Recombinant Interleukin-2 Enhanced the Antitumor Effect of ADV/RSV-HSV-tk/ACV Therapy in a Murine Bladder Cancer Model

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Abstract. *Background: Previous studies demonstrated the antitumor effects of IL-2 and ADV/RSV-HSV-tk in bladder tumor models. In our study, we employed the intramuscular injection of recombinant IL-2 combined with ADV/RSV-HSV-tk gene therapy in the MBT-2 murine bladder tumor model. Materials and Methods: In the in vitro study, after adenoviral gene transduction efficiency had been assessed, the cytotoxicity of ADV/RSV-HSV-tk/ACV was examined. In the in vivo study, ADV/RSV-HSV-tk was injected into MBT-2 subcutaneous tumors, ACV was injected intraperitoneally daily for 13 days and recombinant IL-2 was injected intramuscularly daily for 10 days. Results: The X-gal staining of MBT-2 cells infected with 125 multiplicity of injection (MOI) indicated >20% adenoviral gene transduction efficiency. The cell growth of MBT-2 infected with 125 MOI was significantly inhibited by 40 μM of ACV. In the in vivo study, the combination therapy significantly inhibited tumor growth in the MBT-2 tumor model. Conclusion: The systemic administration of recombinant IL-2 in combination with HSV-tk gene therapy exhibited an enhanced antitumor effect.*

Bladder cancer is prone to recurrence, despite radical surgical procedures and adjuvant chemotherapy or radiotherapy. Although systemic chemotherapy is the main treatment for metastatic bladder cancer, its use is limited by significant toxicity, low complete response rates in the range of approximately 36%, and only 4% long-term survival (1-3). Therefore, advanced bladder cancer warrants novel treatment strategies.

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Key Words: Gene therapy, bladder cancer, adenovirus, recombinant IL-2.

Gene therapy is one such approach. There are two methods of cancer gene therapy. One method involves the direct killing of tumor cells by injecting a therapeutic gene (*i.e.*, suicide gene, tumor suppressor gene) into the tumor cell. The other method is by indirectly inducing immune-mediated cell killing, employing cancer vaccine strategies to convey an immunomodulatory gene that stimulates the immune system to recognize putative tumor antigen (4).

Previously, Sutton *et al.* applied adenovirus-mediated suicide gene therapy and Herpes Simplex Virus-Thymidine Kinase (HSV-tk) gene therapy to the treatment of an experimental bladder cancer model, demonstrating significant tumor growth inhibition and improvement in host survival (5). On the other hand, Conner *et al.* treated a murine bladder cancer model with IL-2, immunomodulatory gene-modified tumor vaccine cells (6). They had shown that the intradermal injection of IL-2 gene-modified MBT-2 cells into tumor-bearing animals was capable of curing mice with a considerable tumor burden. Combining the two strategies, Freund *et al.* tested the efficacy of the adenovirus-mediated suicide and cytokine (mIL-2) gene therapy for the treatment of murine bladder cancer. However, they did not observe any augmentation in the antitumor effect in the combination treatment as compared to the single gene-transduced treatment (4).

In the present study, after examination of the adenovirus-mediated gene transduction efficiency and cytotoxity of ADV/RSV-HSV-tk/ACV gene therapy in MBT-2 cells *in vitro*, we evaluated whether the systemic administration of recombinant IL-2 (intramuscular injection) could enhance the antitumor effect of adenovirus-mediated HSV-tk/ACV gene therapy in a murine bladder cancer model *in vivo*.

Materials and Methods

Recombinant adenovirus vector. The recombinant replication-defective adenovirus vectors used in this study were ADV/RSV-HSV-tk and ADV/RSV-β-gal, with the therapeutic toxic gene, HSV-tk, or the reporter *E.coli* beta-galactosidase gene (β-gal), driven by a universal Rous Sarcoma Virus (RSV) promoter. The adenovirus vectors were prepared, purified and titrated by a standard protocol, as described previously (7). Briefly, the recombinant adenoviruses were propagated in 293 cells and purified by the CsCl centrifugation method. The purified virus stock was then dialyzed against 10 mM Tris buffer, pH 7.5, containing 1 mM $MgCl_2$ and 10% glycerol, and the concentrated virus was titrated, aliquotted and stored at –80˚C. The plaque-forming units (pfu) of the virus were measured by standard biological plaque-forming assay and optical density measurement.

Cell line. The established MBT-2 cells line was purchased from ATCC (Rockville, MD, USA). MBT-2 is a poorly-differentiated murine transitional cell carcinoma cell line (5). It was maintained at 37˚C in a DMEM complete medium (GIBCO, MD, USA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA). The cell was fed twice a week with fresh growth media.

X-Gal staining after infection of ADV-RSV-βgal. After exposure to graded doses of ADV/RSV-β-gal [0-125 multiplicity of infection (MOI)] for 12 h, the cells were grown for 3 days in 2-well chamber slides (Nalge Nunc Co., Naperville, IL, USA) to 80-90% confluence. The media was then discarded and the cells were fixed with 4% paraformaldehyde for 15 min and stained using the X-gal staining assay kit (Gene Therapy Systems Co., San Diego, CA, USA). Gene transfer efficiency was measured by determining the percentage of LacZ-positive (blue-stained) cells by 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) staining by light microscopy. A minimum of 200 cells were counted at each site. Three different sites were investigated and the averages were calculated.

In vitro cytotoxicity assay. The cells were seeded at a density of 500 cells/well in 96-well tissue culture plates before viral infection. After seeding the cells for 24 h, they were infected by directly adding the ADV/RSV-HSV-tk at 125 MOI into each well, which contained 10 μl of the medium. After 6 h, the medium was exchanged with fresh medium containing 400 μM ACV (Burroughs Wellcome, Research Triangle, NC, USA). The medium was changed daily. The viable cell numbers were measured by the Alamar Blue method, according to the manufacturer's instructions (Alamar Biosciences, Inc., Sacramento, CA, USA). Briefly, 10 μl of Alamar Blue was aseptically added to the cultures and returned to the incubator for 3 h. Subsequently, the fluorescence was measured with a 560 nm excitation wavelength and a 590 nm emission wavelength using a fluoroscan (Titertek Fluoroscan II; Labosystems, Tokyo, Japan).

Animal studies. Murine bladder tumor, MBT-2 cells (1.0×10^6 cells/100 μl of medium) were subcutaneously inoculated into C3H mice of 4–6 weeks of age. When subcutaneous tumors with diameters of 15 to 20 mm had developed, 24 animals were randomly assigned to 4 experimental groups: group 1, no treatment (6 animals); group 2, IL-2 alone (6 animals); group 3, ADV/RSV-HSV-tk/ACV alone (6 animals); group 4, ADV/RSV-HSV-tk/ACV plus recombinant IL-2 (6 animals).

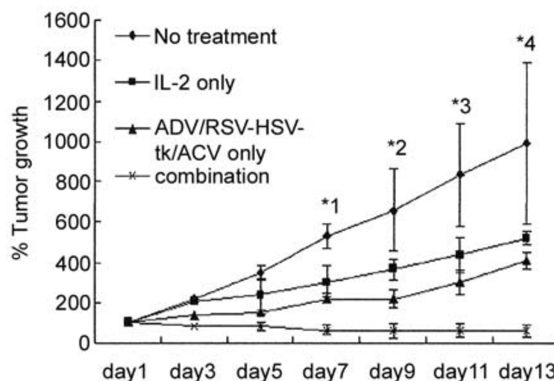

Figure 1. *Attenuation of MBT-2 bladder cancer cell tumor growth by ADV/RSV-HSV-tk plus IL-2. Animals bearing MBT-2 subcutaneous tumors received no treatment, IL-2 alone, ADV/RSV-HSV-tk alone, or ADV/RSV-HSV-tk plus IL-2 treatment, as described in Materials and Methods. The rate of tumor growth was calculated assuming each tumor volume on day 1 to be 100 %. ADV/RSV-HSV-tk plus IL-2 treatment significantly inhibited MBT-2 tumor growth at days 7 (*1), 9(*2), 11(*3), and 13(*4). Each point represents averages of percentage tumor growth with SD bar; ADV/RSV-HSV-tk plus IL-2 treatment group differs from other treatment groups (p<0.05).*

ADV/RSV-HSV-tk (1×10^9 pfu/50 μl of PBS) was intratumorally injected on days 1, 6 and 10 using a microliter syringe fitted with a 26-gauge needle. Starting on day 1, ACV (5 mg/kg body weight) was intraperitoneally injected daily for 13 days and recombinant human IL-2 (1×10^4 U) (Shionogi & Co., Ltd., Osaka, Japan) was intramuscularly injected daily for 10 days. Tumor volume was measured every day and calculated using the following formula: volume (a rotational ellipsoid) = $M_1 \times M_2^2 \times 0.5236$ (M_1, long axis; M_2, short axis) (8). The rate of tumor growth was calculated assuming each tumor volume to be 100% on day 1. The tumor specimens for routine histological examination were fixed in 4% paraformaldehyde and 5 mM $MgCl_2$. Paraffin-embedded tumor sections of 5 μm thickness were cut and stained with hematoxylin and eosin, anti-single-stranded DNA antibody, and anti-CD4 and CD8 antibody. All aspects of the experimental design and procedure were reviewed and approved by the institutional ethics and welfare committees of the Kobe University School of Medicine, Japan.

Statistical analysis. Statistical analysis was performed using the Stat View 4.5 program (Abacus Concepts, Inc., Berkely, CA, USA). ANOVA was applied to compare the results obtained from different treatment groups. Statistical significance was defined as $p<0.05$.

Results

Adenoviral gene transduction efficiency in MBT-2 cells. Adenovirus-mediated gene transduction efficiency was examined by infecting MBT-2 cells with 0, 5, 25 or 125

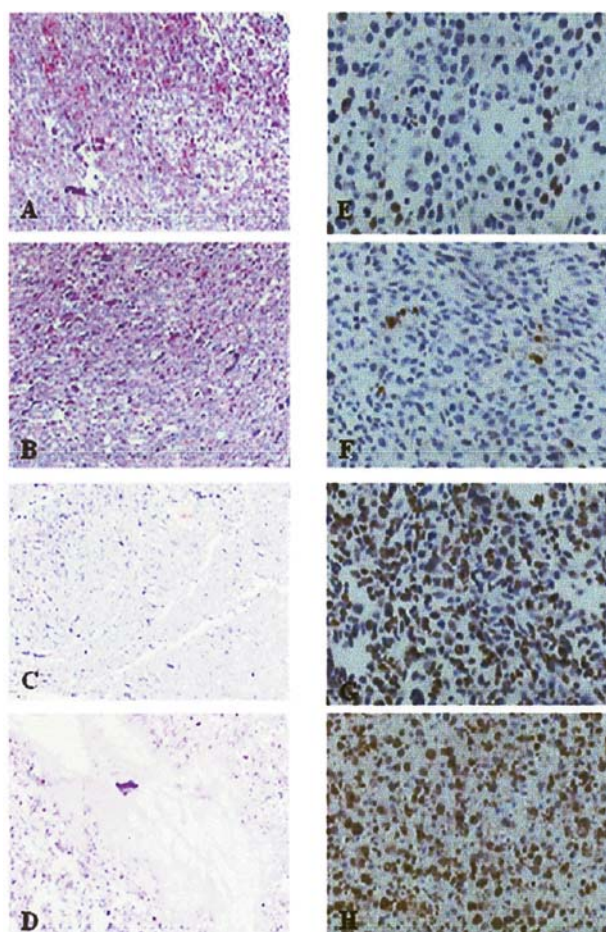

Figure 2. *(A-D) Histological examination by hematoxylin and eosin staining (x100) and (E-H) immunohistochemical examination by anti-single-stranded DNA antibody staining (x400): (A,E) no treatment; (B,F) IL-2 alone; (C,G) ADV/RSV-HSV-tk alone; (D,H) ADV/RSV-HSV-tk plus IL-2.*

MOI of ADV/RSV-β-gal and then performing X-gal staining, as described in Materials and Methods. The adenovirus efficiently transduced the MBT-2 cells in a dose-dependent manner; (2.8±0.3)% were positive with 5 MOI; (5.6±0.7)% with 25 MOI; (20.5±1.6) % with 125 MOI. Thus, the 125 MOI dose was employed in the following *in vitro* cytotoxity studies.

In vitro cytotoxicity of ADV/RSV-HSV-tk /ACV in MBT-2 cells. To determine whether adenovirus-mediated transduction with the HSV-tk gene would make MBT-2 cells sensitive to cell killing by ACV, we tested the toxicity of ACV (40 μM) in ADV/RSV-HSV-tk (125 MOI)-infected or non-infected MBT-2 cells. At day 5, the percentage cell survival with ADV/RSV-HSV-tk alone was (84.6±2.3)%, with ACV alone it was (86.1±3.0)%, while with ADV/RSV-HSV-tk/ACV it was (70.7±1.6)%. ADV/RSV-HSV-tk/ACV

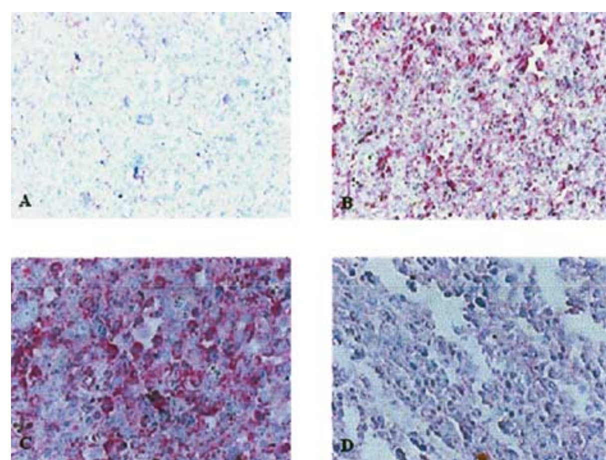

Figure 3. *Immunohistochemical examination of ADV/RSV-HSV-tk plus IL-2 treatment group by anti-CD-4 and anti-CD-8 antibody staining: (A) anti-CD-4 antibody staining (x200); (B) anti-CD-8 antibody staining (x200); (C) anti-CD-8 antibody staining (x400); (D) negative control of anti-CD-8 antibody staining (x400).*

therapy significantly inhibited cell growth compared with no treatment ($p<0.01$), ACV alone ($p<0.01$) and ADV/RSV-HSV-tk alone ($p<0.01$).

Tumor growth inhibitory effect in the MBT-2 subcutaneous tumor models. MBT-2 bladder tumor homografts were induced by subcutaneous injection of MBT-2 cells. After tumor formation, the animals were treated with either recombinant IL-2 alone, ADV/RSV-HSV-tk/ACV alone, or ADV/RSV-HSV-tk/ACV plus recombinant IL-2. Both the recombinant IL-2 alone or ADV/RSV-HSV-tk/ACV alone groups showed slight tumor growth delay, while the ADV/RSV-HSV-tk/ACV plus recombinant IL-2 group demonstrated a significantly decreased growth rate of the MBT-2 tumor up to 13 days (Figure 1).

Histopathological evaluation. To determine whether treatment with ADV/RSV-HSV-tk/ACV plus recombinant IL-2 changed the tumor microenvironment *in vivo*, histopathological studies were performed. Hematoxylin and eosin staining of the tissues revealed a similar histological pattern in the control, recombinant IL-2 alone and ADV/RSV-HSV-tk/ACV alone groups, and infiltration of lymphocytes around the necrotic region was found in the ADV/RSV-HSV-tk/ACV plus recombinant IL-2 group (Figure 2). Analysis of anti-single-stranded DNA antibody staining showed an increased number of apoptotic cells in tumors treated with ADV/RSV-HSV-tk/ACV and ADV/RSV-HSV-tk/ACV plus recombinant IL-2, whereas few cells were positively-stained by anti-single-stranded DNA antibody in the control and recombinant IL-2 alone

groups (Figure 2). Moreover, to assess and detect CD-4 and CD-8 T lymphocytes around the necrotic region in the ADV/RSV-HSV-tk/ACV plus recombinant IL-2 group, immunohistochemical staining was performed. A large number of CD-8 T lymphocytes infiltrated around the necrotic region, whereas few CD-4 T lymphocytes were seen in the necrotic region (Figure 3).

Discussion

The purpose of cancer gene therapy to kill tumor cells either directly, by delivering a therapeutic gene such as a suicide gene to the tumor cells themselves, or indirectly, by using cancer vaccine strategies to convey an immunomodulatory gene stimulating the antitumor immune system (9). The HSV-tk gene directly introduced into tumor cells phosphorylates a non-toxic prodrug ganciclovir (GCV), and the phosphorylated GCV acquires cell toxicity (10). Furthermore, it is known that the HSV-tk/GCV system displays the "bystander effect", defined as an effect in which non-gene-transduced cells nearby undergo cell death, not only because of the direct transfer of metabolic toxic products through gap junctions, but also through disruption of the tumor blood supply, induction of cell apoptosis and cell-mediated tumor immune response (5). In addition, several researchers have reported that the cells killed by the HSV-tk/GCV treatment release soluble factors like cytokines that enable the antitumorigenic immune response in the tumor microenvironments (11, 12). It is suggested that immunotherapy could augment the bystander effects of HSV-tk/GCV gene therapy.

In the present study, intramuscular injection was employed for the systemic administration of recombinant IL-2 cytokine *in vivo*. The administration of the adenoviral vector could result in severe systemic toxicity, and the relationship between the dose of the vector and its toxicity is not linear. The therapeutic dose should be substantially lower than the dose at which toxicity can be seen, such as activation of innate immunity (13). We have designed a method that separately administers a safe dose of adenoviral vector carrying the HSV-tk gene *via* intratumoral local injection to elicit the antitumor effect and recombinant IL-2 *via* intramuscular injection to enhance the systemic antitumor immunity. Our results demonstrated that the intramuscular injection of recombinant IL-2 could synergistically augment the antitumorigenic effect of adenoviral-mediated HSV-tk/GCV gene therapy in the murine bladder tumor model. ADV/RSV-HSV-tk/GCV treatment under the systemic administration of recombinant IL-2 may be able to induce the differentiation and proliferation of naïve CD-8 T cells to cytotoxic T cells and lead to more effective tumor growth inhibition as compared to treatment with ADV/RSV-HSV-tk/GCV alone. ADV/RSV-HSV-tk/GCV plus recombinant IL-2 treatment could induce the synergistic antitumor effect with the minimum dose of adenovirus vector, as compared to treatment with ADV/RSV-HSV-tk/GCV alone.

In conclusion, we demonstrated that ADV/RSV-HSV-tk/ACV plus recombinant IL-2 gene therapy could induce a synergistic antitumor effect in a murine bladder cancer model *in vivo*. This treatment has the potential of becoming a novel therapeutic approach for the treatment of patients with bladder cancer resistant to conventional therapies.

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Received March 30, 2005
Accepted May 26, 2005