Adenoviral-mediated Delivery of the Herpes Simplex Virus Thymidine Kinase Gene Selectively Sensitizes Human Ovarian Carcinoma Cells to Ganciclovir

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ABSTRACT

One strategy used for gene therapy of cancer is molecular chemotherapy. This approach is based on selective expression of an encoded toxin in cancer cells to achieve their eradication. One potential advantage of this strategy derives from a phenomenon, termed the bystander effect, whereby only a fraction of cells needs to be transduced to eradicate a tumor population. Despite the theoretical advantages of this phenomenon, it has only been described in a few cellular targets. Therefore, we undertook strategies to develop a molecular chemotherapy approach for ovarian carcinoma utilizing the herpes simplex virus thymidine kinase (HSV-TK) gene. Initially, we established that human ovarian carcinoma cell lines could be transduced at high efficiency with adenoviral vectors encoding reporter genes. We next determined that the human ovarian cell line SKOV3 could exhibit bystander killing by stably transducing it to express HSV-TK and performing cell mixing experiments with varying percentages of HSV-TK-expressing and HSV-TK-nonexpressing cells. Based on these findings, we constructed a recombinant adenovirus encoding HSV-TK and utilized it to induce human ovarian carcinoma cell lines to the sensitizing effects of ganciclovir. In addition, primary cultures of ovarian carcinoma cells were found to be highly transducible with recombinant adenoviral vectors and could be induced to the sensitizing effects of ganciclovir after induction of HSV-TK expression by the adenoviral vector. These studies indicate that molecular chemotherapy using a recombinant adenoviral vector expressing HSV-TK may provide a rational strategy for human ovarian carcinoma.

INTRODUCTION

Ovarian carcinoma is the leading cause of death from gynecological malignancies in women. It is anticipated that in 1995 there will be 26,600 new cases of ovarian cancer, and it will account for approximately 14,500 deaths (1). This disease has a high fatality rate due to the lack of effective screening strategies and to the lack of symptoms associated with early stage disease. Consequently, 70% of women with ovarian carcinoma present with advanced stage disease at the time of diagnosis. Current standard treatment consists of surgical debulking in combination with chemotherapy. However, extensive intraabdominal disease is difficult to completely eradicate by surgery. Most patients will only transiently respond to adjunctive therapies, and long-term survival for patients with advanced stage disease rarely exceeds 15–30% (1). Based on these survival statistics, a variety of novel therapeutic approaches have been proposed for this disease. These experimental approaches include the use of monoclonal antibodies, immunonconjugates, cytokines, and radioimmunotherapy (2, 3). To date, however, these strategies have not offered evidence of long-term efficacy.

Gene therapy approaches may offer unique methods to achieve anticancer effects. To this end, a variety of distinct strategies have been developed and used. The approach of molecular chemotherapy is designed to achieve selective eradication of carcinoma cells via an expressed toxin gene. In this regard, it has been previously demonstrated that such a gene product can selectively sensitize tumor cells to an agent not ordinarily toxic (4–6). The toxin gene therapy system most widely used to accomplish cell killing utilizes the HSV-TK gene. Expression of the HSV-TK gene allows the tumor to have an enhanced sensitivity to nucleoside analogues such as GCV and acyclovir. The mechanism that allows utilization of viral thymidine kinase for cytotoxic effect is preferential monophosphorylation of GCV by the HSV-TK gene (7). GCV will then be further phosphorylated by cellular kinases to diphosphates and then to triphosphate forms that become incorporated into the cellular DNA. It is the incorporation of the triphosphate form of GCV into cellular DNA that causes inhibition of DNA synthesis and RNA polymerase, which eventually leads to cell death (8). The specificity of this system is the differential metabolism of GCV preferentially by the viral thymidine kinase gene. Because the mammalian enzyme has a much lower affinity for the drug, normal cells tend to be resistant to the toxic effects of GCV.

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3 The abbreviations used are: HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; AdCMVLuc, recombinant adenovirus expressing luciferase; AdCMVLacZ, recombinant adenovirus expressing β-galactosidase; CMV, cytomegalovirus; AdCMVHSV-TK, recombinant adenovirus expressing HSV-TK.
when administered at low levels. Thus, the tumor cells transduced to express the viral gene have enhanced sensitivity for cell killing. In addition to selective sensitization, another advantage of this system was the finding of a bystander effect, in which every tumor cell does not need to express HSV-TK to eradicate a tumor population (9, 10). However, despite the apparent advantage of the bystander effect, this phenomenon has been described in relatively few neoplastic targets such as glioma (9), mesothelioma (11–13), fibrosarcoma (10, 14), and melanoma (15, 16).

From a mechanistic standpoint, previous work has demonstrated the necessity of cell to cell contact as a prerequisite for bystander-mediated cell killing (10, 14). Therefore, the tumor models utilized to date have been confined to body compartments such as the cranial vault and the thoracic cavity, where this phenomenon could be exploited. Because >80% of ovarian carcinomas are confined to the peritoneal cavity, this disease context would theoretically be similar to other compartmentalized tumor models demonstrated to be responsive to molecular chemotherapy strategies. We, thus, investigated the efficiency of gene transfer in human ovarian carcinoma cells in vitro, and examined whether bystander-mediated cell killing occurred. The strategy described here demonstrates that an adenoviral vector is suitable for efficient gene transfer into both ovarian carcinoma cell lines and primary neoplastic cells obtained from women with ovarian cancer. Furthermore, these studies indicate that bystander killing, in the context of ovarian cancer, is feasible and, therefore, provides the rationale for the use of molecular chemotherapy as a gene therapy strategy for human ovarian carcinoma.

MATERIALS AND METHODS

Cell Lines. The human ovarian carcinoma cell lines SKOV3, PA-1, SW626, CaOv3, and the human cervical carcinoma cell line HeLa were obtained from the American Type Culture Collection (Rockville, MD). The human ovarian carcinoma cell line OV-4 was kindly provided by Timothy J. Eberlein (Brigham and Women’s Hospital, Harvard Medical School, Boston, MA) and the SKOV3 derivative line SKOV3.ip1 was kindly provided by Janet Price (Baylor University, Houston, TX). These cell lines were maintained in DMEM (Mediatech, Herndon, VA) supplemented with L-glutamine (200 μg/ml), penicillin (100 μg/ml), streptomycin (25 μg/ml), and 10% heat-inactivated FCS (PAA Laboratories, New Port Beach, CA) (complete media) at 37°C in a humidified, 5% CO2 atmosphere. The human ovarian carcinoma cell line OVCAR-3 was kindly provided by Donald Buchsbaum (University of Alabama at Birmingham, Birmingham, AL) and maintained in RPMI 1640 media (Mediatech) supplemented with 20% heat-inactivated FCS, L-glutamine, antibiotics, and 10 μg/ml bovine insulin (GIBCO Life Technologies, Gaithersburg, MD).

Primary Cells. Human ascites fluid was obtained from patients with ovarian carcinoma at the time of laparotomy or paracentesis. Ovarian carcinoma cells obtained from malignant ascites samples were prepared for short-term maintenance in the following manner. Ascites fluid was aliquoted into 10-ml samples and contaminating RBCs were lysed with 5 ml ACK buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA) for 2 min at room temperature. Lysis was terminated by the addition of 5 ml complete media. Cells were pelleted by a 5-min centrifugation at 190 × g, resuspended, and plated in complete media. Media was changed daily for 7 days during expansion of the carcinoma cells. Ovarian carcinoma cells were harvested by trypsinization and centrifuged through a Ficoll-Hypaque gradient (1077; Sigma Chemical, St. Louis, MO). Viable cells were removed from the interface and replated for subsequent studies.

Recombinant Adenoviruses Encoding Reporter Genes. To analyze gene transfer efficiency, recombinant adenoviral vectors encoding reporter genes were used. AdCMVLuc and AdCMVLacZ are EIA/B-deleted, replication-incompetent, recombinant adenoviruses previously described (17). AdCMVLuc encodes the firefly luciferase reporter gene and is under the control of the human CMV promoter/enhancer (provided courtesy of R. Gerard, University of Texas-Southwestern Medical Center, Houston, TX). AdCMVLacZ encodes the reporter gene Escherichia coli β-galactosidase and is under the control of the CMV promoter/enhancer (provided courtesy of R. Gerard).

Construction of Recombinant Adenoviral Vector Encoding HSV-TK. The adenovirus expressing HSV-TK was prepared using standard homologous recombination techniques (18). In brief, a DNA fragment containing the HSV-TK gene (provided by R. Garver, University of Alabama at Birmingham, Birmingham, AL) was cloned into the polylinker of the adenoviral shuttle vector pACCMVpLpARS (+) (provided courtesy of R. Gerard). This plasmid provides promoter/initiation signals derived from the CMV early promoter/enhancer and polyadenylation signals from SV40.

The resulting recombinant adenovirus shuttle plasmid pAC-HSV-TK was used to derive an E1-deleted, replication-incompetent, recombinant adenovirus using standard methodologies (18). In brief, the shuttle plasmid and the adenoviral packaging plasmid pJM17 (provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) were cotransfected into the E1A trans-complementing cell line 293 using the commercial cationic liposome vector DOTAP (GIBCO Life Technologies). Transfected cells were maintained until the onset of cellular cytopathic effects. The newly generated recombinant adenovirus was plaque purified three times. Validation of single plaques was accomplished by direct PCR. The recombinant adenovirus encoding the HSV-TK gene AdCMVHSV-TK was expanded within 293 cells and purified by CsCl gradient centrifugation (18). Genomic DNA derived from the recombinant adenovirus was subjected to digestion with various restriction endonucleases and analyzed by agarose gel electrophoresis. Wild-type adenovirus WT300 (provided by T. Shenk, Princeton University, Princeton, NJ) was used as a control for analysis of genomic DNA derived from AdCMVHSV-TK. Adenoviral vectors were titrated within the cell line 293 using plaque assay techniques for direct determination of viral plaque-forming units.

Establishment of Stable Clones Expressing the HSV-TK Gene. A stable human ovarian carcinoma cell line expressing the HSV-TK gene from a retroviral vector was produced by standard methods (19). The GP/envAm-12 cell line, kindly provided by Arthur Bank (Columbia University, New York, NY), was utilized with the STK retroviral vector construct...
Sensitivity of HSV-TK-expressing Clones to GCV. To validate the expression of HSV-TK, G418-resistant SKOV3 clonal cells, along with untransduced SKOV3 cells, were analyzed for GCV sensitivity. Cells were plated in triplicate at 5000 cells/well in 96-well plates, and 24 h later complete media containing varying concentrations of GCV (0–100 μM) were added. Cells were cultured at 37°C and in 5% CO2 for 5 days. Sensitivity to GCV was evaluated using a colorimetric cell proliferation assay that measured conversion of tetrazolium salt to formazan by viable cells as described by the manufacturer (Cell Titer 96 Aqueous Nonradioactive Cell Proliferation Assay: Promega, Madison, WI). In brief, 20 μl assay mixture was added to each well of cells, and the plates were incubated for 1 to 4 h at 37°C before absorbance was measured at 490 nm in a 96-well plate reader (Molecular Devices, Menlo Park, CA). The clonal population of cells expressing HSV-TK utilized for all experiments is designated SKRv4 (SKOV3 TK* retroviral clone 4). Cell viability from GCV sensitivity assays were also performed by crystal violet staining. Cells seeded in 6-well plates were infected with the HSV-TK virus as described above. Media were removed 5–7 days postinfection, and cells were fixed with 1 ml 10% formalin (Fisher Scientific, Pittsburgh, PA) for 10 min at room temperature. Cells were stained for 20 min at room temperature with 1 ml crystal violet (70% ethanol containing 1% crystal violet; Fisher Scientific) and then washed five to six times with water to remove dye.

Recombinant Adenoviral Infections. Cells were infected with recombinant adenoviral vectors as previously described (21). In brief, cells were plated in 6-well plates and infected 24–48 h later at a multiplicity of infection of 75–95%. Viral infections were carried out in DMEM media containing 2% FCS and were allowed to proceed at 37°C for 1 h, after which time complete media were added to each culture. For the AdCMVLacZ vector, cells were infected with 1.5 × 10⁹ particles/well (~1500 viral particles/cell). For AdCMVLacZ and AdCMVHSV-TK vectors, cells were infected with 1 × 10⁹ particles/well (~100 viral particles/cell).

Analysis of Reporter Gene Expression. Following adenovirus infection, cells were analyzed for expression of the encoded reporter genes to assess the relative efficiency of gene transfer. Harvesting of cells, cell lysis, and analysis of luciferase gene expression were performed as described by the manufacturer (Promega Luciferase Assay System; Promega, Madison, WI). Lysates from transfected cells were obtained by aspirating the media and adding 150 μl 1 × cell culture lysis reagent to each well of cells. Cells were lysed at room temperature for 10 to 15 min, and cellular debris was removed by centrifugation at 13,000 × g for 3 min. Cell extract (20 μl) was added to 100 μl luciferase assay reagent and analyzed for emitted light in a Lumat LB9501 luminometer (Berthold Systems Inc., Aliquippa, PA). Primary cell extracts from ascites fluid were prepared as above, except extracts were stored at −20°C until several samples could be analyzed concurrently. The primary cell extracts were thawed and quantitated for protein concentration at the time of analysis. For each sample, 15 μg extract were added to 100 μl of the luciferase assay reagent and measured for relative light units as described above.

LacZ reporter gene expression was also utilized to examine transduction frequency. Cells infected with AdCMVLacZ virus were harvested 72 h posttransduction and stained for β-galactosidase expression. Cell monolayers were washed with PBS and fixed with 0.5% glutaraldehyde for 10 min at 37°C. Cells were washed twice for 15 min each in PBS containing 1 mM MgCl₂, then stained for approximately 6 h in a PBS solution containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 1 mM MgCl₂, and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside; Sigma Chemical). After removal of the X-gal solution, cells were overlaid with 70% glycerol and stored at 4°C.

Induction of GCV Sensitivity by an Adenovirus Expressing HSV-TK. The human ovarian carcinoma cell lines and primary cells to be infected were initially seeded into 6-well tissue culture plates. When cells reached the appropriate density, they were infected, as described above, with either the HSV-TK-expressing virus or the luciferase-expressing virus. Twenty-four h postinfection three wells from each sample were treated with 20 μM GCV. Cell viability was determined with crystal violet staining 5–7 days postinfection.

Toxin Gene-killing Experiments. To analyze the bystander effect, cell mixing experiments were performed. These were accomplished by mixing HSV-TK-expressing (SKRV4) and HSV-TK-nonexpressing (SKOV3) cells at varying percentages. Cells were plated in 96-well plates in triplicate wells at 20,000 cells/well to ensure cell to cell contact. One-half of the samples were treated 24 h later with 20 μM GCV; the remaining cells had complete media changed. Cell viability was determined 5 days later using the cell proliferation assay as described above. In these experiments SKOV3 cells were plated on the day of the assay to generate a standard curve. Cells were removed by trypsinization and plated in triplicate wells at the following densities: 100,000, 50,000, 20,000, 10,000, 5,000, 2,000, and 0 cells/well, respectively. From the standard curve, viable cell numbers could be calculated for experimental groups using the SOFTmax computer software (Molecular Devices, Menlo Park, CA).

RESULTS

Human Ovarian Carcinoma Cell Lines Were Transduced at High Efficiencies with Recombinant Adenoviral Vectors. Recombinant adenoviral vectors have been shown to be effective vehicles for the in vivo transduction of a variety of
target cells (22, 23). In this regard, our planned molecular chemotherapy strategy is based on in situ transduction of human ovarian carcinoma cells in the peritoneum. As a first step to establish the efficacy of adenoviral vectors for ovarian cancer gene therapy, we used reporter-encoding recombinant adenoviral vectors to transduce a panel of human ovarian carcinoma cell lines. As an initial screen, cells were infected with a firefly luciferase-encoding adenovirus and evaluated for expression of the luciferase reporter gene at 24 h posttransduction (Fig. 1). It can be seen that all human ovarian carcinoma cell lines examined were transduced and expressed high levels of luciferase when compared to the uninfected control cells. Interestingly, the relative levels of posttransduction expression observed were comparable to that of the cervical carcinoma cell line HeLa, which is known to be highly transducible by recombinant adenoviral vectors.

Since luciferase only measured a relative level of expression, we next sought to more accurately quantitate the transduc-
Demonstration of bystander effect in a human ovarian carcinoma cell line. An in vitro cell mixing experiment was set up by combining varying percentages of HSV-TK-expressing (SKRV4) and HSV-TK-nonexpressing (SKOV3) cells in triplicate wells of 96-well plates. Twenty-four h later cells were treated with 20 μM GCV. After 5 days, cell viability was determined using the cell proliferation assay. Four independent experiments were performed. Columns, mean percentage of cell survival from a representative experiment; bars, ±SD of the mean.

Once selective sensitivity was determined, we quantitated the extent of bystander-mediated cell killing. A retroviral vector was utilized in this experiment to accurately delineate the extent of bystander killing that could be accomplished in these cells. Since the entire population of SKRV4 cells expressed the HSV-TK gene, quantitative cell mixing experiments were undertaken. This assay was accomplished by mixing HSV-TK-expressing, SKRV4, and HSV-TK-nonexpressing, SKOV3, cells together in varying percentages at a high cell density. The cells were treated with GCV 24 h after mixing, and the viability was determined after 5-day exposure to GCV. The results of these experiments illustrated that in a mixed population of SKOV3 and SKRV4 cells, in which as little as 10% of the cells were HSV-TK positive, there was >70% cell death when treated with GCV (Fig. 3). Thus, the level of cell killing exceeded the 10% of the cells that had been transduced to express the HSV-TK gene. These studies further validated the concept that transduced cells expressing HSV-TK not only metabolized GCV and were eradicated, but that they also permitted surrounding cells to become susceptible to the toxic effects of the drug. Although the complete mechanisms of the bystander effect have not been delineated, it is known that cell to cell contact is necessary, but not sufficient, for this phenomenon to occur. It has been postulated that gap junctions may act to pass a toxic metabolite of GCV from the HSV-TK cells to the HSV-TK cells, thus allowing for greater tumor cell eradication then initially envisioned (10). Therefore, we have established that these human ovarian carcinoma cells exhibit the necessary biological properties to elicit in vitro bystander-mediated cell killing.
Human Ovarian Carcinoma Cell Lines Were Susceptible to the Toxic Effects of GCV When HSV-TK Was Delivered by an Adenoviral Vector. To determine whether human ovarian carcinoma cell lines could be selectively sensitized to GCV by adenoviral-mediated delivery of a toxin gene, we undertook the construction of a recombinant adenoviral vector containing the HSV-TK gene. The recombinant virus was produced by standard in vivo homologous recombination techniques (18). The resulting El-deleted, replication-competent, recombinant adenovirus contained the HSV-TK gene downstream of the CMV promoter/enhancer region and also contained the SV40 polyadenylation signal sequence. The construction of the recombinant virus was validated by two independent methodologies. Initially, PCR was performed with two different primer sets. The recombinant vector was found to contain a 600-bp band indicative of the HSV-TK insert while lacking the El-specific band. In contrast, the wild-type virus control (WT300) contained the El-specific fragment. As a second analysis, DNA was isolated from the virus and analyzed by digestion with a panel of restriction endonucleases that resulted in patterns consistent with the predicted size fragments for the HSV-TK gene inserted correctly into the deleted El region of the adenoviral genome (data not shown). These findings indicate the successful construction of a recombinant adenoviral vector containing the HSV-TK gene.

We sought to validate the utility of this adenoviral vector in human ovarian carcinoma cells. Studies were performed to determine whether cells infected with the recombinant viral vector exhibited sensitivity to GCV. Infections were carried out with either AdCMVHSV-TK or a control virus (AdCMVLuc) at a multiplicity of infection of 100 viral particles/cell, as described in ‘‘Materials and Methods.’’ Twenty-four h following infection, one group of cells was treated with 20 \( \mu M \) GCV; the remaining cells had complete media changed. Selective sensitivity to GCV could be demonstrated in the ovarian carcinoma cell lines SKOV3.ip1 and OVCAR-3 that had been infected with the recombinant adenoviral vector encoding HSV-TK (Fig. 4, A and C). In contrast, cells infected with a control virus exhibited no toxicity to GCV (Fig. 4, B). It was important to note that cells infected with the control virus exhibited no sensitivity to GCV, indicating that the observed cytocidal effects were specific for HSV-TK and GCV and were not due to nonspecific viral toxicity. These studies illustrate the efficacy of adenoviral-mediated delivery of HSV-TK as a strategy for molecular chemotherapy for human ovarian carcinoma.

Primary Ovarian Carcinoma Cells Were Highly Transduced with Recombinant Adenoviral Vectors and Could Be Selectively Induced to the Toxic Effects of GCV by HSV-TK-expressing Adenovirus. To determine the clinical relevance of our strategy, primary ovarian carcinoma cells were isolated from ascites fluid from women with ovarian cancer and tested for transduction with the recombinant adenoviral vectors encoding reporter genes. Initially, the primary cultures were transduced with an adenovirus encoding firefly luciferase. Cells were analyzed 24 h postinfection for expression of the luciferase reporter gene. Because samples were obtained at irregular intervals, cell extracts were frozen, then quantitated for protein, and assayed simultaneously for relative light units per 15 \( \mu g \) protein. Primary human ovarian carcinoma cells obtained from women with ovarian cancer were found to express high levels of luciferase when infected with the AdCMVLuc recombinant adenovirus (Fig. 5A). To more accurately delineate the transduction efficiency of the primary carcinoma cells, they were analyzed for \( \beta \)-galactosidase expression after infection with the AdCMVLacZ virus. Cells were stained for \( \beta \)-galactosidase expression 72 h postinfection (Fig. 5, B-D). It could be seen that at least 50% of the cells were positively stained for \( \beta \)-galactosidase, indicating successful transduction of primary cells at relatively high levels.
To further this study, we undertook measures to determine whether the cells derived from the ascites fluid could be sensitized to the toxic effects of GCV when transduced with the recombinant adenoviral construct-encoding HSV-TK. Primary cells were infected with either the AdCMVHSV-TK or the control AdCMVLuc virus. One day following transduction, 20 μM GCV was added, and the cells were assayed for viability after 7 days of GCV treatment (Fig. 6). Selective sensitivity to GCV was demonstrated in this sample of ovarian carcinoma cells only when they were infected with the HSV-TK virus (Fig. 6, A). In contrast, cells infected with the control virus were not eradicated even after treatment with GCV (Fig. 6, B). These data demonstrate that expression of HSV-TK also confers selective sensitivity to GCV in primary ovarian carcinoma cells. Thus, in situ transduction of tumor with a recombinant adenoviral construct encoding a toxin gene will likely be a feasible strategy for gene therapy of ovarian carcinoma.

**DISCUSSION**

A potential strategy for molecular chemotherapy of ovarian carcinoma is in situ transduction of tumor with the HSV-TK gene. This procedure requires a vector system that allows efficient in vivo gene delivery to tumor cells within the peritoneal cavity. In this regard, three vector systems have been described which accomplish in vivo gene delivery: recombinant retrovirus, cationic liposomes, and recombinant adenovirus. With regard to retrovirus, this vector has been utilized in selected circumstances to transduce tumor targets. In these instances, however, direct delivery of viral vector has not been used; rather, direct delivery of retroviral producer cell lines has been accomplished (9, 24). This has been utilized because of the instability of the retrovirus consequent to its susceptibility to humoral-mediated lysis. Within the peritoneal cavity, the use of either retrovirus or retroviral producer cell lines would not likely be feasible due to the dispersed nature of the tumor cells within the ascites fluid.

As an alternative, cationic liposomes have been utilized for in situ gene delivery to a variety of target sites (25, 26) and by the vascular route (27). However, this strategy results in a relatively low level of gene transfer efficiency which would deter their use in ovarian carcinoma, in which high efficiency transduction of tumor is required. Recombinant adenoviral vectors have also been utilized for the direct in vivo delivery of...
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genes into a variety of organs such as the lung, liver, gall bladder, muscle, and bladder (28–32). In addition, they have been used for the direct in situ transduction of tumor (33). In these instances, relatively high efficiency gene transfer can be accomplished. Along these lines, Albelda and his co-workers (11–13) have shown the utility of adenoviral vectors for gene delivery in the molecular chemotherapy of mesothelioma. Thus, the demonstrated advantages of recombinant adenoviral vectors, such as in vivo stability, and high gene transfer efficiency in multiple targets, led us to explore the utility of this vector in human ovarian carcinoma.

In the context of molecular chemotherapy, one principal benefit is the phenomenon of bystander cell killing. Despite the significant advancement in this area, and the number of clinical protocols utilizing HSV-TK, the actual number of cell types that have been documented to exhibit the bystander effect is restricted. In this regard, it is apparent that a certain cellular phenotype must exist in order for a cell to exhibit bystander-mediated cell killing. Pursuant to our strategy, it was not known a priori whether ovarian carcinoma cells exhibit the necessary biological characteristics. We initially chose to utilize a retroviral vector to accurately determine the extent of bystander killing that could be accomplished in this ovarian carcinoma cell line. Use of the adenoviral construct may not provide sufficient, quantitative in vitro data due to the fact that a 100% transduced population could not be obtained, and, therefore, all experiments would only be estimates of HSV-TK-positive to HSV-TK-negative cell ratios. This does not, however, limit the potential for in vivo use of adenoviral vectors since a bystander result will likely occur and allow enhanced cell killing. The data presented here demonstrate that human ovarian SKOV3 cells exhibit the bystander effect and that other subsets of ovarian cells can be selectively sensitized to the effects of GCV by transduction with a recombinant adenovirus expressing HSV-TK. Thus, at least a subset of ovarian carcinoma cells exhibit the cellular requirements necessary for bystander-mediated cell killing.

The components required to allow the bystander effect have not been completely defined; however, certain aspects of the mechanism have been established. For instance, Bi et al. (14) demonstrated that cell to cell contact is a crucial parameter. In addition, Freeman et al. (10) demonstrated that a soluble factor does not contribute to the phenotype required for the bystander effect. Both of these findings are consistent with the concept of transcellular transport of bioactive agents through junctional communication between cells. Interestingly, Wu et al. (34) have reported that the bystander effect may not always require syngeneic cells by demonstrating that cell killing occurred when cells from two different species were mixed. Initially, these authors utilized a murine cerebral melanoma line for stable transfection of a retrovirally expressed HSV-TK gene (C19-STK). They performed mixing experiments with syngeneic, HSV-TK-nonexpressing cells and demonstrated a very potent bystander effect. When the C19-STK cells were mixed with wild-type cells from three other sources (another murine melanoma line, the rat Walker 256 carcinoma, or the human A172 glioblastoma multiforme), bystander cell killing could also be demonstrated. This indicates that the bystander effect may not be a cell line-specific phenomenon, and provides another potential advantage to the molecular chemotherapy approach utilizing HSV-TK and GCV. We show here that human ovarian cells also exhibit the necessary biological properties for bystander-mediated cell killing. Thus, we further extend the tissue types that can be targeted by molecular chemotherapy approaches utilizing HSV-TK and GCV.

The majority of clinical protocols utilizing HSV-TK and GCV use retroviral producer cells for molecular chemotherapy of glioma. A smaller subset of protocols have recently been described which utilize a recombinant adenoviral vector expressing HSV-TK (35). In these instances, the targets are mesothelioma and advanced central nervous system malignancy. All of these approaches target tissues that are within confined body compartments such as the skull and the thoracic cavity. Compartmentalized tumor models are needed for the success of this approach since it is more likely that cell to cell contact would occur within the confines of a specific body compartment, thus allowing one to capitalize on the bystander effect. In this context, ovarian carcinoma would appear to be an ideal candidate for molecular chemotherapy. The peritoneal cavity is the sole site of tumor in >80% of women with this malignancy. In fact, the peritoneum could potentially be a more advantageous site for this type of intervention than the cranial vault. Some patients involved in the human glioma gene therapy protocols have exhibited side effects related to edema, which is of significance in the context of the small enclosed space of the cranium. The peritoneum is a much larger space in which such...
side effects would be minimized but should still be confining enough to allow for bystander killing of cells.

Freeman et al. (36) have recognized the advantages of this model system and have begun a clinical trial for ovarian carcinoma. This study utilizes an allogeneic, lethally irradiated ovarian carcinoma cell line stably expressing HSV-TK from a retroviral construct. In contrast to this, our strategy would rely on the direct delivery of the toxin-encoded gene to the peritoneum of women with ovarian carcinoma in the form of a recombinant adenoviral vector encoding HSV-TK. Although the results of this study demonstrate the utility of an adenoviral vector for delivery of a toxin gene to ovarian cancer cells, it is only the first step in developing a therapeutic strategy for effective delivery in the peritoneum of a human patient. We realize that there will likely be limitations to this strategy, one of which being the large surface area of the peritoneal cavity. Although this vector system is being utilized by a number of investigators in the context of the thoracic space, the central nervous system, and head and neck carcinoma in approved human clinical gene therapy trials (35), it may not be possible to ultimately utilize recombinant adenoviral vectors for in situ delivery in the human peritoneum. In this regard, an ongoing area of research in our laboratory is the development and use of conditionally replicative vectors to obtain even more efficient levels of tumor cell transduction (37, 38). These vectors would have the theoretical advantage of accomplishing genetic modification of tumor cells in the region of vector-transduced cells. Therefore, in a patient, it may be likely that another vector system will prove more efficacious or that the actual amount of virus needed to successfully transfect a majority of tumors would become feasible to accomplish. Despite certain limitations, our strategy would be based on in situ transduction of tumor, probably at a very high efficiency, and capitalizes on the ability to propagate, and concentrate, high-titer viral stocks of the HSV-TK expressing adenovirus. It is also likely that the adenovirus vector could infect the tumor vasculature (39), allowing for eradication of developing tumor neovascularity, providing another advantage to our system. Our strategy would rely on viral stocks and, therefore, would likely be less expensive and labor intensive given the lack of requisite cell culture work needed to derive administered vector reagents required in other systems. In summary, this work provides the rationale for the use of a recombinant adenoviral vector as a vehicle for efficient gene delivery in a molecular chemotherapy strategy for ovarian carcinoma.

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Adenoviral-mediated delivery of the herpes simplex virus thymidine kinase gene selectively sensitizes human ovarian carcinoma cells to ganciclovir.

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