Construction of recombinant human IFNα-2b BCG and its antitumor effects on bladder cancer cells *in vitro*

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ABSTRACT. We constructed recombinant Bacille Calmette-Guérin (rBCG) that secreted human interferon alpha 2b (hIFNα-2b), and investigated its antitumor effects on bladder cancer cells *in vitro*. The recombinant plasmid phIFN-α-2b was constructed using pMAO-4 and transformed into BCG. The supernatant was collected at various times and IFN-γ, interleukin (IL)-12, and tumor necrosis factor (TNF)-α were detected using an enzyme-linked immunosorbent assay. EJ cells were cultivated for 24, 48, and 72 h, together with rBCG, wild-type BCG (wBCG), or wBCG+IFN-α-2b. rBCG capable of secreting cytokine IFNα-2b was constructed. On the 4th day of culture, the IFNα-2b secreted by rBCG reached a maximum. wBCG and rBCG showed no significant difference on cell growth rate over 7 days of incubation in 7H9 medium. wBCG and rBCG were both positive for acid-fast staining, and showed mycobacterial characteristics of intercellular connection in clusters with no clear abnormalities. Higher levels

of IFN-γ, TNF-α, and IL-12 were induced by rBCG compared with wBCG or MAO4-rBCG ($P < 0.05$). rBCG may induce lymphocyte proliferation; the proliferation ratio was higher than those induced by wBCG and wBCG+IFN. rBCG had direct anti-proliferative effects on EJ cells. An MTT assay showed that rBCG inhibited the proliferation of bladder cancer cells and had more activity compared with wBCG ($P < 0.05$). The highest anti-tumor activity of lymphocytes was stimulated by rBCG (20.31-51.22%). rBCG-IFNα-2b induces enhanced cytotoxicity against bladder cancer cells *in vitro* and may be used as an alternative to BCG for bladder cancer patients.

Key words: Bacilli Calmette-Guérin; Bladder tumor; EJ cells; rBCG; IFNα-2b; Peripheral blood monocytes

INTRODUCTION

Immunotherapy with intravesical administration of Bacillus Calmette-Guérin (BCG) is an effective alternative approach to chemotherapy for managing superficial transitional cell carcinoma of the bladder (Alexandroff et al., 1999; Luo et al., 2001). In 1976, Morales et al. first reported the intravesical use of BCG for treating superficial carcinoma of the bladder. BCG has been shown to eradicate residual tumors in ≥60% of patients with papillary disease and ≥70% of patients with carcinoma *in situ*. However, there are numerous limitations to the use of intravesical administration of BCG. For instance, treatment failure including recurrence and progression has been reported depending on the tumor stage and/or histology grade.

The antitumor effects of BCG occur primarily through regulation of the host immune system. In experimental studies, the cytokines interleukin (IL)-2, tumor necrosis factor (TNF), and interferon (IFN)-γ were released in the presence of BCG stimulation, which played a crucial role in the T-cell and natural killer cells effects on bladder cancer. However, there are some adverse events related to BCG immunotherapy. Local adverse reactions with an incidence of 27-95% were observed in patients who underwent immunotherapy. Moreover, severe systemic side events can occur, which caused great threats to the safety of the patients.

In cultured murine MB49 bladder tumor cells, combination of BCG and IFN-α had superior and earlier antitumor activity than BCG alone. It has been reported that recombinant BCG (rBCG)-S1PT immunotherapy resulted in bladder weight reduction compared with a single BCG treatment group and control group. Recently, the use of rBCG technology has increased for developing immunotherapeutic agents with specific targets (Sarosdy and Kierum, 1989; Gan et al., 1999). Genetic engineering technology can be used to transform wild-type BCG into rBCG that can secrete cytokines. rBCG can reduce not only the BCG dosage but also side effects through the continuous secretion of cytokines to overcome the disadvantages associated with the direct use of cytokines including perfusion because of its short half-life, water-solubility, ease of urine loss, need for a large number of perfusions, and higher cost. Other than BCG, IFNα-2b has been used with some success as an intravesical agent for superficial bladder cancer. However, its response rate of 40% has been overshadowed by that of BCG (Luo et al., 1996; Gan et al., 1999). Nevertheless, a significant number of BCG non-responders have been cured with IFNα-2b (Lamm, 1992). In contrast to BCG, intravesical IFNα therapy has few and typically mild side effects (Luo et al., 1996; Gan et al., 1999).

In this study, hIFNα-2b-expressing rBCG was constructed to confirm whether rBCG promotes the secretion of Th1-type immune factors in peripheral blood mononuclear cells (PBMCs). In addition, we investigated the direct inhibition effect of hIFNα-2b-rBCG on a bladder cancer cell line and the lethal effects of the activated peripheral blood lymphocytes on EJ bladder tumor cells.

MATERIAL AND METHODS

Plasmids and bacterial strains

Escherichia coli DH5α cells were provided by Tianjin Institute of Urology (Tianjin, China). Danish BCG D2BP302 was purchased from the Beijing Institute of Biological Products (Beijing, China). Plasmid pMAO-4 and the plasmid containing hIFNα-2b cDNA were kindly provided by Prof. Y. Luo from the University of Iowa (Iowa City, IA, USA).

Main reagents

Middlebrook 7H9 Broth (Difco), Middlebrook 7H10 Agar (Difco), T4DNA Ligase, *Bam*HI, *Eco*RI, *Nco*I, *Bsp*HI, *Cla*I, *Nla*III, *Taq* enzyme, the E.Z.N.A Gel Extraction Kit, DNA Fragment Purification Kit Ver 2.0, and the Wizard Purification Plasmid DNA Purification System were purchased from TaKaRa Co., Ltd. (Dalian, China). Human IFN-α, IFN-γ, TNF-α, IL-12, and the enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience, Inc. (San Diego, CA, USA). IFNα-2b and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

Human bladder cancer cell line and effector cell preparation

Human bladder cancer EJ cells were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-G, and 100 g/mL streptomycin in a humidified air/5% CO_2 incubator at 37°C.

PBMCs were isolated from heparinized blood samples using standard Ficoll-Paque centrifugation. The cellular viability of PBMCs was determined using a trypan blue exclusion assay. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM β-mercaptoethanol, and 30 g/mL kanamycin at 37°C in 5% CO_2. To define the type of effector cells involved in the enhancement of rBCG-mediated PBMC cytotoxicity on bladder cancer cells, CD8+ T lymphocytes were isolated from PBMCs after BCG stimulation using the MACS negative selection kits (130-094-156 for CD8+ T lymphocyte cells), and then used as effector cells in the antitumor assay.

Construction of pMAO-4-hIFNα-2b

cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. Amplification of hIFNα-2b cDNA was carried out using a Geneamp PCR 2400 system (PerkinElmer Inc., Waltham, MA, USA). The primers used for hIFNα-2b were 5'-CAAGggatccTGTGATCTGCCTCAAACCCA-3'and

5'-GCCGgaattcTCATTCCTTACTTAAACTTT-3'. The cDNA of Nrf2 was amplified by polymerase chain reaction (PCR) (predenaturation at 94°C for 5 min; 30 cycles: 94°C, 30 s; 55°C, 50 s; and 72°C, 50 s; and a final extension at 72°C for 7 min) using 150 μg/mL cDNA template according to manufacturer instructions. Subsequently, the mixture was digested with *Bam*HI and *Eco*RI in a 40-μL total reaction volume containing 50 μg/mL pMAO-4 and 30 μg/mL hIFNα-2b cDNA at 37°C for 10 h.

DNA purification was performed according to manufacturer instructions. Subsequently, hIFNα-2b cDNA was linked to the pMAO-4 plasmid by T4 DNA Ligase. Next, 100 μL DH5α competent bacteria was mixed with 1 μL recombinant plasmid and placed in an ice bath for 30 min, heat-shocked at 42°C for 45 s, and placed in an ice bath for 5 min. After adding 0.9 mL Luria-Bertani (LB) medium, the mixture was cultured at 37°C for 90 min. Finally, the mixture was inoculated onto LB solid medium containing 30 μg/mL kanamycin, and incubated at 37°C for 24 h. To validate the DNA sequence of pMAO-4-hIFNα-2b, gene sequencing was carried out.

Construction of rBCG-hIFNα-2b

BCG was cultured in Middlebrook 7H9 medium in a shaker at 37°C until an optical density at 600 nm (OD_{600}) of 0.5 was reached. A total of 100 μL competent BCG and 10 μg pMAO4-IFNα-2b were mixed to construct recombinant BCG according to the previous description (Figure 1). After 1.5 h of incubation in an ice bath, the sample was centrifuged at 2800 *g* for 10 min. Next, 20 mL 4°C-precooled 10% glycerol was used to wash the precipitate, the sample was centrifuged, and the wash was repeated. The pellet was resuspended in 10% precooled glycerol and the resulting mixture containing BCG was divided into several 1.5-mL tubes for immediate electroporation or storage at -70°C for later use. Approximately 5-15 μg prepared plasmid DNA (dissolved in 10-20 μL) was added to 100 μL competent BCG. The total volume of ≤150 μL was mixed for electro-transduction at a voltage of 1.8 kV. Next, 1 mL 7H9 medium was added and the sample placed in a shaking incubator for 5 h at 35°C. The culture was coated onto 7H10 solid medium with 30 μg/mL kanamycin for incubation at 37°C. After 4-6 weeks, colony selection was carried out. Positive colonies were picked after 3-4 weeks and verified by acid-fast staining. Acid-fast staining is used to observe morphological changes. rBCG-IFNα-2b plasmids were extracted and human IFNα-2b was amplified using the same primer pairs. The size of the insert was confirmed by electrophoresis. rBCG-IFNα-2b was induced by temperature-shift induction and hydrogen peroxide. After electrophoresis, PCR products were purified and prepared for sequencing.

Identification of positive colonies and growth characteristics

A single colony grown on the selection plate was picked for inoculation into 10 mL 7H9 liquid medium containing 30 μg/mL kanamycin at 37°C at 150 rpm with shaking. After reaching an OD_{600} of 1, 1 mL sample was centrifuged at 10,000 rpm for 10 min. The pellet was washed 3 times with deionized distilled H_2O (ddH_2O) and then 30 μL ddH_2O was added to dissolve the sediment. The sample was boiled for 10 min, centrifuged at 10,000 rpm for 10 min, and 20 μL supernatant was used as a template for PCR analysis. The protocol was the same as that used for hIFNα-2b-cDNA. After electrophoresis, the PCR products were removed from the gel to purify the sequencing templates. The primer 5'-CAAGGGATCCTGTGATCTGCC

TCAAACCC-3' was used for sequencing.

hIFNα-2b-rBCG, MAO4-rBCG, and BCG cultures were separately inoculated into 50 mL 7H9 medium, each in 3 bottles, and OD was measured. Microscopic morphology was observed after acid-fast staining. To 1 mL bacterial culture solution, 9 mL 7H9 medium was added, and this dilution procedure was repeated an additional 4 times (i.e., 1:10,000 and 1:100,000). Next, the diluted solution was used for inoculation onto 7H10 solid medium containing 30 μg/mL kanamycin, and then cultured at 37°C for 4 weeks. The average after counting the results of 3 dishes of the same concentration was used to calculate the number of viable cells per mL BCG. Viable BCG count = (colony number of 1:10,000 x 10^5 + colony number of 1:100,000 x 10^6) / 2. When A_{600} = 1, rBCG was approximately 1.4 x 10^8 colony-forming units (CFU).

hIFNα-2b expression detection

The bacterial culture of hIFNα-2b-rBCG or MAO4-rBCG was centrifuged at 10,000 rpm for 10 min. Expression of secreted extracellular hIFNα-2b was detected. The pellet was washed 3 times, 100 μL ddH$_2$O was added, and the sample was ultrasonicated in an ice bath until the solution was clear. Next, the sample was centrifuged at 10,000 rpm for 10 min to collect the supernatant containing hIFNα-2b. The level of hIFNα-2b was determined using an ELISA according to manufacturer instructions. Each sample was taken in triplicate and each specimen was tested in 2 wells. Sample content was determined according to the standard curve.

Changes in hIFNα-2b secretory dynamics *in vitro*

rBCG single colonies were cultured in 7H9 medium in 200-mL Erlenmeyer flasks, and were placed in a shaking incubator at 38°C and 150 rpm under constant humidity. Next, 1 mL sample was acquired every day for 10 days. The OD$_{600}$ was measured using a spectrophotometer. Bacteria in the medium were centrifuged at 4000 rpm to collect the supernatant. The supernatant was stored at 20°C and used to detect changes in hIFNα-2b secreted from rBCG at different growth cycles. IFNα-2b protein level was determined by ELISA according to manufacturer instructions.

Th1-type cytokines secreted by PBMCs

PBMC extracts were freshly prepared from the peripheral blood of healthy subjects. hIFNα-2b-rBCG, MAO4-rBCG, and BCG were co-cultured with PBMCs on 24-well plates. The final concentration of bacteria was 1.4 x 10^6 CFU/mL and that of the cells was 1.0 x 10^5 cells/mL. A sample containing only PBMCs was used as a blank control. After culturing in a CO$_2$ incubator, the supernatant was collected at 12, 24, 48, and 72 h, as well as at 5 and 7 days and stored at 70°C. The cytokines IFN-γ, TNF-α, and IL-12 were detected by ELISA according to the manufacturer protocol.

Lymphocyte proliferative activity detection

Lymphocytes were isolated from PBMCs as effector cells and divided into a control group and 3 experimental groups (rBCG group, wild-type BCG group, and wild-type BCG plus IFN-α group). Lymphocytes were cultured for 2 h. Lymphocytes unattached to a 24-well

cell culture plate were collected and the concentration adjusted to 2×10^5/mL. The final concentration of BCG was 1.4×10^6 CFU/mL, with 1 mL in each well. After co-culture for 0, 24, 48, and 72 h, 100 μL was added to a 96-well plate.

Measurement of bladder tumor growth inhibited directly by rBCG

Bladder tumor EJ cells (1×10^5/mL density) were cultured during the logarithmic phase and inoculated on a 96-well plate, cultured for 16 h to allow complete attachment to the plate, and rBCG, wild-type BCG, and wild-type BCG plus IFN-α in mixed composition were added to the plate. The 3 groups were cultured at 37°C in saturated humidity and 5% CO_2 for 24 and 72 h. Next, 20 μL 5 mg/mL MTT was added to the wells and the plate was cultured for 4 h. The plate was centrifuged and the supernatant was discarded. Next, dimethyl sulfoxide was added to dissolve the pellet. An enzyme-linked immune detector was used to measure the concentration at 570 nm. Six wells were measured to calculate the mean. The following equation was used to determine the cell growth inhibition rate: cell growth inhibition rate (%) = (control group A value - treatment group A value) / control group A value x 100%.

Measurement of the killing effect of BCG-activated lymphocytes on tumor cells by a lactate dehydrogenase (LDH) release test

Lymphocytes were cultured for 2 h, after which the concentration was adjusted to 4×10^5/mL; rBCG, wild-type BCG, and wild-type BCG plus IFN-α were added and then cocultured at 37°C in 5% CO_2 in a saturated humid atmosphere for 72 h. In the LDH release experiment, the effector cells were lymphocytes that had been divided into experimental and control groups, while the target cells were EJ bladder cancer cells. The effector to target ratio was 50:1. Next, 0.1 mL of each proliferating lymphocytes and EJ cells were mixed and incubated at 37°C in 5% CO_2 for 4 h; incubation was carried out at 37°C for 10 min, after which additional substrate liquid was added and an ultraviolet spectrometer at 340 nm was used for measurements at 30 s, 1 min, 2 min, and 3 min to determine the A value. The following formula was used to determine kill activity: kill activity (%) = (LDH-L - natural release LDH-L) / (maximum release LDH-L - natural release LDH-L) x 100%.

Statistical analysis

All data are reported as means ± standard deviation. SPSS18.0 was used for statistical analysis (SPSS, Inc.; Chicago, IL, USA). The chi-square test was used for intra-group comparisons, whereas the Student *t*-test was performed for inter-group comparisons. $P < 0.05$ was considered to indicate a significant difference.
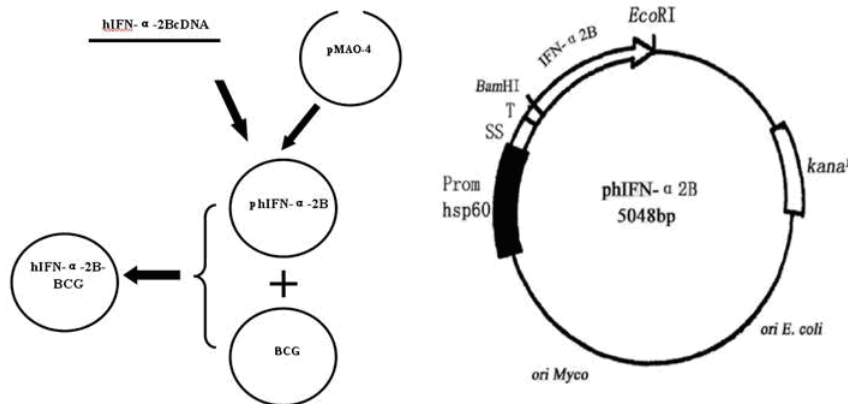
RESULTS

### Construction and characterization of pMAO4-hIFNα-2b

The upstream primer contained *Bam*HI restriction sites, while the downstream primer contained *Eco*RI restriction sites. The plasmid containing hIFNα-2b cDNA was used as a template for the PCR. A 500-bp hIFNα-2b cDNA fragment was obtained after *Bam*HI and *Eco*RI

digestion as indicated in Figure 1. Double-digestion using *Bam*HI and *Eco*RI was carried out for the PCR products, and a 500-bp fragment was identified after electrophoresis (Figure 2, left). A DNA fragment (approximately 5 kb) was obtained after *Bam*HI and *Eco*RI digestion of pMAO-4 (Figure 2, right). The recombinant plasmid phIFN-α-2b formed using the digested fragments was transformed into *E. coli*, which showed many scattered, smooth, yellow-white colonies after culture. After double-digestion and electrophoresis, a 500-bp band (hIFN-α-2b cDNA) and a 5-kb band (pMAO-4 digested fragments) were identified (Figure 2, left). After PCR with both primers designed based on the sequences of the cloning site ends, a band of approximately 500 bp appeared on the gel after electrophoresis (Figure 2, right). Sequencing demonstrated that the hIFNα-2b sequence cloned into pMAO4-hIFNα-2b was identical to that in GenBank.

Figure 1. Construction strategy of recombinant BCG and plasmid profile of phIFN-α-2B.
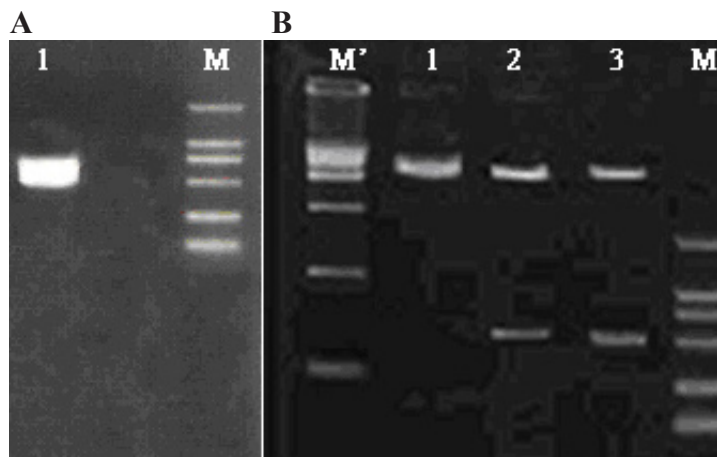
Figure 2. A. Electrophoresis results of digested PCR product of hIFN-α-2B-cDNA. *Lane 1*, PCR products of hIFN-α-2B-cDNA were treated by *Bam*HI, *Eco*RI double digestion reaction, to produce 500 bp fragment with sticky ends; *lane M* = DNA marker DL2000 (bands from bottom: 100, 250, 500, 750, 1 k, 2 kbp). **B.** Electrophoresis results of phIFN-α-2B before and after digestion: *lane M* = DNA marker DL15000 (from bottom 250, 1 k, 2.5 k, 5 k, 7.5 k, 10 k, 15 kbp); *lane 1* = pMAO-4 by *Bam*HI and *Eco*RI double digestion; *lanes 2-3* = double-digested plasmids phIFN-α-2B, yield two bands about 500 bp (hIFN-α-2B cDNA) and 5kb (cutting-off pMAO-4); *lane M* = DNA marker DL2000.

A   **B**
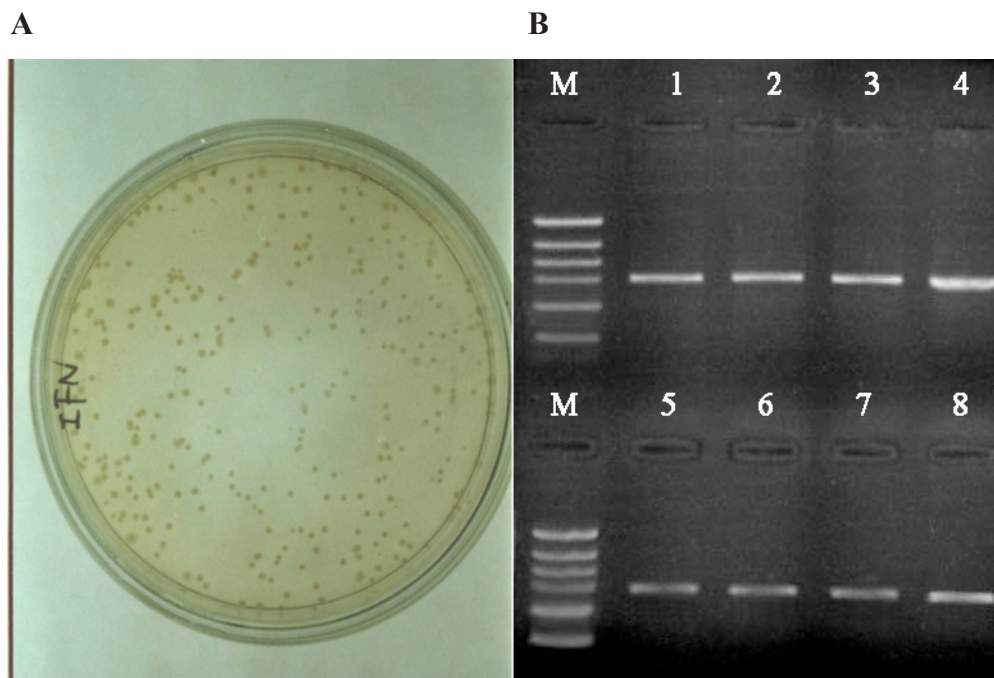
Figure A. Formation of single colony after phIFN-α-2b transformed into *Escherichia coli*. The smooth yellow-white colony appeard on LB solid medium in the presence of kanamycin, after culture for 10 h. **B.** Electrophoresis results of phIFN-α-2B PCR products. *Lane M* = DNA marker DL2000; *lanes 1-7* = hIFN-α-2B PCR product of recombinant plasmid phIFN-α-2B multiplicated in electrotransducted *E. coli* growed on the above LB plate, a band of about 500 bp; *lane 8* = the PCR result of plasmid containing hIFN-α-2B, as positive control.

Construction of rBCG- hIFNα-2b

Four weeks after electrotransformation, several white, scattered, dry bacterial colonies were identified on the culture dish containing kanamycin. After 6 weeks, colony diameters were 1 mm as indicated in Figure 3. A PCR with a pair of primers specific to the hIFNα-2b sequence showed a 500-bp band (Figure 3, right). DNA sequencing results showed that the sequence was identical to that of hIFNα-2b cDNA in GenBank.

### Identification of hIFNα-2b-rBCG and its growth characteristics

No significant difference was noted in the growth curves of BCG, MAO4-rBCG, and rBCG from days 1-7. On day 8, BCG and MAO4-rBCG decreased slightly to 1.22 ± 0.10 and 1.21 ± 0.09; rBCG reached a plateau of 1.22 ± 0.08 (Figure 4).

No obvious difference was noted between groups (all $P > 0.05$). In this study, BCG and rBCG showed OD_{600} values of 1 and were 1.4×10^8 CFU/mL. Similar colony patterns, acid-fast staining characteristics, morphology, and interconnected cluster features were observed, although the microscopic rBCG body was slightly larger than that of BCG (Figure 5).

A   **B**
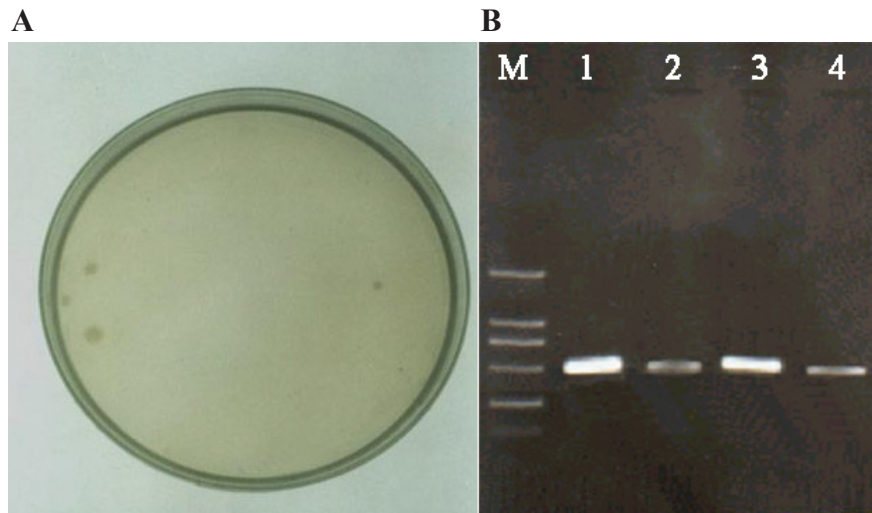
Figure 4. A. Formation of single colony of recombinant BCG transformed by phIFN-α-2B. After electrotransformation, culturing for 4 weeks on 7H10 plate in presence of kanamycin, several yellow-white, scattered, dry surface of recombinant BCG colonies appeared. **B.** Electrophoresis results of rBCG PCR product: *lane M* = DNA marker DL2000; *lane 1-4* = PCR product of hIFN-α-2B-rBCG collected from the 7H10 plate, a band of about 500 bp.
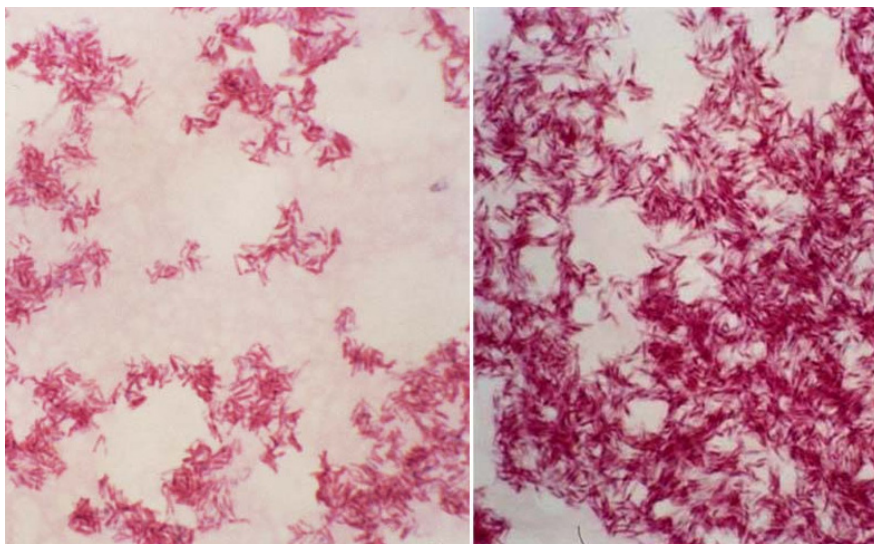

Figure 5. Morphology of anti-acid stained rBCG and wBCG: wild-type BCG (left), hIFN-α-2b rBCG (right). Similar acid-fast staining characteristics, morphology, and interconnected cluster features, while microscopy rBCG slightly larger than the wild one. (LMC 1000X).

hIFNα-2b expression

When rBCG was incubated to approximately 1.4×10^8 CFU/mL, the content of hIFN-α-2b secreted by hIFN-α-2b-rBCG into the supernatant was 998.0 ± 9.3 pg/mL, while the intracellular content was 99.2 ± 3.1 pg/mL. Expression of hIFN-α-2b by both wild-type BCG

and MAO4-rBCG was negative. Approximately 100 pg IFN secreted by rBCG was equal to 1 IU in the activity titer. The dynamics of IFNα-2b in the supernatant with the growth of rBCG are shown in Figure 6. From days 4-7 of culture, rapid growth of rBCG was observed, followed by a slow growth period. The initial increase in interferon production was noted on the 3rd day, reaching a plateau (initial 0_{600} = 0.2) from days 6-10.
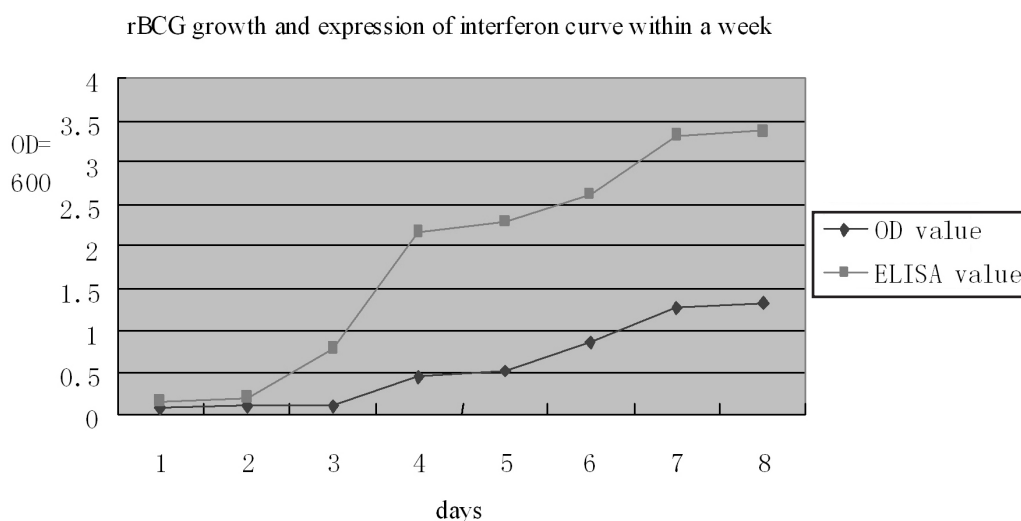
Figure 6. rBCG growth and expression of interferon curve within a week.

Th1-type cytokines secreted by activated PBMCs

The level of hIFN-γ produced by rBCG-induced PBMCs was significantly increased compared to that in the wild-type BCG (wBCG) and MAO4-rBCG groups ($P < 0.05$, Figure 7). No significant difference was observed in the levels of IL-12 observed at 12 and 24 h in the wBCG group, MAO4-rBCG group, and rBCG group ($P > 0.05$). Subsequently, a slow downward trend was observed, during which IL-12 expression in the rBCG group was significantly higher compared with that of the other 2 groups ($P < 0.05$). The levels of TNF-α were remarkably increased at 12 h in each group; however, no significant difference was identified ($P > 0.05$). In addition, TNF-α levels in the rBCG group were higher than those in the other 2 groups ($P < 0.05$).
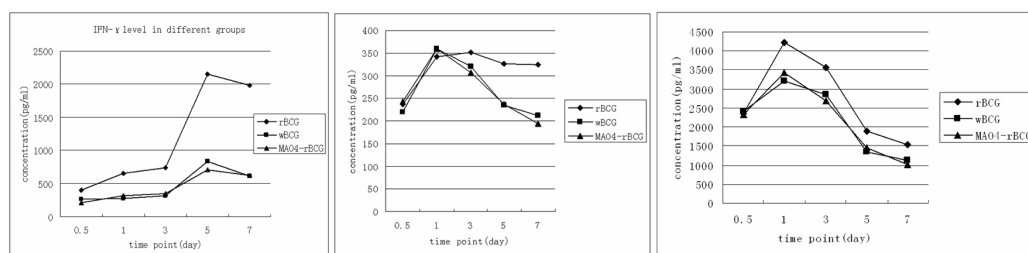
Figure 7. IFN-γ, IL-12, TNF-α levels in different groups.

Lymphocyte proliferation induced by rBCG

Figure 8 summarizes the proliferation of lymphocytes as determined by the MTT test, which indicated that the proliferation of lymphocytes in the rBCG group, BCG group, and wBCG plus IFN-α-2b group was higher than that in the lymphocyte group. The wBCG plus IFN-α-2b group showed a higher multiplication effect than the BCG group at 48 h, but lower than the rBCG group (P < 0.05).
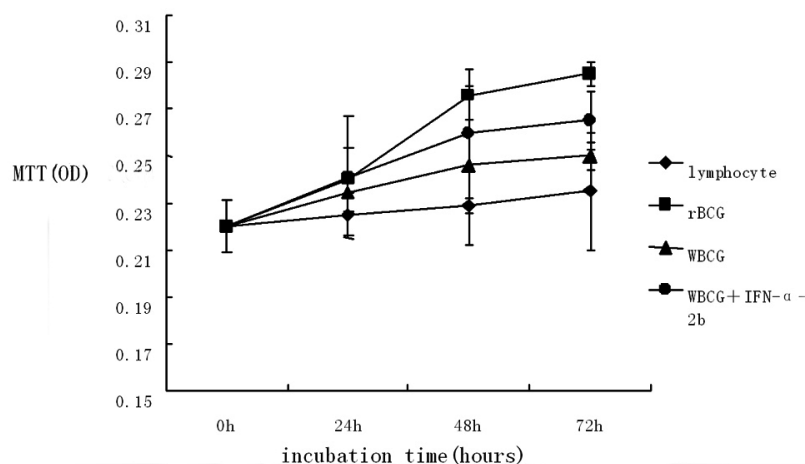
Figure 8. Comparison of the proliferation effect of lymphocyte with different group stimulation.

Inhibition of bladder tumor cell growth

Table 1 summarizes the inhibition rates of cancer cells in each group. Compared with the BCG group, remarkable inhibition was noted in the rBCG group and the BCG plus IFN-α-2b group (P < 0.05). In each group, the cell growth inhibition rate increased with treatment time. There was no significant difference in tumor cell suppression between the BCG plus IFN-α-2b group and the rBCG group (P > 0.05).

Table 1. Comparison of the EJ cell growth inhibition rate (%).

Time	rBCG	wBCG	BCG+IFN-α-2b
24 h	31.38	21.73	32.40
48 h	60.04	44.70	58.17
72 h	79.21	56.21	69.23

Killing activity of rBCG-activated lymphocytes on bladder cancer cells

The killing activity of lymphocytes activated by rBCG and BCG is detailed in Table 2. The killing activity of lymphocytes activated by rBCG was 2.67-6.74 times higher than that observed in the blank group (simple lymphocytes), 1.64-2.45 times higher that in the BCG group, and 1.25-1.66 times higher than that in the BCG plus IFN-α-2b than group. The

activity of killing EJ cells of simple lymphocytes without stimulation was 7.60%; this value is much lower than that resulting from BCG stimulation after co-incubation (12.38-20.89%) ($P < 0.05$). The killing effects of activated lymphocytes stimulated with rBCG was highest (20.31-51.22%) ($P < 0.05$) among all groups, and that of the BCG plus extrinsic IFN-α-2b group was 15.78-34.72%, which is significantly higher than that of the BCG group ($P < 0.05$), with a dose increase from 2.5 μL to 10 μL. BCG-activated killer cell (BAK) killing activity also increased in each group, in which the peak was obtained with 10 μL, showing a significant difference between the mixed group and the rBCG group ($P < 0.05$).

Table 2. Different concentrations of various components stimulate lymph cell damage is active (%).

Concentrations	rBCG	wBCG	BCG+IFN-α-2b
2.5 μL	20.31	12.38	15.78
5.0 μL	32.73	16.24	22.92
10.0 μL	51.22	20.89	34.72
20.0 μL	30.19	17.46	24.11

DISCUSSION

Intravesical BCG is widely used for treating superficial transitional cell carcinoma of the bladder (Lamm, 1992; Morales and Nickel, 1992; O'Donnell et al., 1994); however, the exact mechanism of how BCG mediates antitumor activity remains unclear. Non-muscle invasive bladder cancer shows significant recurrence and progression rates despite transurethral resection. Currently, the standard method to decrease the risk of recurrence and progression is adjuvant BCG treatment followed by BCG maintenance. Unfortunately, a significant number of patients experience recurrence and progress to invasive cancer. Thus, several randomized trials have been carried out using combination therapy to reduce recurrence and progression rates (Houghton et al., 2013). Recently, IFNα-2b has been used as second-line chemotherapy for bladder cancer. Some patients with no response to BCG are sensitive to IFN. In addition, IFN shows lower local and systemic toxicity in clinical practice. Because of the short intravesical retention period of bladder perfusion, extraneous IFN fails to induce the desired immune responses. The amount of IFN used in single therapy or in combined therapy during intravesical perfusion are far beyond that required to induce local antitumor immunity, resulting in repeated perfusion and high costs.

To overcome these limitations, recombinant BCG vaccines were developed using modern genetic engineering techniques in our study to sustain secretion of hIFN-α-2b. BCG may bind to the bladder epithelium through fibronectin and be maintained in cells for several days or even months. Thus, recombinant interferon may be expressed *in vivo*. The growth curves of single hIFN-α-2b and wild-type BCG plus hIFN-α-2b showed no significant difference, indicating that hIFN-α-2b did not affect the growth of BCG.

It has been well established that human PBMCs stimulated by BCG can generate effector cells that are cytotoxic to bladder cancer cells *in vitro*. In this study, we found that rBCG showed a strong ability to activate PBMCs. In addition, the levels of IFN-γ, TNF-α, and IL-12 in the rBCG group were higher than those in the other 2 groups ($P < 0.05$), demonstrating that rBCG-IFNα-2b augmented T helper type 1 (Th1) cytokine IFN-γ production by PBMCs. Furthermore, the level of hIFN-γ produced by rBCG-induced PBMCs was significantly higher than that in the other 2 groups. Previous studies have demonstrated that IFN-γ, TNF-α, and

IL-12 are antiproliferative to cancer cells, as well as induce apoptosis (Thanhäuser et al., 1995; Brandau et al., 2000a). In addition, Luo et al. (2001) showed that the restructuring of hIFNα-2b-rBCG effectively induced generation of hIFN-γ, hTNF-α, and hIL-12, which was clearly higher than that in wild-type BCG and wild-type BCG plus IFN.

Our results showed that BCG plus IFNα-2b reinforced BCG immunogenicity and promoted lymphocyte proliferation and differentiation. This is critical for BCG differentiation for IFNα-2b to enhance the cytokine network control effect. As a cytokine regulating network start node, IFNα plays an indispensable role, and even a small amount of IFNα can produce network feedback.

The results of the MTT test showed lower proliferation of lymphocytes in the wild-type BCG plus IFNα-2b group. After 24 h in the BCG plus IFNα-2b group, lymphocyte proliferation was higher than that in the wild-type BCG group, and the MTT values showed that proliferation of the rBCG group was greater than that in the wild-type BCG group; rBCG group showed sustained release of IFNα-2b under the action of IFN and BCG synergy and showed a strong stimulatory effect; lymphocyte proliferation also showed a strong growth trend. Exogenously added IFNα-2b showed a reduced concentration, while wild-type BCG synergy was also reduced. Therefore, the effect of lymphocyte proliferation induced by rBCG was superior to that of BCG *in vitro*.

It is well known that stimulation of human PBMCs by viable BCG *in vitro* leads to the generation of a specialized cell population known as BAK cells (Bohle et al., 1993; Thanhäuser et al., 1993; Brandau et al., 2000b). BAK cells play an important role in the immune mechanism of the BCG anti-tumor effects and may kill bladder cancer cells through the perforin-mediated lysis pathway, which is effective for lysing natural killer cell-resistant bladder cancer cells. After BCG stimulation, lymphocytes were activated to begin proliferation, differentiation, and cytokine secretion, as well as to enhance immune activity and tumoricidal activity. rBCG and BCG plus IFNα-2β showed a stronger ability to induce lymphocyte proliferation, differentiation, and cytokine secretion. Thus, immune activity was sharply enhanced. In our study, rBCG had more obvious antitumor effects than the mixed group. The addition of external IFN to BCG showed an increased killing effect of tumor cells. However, along with its consumption, administration of the large dose of IFN for bladder perfusion in clinical practice is not practical. According to our experimental results, the killing activity of activated rBAK increased with increasing concentration. However, a higher concentration of BCG may damage the activity by influencing lymphocyte survival and inhibit its action. rBCG-IFNα-2b is an improved BCG agent that induces enhanced PBMC cytotoxicity against bladder cancer cells *in vitro*.

Whether rBCG can maintain the growth characteristics of BCG is unknown. Previous studies reported no significant difference in the growth and reproduction between a single copy of rBCG and BCG strains. However, multiple copies of the rBCG showed significantly lower replication. By detecting IFN during *in vitro* culture, we found that IFN is secreted and had complete activity. ELISA cannot be used to determine whether a protein has a complete structure and biological activity. Thus, after ELISA, biological activity was determined using a cytopathic inhibition assay. Based on the number of passages, the plasmid stability of rBCG was >92%, and no change was observed in the capacity of IFN secretion.

In our study, rBCG-IFNα-2b was constructed and its biological activity was validated, revealing the immunological activities and antitumor effects on the bladder cancer cell line EJ. Our data showed that rBCG-IFNα-2b significantly increased the antitumor effects of BCG

against bladder cancer and has advantages over traditional treatment methods combining BCG and exogenous IFN-α-2b. Thus, this rBCG strain may serve as an alternative to BCG for treating superficial bladder cancer.

CONCLUSIONS

rBCG-IFNα-2b is an improved BCG agent that can induce enhanced cytotoxicity against bladder cancer cells *in vitro*. rBCG-IFNα-2b may be an alternative to BCG for treating bladder cancer patients and reduce both the clinical dosage and side effects of BCG immunotherapy. Further studies are needed to validate the clinical applications of rBCG-IFNα-2b and to reduce BCG dosage as well as to attenuate side effects and post-operation recurrence.

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REFERENCES

Alexandroff AB, Jackson AM, O'Donnell MA and James K (1999). BCG immunotherapy of bladder cancer: 20 years on. *Lancet* 353: 1689-1694.

Bohle A, Thanhäuser A, Ulmer AJ, Ernst M, et al. (1993). Dissecting the immunobiological effects of Bacillus Calmette-Guérin (BCG) *in vitro*: evidence of a distinct BCG-activated killer (BAK) cell phenomenon. *J. Urol.* 150: 1932-1937.

Brandau S, Böhle A, Thanhäuser A, Ernst M, et al. (2000a). In vitro generation of Bacillus Calmette-Guérin-activated killer cells. *Clin. Infect. Dis.* 31 Suppl 3: S94-S100.

Brandau S, Suttmann H, Riemensberger J, Seitzer U, et al. (2000b). Perforin-mediated lysis of tumor cells by *Mycobacterium bovis* Bacillus Calmette-Guérin-activated killer cells. *Clin. Cancer Res.* 6: 3729-3738.

Gan YH, Zhang Y, Khoo HE and Esuvaranathan K (1999). Antitumour immunity of Bacillus Calmette-Guérin and interferon alpha in murine bladder cancer. *Eur. J. Cancer* 35: 1123-1129.

Houghton BB, Chalasani V, Hayne D, Grimison P, et al. (2013). Intravesical chemotherapy plus bacille Calmette-Guérin in non-muscle invasive bladder cancer: a systematic review with meta-analysis. *BJU Int.* 111: 977-983.

Lamm DL (1992). Long-term results of intravesical therapy for superficial bladder cancer. *Urol. Clin. North Am.* 19: 573-580.

Luo Y, Szilvasi A, Chen X, DeWolf WC, et al. (1996). A novel method for monitoring *Mycobacterium bovis* BCG trafficking with recombinant BCG expressing green fluorescent protein. *Clin. Diagn. Lab Immunol.* 3: 761-768.

Luo Y, Chen X, Han R, and O'Donnell MA (2001). Recombinant bacille Calmette-Guérin (BCG) expressing human interferon-alpha 2B demonstrates enhanced immunogenicity. *Clin. Exp. Immunol.* 123: 264-270.

Morales A and Nickel JC (1992). Immunotherapy for superficial bladder cancer. A developmental and clinical overview. *Urol. Clin. North Am.* 19: 549-556.

Morales A, Eidinger D, Bruce AW (1976). Intracavitary bacillus Calmette-Guérin in the treatment of superficial bladder tumors [J]. *J. Urol.* 116: 180-183.

O'Donnell MA, Aldovini A, Duda RB, Yang H, et al. (1994). Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. *Infect. Immun.* 62: 2508-2514.

Sarosdy MF and Kierum CA (1989). Combination immunotherapy of murine transitional cell cancer using BCG and an interferon-inducing pyrimidinone. *J. Urol.* 142: 1376-1379.

Thanhäuser A, Bohle A, Flad HD, Ernst M, et al. (1993). Induction of bacillus-Calmette-Guérin-activated killer cells from human peripheral blood mononuclear cells against human bladder carcinoma cell lines in vitro. *Cancer Immunol. Immunother.* 37: 105-111.

Thanhäuser A, Böhle A, Schneider B, Reiling N, et al. (1995). The induction of bacillus-Calmette-Guérin-activated killer cells requires the presence of monocytes and T-helper type-1 cells. *Cancer Immunol. Immunother.* 40: 103-108.