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Antitumor activity of recombinant Bacille Calmette-Guérin secreting interleukin-15-Ag85B fusion protein against bladder cancer

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ABSTRACT

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is used for the treatment of bladder cancer. The recruitment of neutrophlis to the bladder after BCG instillation exerts anti-tumor activity against bladder tumor. We have recently demonstrated that interleukin (IL)-17A produced by γδ T cells played a role in the recruitment of neutrophlis to the bladder after BCG instillation. IL-15 is known to play an important role in neutrophil migration during inflammation. We previously constructed a recombinant BCG strain expressing the fusion protein of IL-15 and Ag85B (BCG-IL-15) for prevention of *Mycobacterium tuberculosis* infection. Here we compared the efficacy of the BCG-IL-15 in protection against bladder cancer with that of rBCG-Ag85B (BCG).

Six-week-old female C57BL/6 mice were inoculated with MB49 bladder tumor cells in the bladder and subsequently intravesically inoculated with BCG or BCG-IL-15.

BCG-IL-15 treatment significantly prolonged survival of mice inoculated with bladder cancer cells compared with BCG treatment. Infiltration of neutrophils was significantly elevated in BCGB-IL-15 treated mice accompanied by increased chemokines (MIP-2 and MIP-1α) in the bladder. Thus, BCG-IL-15 exerted additive effect on Infiltration of neutrophils in the bladder. BCG-IL-15 may be a promising drug for non-muscle invasive bladder cancer.

1. Introduction

Intravesical inoculation of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) was first reported as an effective adjuvant therapy for bladder cancers in 1976s [1]. Transurethral resection of bladder tumor, together with intravesical BCG management is presently considered the most effective management for intermediate and high-risk non-invasive tumors. A recent study demonstrated that neutrophils that infiltrated in the bladder after BCG treatment played a key role in the antitumor effect [2]. Expression of TNF-related apoptosis-inducing ligand (TRAIL) on neutrophils in voided urine following BCG therapy suggests a direct antitumor effect of neutrophils [3,4]. In addition, neutrophils isolated from BCG-treated bladder produced CC (e.g. MIP-1α) as well as CXC chemokines (e.g. IL-8 and GRO-α). The chemokines released by activated neutrophils attract further neutrophils. We have recently reported that BCG treatment in murine bladder induced interleukin (IL)-17A production by γδ T cells, which play an essential role in local neutrophil infiltration [5]. Thus, both IL-17A and chemokines derived from γδ T cells and neutrophils, respectively, may exert antitumor effects on bladder tumor.

IL-15 is a pro-inflammatory cytokine with structural similarities to IL-2 [6]. The receptors of these cytokines share the β and γ chains, while the unique IL-15Rα subunit displays broader tissue distribution such as monocytes, macrophages, dendritic cells and neutrophils, and endothelial, epithelial [6]. IL-15 plays an important role in the therapy of neoplasia and has been studied as an anti-cancer agent [6,7]. IL-15 is involved not only in maintenance of NK and memory CD8T cells but also in the activation and migration of neutrophils [8–11]. Verri et al. showed that IL-15 mediated neutrophil migration by triggering IL-18 production. Their report demonstrated that IL-15 played an important role in neutrophil migration during inflammation, triggering a sequential IL-15, IL-18, MIP-2, MIP-1α, TNFα, and LTB4 signaling cascade for neutrophil migration [8].

In our previous report, we constructed a recombinant BCG strain expressing IL-15 and Ag85B fusion protein (BCG-IL-15), which conferred much stronger protection against *M. tuberculosis* challenge in the lung [12,13]. In the present study we examined whether BCG-IL15 treatment could show enhanced anti-tumor effects against bladder cancer compared with BCG treatment.

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2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Japan SLC (Hamamatsu, Japan). CδKO (gd T cells-deficient) mice, which was maintained in B6 background, were kindly provided by Dr. S. Itohara. The mice were bred in specific pathogen-free conditions in our institute. We used 6- to 8-week-old female mice for the experiments. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine, Kyushu University. Experiments were conducted under the control of the Guideline for Animal Experiment.

2.2. BCG

The construction of BCG-IL-15 was previously reported [12]. Briefly, after the mIL-15 sequence was confirmed by DNA sequencing, the DNA fragment was cloned into the pKH20 that contains Ag85B gene. The DNA fragment of Ag85B and IL-15 was inserted into the pSO246, a *E. coli*-mycobacteria shuttle vector which possesses a kanamycin-resistance gene as a selection marker. pSO246-mIL-15-Ag85B were then introduced into BCG (Tokyo 172 strain) by electroporation. The transformed BCG was plated on Middlebrook 7H10 agar supplemented with 10% OADC, 20 μg/ml kanamycin, 100 U/ml penicillin G and 100 μg/ml cycloheximide. After growing for 3 weeks at 37 °C, some single colonies were picked up and grown in Sauton media for 3 weeks at 37 °C. In the present study, we used BCG strain expressing the fusion protein of Ag85B (BCG) as control.

2.3. Tumor

The MB49 murine bladder cancer cell line was kindly provided by Dr. Timothy L. Ratliff. The cells were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES. They were maintained in 75 cm^2 tissue flasks at 37 °C in a humidified 5% CO_2 atmosphere and passaged 2–3 times weekly.

2.4. Treatment protocol

We used a well-defined murine syngeneic bladder tumor model for BCG immunotherapy [2,5]. Briefly, mice were catheterized to receive an intravesical inoculate of 1×10^5 MB49 tumor cells on day 0. On days 1, 8, 15, and 22 following tumor cell implantation, mice were treated intravesically with either 3×10^6 CFU of BCG, BCG-IL-15 or PBS for a total of four instillations. Immediately after BCG injection, the urethras of the mice were ligated by 3-0 silk and released 3 h later.

2.5. Isolation of bladder neutrophils and lymphocytes and flow cytometric analysis

Bladders were dissected from mice and minced to yield 1–2 mm pieces. To harvest neutrophils and lymphocytes, the bladder pieces were incubated in a mixture of 1 mg/ml collagenase (Invitrogen, Carlsbad, CA, USA) and 20 μg/ml DNase (Sigma-Aldrich, St. Louis, MO, USA) in RPMI 1640 containing 10% FCS for 90 min at 37 °C. Neutrophils and lymphocytes were analyzed by flow cytometry (FACS) using various antibodies as follows: FITC-conjugated anti-TCRγδ (GL3), anti-TCRγδ (H57-597), and anti-I-A/E (M5/114.15.2) mAbs; allophycocyanin-conjugated anti-CD3ε (145-2C11) mAb; and allophycocyanin-conjugated streptavidin (all purchased from eBioscience, San Diego, CA, USA). Stained cells were run on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using Cell Quest software (BD Biosciences).

2.6. In vivo depletion of neutrophils

Groups of 5 mice were treated intravesically with BCG-IL-15, BCG or PBS for a total of four instillations. 200 μg of anti-Gr-1 mAb (RB6-8C5) or the isotype control mAb was given i.p. every 7 days. Depletion of each cell subset was confirmed by flow cytometric analysis (data not shown).

2.7. Reverse transcription (RT)-PCR for MIP1a and MIP2 production

We used the supernatant from dissected bladders as described for ELISA assays to evaluate MIP1α and MIP2 mRNA levels by RT-PCR. Total cellular RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY, USA). First-strand cDNA was synthesized using reverse transcriptase (SuperScript RT; Invitrogen Life Technologies) with random primers. The PCR primers were as follows: β-actin sense, 5′-TTCTGCATCCTGTCAGCAAT-3′ and antisense, 5′-TAAA ACGCAGCTCAGTAACAGTCCG-3′; MIP-2 sense, 5′-ACTTCAGCCTAGCG CCATGG and antisense, 5′-AGGTCAGTTAGCCTTGCCTT-3; and MIP-1α sense, 5′-AACATCATGAAGGTCTCCAC-3′ and antisense, 5′-CCAAGACT CTCAGGCATTCA-3′. The PCR products were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. Statistical analyses

Survival of mice was evaluated using Kaplan–Meier plots and the log-rank test. Statistical significance was calculated by Student's *t*-test using GraphPad Prism 5.0 software (Prism Graphpad, San Diego, CA, USA). Differences with p values of <0.05 were considered statistically significant.

3. Results

3.1. BCG-IL-15 treatment showed enhanced antitumor effects compared with rBCG-Ag85B and suppressed growth of murine vesical tumor

We first examined the antitumor effect of BCG-IL-15. As shown in Fig. 1A, mice treated with BCG-IL-15 exhibited significantly longer survival compared with BCG-treated mice. Next, we harvested bladders and measured the tumor volume on day 20 after vesical administration with MB49 tumor cells to evaluate the antitumor effect of BCG-IL-15. As shown in Table 1, tumor growth was significantly suppressed in BCG-IL-15-treated mice compared with mice treated with either BCG or PBS.

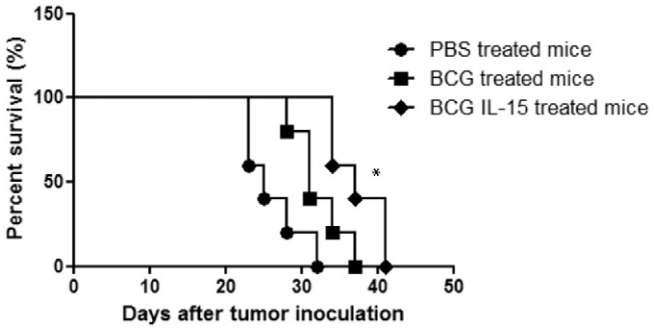

Fig. 1. Efficacy of BCG-IL-15 treatment against bladder cancer. C57BL/6 (B6) mice were intravesically inoculated with 1×10^5 MB49 tumor cells on day 0 and received repeated intravesical injections of BCG-IL-15, BCG or PBS (♦, BCG-IL-15-treated C57BL/6 mice; ■, BCG-treated C57BL/6 mice; ●, PBS-treated mice) on days 1, 8, 15, and 22 following tumor cell inoculation. C57BL/6 mice treated with BCG-IL-15 after tumor cell intravesical inoculation showed significantly longer survival compared with mice treated with BCG or PBS. $^*p < 0.05$ compared with the other groups, by Kaplan–Meier analysis. Each group consisted of five mice. This panel shows representative findings of three separate experiments, which all showed similar results.