Combination Gene Therapy for Oral Cancer in a Murine Model

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Abstract

Combination therapy involving adenovirus-mediated transfer of the genes for herpes thymidine kinase (tk) and murine interleukin 2 (mIL-2) was used to treat head and neck cancer in C3H/HeJ mice. Tumors were generated by transcutaneous injection of $5 \times 10^5$ murine squamous carcinoma cells into the floor of the mouth of these syngeneic mice. After 1 week, recombinant adenoviral vectors containing both therapeutic and control genes in various combinations were injected directly into the established tumors, and subsequently all mice were administered ganciclovir twice daily (25 mg/kg) for 6 days. Animals receiving either tk alone or tk + mIL-2 demonstrated significant tumor regression compared to established tumors, and subsequently all mice were administered ganciclovir twice daily (25 mg/kg) for 6 days. Animals receiving either tk alone or tk + mIL-2 demonstrated significant tumor regression compared to mIL-2 alone or control vector-treated mice ($P < 0.008$). Mice receiving both tk + mIL-2, however, also demonstrated a significantly greater regression of tumors compared to those treated with tk alone ($P < 0.008$), indicating a synergistic effect of the combination gene therapy. This synergism was confirmed in survival studies because tk + mIL-2-treated mice showed increased survivals ($P = 0.0002$). Clinical and microscopic exam of regional surrounding tissues and distant organs showed no evidence of cytotoxicity for representative animals in each experimental group. These results suggest that combination tk and mIL-2 gene therapy may provide a powerful new modality for the treatment of head and neck cancer.

Introduction

Oral cavity and oral pharyngeal tumors are among the most morbidd of human cancers and compromise the majority of the estimated 42,800 new cases of head and neck cancer annually (1). Standard therapy includes deforming radical surgical procedures coupled with radiotherapy and possibly chemotherapy. In addition to the severe cosmetic deformity, surgical resection frequently results in significant functional deficits in speech, swallowing, and upper extremity strength. Radiotherapy also brings substantial morbidity with mandible and laryngeal cartilage radionecrosis, soft tissue fibrosis, and mucosal atrophy, pain, and xerostomia (2). Chemotherapy regimens are not innocuous as well, and frequent complications from their use include gastrointestinal, bone marrow, ototoxicity, and renal toxicity (2). Furthermore, the efficacy of adjuvant or induction chemotherapy in head and neck cancer remains questionable, with no clinical trial to date demonstrating improved survival (1). Despite years of investigation of the treatment of head and neck cancer, outcomes remain consistently poor with 2-year survivals rarely exceeding 30% in patients with advanced stage III and IV disease (1, 3). Because of the dismal results of conventional therapy, a new thrust in treatment strategies has focused on the use of gene therapy. Two of the most promising strategies to date include the use of “suicide genes” and cancer vaccines based on immunotherapies.

The herpes simplex virus tk gene is a prototype “suicide gene” because it encodes a viral enzyme that is foreign to mammalian cells, which will convert an inactive prodrug to a toxic product. Upon viral transduction, the tk gene selectively kills dividing cells (cancer cells) by converting GCV into a phosphorylated compound that terminates DNA synthesis (4). Since GCV is an excellent substrate for viral tks and a poor substrate for mammalian tks, concentrations can be achieved that are lethal to cells expressing tk but are nontoxic to normal mammalian cells (5–7). An additional advantage of tk gene transfer is the presence of a “bystander effect.” In both murine and human tumor models, surrounding nontransduced dividing cells are also killed, presumably from the transfer of a toxic metabolite of GCV via gap junctions or endocytosis of apoptotic vesicles from virally transduced dying tumor cells (8, 9).

Recently, the evolution of cancer vaccine research has led to the transplantation of genetically modified tumor cells that generate cytokines locally and elicit an antitumor immune response. The most common strategies developed in animal models involve the transduction or transfection of established tumor cell lines with various cytokine genes in vitro, followed by the implantation of these modified cells into immune competent animals (10, 11). Several studies have shown that the transduction of murine tumor cells with the genes for mIL-2 (10, 12) have resulted in the rejection of these tumor cells after implantation into syngeneic hosts. In addition to the local tumoricidal inflammatory effect, injection of cytokine-secreting tumor cells generates systemic immunological responses capable of protecting animals from subsequent challenge with the same tumor at a distant site (11).

For the suicide gene therapy and cytokine vaccine strategies, the use of a recombinant adenoviral vector system has many advantages. Unlike the low efficiency of previously described retroviral systems (13, 14), adenoviral vectors have very high titters and efficiency for direct delivery into cancer cells in vivo (15). As a safety factor for the suicide gene therapy strategy, the rapidly dividing tumor cells will be selectively targeted for adenoviral transduction over the surrounding normal tissues. For cytokine therapies, the use of recombinant adenoviral vectors should prove valuable in eliminating the need for daily local cytokine suspension injections while still providing the important paracrine cytokine function. This will also greatly reduce the moderate to severe toxicities including fever, chills, headaches, and capillary leak syndrome associated with systemic mIL-2 delivery (16). With respect to cancer vaccine strategies, the adenoviral vector system would eliminate the time-consuming and costly procedures of harvesting tumor specimens, isolating cancer cells, culturing cells, treating with radiation and gene transfer in vitro, and then re-introducing the engineered tumor cells back into the patients as a vaccine (11).

Adenoviral vectors containing the tk and mIL-2 genes can also be incorporated into a single injection procedure into primary tumors to assess the potential benefit of both a direct cytotoxic and immune-enhancing gene therapy strategy. For these reasons, we have chosen to evaluate single gene versus combination adenoviral gene transfer

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4. To whom requests for reprints should be addressed, at Department of Otolaryngology–Head and Neck Surgery, Johns Hopkins University, P. O. Box 41402, Baltimore, MD 21203-6402.
5. The abbreviations used are: tk, thymidine kinase; GCV, ganciclovir; mIL-2, murine interleukin 2; ADV, adenovirus; RSV, Rous sarcoma virus; ADV/RSV-tk, recombinant ADV vector containing the tk gene driven by the RSV long terminal repeat; ADV/RSV-β-gal, same vector as above but containing the gene for β-galactosidase; SCC, squamous cell carcinoma; MOI, ratio of viral plaque-forming units to tumor cell.
In Vitro Experiments. The SCC line, SCC VII, was used in all experiments. Originally, SCC VII arose spontaneously in C3H/HeJ mice and has subsequently been propagated in vivo (19). The cells were cultured in T-75 tissue culture flasks (Corning) containing 30 ml of RPMI 1640 (Sigma Chemical Co.), 12% bovine calf serum, 1% penicillin-streptomycin, and 1% L-glutamine. Cells were maintained in 10% CO₂ incubators.

In Vivo Treatment and Survival Experiments. All animal experiments were performed on C3H/HeJ mice (Jackson Laboratories) using sterile technique under a laminar flow hood. Mice 6–10 weeks old were anesthetized using the inhalational agent Metophane, and a 0.1-ml suspension of 5 x 10⁵ SCC VII cells in HBSS were injected directly into the floor of the mouth. The animals were then maintained in standard housing conditions.

Six days after cell implantation, mice were anesthetized with 0.5 ml of avertin at a concentration of 20 mg/ml with the depth of anesthesia determined by toe pinch. A skin incision was made in the lower neck, and surgical dissection revealed the established floor of mouth tumors. Tumors were measured in three dimensions with calipers. Using a 100-μl syringe (Hamilton, Reno, NV) and 26-gauge needle, 1.0 x 10⁶ total plaque-forming units of either ADV/RSV-tk, ADV/RSV-β-gal control, ADV/RSV-tk + ADV/RSV-mIL-2 (2.0 x 10⁷), or ADV/RSV-mIL-2 (2.0 x 10⁶) + ADV/RSV-β-gal in 60 μl solution were injected directly into the tumors. Neck incisions were closed with 4–0 silk suture (Ethicon). Eighteen h after adenoviral injection, the mice were administered GCV i.p. at a regimen of 25 mg/kg twice daily. Mice were sacrificed on the seventh day after adenoviral delivery, the neck incision was reopened, and the tumor sites were examined. Residual tumor masses were measured, and specimens including normal surrounding tissues were sent for routine histology. The pathologists were blinded to the treatment regimens until after interpretation of the results. The significance of outcomes was determined by Mann-Whitney analysis and was further confirmed by Dunnett's test of multiple comparisons.

A survival experiment was performed under the same conditions, and animals were evaluated daily. Tumor size was assessed every 3 days using caliper measurements. The significance of survival outcomes was determined using identical statistical analysis as in the preceding experiments.

Histology. At the time of necropsy, harvested tumor, surrounding tissues, and distant organs were placed in 10% buffered formalin for fixation. The specimens were then embedded in paraffin, sectioned, and stained with H&E.

Results

Efficacy of Adenoviral Transduction in Vitro. ADV/RSV-tk was delivered to SCC VII cells in vitro to assess the efficacy of cancer cell regression after subsequent administration of GCV (Fig. 1A) After using tk and mouse mIL-2 vectors in a recently developed immunocompetent murine model for head and neck SCC. This study investigates the efficacy and synergism of this combination gene therapy strategy on primary tumor regression and animal survival.

Materials and Methods

Construction of Recombinant Adenoviral Vectors. Construction of a replication-defective adenoviral vector containing the tk gene under transcriptional controls of the RSV long terminal repeat (ADV/RSV-tk) has been reported previously (17, 18). A replication-defective adenoviral vector containing the mIL-2 cDNA under the transcriptional control of the RSV long terminal repeat promoter (ADV/RSV-mIL-2) was similarly constructed and plaque purified. The viral titer (plaque-forming units/ml) was determined by plaque assay.
Fig. 3. Photomicrographs of tumors from various treatment groups. A, completely viable tumor in control animal after ADV/RSV-β-gal transduction with GCV, demonstrating poorly differentiated SCC (H&E, ×400). B, ADV/RSV-tk + mIL-2 and GCV-treated tumor showing extensive zonal necrosis (right to left) with inflammatory infiltrate (×200). C, another ADV/RSV-tk + mIL-2 and GCV-treated tumor depicting intermingled necrosis, swollen dying cells with pyknotic nuclei, some viable tumor cells, and inflammatory infiltrate (×400).
transduction with ADV/RSV-tk and GCV treatment, less than 10% viable cancer cells remained at a MOI of 25 and 98% cell death was achieved at a MOI of 100. ADV/RSV transduction followed by PBS as a control, however, showed no cell death. The presence of a bystander effect was exemplified by mixing ADV/RSV-tk-transduced SCC VII cells with nontransduced tumor cells in various concentrations. Only 3–33% ADV/RSV-tk-transduced SCC VII cells were required to confer GCV toxicity to 90–100% of the parental tumor cells in vitro (Fig. 1B). Efficacy of the replication-defective ADV/RSV-mIL-2 vector at an MOI of 20 had been established previously in vitro on B16 cells using a T-cell proliferation assay on conditioned media (data not shown).

**Regression of Established SCCs after Combination Gene Therapy in Vivo.** After implantation of 5 × 10³ tumor cells into 36 animals, the mice showed rapid tumor growth in the floor of the mouth over 5 to 7 days. There were no signs of cachexia during this period, and all animals appeared clinically healthy at the time of adenovirus injection. Four experimental groups were designed as follows: group 1, ADV/RSV- β-gal control virus infection (β-gal); group 2, ADV/RSV-mIL-2 + ADV/RSV- β-gal (β-gal + mIL-2); group 3, ADV/RSV-tk alone (tk); and group 4, ADV/RSV-tk + ADV/RSV-mIL-2 (tk + mIL-2). After direct intratumoral viral delivery, all animals were treated with GCV at 25 mg/kg twice daily for 6 days and then were sacrificed on the seventh day after adenoviral delivery. Any residual tumors were measured and harvested for histopathological evaluation. All animals showed no signs of cachexia or change in eating habits during the treatment period, and all were clinically healthy at the time of necropsy.

Pretreatment tumor volumes ranged from 70–160 mm³ with an overall average size of 122.5 mm³. There were no significant variations in pretreatment tumor sizes between each tumor group, as determined by Mann-Whitney analysis (data not shown). Animals treated with either tk or combination tk + mIL-2 demonstrated significant tumor regression as compared to the large tumors found in mIL-2 alone or β-gal control injection mice (P < 0.008; Fig. 2). Three of the combination tk + mIL-2-treated animals had near complete tumor eradication with residual necrotic masses less than 2 mm³; however, none of the remaining groups demonstrated such dramatic effects. Overall, the combination of tk + mIL-2 showed the greatest effect on tumor regression and was significantly better than tk alone (P < 0.008). Although the tumor sizes in the β-gal + mIL-2 animals showed less tumor progression than the β-gal alone controls, they were not significantly different based on 95% confidence limits.

Histopathological analysis revealed poorly differentiated SCC in residual tumors and controls. There was substantial tumor necrosis, including regions of zonal necrosis abutting areas of mixed necrotic and viable cells noted especially in the tk + mIL-2 and tk alone treatment groups (Fig. 3). The residual tumors had low levels of macrophage infiltrates and variable levels of lymphocyte infiltration noted more commonly in the mice receiving mIL-2 gene transfer. Distant organs including lung, liver, bowel, and kidneys were examined from three animals in each experimental group, and no evidence of necrosis or cytotoxicity or viral inclusion bodies were seen (data not shown).

**Survival Experiment.** To evaluate the long-term effects of combination gene therapy, a survival experiment was performed on 38 animals with established SCC VII tumors using identical treatment regimens as in the preceding experiment. The effects of adenovirus treatment were assessed by visual inspection, palpation, and caliper measurements of tumors three times a week (Fig. 4.). Survival was calculated in days after original adenovirus injection, and end point was assessed by either animal demise or when tumors increased to 2 cm in any dimension (cachexia is present in all animals with tumors of this size), as required by the Institution's Animal Care Committee. Mice receiving tk + mIL-2 combination therapy demonstrated significant survival over both the tk alone and remaining groups (P = 0.0002). The tk alone-treated animals showed significant survival over the β-gal + mIL-2 and control β-gal-alone group. Animals receiving β-gal + mIL-2 showed no significant survival over the control group.

**Discussion**

These experiments demonstrate three important aspects and effects of adenovirus-mediated gene transfer on SCC regression in a murine animal model:

(a) Initial in vitro studies established that tk gene delivery and GCV administration prove directly cytotoxic to SCCs and initiate a bystander effect, whereby surrounding nontransduced cells are destroyed. Only 3–33% of transduced tumor cells were required to kill 90–100% of the total tumor cells in culture. The presence of this effect is a major strength of this suicide gene therapy strategy and an essential therapeutic component when considering actual clinical application. Known limited diffusion of adenovirus after direct in vivo injection (17, 18) could greatly reduce therapeutic benefit in larger human tumors. Incorporation of a bystander effect, however, may circumvent unforeseen limitations of viral titers, delivery volumes, or actual access to complete tumor injection in the human patient.

(b) The delivery of tk to established tumors in vivo followed by GCV administration results in significant tumor regression and improved survivals over animals treated with adenovirus carrying the murine mIL-2 gene alone as well as control animals. This finding shows the strength of tk over a cytokine approach when considering single gene therapy for cancer treatment. Upon considering the lack of efficacy of mIL-2 alone despite a notable increased inflammatory infiltrate in mIL-2-treated animals, issues of injection volume or total viral particle delivery cannot be raised because each of these variables were constant among the treatment groups. Furthermore, the viral titer for mIL-2 delivered to the murine tumors was at the high end of safe dosing based on previous toxicity studies for this vector (data not shown). In this tumor model, the use of mIL-2 alone as a adenoviral strategy paralleling classic cancer vaccine approaches is not an acceptable choice of therapeutic regimens.
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(c) The most important aspect of this work is the demonstration of a synergistic effect after combination tk and mIL-2 gene transfer on primary oral cancer. Although a recent report by Chen et al. (20) described a similar short-term effect of combined tk and mIL-2 therapy on regression of metastatic colon carcinoma, this previous study did not demonstrate a survival advantage (20). In the oral cancer model, the combining of tk and mIL-2 followed by GCV administration was more effective than tk alone, mIL-2 alone, and β-gal virus controls for both short-term and survival studies. This work introduces a merger between a strong direct in vivo gene therapy strategy with a standard ex vivo cancer vaccine (immune-enhancing) modality for the treatment of primary head and neck cancer. The results suggest that the presence of cell debris or local tumor cytotoxic effects (from tk) controls for both short-term and survival studies. This work establishes efficacy on tumor regression and survival and provides a foundation for advancing combination gene therapy for the treatment of head and neck cancer.

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References

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