Adenovirus-mediated Gene Therapy for Human Head and Neck Squamous Cell Cancer in a Nude Mouse Model

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ABSTRACT

Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene followed by ganciclovir administration was used to treat human head and neck cancer in nude mice. Tumors were generated by transcutaneous needle injection of 6 x 10^6 human squamous carcinoma cells into the floor of the mouth. After 14 days, 10^10 particles of a replication-defective recombinant adenovirus containing the herpes simplex virus thymidine kinase gene (ADV/RSV-tk) were injected directly into the tumors. The mice subsequently received ganciclovir injections for six consecutive days and were sacrificed at 21 days post tumor cell implantation. Clinical response to the treatment was assessed by computer-imaged morphometric analysis of cross sectional area of nonnecrotic tumor and mitotic activity, which were used for the calculation of a tumor index. The median tumor index value of the treatment group was 2800- to 2400-fold smaller than controls which did not receive the therapeutic gene (P < 0.001-0.0016) and three-quarters of the treatment group had tumor index values that were indicative of near total tumor regression. Survival studies showed that 50% of the ADV/RSV-tk-treated mice are free of tumor at 160 days post adenovirus injection, while all controls died or required sacrifice within 43 days. These results demonstrate that clinically effective in vivo treatment of human squamous cell cancer can be achieved using adenovirus-mediated gene therapy.

INTRODUCTION

The incidence of squamous cell cancer of the head and neck is approximately 42,800 cases per year in the United States with worldwide projections of more than 500,000 annually (1). Squamous cell carcinoma of the head and neck is among the most morbid of human cancers. Present therapy includes deforming surgical procedures coupled with radiotherapy and possibly chemotherapy. Aside from the notable cosmetic defect from surgical resection, radiotherapy brings associated morbidity of bone and soft tissue necrosis, tissue fibrosis and atrophy, pain, and xerostomia (2). In considering chemotherapy regimens for head and neck squamous cell cancer, frequent complications include variable degrees of gastrointestinal, bone marrow, and renal toxicity (2). Furthermore, the efficacy of adjuvant or induction chemotherapy remains questionable, with no clinical trial to date demonstrating improved survival (1). Despite many years of studying squamous cell cancer, the present therapies can rarely hope to provide 2-year survivals of more than 30% in patients with advanced stage III and IV disease (1, 3).

The purpose of this study was to define a new treatment modality by demonstrating the cytotoxic and overall therapeutic effects of viral-mediated gene transfer with concurrent antiviral pharmacological therapy on human head and neck squamous cell cancer implants in nude mice. The nude mouse is the standard for in vivo investigations of investigating the isolated effects of gene transfer treatment since there is no component of T-cell-mediated activity on tumor implants. Retroviral-mediated transfer of the herpes simplex virus tk gene has been described in studies involving animal tumor cell lines in vitro and in vivo (5-8). The importance of herpes thymidine kinase gene transfer centers on its ability to render cells sensitive to the nucleoside analogue GCV. Upon viral transduction, the tk gene selectively kills dividing cells by converting GCV into a phosphorylated compound that terminates DNA synthesis. This property proves valuable for the treatment of rapidly growing tumors which invade normal surrounding tissues that are not dividing. Prior in vivo reports have shown regression of rat gliomas after retroviral-mediated transfer of the tk gene (9, 10). These studies, however, required the implantation of retrovirus-producing mouse fibroblasts into the rat brain tumors in order to achieve adequate retroviral titers and improve the efficiency of tumor cell transduction. Our new system for treating squamous cell cancer is based upon our recent studies demonstrating regression of rat gliomas after delivery of a recombinant adenoviral vector containing the tk gene (11). A major advantage of this system is that the need for mouse fibroblast implantation is eliminated by using the highly efficient adenoviral vector to transfer the tk gene directly into tumors via simple needle injection. We now demonstrate significant tumor regression and long-term survival after adenoviral-tk transduction and GCV administration in a human squamous cell cancer that has proven difficult to treat with surgery, radiation, or chemotherapy. Unlike prior studies which have described gene transfer into monoclonal animal tumor cell lines, the following report pioneeers investigation in a human head and neck cancer model that closely parallels true clinical disease.

MATERIALS AND METHODS

Construction of Recombinant Adenovirus. The plasmid pADL1/RSV was created by inserting the RSV long terminal repeat promoter into the XbaI and CiaI sites of pXCJL1 (kindly provided by Dr. Frank Graham). The 2.8-kilobase pair BglII to BamHI fragment containing the herpes simplex virus thymidine kinase gene and poly(A) tail was inserted into the BamHI site of pADL1/RSV as described previously (11). The resulting plasmid pADL1/RSV-tk uses the RSV promoter to control transcription of the thymidine kinase gene. To generate a recombinant adenovirus, pADL1/RSV-tk and pM17 (a plasmid containing the complete adenovirus genome) were cotransfected into the 293 transformed kidney cell line by calcium phosphate precipitation. Recombinant adenovirus was isolated from a single plaque, expanded in the 293 cell line, and purified by double cesium gradient ultracentrifugation (12). The viral titers were then determined by optical absorbance at 260 nm.
**RESULTS**

**Efficiency of Adenoviral Transduction of HLaC-79 Cells in Vitro.** For the ADV/RSV-β-gal experiments, 85% of the human squamous cancer cells were transduced at a M.O.I. of 8 as demonstrated by positive blue X-gal staining (Fig. 1), and 100% of cells were transduced at an M.O.I. of 16 (data not shown). There was no apparent toxicity at this adenovirus concentration, and the cancer cells showed no morphological changes compared to controls. For the ADV/RSV-tk experiments, transductions were performed using a range of 0 (control) to 30 M.O.I., followed by either PBS or GCV treatment in the media. Effective cancer cell killing in the GCV group was achieved at a very low M.O.I. of 3, with 96% cell death achieved at an M.O.I. of 30. There was no toxicity in the PBS control group up to an M.O.I. of 30 (Fig. 2). These findings indicate that ADV/RSV-tk is an efficient vector system that is effective in killing human squamous cancer cells in vitro in combination with GCV.

**Regression of Human Squamous Cell Cancers after Adenoviral Transduction.** After implantation of $6 \times 10^6$ tumor cells, the mice showed slow clinical tumor growth in the floor of mouth with extension into the anterior neck over the following 2 weeks. There were no signs of cachexia during this period, and all animals appeared healthy at the time of adenovirus injections. One control group of animals was sacrificed at 2 weeks, and histopathological examination revealed a poorly differentiated squamous cell cancer with many mitotic figures and without keratin formation or necrosis. There was also clinical and histopathological evidence of surrounding soft tissue, muscle, and bony invasion. A second group of control animals eventually developed cachexia and died after 35–45 days.

In the clinical experiment, 35 animals were divided into four control groups and one complete treatment group: group 1, PBS intratumor injection plus GCV treatment (PBS+/G+); group 2, ADV/RSV-tk or ADV/RSV-β-gal vector containing the bacterial β-galactosidase gene (ADV/RSV-β-gal; kindly provided by L.D. Stratford-Perricaudet; Ref. 13) was added at various multiplicities of infection. The transduced cells were then stained with X-gal 24 h after transduction. Under identical conditions, separate cell culture experiments were performed using the ADV/RSV-tk vector, followed by either PBS or GCV treatment at a concentration of 10 μg/ml. Sixty-eight h later, the surviving cells which remained attached to the tissue culture plate were counted and compared to the PBS control plates.

**In Vitro Experiments.** All animal experiments were performed on athymic nude (nu/nu) mice (Harlan Sprague-Dawley) using sterile technique under a laminar flow hood. Nude mice, aged 6–10 weeks, were anesthetized by i.p. injection of 0.5 ml avertin at a concentration of 20 mg/ml. Using a 100 μl syringe (Hamilton, Reno, NV) and 26 gauge needle, a 50-μl solution containing $6 \times 10^6$ human HLaC-79 squamous cells in Hank’s balanced salt solution was injected into the floor of the mouth of nude mice. The cell suspension was slowly injected at the depth of the mylohyoid muscle and then the needle was removed with no apparent leakage. The animals were then maintained in standard housing conditions for 14 days.

For the adenovirus injection, the nude mice were anesthetized as before, and the neck skin was incised with scissors. The tumors were exposed by careful surgical dissection, and size was measured in three dimensions using calipers. A microliter syringe fitted with a 25 gauge needle was then used to directly inject a 75-μl solution containing $1 \times 10^6$ adenoviral particles of either ADV/RSV-tk or ADV/RSV-β-gal. Another control group received only 75 μl of PBS. The actual adenovirus or PBS delivery was performed with four separate needle passes, two parallel to the long axis of the tumor and two perpendicular to this axis. Neck incisions were closed with 4-0 silk (Ethicon). Eighteen h after virus injections, the mice were begun on i.p. GCV treatments at 100 mg/kg twice daily or PBS at the same volume for six days. The treatment mice showed no change in eating or other behavior habits during the course of the GCV treatment.

The mice were sacrificed 21 days after original tumor implantation, and the lesions were carefully excised to include only the necrotic or residual tumor. The tumor masses were measured with calipers immediately after excision, and then repeat measurements were made by a second independent examiner prior to embedding for histological evaluation. For X-gal studies, excised tumor was embedded in O.C.T. and snap frozen over dry ice. It was stored at −80°C until preparation of frozen sections, which were stained with X-gal (14). For all other histological studies, tissue was fixed in 10% buffered formalin and embedded in paraffin; serial 3-μm sections were cut and stained with hematoxylin and eosin. Histological sections were examined by a single individual (M. R. S.), who was blinded to the particular treatments of each animal. Assessment of tumor grade, circumscription, necrosis, fibrosis, inflammatory response, and mitotic counts were done using standard microscopic equipment. Quantitative morphometric measurements of maximum tumor cross-sectional area, percentage tumor necrosis per cross-sectional area, and mitotic figures per 100 high power fields were performed using a computer-assisted image analyzer. The system includes a Nikon Microphot-FXA microscope, Ikegami 370 TV high resolution color camera, Sony Triniton high resolution color video monitor, and a Compudyne 486 computer with Bioscan Optimas software (Edmonds, WA).

**Survival Experiment.** In order to assess long-term outcome, 30 nude mice received floor of mouth injections with the human squamous cancer cells, and tumors were identified in 27 of these animals at day 11. Using the same conditions as in prior experiments, the tumors were injected with either the ADV/RSV-tk or the ADV/RSV-β-gal virus, and all animals received treatment with GCV according to protocol. Tumor size was assessed weekly using caliper measurements in two dimensions.

![Fig. 1. Efficient adenovirus transduction in HLaC-79 human squamous cancer cells in vitro using a recombinant vector containing the β-galactosidase gene (ADV/RSV-β-gal). Gene expression is depicted by a dense nuclear blue staining 24 h after transduction when exposed to X-gal stain. A, transduced cells showing 85% positive nuclear staining at M.O.I. = 8. B, control cells (M.O.I. = 0) with no positive staining.](image-url)
In order to objectively analyze these findings, a tumor index value indicating overall clinical response was determined for each group using a modification of the previously described cancer cell index calculation (8, 15). The tumor index was based on morphometric measurements of maximum cross-sectional tumor area multiplied by the mitotic activity of nonnecrotic tumor mass and change in macroscopic size. The majority of animals in the tk+/G- group had a tumor index of “30” or less, which reflects near total tumor regression with only rare viable tumor cells noted on microscopic analysis (Fig. 5). A value of “0” occurred in four tk+/G+ animals and correlates not only with an absence of mitotic figures but also a lack of characteristic viable tumor cells upon histological examination. The mean tumor index for the complete treatment group (tk+/G+) was 10–100-fold smaller, and the median value was 280–2400-fold smaller than values for the control groups which did not receive the therapeutic tk gene. When compared to the tk group that did not receive GCV (tk+/G-), the tk+/G+ treatment group had mean and median tumor index values which were 6- and 55-fold smaller. Using Mann-Whitney analysis, statistical significance was determined by comparing the tk+/G+ group to each of the controls with values ranging from P < 0.001 to P < 0.02 (Fig. 5).

Local and Systemic Effects of Adenovirus Gene Transfer and GCV Treatment. Two of the animals that received ADV/RSV-tk and GCV treatment were chosen at random, and samples of local tissue and distant organs were evaluated for any histological abnormalities. Surrounding muscle, soft tissue, and salivary glands in the floor of mouth and neck regions did not show any evidence of necrosis, dying cells, or morphological changes. Distant organs were also harvested at necropsy and included small intestine, bladder, ovaries, spleen, heart, brain, kidney, lung, and liver. All specimens were normal on gross examination and histological analysis revealed no metastatic tumor, necrosis, fibrosis, or other abnormal morphology.

Survival Experiment. Ten tk+/G+ treatment mice and 15 β-gal+/G+ control mice had tumors ranging from 21–75 mm³ at the time of adenovirus injection. The effects of the adenovirus treatment were assessed by visual inspection, palpation, and caliper measurements of tumors on a weekly basis (Fig. 6). All of the β-gal+/G+ control mice developed large tumors, became cachectic, and died or required sacrifice within 43 days of treatment. In the tk+/G+ treatment group, 50% of the mice have no evidence of residual or recurrent...
tumor and are clinically healthy at 160 days post viral injection. The remaining treatment animals developed slow recurrence of their tumors over the following 70 days and eventually required sacrifice at 115 days.

DISCUSSION

These experiments are the first successful demonstration of adeno-viral-mediated gene transfer used for the treatment of human head and neck squamous cell carcinoma in an animal model. The effectiveness of the treatment scheme is depicted by the very low M.O.I needed for in vitro killing and results of the two in vivo therapeutic indices analyzed. This human cancer cell line is highly susceptible to transduction via the adenovirus vector system as dramatic killing occurred at an M.O.I. as low as 3 with 96% tumor cell death achieved at an M.O.I. of 30. The sensitivity and response of this human squamous cell cancer to the adenoviral and GCV treatment is at least 50 times greater than the response noted in previously reported rat glioma cell line experiments (11). This susceptibility to adenoviral transduction should prove advantageous in treating head and neck squamous cell cancer by providing effective clinical eradication at lower concentrations of ADV/RSV-tk.

The first in vivo therapeutic index is mean tumor necrosis which was high for the ADV/RSV-tk plus GCV group (tk+/G+), indicating a substantial cytotoxic effect of the coupled therapy. Treatment with GCV alone (PBS+/G+) or in conjunction with the β-gal vector (β-gal+/G+) showed no tumor necrosis, and two of the seven tumors injected with the tk vector alone (tk+/G−) showed only microscopic regions of focal necrosis. The remaining experimental group, β-gal vector alone (β-gal+/G−), contained microscopic focal necrosis in each tumor which was consistent with the sites of needle injection. Thus, the combination of thymidine kinase gene transfer plus GCV is essential in achieving direct tumor eradication.

The second therapeutic index is based on morphometric analysis and histological characteristics of the tumors and has been designated the tumor index. The importance of this calculation method for tumor index is that it provides a completely objective means of assessing any apparent residual tumor as well as the overall treatment outcome, thereby eliminating possible examiner bias in interpreting tumor histology. The majority of animals in the complete treatment group (tk+/G+) had tumor index below “30,” indicating near total tumor eradication, and four animals had values of “0.” These findings demonstrate a definite therapeutic effect of the tk gene transfer and GCV treatment.

There were two high clinical response values in the tk+/G+ treatment group which result from incomplete tumor killing and areas of residual viable cancer. Upon reviewing the pretreatment gross size, the tumor volumes for these animals were 324 and 220 mm³, compared to an average volume of 95 mm³ for the other tumors in the tk+/G+ group. These findings suggest that a critical tumor volume exists which limits the response of “one time” gene transfer therapy. Further studies will be performed to define a therapeutic tumor size limit and to determine whether increasing the concentration of

Fig. 4. Photomicrographs of tumor from various treatment groups. A, X-gal stain of tumor injected with ADV/RSV-β-gal shows focal strong nuclear staining indicating transduction (× 110). B, completely viable tumor after ADV/RSV-tk transduction without GCV, demonstrating viable poorly differentiated squamous carcinoma cells with numerous mitotic figures (hematoxylin and eosin, × 440). C. ADV/RSV-tk + GCV tumor showing extensive early (left) and late (right) necrosis (hematoxylin and eosin, × 110). D. another ADV/RSV-tk + GCV tumor demonstrating almost total necrosis with only a few dying swollen tumor cells at left (hematoxylin and eosin, × 440).

Fig. 5. Tumor index values (dots) depicting overall clinical response for each animal in the experimental groups. Data at or below the horizontal line (tumor index of 30 or less) indicate near total tumor regression. Bars, median values for each group. Dramatic clinical response was seen in the complete treatment animals (TK+ G+) compared to each control group (P < 0.001–0.02 per Mann-Whitney analysis).
injected virus provides any clinical advantage. There were also two low clinical response values in the tk+/G− control group, but on histological review, there was no evidence of necrosis, and the tumor contained large numbers of mitotic figures. These low values were a direct result of the very small cross-sectional area of the two tumors and could simply be a factor of the known variable cancer growth in this model (4). The possibility of an inhibitory effect on tumor growth from tk gene transfer must be considered, however, since the tumor indices of the tk+/G− group were overall smaller than both the PBS and β-gal adenovirus gene transfer control animals (P < 0.010–0.016). The β-gal+/G+ and β-gal+/G− control animals also showed no statistical differences in tumor response from the PBS+/G+ control group.

Athymic mice which lack T cells (CD4+ and CD8+ lymphocytes) were selected in these experiments for the purpose of eliminating the cellular immune response which has been implicated as a major component of tumor regression after viral transduction (8–11). Previous studies on retroviral-mediated tk gene transfer into rat glioma tumors in immune-competent animals have shown that infiltration of macrophages and lymphocytes occurs in these tumors (9–11). It is believed that this immune reaction enhances general tumor killing after viral transfection. In our experiments, there was no inflammatory or immune cell response in the tk+/G+ treatment group or any of control groups. Therefore, the tumoricidal response directly results from the tk gene transfer coupled with GCV administration.

The findings in our studies do support the concept of a contribution from what has been called “the bystander effect.” In both murine and human sarcoma tumor models, the transfer of a toxic metabolite of GCV, presumably a phosphorylated form, via gap junctions or endocytosis of apoptotic vesicles from virally transduced dying tumor cells to nearby nontransduced cells has resulted in killing of these “bystander” cells (16, 17). In our experiments with human squamous cell cancers in vivo, ADV/RSV-β-gal delivery resulted in only a 1–10% transduction as detected by X-gal staining, whereas the same quantity of virus injection with ADV/RSV-tk showed diffuse tumor killing and necrosis in the experimental group. The localized β-gal staining indicates that the adenovirus does not readily diffuse throughout the solid tumor to affect cancer cells distant to the site of delivery. It should also be noted that, in vitro, similar transduction efficiencies occurred at low M.O.I. for both the β-gal and tk adenovirus. Furthermore, the presence of an

**ACKNOWLEDGMENTS**

We thank Lois Brandon for her expert technical assistance with tissue culture experiments and Annetta Walker of Baylor’s Dermatopathology Laboratory for preparation of histological slides.

**REFERENCES**

ADENOVIRUS-MEDIATED GENE THERAPY FOR HEAD AND NECK SCC


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