A novel immunotherapy for superficial bladder cancer by the immobilization of streptavidin-tagged bioactive IL-2 on the biotinylated mucosal surface of the bladder wall

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[Abstract] Background and Objective: Intravesical administration of Bacillus Calmette-Guérin (BCG) after transurethral resection is by far the most effective local therapy for superficial bladder cancer, the fifth most common cancer in the world. However, approximately one-third of patients fail to respond and most patients eventually relapse. In addition, there are pronounced side effects of BCG therapy, such as BCG sepsis and a high frequency of BCG-induced cystitis. This study established a novel Immunotherapy through Immobilization of streptavidin-tagged human IL-2 (SA-hIL-2) on the biotinylated mucosal surface of bladder wall. Methods: A mouse orthotopic model of MB49 bladder cancer was established by perfusing MB49 cells into mouse bladders. The SA-hIL-2 fusion protein was immobilized on the biotinylated mucosal surface of the bladder wall. Treatment began on day 1 after MB49 Implantation, once every 3 days for 6 times. Immunohistochemical assay was performed to assess the persistence of SA-hIL-2 Immobilized on the biotinylated mucosal surface of the bladder wall. The mice were monitored for tumor growth and survival. On day 60 after MB49 implantation, the SA-hIL-2-cured mice, which were found to have no hematuria or palpable tumors, were challenged with wild-type MB49 cells implanted into the pretreated bladder and monitored for survival. Results: SA-hIL-2 could be immobilized efficiently and durably on the bladder mucosal surface as long as 7 days. On day 60 after MB49 implantation, 9 out of 20 SA-hIL-2-treated mice survived, but all mice in PBS control group died. More importantly, 5 out of 9 tumor-free mice in the SA-hIL-2 group were protected against a second intravesical wild-type MB49 tumor challenge. Conclusions: SA-hIL-2 fusion protein could significantly inhibit tumor growth and extend the survival time in the orthotopic model of MB49 bladder cancer.

Key words: Interleukin-2, streptavidin, Immunotherapy, superficial bladder cancer

Bladder cancer is one of the most common cancers in the world. Among the cases, most (75%) are superficial bladder cancer (SBC). Patients with SBC are at high risk of relapse after surgery with concomitant radiotherapy and chemotherapy. Thus it is necessary to find an efficient immunotherapy for preventing relapse of bladder cancer.

Human interleukin-2 (hIL-2) is a T cell growth factor and signaling modulator. It plays an important role in immunoregulation(1). Streptavidin (SA) has an extraordinarily strong affinity for biotin. This strong noncovalent interaction is widely made use of in many areas of biomedicine(2). Based on this strong interaction and the ability of biotin to be incorporated easily onto the tumor cell surface, we have developed a bifunctional fusion protein, streptavidin-tagged hIL-2 (SA-hIL-2), which may efficiently bind to the biotinylated tumor cells via its SA moiety, anchor the hIL-2 moiety at the local site of SBC, trigger a more effective antitumor immune response, and thus be applied in the clinical treatment of patients with SBC. In this study, based on a mouse orthotopic model of SBC that we successfully established(3), we assessed the antitumor effects of SA-hIL-2 by its immobilization on the mucosal surface of the mouse bladder to provide some experimental basis for the application of SA-hIL-2 in bladder cancer therapy.

Materials and Methods

Materials

The mouse bladder cancer cell line (MB49) was kept by our
SA-hIL-2 activity assay after in vitro immobilization in MB49 cells

MB49 cells were detached with trypsin and washed 3 times with phosphate buffered saline (PBS) (pH 7.4). Cell density was then adjusted to $5 \times 10^6$/mL. Sulfo-NHS-LC-LC-Biotin was added to reach an ultimate concentration of 1 mg/mL. The cells were incubated at 37°C for 30 min and then washed 3 times with PBS. Then the cells were incubated with SA-hIL-2 (100 ng/mL) at room temperature for 60 min. SA-GFP (100 ng/mL) was used as a negative control. After PBS washing (800 r/min x 5 min centrifugation), RPMI-1640 was added. Freezing and thawing of the cells in liquid nitrogen was repeated 3 times. The suspension of broken cells, now being the sample, was tested for activity of SA-hIL-2 according to the method in subsection mentioned above.

Establishing the mouse orthotopic model of SBC

When in the log phase, the cultured MB49 cells were detached with 0.25% trypsin and washed with PBS 3 times. Cell density was adjusted to $1 \times 10^6$/mL with RPMI-1640 containing 1% fetal bovine serum. After the female C57BL/6 mice were anesthetized by intraperitoneal 0.6% pentobarbital sodium at 60 mg/kg, sterile procedures of urethral catheterization were performed with a venous catheter. Bladder perfusion started with 0.1 mL of PBS to wash away urine. A volume of 0.1 mL hydrochloric acid (HCl) (0.1 mol/L) was then given and after 15 s drained. A volume of 0.1 mL sodium hydroxide (NaOH) (0.1 mol/L) was then given and after 5 s drained. After 3 times of PBS washing, 0.1 mL of MB49 (about $1 \times 10^6$ cells) was given in a 2-h perfusion for implantation of the tumor cells[9].

Immunohistochemical assay of immobilization of SA-hIL-2 on biotinylated bladder mucosa

On days 1, 3, 5, and 7 after the bladder mucosa was biotinylated and perfused with SA-hIL-2, 1 mouse was killed, and the tissue of the bladder mucosa was sampled for immunohistochemical assays to assess the persistence of the SA-hIL-2 immobilized on the biotinylated mucosal surface of the bladder wall.

Experimental therapy for mouse bladder cancer by SA-hIL-2 immobilization

A total of 125 female C57BL/6 mice was divided into five groups: the PBS control group, the soluble IL-2 group, the SA-GFP group, the SA-hIL-2 group, and the untreated group (blank control group); each with 25 mice. Treatment started on day 2 after the model had been established: Sulfo-NHS-LC-LC-Biotin (1.25 mmol/L) was given in a 30-min bladder perfusion. Then mice of each group received bladder perfusion with 0.1 mL of PBS, hIL-2 (0.15 mg/mL), SA-GFP (0.15 mg/mL), and SA-hIL-2 (0.15 mg/mL), respectively. A total of 6 continuous cycles of this treatment were conducted, 3 days per cycle. Survival states (mental state, diet, weight) and tumor growth (formation of tumor mass, tumor size, hematuria, distant metastasis) of the mice were observed every day. Survival time was recorded.

On day 60 after the establishment of the model, the SA-hIL-2 mice survived and the untreated mice were each challenged with $1 \times 10^6$ MB49 cells. Tumor formation and survival time were recorded.

Detection of IL-2 receptor in bladder mucosa

After the 6 cycles of treatment, mice in the SA-hIL-2 group were killed and their
bladders were sampled for immunochemical assay to demonstrate the distribution of IL-2 receptor in bladder mucosa. The PBS control group served as the negative control.

**Tumor-specific lymphocyte cytotoxicity assay** Splenocytes from the tumor-free or tumor-bearing mice in the SA-hIL-2 group were harvested and stimulated by inactivated MB49 for 5 days. IL-2 (20 U/mL) was added to maintain cell growth. These splenocytes served as effector cells and were adjusted to the density of 1 x 10⁶/mL. MB49 cells served as target cells and were adjusted to the density of 1 x 10⁴/mL. Both were added to a 96-well plate at the effector to target ratios of 1:1, 25:1, and 50:1, 3 wells for each ratio. After 6 h of incubation, the supernatant fluids were collected (100 μL/well). The LDH method was used to assess the cytotoxicity of tumor-specific cytotoxic T lymphocytes (CTL) under the wavelength of 490 nm. CTL = (experimental value – spontaneous release value of effector cells – spontaneous release value of target cells)/ (maximum release value of target cells – release value of target cells) x 100%. The PBS control group served as the negative control, and the untreated group as the blank control.

**Statistical analysis**

SPSS version 17.0 was used for statistical analysis. Survival was analyzed by the Kaplan-Meier method. P < 0.05 was considered as significantly different.

**Results**

**Identification of SA-hIL-2**

SA-hIL-2 fusion protein was found in the 12% SDS-PAGE to have a polymeric structure in its nonreduced state (Figure 1). The result of Western blot analysis showed that both renatured monomeric forms and polymeric forms of SA-hIL-2 could bind to IL-2 monoclonal antibodies and have a color reaction (Figure 1).

**Extent of modification of MB49 cells by SA-hIL-2**

SA-hIL-2 on the biotinylated MB49 cell surface was detected by IL-2 monoclonal antibody and a fluorochrome-conjugated second antibody using flow cytometry. The results showed that biotinylated MB49 cells could be modified efficiently by SA-hIL-2 and the modification rate reached more than 90% (Figure 2).

**hIL-2 activity assay of SA-hIL-2**

SA-hIL-2 could stimulate the proliferation of PHA-activated hPBL, which was concentration-dependent. The biologic activity of SA-hIL-2 was 1 x 10⁷ U/mL. Activity of immobilized SA-hIL-2 on the biotinylated MB49 cells was also assessed after the cells were broken apart by repeated freezing and thawing and the results showed that this immobilized protein retained the bioactivity of its hIL-2 moiety that stimulates hPBL proliferation (Figure 3).

**Immobilization of SA-hIL-2 on biotinylated bladder mucosa**

On days 1, 3, 5, and 7 after the bladder mucosa was biotinylated and perfused with SA-hIL-2, 1 mouse was killed, and the tissue of the bladder mucosa was sampled for immunohistochemical assay. The result showed that after SA-hIL-2 perfusion, SA-hIL-2 could be durably immobilized on the bladder mucosa as long as 7 days (Figure 4).

**Distribution of IL-2 receptor-positive lymphocytes at the site of mouse bladder cancer**

After the 6 cycles of treatment, mice in the SA-hIL-2 group were killed and their bladders were sampled for immunochemical...
 assay. A significant increase in IL-2α receptor-positive lymphocytes was observed in the SA-hIL-2 group compared with the PBS control group (Figure 5).

**General status of the tumor-bearing mice**

In the PBS control and the SA-GFP groups, palpable masses in the bladder region were found on days 7–9, and gross hematuria, a gradual decline in body weight, and gradually worsening mental states started on days 17–20. Some of these mice died on day 19 and day 23. In the soluble IL-2 and the SA-hIL-2 groups, some mice died or decreased in body weight on day 31 and day 35, respectively. These mice, compared with those in the PBS control group, had longer survival times and were more active. No significant change was observed in other mice.

**Survival of the mice**

On day 60 after MB49 implantation, all mice in the PBS control group died. Two of the 25 SA-GFP-treated mice survived. Six of the 25 mice treated with soluble hIL-2 survived. A total of 9 SA-hIL-2-treated mice survived. Significant differences in survival time existed between the SA-hIL-2 group and the other 3 groups \( (P < 0.05) \) (Figure 6A). Furthermore, 5 out of 9 tumor-free mice in the SA-hIL-2 group still survived 60 days after a second intravesical wild-type MB49 tumor challenge, which was significantly different from the situation of the control group \( (P < 0.05) \) (Figure 6B). This indicates that the SA-hIL-2-treated mice established an immune memory mechanism.
Tumor-specific lymphocyte cytotoxicity assays

Inactivated MB49-stimulated splenocytes from both the tumor-free or tumor-bearing mice in the SA-hIL-2 group and the normal untreated mice were found to have a cytotoxic effect on MB49 cells. The result showed that cells from the tumor-free mice in the SA-hIL-2 group had a significantly stronger cytotoxic effect than the tumor-bearing or the normal untreated mice ($P < 0.01$) (Figure 7).

Discussion

In recent years, there has been a rise in the incidence of bladder cancer. The most common type of bladder cancer is transitional cell carcinoma. Among patients with bladder cancer, about 75% have SBC, which is characterized by a high risk of relapse after surgery. Hence postoperative adjuvant therapy plays a key role and has become a hot research topic. Intravesical administration of Bacillus Calmette-Guérin (BCG) after transurethral resection is by far the most effective local therapy for SBC[9]. However, BCG, as an attenuated vaccine, has high antigenicity, allergenicity, and residual toxicity. Intravesical administration of BCG may lead to various complications. Some patients also develop immunotolerance against BCG therapy[9]. Activation of the production of cytokines is believed to be responsible for the antitumor effects of BCG. Application of recombinant cytokines in the treatment patients with bladder cancer has shown some experimental effects. Sosnowski et al.[7] reported that significant reductions in tumor volume occurred when IL-2 was intralesionally administered to subcutaneously transplanted tumors of mouse transitional cell carcinoma. Intravenous administration of IL-2 in patients with advanced cancer has shown some clinical effects but also may lead to severe side effects due to its short half-life and thus the high dose required[9]. Moreover, the efficiency of gene transfection (or transduction) in situ in SBC is usually low, and the protein product of therapeutic genes has difficulty reaching an effective concentration and persisting for enough time at the local lesion. Potential safety concerns and immunogenicity of viral vectors are also problems yet to be solved in application[9].

In recent years, based on the ability of biotin to be incorporated easily onto the tumor cell surface and the strong interaction between biotin and streptavidin, we have developed a platform of cell-surface immobilization and modification that allows cytokines that serve as adjuvants to be immobilized on the tumor cell surface and thus enhance the effect of antitumor vaccines that induce active immunity. The SA-GM-CSF-modified mouse melanoma whole-cell vaccine developed by us has shown promising effects in animal experiments[9].

In this study, the antitumor effect of the SA-hIL-2 bifunctional fusion protein, which may efficiently bind to biotinylated bladder mucosa via the extraordinarily strong affinity of SA for biotin, was studied in a mouse SBC model. The results showed that SA-hIL-2 could be immobilized efficiently and durably on the bladder mucosal surface for as long as 7 days. Immunochemistry suggested that SA-hIL-2 would significantly decrease 3 days after immobilization, which might be the result of the repair and renewal of the bladder mucosa and the degradation of SA-hIL-2 by urine protease. Thus the treatment cycles we adopted (3 days per cycle, totaling 6 continuous
immunotherapy. This novel treatment method may become a promising approach. Mice established an antitumor immune memory mechanism and showed effects on MB49 cells. All these indicate that the SA-hIL-2-treated group and the untreated group were found to have cytotoxicity inactivated MB49-stimulated splenocytes from both the SA-hIL-2 group compared with the PBS control group. Therefore, we suppose that there are T cells and natural killer (NK) cells infiltrating in the tumor microenvironment, their proliferation and activation can be stimulated, and their cytotoxic effects can be enhanced by SA-hIL-2 perfusion and immobilization at the tumor focus.

The results also showed that SA-GFP treatment could increase the survival rate of tumor-bearing mice in comparison with the PBS control group ($P < 0.05$). This may be because streptavidin, as a bacterial protein, is highly immunogenic and able to stimulate the mice to mount a specific immune response [59].

On day 60 after MB49 implantation, 7 mice in the SA-hIL-2 group survived without tumor formation and received a second intravesical MB49 tumor challenge. A total of 4 of them still survived without tumors 60 days after the challenge. Moreover, inactivated MB49-stimulated splenocytes from both the SA-hIL-2 group and the untreated group were found to have cytotoxic effects on MB49 cells. All these indicate that the SA-hIL-2-treated mice established an antitumor immune memory mechanism and this novel treatment method may become a promising immunotherapy.

In summary, this study reported a novel immunotherapy for SBC. Immobilization of SA-hIL-2, a bifunctional fusion protein, on mouse bladder mucosa could inhibit tumor growth and increase the survival rate of tumor-bearing mice. These findings may provide some experimental basis for the application of SA-hIL-2 in human bladder cancer treatment.

References