Use of Recombinant Adenovirus to Transfer the Herpes Simplex Virus Thymidine Kinase (HSVtk) Gene to Thoracic Neoplasms: An Effective in Vitro Drug Sensitization System

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Abstract

Transfer of the herpes simplex virus thymidine kinase (HSVtk) gene into tumor cells using retroviral vectors followed by administration of ganciclovir provides a potential strategy for the treatment of malignancy. Because of the limitations of using retroviral vectors for clinical application, the feasibility of using a recombinant adenovirus containing HSVtk was examined. Cell lines derived from human malignant mesotheliomas and non-small cell lung cancers infected with a recombinant adenovirus containing HSVtk showed strong expression of HSVtk protein as determined by immunohistochemical staining. Infection with a recombinant adenovirus containing HSVtk rendered cells sensitive to doses of ganciclovir that were 2–3 logs lower than uninfected cells or those infected with a control virus. A strong “bystander effect” was noted in mesothelioma lines; there was no diminution in the efficacy of ganciclovir treatment until the ratio of infected:uninfected cells fell below 1:10. This study thus demonstrates in vitro efficacy of an adenovirus-transduced HSVtk drug sensitization gene therapy system in thoracic malignancies. Recombinant adenovirus transfer of the HSVtk gene followed by ganciclovir may have promise as an in situ treatment for tumors.

Introduction

Malignant mesothelioma is a tumor arising primarily from the normal mesothelium lining the thoracic cavity. Although mesothelioma is relatively uncommon compared to a number of other neoplasms, it is a frustrating clinical problem due to its extremely poor response to treatment. Aggressive multimodality therapies, including radical resection with adjuvant chemotherapy and radiation, result in survivals of only 18–24 months from diagnosis (1). Despite this, there are a number of characteristics that make mesothelioma an attractive target for gene therapy. Residing in the potential space of the thoracic cavity, the tumor is easily accessible, facilitating administration of substances such as recombinant genetic material. In addition, local extension of disease rather than distant metastasis is responsible for the majority of the morbidity and mortality associated with this neoplasm. Thus, even small increments of improvement in local control could lead to significant improvement in survival or palliation. NSCLC is a much more common malignancy, and combined chemotherapy/radiation therapy for advanced stage disease may not significantly improve survival beyond what is offered by supportive care (2). Local complications of advanced disease such as malignant effusion and bronchial obstruction may also be reasonable targets for somatic gene therapy.

Various systems of somatic gene therapy for malignancy have been investigated, including transfer of drug susceptibility genes. This paradigm allows recipient cells to selectively produce a novel substance, usually an enzyme, which sensitizes them to a normally nontoxic drug. One such combination is transfer of the HSVtk gene followed by administration of GCV (3). GCV is an acyclic nucleoside that is normally metabolized at very low levels by mammalian cells (4). GCV is transformed by viral thymidine kinase into a monophosphate form that is subsequently converted by endogenous mammalian kinases to GCV triphosphate, a potent inhibitor of viral DNA polymerase and a purine analogue competing with normal nucleotides for viral DNA replication (4). In addition, GCV triphosphate also competes with normal nucleotides for DNA replication in mammalian cells and can cause inhibition of cell growth and cell death. By these mechanisms, tumor cells expressing the HSVtk enzyme become susceptible to the toxic effects of GCV. One of the more promising aspects of the use of HSVtk for gene therapy is the existence of a “bystander effect.” Tumor cells not actually expressing the HSVtk gene, but in proximity to neighboring HSVtk-transfected cells, are killed after exposure to GCV (5, 6). The presence of the bystander effect means that effective tumor treatment may then occur after transfer of genetic material to a minority of the tumor cells.

Recent reports describing the use of the HSVtk-GCV system for the treatment of tumors have used retroviral vectors for gene transfer (6–8). Although retroviruses have the advantage of mediating stable gene transfer with a low potential for immunogenicity, this vector delivery system has a number of characteristics which make in vivo application problematic, including: (a) difficulties in producing high titers of retrovirus for in vivo use; (b) the fact that only actively dividing cells are infected; and (c) the possibility of insertional mutagenesis (9).

For these reasons, an alternative gene delivery system would be desirable. In contrast to retroviruses, adenoviral vectors can be experimentally produced in very high titers and have no significant known potential for integration and insertional mutagenesis (10). A variety of dividing and nondividing cells have been successfully infected in vivo with recombinant adenovirus allowing the transfer of either marker or therapeutic genes into both animal and human tissues (11–14). In addition, we have recently shown that a recombinant adenovirus vector can transfer a marker gene into human mesothelioma and NSCLC cells in vitro, as well as in an animal model of human mesothelioma.
mesothelioma in immunodeficient mice. The purpose of this report is to describe experiments demonstrating that transfer of the HSVtk gene to human NSCLC and malignant mesothelioma cells can be accomplished using a recombinant adenovirus. In addition, cellular killing with bystander effect occurs in vitro following GCV administration. These results suggest that an adenovirus-HSVtk system may be an effective alternative for gene therapy of malignant mesothelioma and NSCLC, as well as other human tumors.

Materials and Methods

Cell Lines. The 1-45 malignant mesothelioma cell line was the generous gift of Dr. Joseph Testa (Fox Chase Institute, Philadelphia, PA). Human mesothelioma cell lines REN and HeMe-B were isolated in our laboratory from patient tumor specimens with development and characterization described elsewhere. Cells were grown in RPMI containing 10% fetal bovine serum, nonessential amino acids, and penicillin/streptomycin (RPMI 10). All three mesothelioma cell lines were further characterized by conventional immunohistochemistry and exhibited strong expression of the Wilms tumor gene (15). The human non-small cell carcinoma cell lines A549 (carcinoma) and Calu-6 (anaplastic adenocarcinoma) were obtained from the American Type Culture Collection (Rockville, MD).

Recombinant Adenovirus. The production of recombinant replication-deficient adenoviral vectors has been described in detail elsewhere (13). Briefly Ad.RSVtk was constructed from an adenovirus type 5 mutant, that lacks most of the viral sequence from the Ela, Elb, and a portion of the E3 region. With the use of homologous recombination techniques, the herpes simplex type I thymidine kinase gene and the Rous sarcoma virus 3' promoter region were inserted into the viral genome. Viral stocks were propagated in 293 cells and titers were quantified by 293 plaque assays and spectrophotometric density.

Cell Culture Infection with Ad.RSVtk. For initial in vitro experiments, all cell lines were plated and grown to confluence in T25 cell culture flasks. Stock Ad.RSVtk in 5% glycerol was diluted to 5 x 10^5 particles/ml in RPMI 10 and 2.0 ml (corresponding to ~100 viral particles/tumor cell) of viral suspension were placed on cell monolayers. Flasks were then incubated at 37°C and 5% CO2 for 3 h, after which time the viral suspension was removed and replaced with fresh media.

In Vitro Ganciclovir Sensitivity Assay. Initial studies examining the effect of Ad.RSVtk were performed in 24-well culture plates. Infected and uninfected cells were plated at a density of 20,000 cells/well. After incubation for 2 days, the cell culture medium in each well was removed and replaced with RPMI 10 with varying concentrations (0—2000 µM) of ganciclovir (Syntex Laboratories, Inc., Palo Alto, CA). The cells were incubated at 37°C and 5% CO2 for 5 days. Sensitivity to ganciclovir treatment was measured by direct cell counts using a Coulter Counter (Coulter, Inc., Hialeah, FL). Cell number was also assessed using a colorimetric cell proliferation assay that measures viable cell dehydrogenase activity (16) (CellTiter 96 Aqueous Non-radioactive MTT Cell Proliferation Assay, Promega, Madison, WI). Infected and/or uninfected cells were plated at a concentration of 6000 cells/well in 96-well culture plates (100 µl media/well). After a 24-h incubation period, medium was removed and replaced with RPMI 10 containing varying concentrations of ganciclovir (0—2000 µM). After 5 days of incubation at 37°C, 20 µl of assay mix were added to each well and the plates were incubated for 3 additional h and absorbance at 490 nm was measured.

The bystander effect was determined by mixing infected and uninfected cells at varying ratios. Cells were then plated into 96-well plates at 20,000 cells/well to ensure cell-cell contact. Twenty-four h later, the cells were treated with 20 µM GCV for 5 days. Cell number was then determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described above.

Immunohistochemistry. Confluent cell monolayers on coverslips were infected with either 100 or 500 viral particles/tumor cell of Ad.RSVtk. Forty-eight h later, the monolayers were fixed in 3% paraformaldehyde, permeabilized with 0.5 % NP40, and blocked with 0.1M glycine. The monolayers were then treated with 5% goat serum, washed in phosphate-buffered saline, and reacted with a polyclonal rabbit anti-HSVtk antibody (courtesy of W. C. Summers, Yale University) at room temperature for 1 h. After a washing in phosphate-buffered saline, the bound primary antibody was detected with the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) using 3-amino-9-ethylcarbazole as chromogen. An anti-human FVIII-related antigen was used as a positive control.
ADENOVIRUS TRANSFER OF HSVtk

Fig. 2. Comparison of ganciclovir dose response in three human mesothelioma and two human non-small cell lung carcinoma cell lines. Ad.RSVtk-infected cells are compared to uninfected cells or cells infected with Ad.RSVlacZ. Points on the ordinate correspond to the percentage of cells present at each dose compared to cells treated with no ganciclovir (untreated cells). IC₅₀, dose required to eliminate 50% of cells present compared to untreated cells. (A) REN mesothelioma cell line. IC₅₀ for Ad.RSVtk-infected cells is ~0.2 µM, with uninfected cells having an IC₅₀ of ~1500 µM. Ad.RSVlacZ-infected cells do not exhibit increased sensitivity. (B) 1-45 mesothelioma cell line. IC₅₀ for Ad.RSVtk-infected cells is ~0.08 µM, with uninfected cells having an IC₅₀ of ~500 µM. Ad.RSVlacZ-infected cells do not exhibit increased sensitivity. (C) HeMe-B mesothelioma cell line. IC₅₀ for Ad.RSVtk-infected cells is ~2.0 µM, with uninfected cells having an IC₅₀ of ~2000 µM. (D) Calu-6 non-small cell lung carcinoma cell line. IC₅₀ for Ad.RSVtk-infected cells is ~1.0 µM, with uninfected cells having an IC₅₀ of ~1000 µM. (E) A549 non-small cell lung carcinoma cell line. Uninfected cells exhibit an IC₅₀ of >2000 µM, and Ad.RSVtk-infected cells at ratios of 500, 1000, and 2000 viral particles/tumor cell exhibit an IC₅₀ of ~100 µM.

Results

Ad.RSV.tk Transfers the Thymidine Kinase Gene to Human Thoracic Tumor Cells. We have previously shown the Ad.RSVlacZ vector infects 90–100% of human thoracic tumor cells in vitro at 50–100 viral particles/tumor cell. Previously, estimates have been made of the efficiency of the HSVtk gene transfer by extrapolation from data using the lacZ marker gene (5). In this study, however, we determined the efficiency of HSVtk gene transfer directly by staining coverslips containing Ad.RSVtk-infected mesothelioma cells with a rabbit polyclonal antibody specifically recognizing the HSVtk protein. At an infection ratio of 100 viral particles/tumor cell, approximately 40% of both REN (Fig. 1A) and 1-45 (data not shown) cells expressed clearly detectable HSVtk enzyme in the nucleus and/or cytoplasm by...
immunohistochemical staining. Faint staining was seen in many other cells and may represent gene transfer at lower levels. Increasing the virus concentration to 500 particles/cell did not appear to increase the percentage of cells strongly expressing HSVtk protein. Uninfected cells from both lines did not stain with HSVtk antibody (Fig. 1B). Neither infected nor uninfected mesothelioma cells stained positively with the FVIIIra antibody (data not shown).

Recombinant Adenovirus-mediated Transfer of the HSVtk Gene to Mesothelioma and Non-Small Cell Carcinoma Cell Lines Sensitizes Them to Ganciclovir Treatment in Vitro. To test the ability of Ad.RSVtk to confer sensitivity to GCV, all cell lines were infected at a concentration of 100 viral particles/tumor cell with both Ad.RSVtk and Ad.RSVlacZ vectors and exposed to varying doses of GCV continuously for 4 days. The IC50 of GCV for the mesothelioma cell lines 1-45, REN, and HeMe-B was determined by either direct cell counting (data not shown) or by using a chromogenic assay that measures the number of viable cells in each group at the end of the treatment period (Fig. 2, A—C). Both counting methods were in close agreement. Cells of all three lines infected with Ad.RSVtk were rendered sensitive to doses of GCV that were 3–4 logs lower than uninfected cells, with an IC50 reached at concentrations between ~0.08 and 2.0 μM. Fig. 2, D and E show the IC50 data for the lung cancer lines Calu-6 and A549 infected with Ad.RSVtk following exposure to GCV. The Calu-6 cells exhibited a sensitivity profile similar to those of the mesothelioma lines with an IC50 at a GCV dose of ~2.0 μM; however, the A549 NSCLC line had a much higher IC50 of approximately 100 μM GCV. This comparatively high IC50 was also noted in the A549 cells at higher concentrations of viral particles/tumor cell of 500, 1000, and 2000 (Fig. 2E) and thus does not appear to be due to inefficient infection.

In addition to cellular death, HSVtk-infected residual viable cells of all lines treated with GCV were noted to exhibit increased size, vacuolization, and granular cytoplasm. These morphological changes were not noted in uninfected or Ad.RSVlacZ-infected cells at identical GCV doses. Of note is the fact that infection with an adenoviral vector containing a control marker gene (Ad.RSVlacZ) did not sensitize cells to GCV (Fig. 2, A and B). GCV sensitivity of 1-45 cells infected with Ad.RSVlacZ appeared to be similar to uninfected control cells, while REN cells infected with this marker virus actually appeared less sensitive.

A Bystander Effect Occurs in Ad.RSV.tk-infected Mesothelioma Cells in Vitro. To determine if Ad.RSVtk-infected mesothelioma cells would exhibit a bystander effect (where uninfected cells adjacent to those expressing the HSVtk gene were killed after exposure to GCV), varying ratios of Ad.RSVtk-infected (100 viral particles/cell) to uninfected cells were prepared and treated with 4 days of continuous GCV in vitro. A dose of 20 μM GCV was chosen because this approximates plasma concentrations of patients receiving GCV therapy. Fig. 3, A and B display the results from experiments performed with both the 1-45 and REN lines. There was no diminution in the efficacy of GCV treatment until the infected:uninfected cell ratio fell below 1:10 (i.e., a maximum of 10% of cells Ad.RSVtk infected). A partial but significant effect was seen at an infected:uninfected cell ratio of up to 1:100 (Fig. 3).

Discussion

In this report, we demonstrate transfer of a therapeutic gene construct and successful in vitro drug sensitization of human malignant thoracic tumor cell lines using a recombinant replication-deficient adenovirus vector. In addition, we characterize transfer of the HSVtk gene by use of immunohistochemical techniques and illustrate the presence of a bystander effect. These data suggest that recombinant adenovirus transfer of a drug susceptibility gene offers an alternative to recombinant retroviruses.

Application of the HSVtk drug susceptibility system to malignancy was pioneered by Moolten et al. (7, 8) and others (5, 6). A number of reports have recently been published illustrating efficacy in both in vitro and in vivo tumor models. To date, however, all of these studies have used recombinant retroviral vector gene transfer technology (6–8, 17, 18). Although the ability to infect rapidly dividing cells may be a useful feature of retroviral vectors, this potential advantage is limited by the fact that direct inoculation of tumor tissue in situ may require that retroviral "producer" cells be used. Ram et al. (17) showed that experimental brain tumors could be eliminated by administration of GCV to rats following injection of 3T3 cells actively producing recombinant retrovirus carrying the HSVtk gene. However, no effect was noted with intratumoral injection of infectious viral supernatant alone. Because recombinant adenovirus has the ability to primarily infect mesothelioma cells in vivo after intratumoral injection and after instillation into the peritoneal cavity of mice with established tumor, we have evaluated recombinant adenovirus as an alternative vector for transfer of the HSVtk gene.

The in vitro sensitivity of three Ad.RSVtk-transfected mesothelioma cell lines to GCV reported in this study is similar to reports using the retrovirus. The IC50 of GCV was less than 2.0 μM. These doses are certainly acceptable with regard to considering eventual in vivo efficacy, as nontoxic peak serum levels for patients receiving GCV therapy for viral infections reach 15–18 μM (19). Although one NSCLC lung cancer line was highly sensitive to this Ad.RSVtk-GCV treatment, it appears that the adenovirus is a useful alternative.
system, the relative insensitivity of the A549 NSCLC line to this treatment system suggests that some degree of variability of response can be anticipated when treating in situ tumors. The explanation for this HSVtk insensitivity is not evident but is probably not due to an inability of the adeno virus to infect the cells since this line expresses large amounts of the β-galactosidase after infection with Ad.RSV- 

lacZ. Resistance to HSVtk has also been noted in some neoplastic cells in an in vitro retrovirus-transduced system (8).

Perhaps the most important characteristic of the HSVtk-GCV drug susceptibility paradigm is the ability of HSVtk-transfected cells to kill adjacent untransfected tumor cells after exposure to GCV, the so-called bystander effect (6). This becomes an extremely important consideration in the treatment of preexisting solid tumors by this system since adeno viral transfer of new genetic material into 100% of cells in such lesions is not likely. Other groups, using rat fibrosarcoma, murine sarcoma, and rat glioma tumors arising from cells infected ex vivo with retroviral HSVtk, have shown marked tumor growth inhibition, and even elimination, after systemic GCV therapy, even when as few as 10% of cells were originally transduced (5, 6, 17). Explanations offered for this phenomenon include continued retroviral infection in situ, transfer of an integrated retroviral HSVtk gene during mitosis, transfer of the toxic purine analogue GCV triphosphate via gap junctions or apoptotic vesicles, or immune mediated effects (5, 6, 17, 18). The data from this study argue against retroviral-specific or immune mechanisms, since a strong bystander effect was demonstrated in this adeno virus HSVtk-transduced in vitro tumor system.

In summary, this is the first report, to our knowledge, that the HSVtk gene can be efficiently transferred to human tumor cells using a recombinant adeno virus vector with impressive cytotoxicity following GCV exposure. Transfer is associated with a strong bystander effect, suggesting that recombinant adeno virus transfer of this gene could potentially be effective for the in situ treatment of solid neoplasms.

References


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