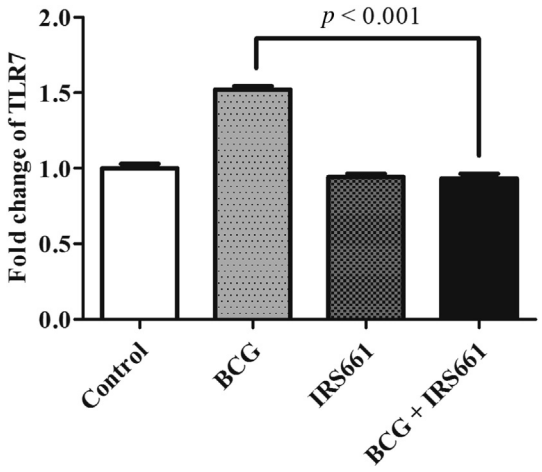

Figure 4. TLR7 expression in TSGH8301 cells after treatment with BCG (0.5 mg/mL) and TLR7-inhibitor IRS661 for 24 hours. BCG = Bacille Calmette-Guerin; TLR = toll-like receptor.

treatment showed a significantly increased apoptotic process of phagocytosis, including beclin 1, ATG2, and LC3, in UC cells. A reverse change in the case of bcl-2 was seen in treatment of UC cells with a higher dosage of BCG; this indicates that a high dosage of BCG may induce a reverse effect on the subsequent cytotoxicity to tumor cells. Our observation demonstrated that BCG-activated TLR7 significantly leads to activation of IRAK2 and IRAK4, both critical signaling mediators of the TLR/IL1-R superfamily, and production of caspase 8 in TSGH8301 cells. It is an intrinsic apoptosis pathway rather than a typical pathway with bcl-2 reduction and caspase 3 and 9 activation.

The current study demonstrates that activation of TLR7 by BCG can lead to a proapoptotic effect with increased phagocytic activity on UC cells. Although TLR4 was also functionally expressed in TSGH8301 cells, TLR4 signaling did not promote apoptotic activity of tumor cells; instead NF-κB activity was promoted to enhance the proliferating ability of UC cells (data not shown). The effect of TLR7 activation on the proliferation of TSGH8301 cells was examined; we found that overexpression of TLR7 by BCG treatment dramatically inhibits UC cellular proliferation. These results indirectly indicate that TLR7 activity in nonmuscle invasive bladder cancer cells is highly related to tumor behavior with inhibitory effects and on the contrary to TLR4.

In addition to the direct killing of tumor cells through an apoptotic pathway, TLR activation will also promote the maturation of immune cells and the production of cytokines such as interleukin-12 within the host, which signals naive T cells to mature into Type 1 helper T cells [24]. Although TLRs are more heavily expressed in normal urothelial cells, their expression and activity persist in tumor cells, offering the possibility of therapeutic use of TLR ligands. In recent years, three TLR agonists have been approved by the Food and Drug Administration for use in humans: BCG, monophosphoryl lipid A, and imiquimod [25,26]. Cherfils-Vicini et al [27] have reported that TLR7 and TLR8 agonists have been shown to increase the resistance of cultured nonsmall cell lung cancer cells to chemotherapy, an effect that followed the activation of an MYD88-NFκB signaling axis resulting in the upregulation of antiapoptotic bcl-2 proteins. The success of TLR-mediated BCG immunotherapy for nonmuscle invasive bladder cancers, such as TLR7-induced apoptotic pathway proved in our study, suggests that alternative TLR-based immunotherapies might still be successful for these cancers. In this study, the direct cytotoxicity of BCG to UC cells has been reconfirmed and TLR7-related apoptotic pathway enhancement has been demonstrated.

Conclusion

Our results indicated that the intrinsic expression of TLR7 in UC cells may be useful as a predictor of BCG therapy, and the upregulation of TLR7 using agonists can be considered as effective therapeutic alternatives for treating superficial bladder cancer in the future.

Acknowledgments

This study was supported by grants from the National Science Council (NSC101-2314-B-016-005), Tri-Service General Hospital (TSGH-C100-069, TSGH-C101-057), and Chi-Mei Research Foundation (CMNDMC-10205).

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