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Treatment of Experimental Human Mesothelioma Using Adenovirus Transfer of the Herpes Simplex Thymidine Kinase Gene

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Objective

The authors demonstrate the ability of an adenovirus vector expressing the herpes simplex thymidine kinase (HSV*tk*) gene to treat human malignant mesothelioma growing within the peritoneal cavity of severe combined immunodeficient (SCID) mice.

Background Data

Introduction of the HSV*tk* gene into tumor cells renders them sensitive to the antiviral drug ganciclovir (GCV). This approach has been used previously to treat experimental brain tumors. Although malignant mesothelioma is refractory to current therapies, its localized nature and the accessibility of the pleural space make it a potential target for a similar type of *in vivo* gene therapy using adenovirus.

Methods

An adenovirus containing the HSV*tk* gene (Ad.RSV*tk*) was used to transduce mesothelioma cells *in vitro*. These cells were then injected into the flanks of SCID mice. Ad.RSV*tk* was also injected directly into the peritoneal cavity of SCID mice with established human mesothelioma tumors. Mice were subsequently treated for 7 days with GCV at a dose of 5 mg/kg.

Results

Mesothelioma cells transduced *in vitro* with Ad.RSV*tk* formed nodules when injected in the subcutaneous tissue. These tumors could be eliminated by the administration of GCV, even when as few as 10% of cells were transduced to express HSV*tk* (bystander effect). Administration of Ad.RSV*tk* into the peritoneal space of animals with established multifocal human mesothelioma followed by GCV therapy resulted in the eradication of macroscopic tumor in 90% of animals and microscopic tumor in 80% of animals when evaluated after 30 days. The median survival of animals treated with Ad.RSV*tk*/GCV was significantly longer than that of control animals treated with similar protocols.

Conclusion

These results indicate that an adenoviral vector containing the HSV*tk* gene is effective in treating established malignant mesothelioma in an *in vivo* setting and raise the possibility of using adenovirus transfer of HSV*tk* for clinical trials in mesothelioma and other localized tumors.

Malignant mesothelioma is a neoplasm of the mesothelial lining of the pleural or peritoneal cavity linked to prior exposure to asbestos. Although relatively rare, mesothelioma accounts for approximately 4000 deaths per year in the United States. Morbidity and mortality in mesothelioma primarily are the result of aggressive local spread rather than from distant metastases, as is characteristic of most malignancies. Unfortunately, mesothelioma is extremely resistant to aggressive multimodality therapies, with reported median survivals of only 18 to 24 months after diagnosis.[1,2] Although unresponsive to conventional treatments, this combination of extremely poor prognosis, lack of a clinically important metastatic component, and the fact that the tumor grows in the accessible potential space of the thoracic cavity make mesothelioma an attractive candidate for local somatic gene therapy.

Culver et al.[3] have reported success in treating localized brain tumors by injection of fibroblasts producing retroviruses engineered to contain the herpes simplex thymidine kinase (HSV*tk*)[1] gene. These studies and others have demonstrated that introduction of the HSV*tk* gene into mammalian cells renders them highly sensitive to subsequent treatment with the relatively nontoxic nucleoside analog ganciclovir (GCV).[4–9] One key feature of this system is a "bystander effect," in which tumor growth may be slowed, or tumor tissue eliminated, after GCV administration, even when only 10% to 15% of cells are transfected with HSV*tk*. This effect is thought to be caused by intercellular transfer of toxic GCV metabolites.[3–9]

To date, most of the reported studies using transfer of HSV*tk* have used recombinant retrovirus.[3,6–9] Although potentially effective, certain features of retrovirus-mediated gene transfer are not optimal for *in vivo* gene therapy. These include difficulties in producing and purifying high-titer viral stock, the fact that only actively dividing cells allow retroviral integration and transgene expression, and the possibility of insertion mutagenesis.[10] In addition, because recombinant retroviral supernatant may not reliably infect tissues, *in vivo,* "packaging cells" are required,[9] confounding interpretation of subsequent effects and raising potential safety issues.

For these reasons, we have explored the use of recombinant adenovirus to deliver the HSV*tk* gene to treat malignant mesothelioma.[11,12] In contrast to retroviruses, adenoviruses are very efficient in directly transducing tissues *in vivo,* can easily be produced in high titer, do not require cell division for transduction of cells, and have an established record of safety as vaccination material.[13] Although the duration of gene expression is relatively short with regard to treatment of genetic disorders,[14] this limitation may be less important when the goal of therapy is rapid eradication of tumor cells.

We have recently completed a series of studies that demonstrate that the delivery of HSV*tk* via adenovirus followed by the administration of GCV may be an effective therapeutic approach. These studies have shown that 1) a replication-deficient recombinant adenovirus carrying the *Escherichia coli lacZ* marker gene driven by a heterologous promoter effectively infected mesothelioma cells *in vitro*[11]; 2) introduction of recombinant adenovirus directly into the peritoneal cavity of severe combined immunodeficiency (SCID) mice with established human mesothelioma tumor nodules resulted in extensive gene transfer at the tumor surfaces and deeper within the tumor nodules[11]—these findings are similar to those recently reported by Brody et al.[15]; 3) recombinant adenovirus Ad.RSV*tk* successfully transferred the HSV*tk* gene into human mesothelioma cells *in vitro* and resulted in expression of enzyme as assessed by immunohistochemical staining[12] 4); infection with Ad.RSV*tk* rendered human mesothelioma cells sensitive to doses of GCV that were 2 to 4 log doses lower than that required to kill cells infected with control virus *in vitro*[12]; and 5) infection of mesothelioma cells *in vitro* with Ad.RSV*tk* was accompanied by a strong "bystander effect" with no diminution in the efficacy of GCV treatment until the ratio of infected:uninfected cells was less than 1:10.[12]

Based on these preliminary studies, experiments were conducted to determine if a recombinant adenovirus carrying the HSV*tk* gene could be used to treat tumors in animal models of human mesothelioma. The results demonstrate that an adenoviral vector containing the HSV*tk* gene is effective in treating an established, localized, malignancy in an *in vivo* setting and raise the possibility of using adenovirus transfer of HSV*tk* for the treat-

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ment of human tumors, such as malignant mesothelioma, that exert morbidity by growing within localized body cavities.

METHODS

Adenoviral Vectors

The production of recombinant replication-deficient adenoviral vectors has been described in detail elsewhere.[12,16,17] Briefly, the vectors were constructed from an adenovirus type 5 (Ad5) mutant that lacks most of viral sequence regions E1a and E1b and a portion of E3. By homologous recombination techniques, either the *E. coli lacZ* marker gene or the herpes simplex type I thymidine kinase gene driven by the Rous sarcoma virus 3′ promoter region were inserted into the viral genome. The resultant vectors were termed Ad.RSV*lacZ* and Ad.RSV*tk*., respectively. Viral stocks were propagated in 293 cells, and titers were quantified by 293 plaque assay or spectrophotometric density.

Cell Lines and Animal Models

Two cell lines derived from human mesothelioma, 1-45 and REN were used in this study. The characterization of these cells is described elsewhere.[12]

Two animal models of mesothelioma were developed in SCID mice, as described previously.[12] All protocols were approved by the animal use committees of the Wistar Institute and the University of Pennsylvania in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health No. 85-23, revised 1985).

Briefly, to study the growth of subcutaneous tumors, 2×10^7 cells from the 1-45 human malignant mesothelioma cell line were injected subcutaneously into the flanks of SCID mice. Tumor length and width were measured using calipers, and tumor volumes were calculated using the formula: tumor volume = $L \times W^2/2$.

To study a more clinically relevant model of mesothelioma, 4×10^7 cells from the REN cell line were suspended in 1 mL of RPMI medium and injected with a 23-gauge needle into the peritoneal cavities of 5-week old SCID mice. A peritoneal model was chosen rather than a pleural model because it is difficult to introduce cells into the pleural cavity of mice without causing death due to iatrogenic pneumothorax. Within 5 days after cell instillation, 1- to 2-mm macroscopic tumor nodules were obvious on peritoneal surfaces. By 30 days, large tumor nodules were present on the lesser omentum, adherent to the pancreas, in the small bowel mesentery, in the area of the porta hepatis, and in smaller scattered deposits on the diaphragm and lateral abdominal wall. Animals usually succumbed to bowel or portal obstruction within 5 to 6 weeks (median survival 35 days; Table 1). In contrast to other mesothelioma cell lines,[15,18] the cells used in these studies produced minimal amounts of ascites.

Experimental Protocols

Subcutaneous Nodule Experiments

To determine if the adenovirus HSV*tk* system would be effective in animal models after systemic GCV administration, confluent flasks of 1-45 human mesothelioma cells were treated with either Ad.RSV*tk* or Ad.RSV*lacZ* at a ratio of 100 particles per cell (2 plaque-forming units per cell) for 6 hours. At this ratio, virtually all cells were transduced.[12,13] The cells were washed and allowed to incubate overnight. After 24 hours, the cells were removed with trypsin/EDTA and injected into the flanks of SCID mice. For each animal, one side was injected with Ad.RSV*tk*-infected cells, and the contralateral flank was injected with either uninfected cells or cells infected with Ad.RSV*lacZ*. To study the bystander effect, some animals were injected with Ad.RSV*tk*-infected cells mixed with varying numbers of uninfected cells to achieve ratios of infected:uninfected cells of 1:1, 1:5, and 1:10. Six days after injection, when all tumors measured 4 to 6 mm in diameter, the animals were treated with intraperitoneal GCV (250 mg/kg/day) or saline daily for 7 days. Tumor growth was measured for the next 6 weeks.

Intraperitoneal Experiments

Four $\times 10^7$ REN cells were injected into the peritoneal cavities of 33 SCID mice. Five days later, when macroscopic tumor nodules of 1 to 2 mm were present, 3×10^{11} particles (6×10^9 plaque forming units) of virus, diluted in 0.5 mL of sterile saline, were injected into the peritoneal cavity using a 23-gauge needle. Experimental groups were injected with a control vector (Ad.RSV*lacZ*; 6 mice), the therapeutic gene construct (Ad.RSV*tk*; 19 mice), and diluent (sham-infected; 6 mice). Selected groups of animals were treated 2 days later with intraperitoneal GCV at a dose of 5 mg/day (250 mg/kg) or saline for 7 days. One month after initial tumor inoculation, animals underwent necropsies, and the extent of tumor was quantified.

To evaluate the effects of treatment on survival using the same protocol, 4×10^7 REN cells were injected again into the peritoneal cavities of 33 SCID mice. Five days later, animals received either control vector (Ad.RSV*lacZ*; 7 mice), the therapeutic gene construct (Ad.RSV*tk*; 14 mice), diluent (sham-infected; 7 mice), or no treatment (5 mice). Selected groups of animals were treated 2 days later with intraperitoneal GCV at a dose of 5 mg/day (250 mg/kg) or saline for 7 days. The animals were carefully observed and underwent necropsy as soon as possible after death.

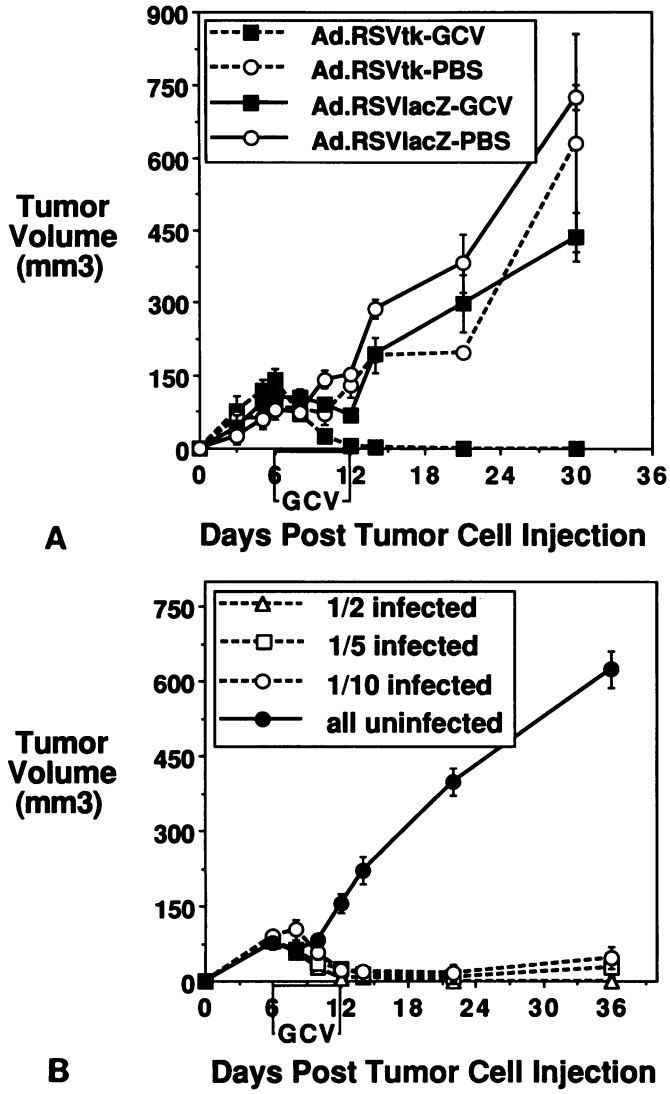

Figure 1. Ganciclovir-induced regression of subcutaneous human malignant mesothelioma tumors developing from cells transduced *in vitro* with Ad.RSV*tk*. (A) Animals received cells infected with Ad.RSV*tk in* one flank and Ad.RSV*lacZ* in the other. One group then received intraperitoneal GCV with the other receiving saline. Tumors arising from Ad.RSV*tk*-infected cells were eliminated in animals receiving GCV, whereas tumors arising from Ad.RSV*lacZ*-infected cells continued to grow (Error bars = standard error of the mean [SEM]). (B) Cells infected with Ad.RSV*tk* were mixed with uninfected cells at ratios of 1:1, 1:5, and 1:10 and injected subcutaneously in one flank. All animals received uninfected cells in the contralateral flank as a control. After GCV treatment, all tumors developing from mixtures of infected and uninfected cells exhibited complete regression. Tumors arising from the uninfected cells continued to grow (Error bars = SEM).

Histochemistry and Evaluation of *LacZ* Gene Expression

To detect microscopic tumor growth, portions of tumor and intra-abdominal organs were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. As a more sensitive test for deposits of human tumor, other portions of tumor and all intra-abdominal organs were removed and snap-frozen in OCT-embedding media (Miles Inc. Elkhart, IN) at −70 C. Frozen sections were cut and stained with an antibody (W632-PelFreeze Biologicals, Rogers, AR) against the human lymphocyte antigen (HLA)-1 shared determinant using the Vectastain Elite kit (Vector, Burlingame, CA).[13]

To detect areas of *lacZ* expression, frozen tumor sections were cut, placed on glass microscope slides, and stained using the X-gal protocol described previously.[12] Before mounting, the sections were counterstained with hematoxylin for 30 seconds. Sections then were evaluated for *lacZ* gene expression by light microscopy.

Statistics

Survival data among all groups were compared using analysis of variance. Comparison of treatment survival data to the combined control group was performed by use of a log-rank test.

RESULTS

Systemic Administration of Ganciclovir Induces Tumor Regression of Mesothelioma Cells Transduced with Ad.RSV*tk In Vitro*

To test if the adenovirus HSV*tk* system would be effective in animal models after systemic GCV administration, we infected 1-45 human mesothelioma cells *in vitro* with Ad.RSV*tk* or Ad.RSV*lacZ* and injected these cells subcutaneously into the flanks of SCID mice. Six days later, when all tumors measured 4 to 6 mm in diameter, the animals were treated with intraperitoneal GCV or saline.

As shown in Figure 1A, a 7-day course of GCV rapidly eliminated all Ad.RSV*tk*-infected tumors, whereas tumors composed of cells treated with Ad.RSV*lacZ* continued to grow to large size. No inhibition of growth was seen when animals containing tumors composed of cells transduced with Ad.RSV*tk* were treated with saline. There was no regrowth of the Ad.RSV*tk*-infected tumors after more than 6 weeks of observation. In addition, there was no evidence of injury to normal subcutaneous tissues. Microscopic examination of the tumor site in the Ad.RSV*tk*/GCV animals after 6 weeks showed no evidence of microscopic disease.

A similar degree of tumor regression was seen in these models at a lower dose of GCV (10 mg/kg/day), a dose that approximates that used clinically to treat viral infection[19] (data not shown).

A Strong Bystander Effect Exists *In Vivo*

To determine if a bystander effect occurred in tumors growing *in vivo*, cells were infected with Ad.RSV*tk in vitro* and mixed with various concentrations of nontransduced cells 24 hours after infection (under these conditions, no contamination of cells via residual adenovirus could occur). The cell mixtures were injected into the flanks of SCID mice and allowed to grow to measurable size. The animals were then treated with intraperitoneal GCV for 7 days. As shown in Figure 1B, all tumors containing Ad.RSV*tk*-transduced cells demonstrated equivalent regression, even those derived from mixtures of infected:uninfected cells at ratios 1:1, 1:5, and 1:10. These experiments show that a strong bystander effect is operative in adenovirus-transduced solid tumors *in vivo*.

Ad.RSV*tk*/GCV Treatment Induces Regression of Established Intraperitoneal Mesothelioma Tumor at 30 Days

Based on these results, we designed experiments to evaluate *in vivo* transduction of pre-existing mesothelioma in a model that approximated human disease. Four groups of SCID mice were injected intraperitoneally with 4×10^7 REN tumor cells. Five days later, two animals were killed to confirm the presence of tumor. Multiple small macroscopic (1–2 mm) tumor nodules were present throughout the peritoneum and were especially prominent in the mesentery (Fig. 2A). At this time point, animals were injected intraperitoneally with 3×10^{11} particles of Ad.RSV*lacZ* virus, Ad.RSV*tk* virus, or saline (sham infection control). Two days later, groups of animals were treated intraperitoneally with either saline or with GCV (Table 1).

Efficient gene transfer was confirmed by necropsy of one of the animals injected with Ad.RSV*lacZ* 2 days after viral injection. As previously observed with an adenovirus vector containing the *lacZ* gene driven by a cytomegalovirus promoter,[12] the Ad.RSV*lacZ* vector also readily infected tumors and showed β-galactosidase activity on the surface of tumor nodules, as well as much deeper within the tumor mass (Fig. 2B).

Two weeks after the intraperitoneal GCV or saline treatments were completed (i.e., 28 days after the initial tumor cell inoculations), the animals were killed and carefully examined for the presence of gross and microscopic disease (Table 1). There was no grossly detectable disease in nine of the ten mice in the Ad.RSV*tk*/GCV group (Figs. 3B, 3D, and 3F) The one exception was an animal with a solitary 3-mm macroscopic tumor nodule. In contrast, all but one of the 20 animals in the other groups had an extensive burden of intraperitoneal tumor (Table 1 and Figs. 1 and 3). Scattered tumor nodules of up to 1.5 cm in diameter were noted in these animals, many of which were partially obstructing the bowel or biliary tree (Figs. 3A, 3C, and 3E). In addition to macroscopic disease (found primarily in the upper abdomen, pancreatic mesentery, region of the porta hepatis, and diaphragm), all control animals exhibited microscopic tumor uniformly throughout the upper abdomen (Figs. 2D–2F).

The intra-abdominal organs and tissues removed from animals in the HSV*tk*/GCV group were examined for tumor microscopically by conventional H&E staining of paraffin sections and by a more sensitive staining technique using an antibody against the human HLA-class 1 shared determinant. Examination of stained sections (Table 1 and Figs. 2D–2F) showed evidence of tumor in only two of the ten mice. In one animal (which also had the macroscopic nodule), the disease was detected in one small area in the porta hepatis. In the other mouse, there was one small nest of tumor cells (smaller than those shown in Fig. 2F) adherent to the surface of the small intestine, detectable only by antihuman HLA immunostaining.

Animals with Established Intraperitoneal Mesothelioma Exhibit Significant Survival Benefit When Treated with Ad.RSV*tk*/GCV

To evaluate longer-term benefit from HSV*tk*/GCV treatment of SCID mice with established intraperitoneal human mesothelioma, and to assess tumor regrowth, a survival experiment was performed. Five groups were studied which included animals receiving no treatment, as well as those that received no virus (sham)/GCV, Ad.RSV*tk*/GCV, Ad.RSV*tk*/no GCV, and Ad.RSV*lacZ*/GCV. After treatment, survival of each group of mice was observed closely. Animals underwent careful necropsy as soon as possible after death.

The mean and median survival times for all animals are reported in Table 2. Median survival of control animals ranged from 35 to 51 days; however, Ad.RSV*tk*/GCV-treated animals exhibited a median survival of 76.5 days. Because the survival data did not significantly differ among control groups, the data from all controls were combined for further analysis. When the survival data of treatment animals were compared with control animals, a significant survival advantage was noted (Fig. 4, $p < 0.005$, log rank). Necropsy of animals in all control groups revealed large intraperitoneal tumor burdens consisting of 1) large pancreatic mesentery tumors, 2) multiple small bowel mesenteric nodules, and 3) extensive portal tumor. Death of these animals appears to have been caused by small bowel or portal tumor mass obstruction. Additionally, several of the control animals exhibited diaphragmatic and retroperitoneal tumor growth. On autospy, no treatment animal appeared to have died a direct tumor-related death. Necropsy of

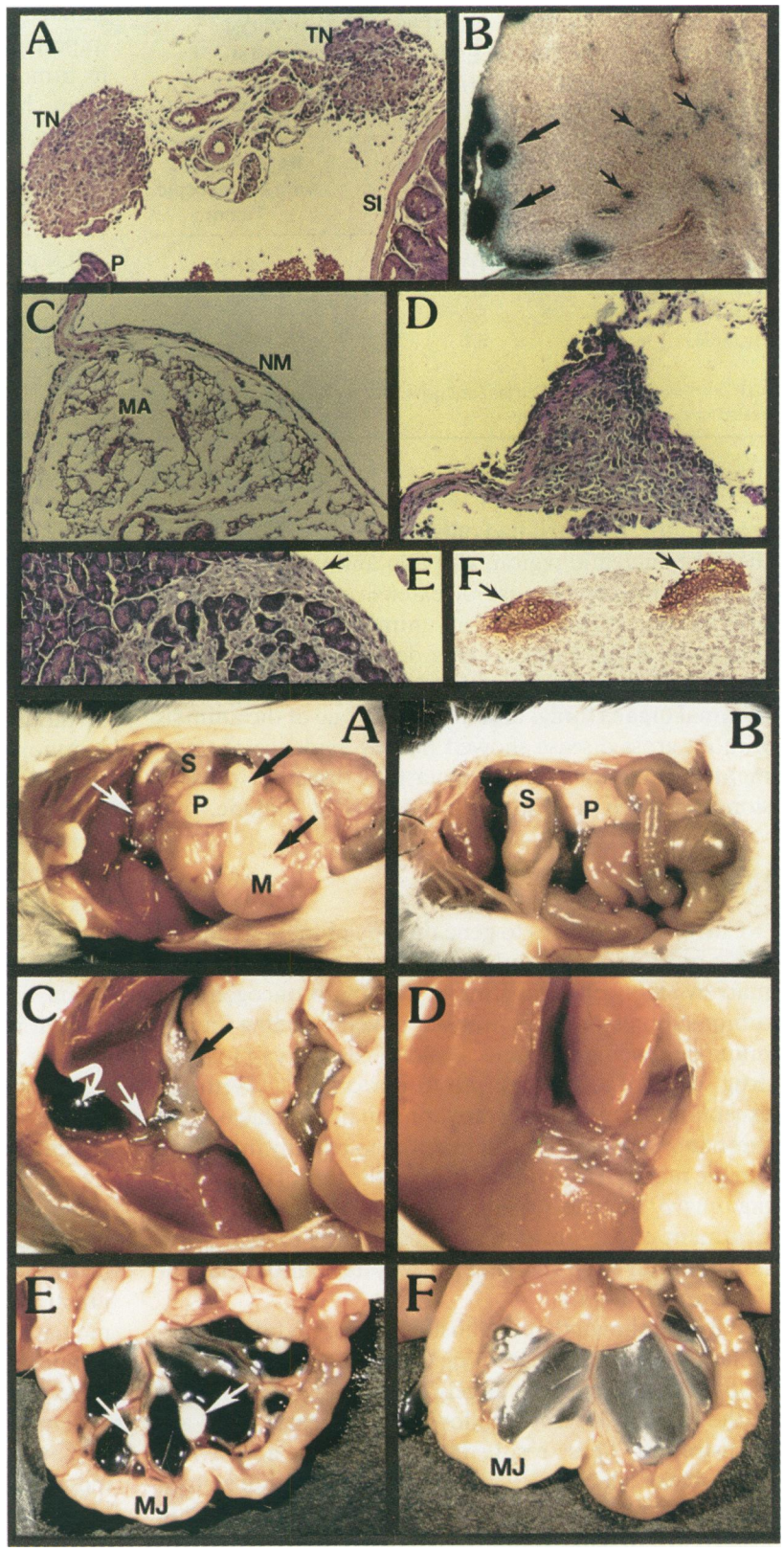

Figure 2. Microscopic evaluation of human mesothelioma tumor growing in SCID mice. (A) Appearance (H&E staining) of tumor nodules (TN) growing within the mesentery adjacent to the small intestine (SI) and pancreas (P) within the peritoneum 5 days after tumor cell injection (200x). (B) Frozen sections of tumor in mesentery (7 days after tumor cell injection) stained with X-gal to identify the transduced β-galactosidase gene. Blue areas mark expression of *lacZ* in tumor cells at the surface of the tumor (large arrows) and deep within the tumor (small arrows; 100×). (C) Histologic appearance of normal mesothelium (NM) and mesenteric adipose (MA) tissue 30 days after mesothelioma cell injection and after treatment with Ad.RSV*tk*/GCV, demonstrating no microscopic tumor or mesothelial damage (200×). (D) Corresponding region of mesentery of animal treated with Ad.RSV*lacZ* shows clear tumor involvement (200×). (E) Histologic appearance of pancreas in an animal 30 days after mesothelioma cell injection after treatment with Ad.RSV.*lacZ* shows extensive tumor infiltration (arrow). Corresponding sections from animals treated with Ad.RSV*tk* showed no evidence of tumor. (F) Appearance of a pancreatic mesothelioma micrometastasis (arrows) detected by immunohistochemical staining of frozen sections (hematoxylin counterstain) with an antibody against the human HLA-1 shared determinant (200×).

Figure 3. Effect of Ad.RSV*tk*/ganciclovir treatment on intraperitoneal growth of human mesothelioma in SCID mice. (A) Photomicrograph of upper abdominal organs of an animal treated with Ad.RSV*lacZ*/GCV. Tumor nodules (arrows) are noted in the upper abdomen near the stomach (S), pancreatic mesentery (P), porta hepatis (white arrow), and in the mesentery (M) of the small bowel. (B) An animal treated with Ad.RSV*tk*/GCV shows complete absence of tumor in these same regions. (C) Higher power view of the hepatic portal region in an animal treated with Ad.RSV*lacZ*/GCV shows tumor (arrows) in the porta hepatis (black arrow) with common bile duct obstruction (straight white arrow) and enlargement of the gallbladder (curved white arrow). (D) Similar view of an animal treated with Ad.RSV*tk*/GCV illustrates lack of tumor. (E) Higher power view of the jejunal mesentery (MJ) in an animal treated with Ad.RSV*lacZ*/GCV shows numerous tumor nodules (arrows). (F) A comparable view in an animal treated with Ad.RSV*tk*/GCV with no visible tumor.

Ad.RSV*tk*/GCV-treated animals was most remarkable for extensive benign-appearing intra-abdominal adhesions. All animals necropsied had obstruction of either the large or small intestine with these adhesions, but no obstructing tumor nodules were noted. Of the animals that were necropsied to date, two had no evidence of

Table 1. EFFECT OF GENE THERAPY ON THE GROWTH OF ESTABLISHED HUMAN MESOTHELIOMA IN THE PERITONEAL CAVITY OF SCID MICE

Treatment Group	No. of Animals with Gross Tumor	No. of Animals with Microscopic Tumor
Ad.RSV*tk*/GCV	1/10	2/10
Ad.RSV*tk*/saline	9/9	9/9
Ad.RSV*lacZ*IGCV	5/5	5/5
Sham/GCV	5/6	5/6

Ad.RSV*tk* = adenovirus containing the *LacZ* gene; GCV = ganciclovir; Sham = mice treated intraperitoneally with saline.

gross tumor, two each had only one 2 × 2-mm nodule in the peripancreatic region, and two animals did not undergo necropsies because of extensive tissue autolysis. Microscopic evaluation of all animals was not possible because of tissue autolysis after death and preceding autopsy; however, microscopic examination of extra-abdominal organs (lung, heart, brain) of one of the animals without gross disease showed neither evidence of treatment regimen toxicity nor metastatic disease. No adhesions were seen in animals treated with GCV alone.

DISCUSSION

Current approaches to gene therapy of malignancy include transfer of chemotherapy resistance genes to bone marrow stem cells, targeting of dominant oncogenes or tumor suppressor genes, transfer of genes coding for immune-enhancing substances, and the delivery of toxic or drug susceptibility genes into tumor cells.[20–23] With the exception of immunotherapy, one major obstacle to all these approaches will be an inability to reliably introduce genetic material into most, or all, of the tumor cells growing *in vivo*. For this reason, successful approaches to the treatment of solid tumors will require the careful selection of tumor type, vector delivery system, and therapeutic gene paradigm.

We have shown that established tumor nodules of malignant mesothelioma growing within the peritoneal cavity of SCID mice can be largely eradicated by instillation of the Ad.RSV*tk* vector followed by systemic GCV administration. These results indicate that the adenovirus is capable of effective HSV*tk* gene delivery within a physiologically relevant milieu and are able to penetrate deeply within solid tumor after topical administration. Brody et al.[15] also have shown efficient delivery of transgene via adenovirus into mesothelioma tumor growing within the peritoneal cavity of immunodeficient mice. We theorize that the adenoviral particles may have gained access to deeper tumor areas via morphologically imperfect tumor capillaries. Newly formed microvessels in tumor tissue often lack a complete pericyte investment, have poorly-formed or absent basement membranes, and have larger-than-normal endothelial fenestrations.[24] Adenovirus could have entered these microvessels at the tumor surface, then exited the vessels in deeper regions to infect tumor cells.

It is pertinent to address the issue of potential viral dissemination in this experimental model. In previous studies using transfer of the *lacZ* gene in this intraperitoneal mesothelioma model, we did not find evidence of disseminated gene transfer because the normal mesothelium appeared to form a barrier.[11] The only exception to this observation was infrequent β-galactosidase positive-staining hepatic cells. In this study, careful review of hepatic histology in HSV*tk*/GCV-treated animals at 30 days did not reveal evidence of hepatocyte damage. These data are consistent with those of Setoguchi et al., who, after intraperitoneal administration of recombinant adenovirus carrying the α1-antitrypsin gene, were not able to detect transgene in other organs, even by sensitive reverse transcription-polymerase chain reaction methods.[25] Our preliminary studies using reverse transcription-polymerase chain reaction to detect the HSV*tk* gene in other organs have confirmed this observation. Although significant benign intraperitoneal adhesions formed in the survival experiment, the normal mesothelium appeared intact at 30 days (Fig. 2C), suggesting that this tissue is not irreversibly damaged. The development of adhesions should not pose a significant problem in the treatment of intrathoracic malignancy, such as mesothelioma, but may impact on the potential use of this system to treat intraperitoneal malignancy such as ovarian carcinoma.

This study also demonstrates that not all tumor cells need to be infected with the Ad.RSV*tk* vector to effect tumor eradication, thus illustrating the power of the bystander effect. Explanations for the bystander effect have included continued viral infection *in situ*,[3] transfer of an integrated retroviral HSV*tk* gene during mitosis, transfer of the toxic purine analog GCV-triphosphate via gap junctions or apoptotic vesicles,[6,9] or primarily immune-mediated effects.[8] Although the mechanisms of the bystander effect were not specifically examined, our results clearly argue against retroviral-specific or T cell-mediated immune mechanisms. Of course, an immune component to the bystander effect via natural killer cells, macrophages, eosinophils, and neutrophils still is possible because SCID mice are not deficient in these cell types. Regardless of mechanism, however, the presence of a strong bystander effect using adenovirus *in vivo* is particularly important in the treatment of solid tumors because the infection rate of a mass of tumor cells is not likely to be high.

Table 2. SURVIVAL AFTER TREATMENT FOR ESTABLISHED HUMAN MESOTHELIOMA

Treatment Group	Mean Survival (Days ± S.E.M.)	Median Survival (Days)
No treatment (n = 5)	35.8 ± 1.5	35
Ad.RSV*tk*/saline (n = 7)	39.9 ± 2.7	37
Ad.RSV*lacZ*/GCV (n = 7)	48.6 ± 4.5	51
Sham/GCV (n = 7)	50.7 ± 14.3	39
Ad.RSV*tk*/GCV (n = 7)	90.8 ± 15.8	76.5

Ad.RSV*tk* = adenovirus containing the HSV*tk* gene; Ad.RSV*lacZ* = adenovirus containing the *LacZ* gene.

An interesting issue that will likely have an impact on the clinical utility of the Ad.RSV*tk*/GCV approach is the immune response generated against recombinant adenovirus in immunocompetent organisms. Transduction of cells with recombinant adenovirus results in the production of both neutralizing antibodies and cytotoxic T lymphocytes[14]; however, it is difficult to predict how these responses will affect therapeutic efficacy. It is possible that the generation of cytotoxic T lymphocytes against adenovirally transduced tumor cells actually may enhance tumor destruction.[26] On the other hand, too rapid a destruction of transduced tumor cells will limit the expression of the HSV*tk* gene and diminish the resultant bystander effect. Comparison of Ad.RSV*tk* therapy in animals in which the same rat mesothelioma cell line can be grown in syngeneic immunocompetent Fischer rats and in immunodeficient nu/nu rats currently are underway and will help answer this question. The generation of neutralizing antibodies against adenovirus also may provide a potential problem and limit the possibility of repeated administration of vector. Clearly, a better understanding of the immune response to recombinant adenovirus will be helpful in designing useful clinical approaches (i.e., immunosuppresive therapy), and guiding development of new vector technology (i.e., less immunogenic adenoviruses, adenoviruses of different serotypes, or alternative vectors such as adeno-associated virus).

In addition to issues of immune response, there are a number of additional questions raised by this study. Selective destruction of tumor *versus* normal tissues is one such issue. Because adenoviruses have the ability to infect nondividing cells, they are not dependent on the tumor cells being in active cell cycle during the period of infection. This is important because only a minority of cells within a tumor mass may be dividing at any one point in time. Selective destruction of tumor cells apparently is achieved by the fact that GCV preferentially destroys cells undergoing division.[27] The recent report of Chen et al., showing that direct intratumoral injection of an adenovirus vector carrying an HSV*tk* gene in gliomas caused tumor regression, supports the potential use of this system in the treatment of cancer.[28] Another potential limitation of this study is that the size of the intraperitoneal tumors was relatively small, although clearly macroscopically visible. Although this animal model does not simulate the massive tumor burden of a patient with advanced mesothelioma, it suggests the possibility of using gene therapy as adjuvant to a surgical debulking procedure. We currently are evaluating the possibility of single and multiple administrations of Ad.RSV*tk*/GCV for the treatment of much bulkier disease. Currently, the optimal dose of virus for use in *in vivo* delivery also is unclear. For reasons not yet well understood, adenoviral transduction of cells *in vitro* usually is much more efficient than when the same virus is administered *in vivo*.[29] In this report, we used a dose of virus in the mouse that would be the equivalent of 2×10^{12} plaque-forming units in a 70 kg human subject. Administration of this dose of virus is feasible in a clinical setting, but would be larger than those used to date in trials for the treatment of cystic fibrosis. Preliminary dose response experiments in the SCID mouse model have shown significant antitumor effects at doses of virus 100-fold lower than those used in these studies. Finally, although we saw no gross evidence of local or systemic damage from this treatment, more careful analysis of the toxicity of this therapy is warranted.

This study demonstrates that recombinant adenovirus transfer of HSV*tk*, followed by systemic administration of GCV, can effectively treat established intracavitary tumor *in vivo*. In conjunction with the only other published report describing recombinant adenovirus-mediated *in vivo* tumor treatment,[28] these results raise the possibility of using this system for the treatment of human

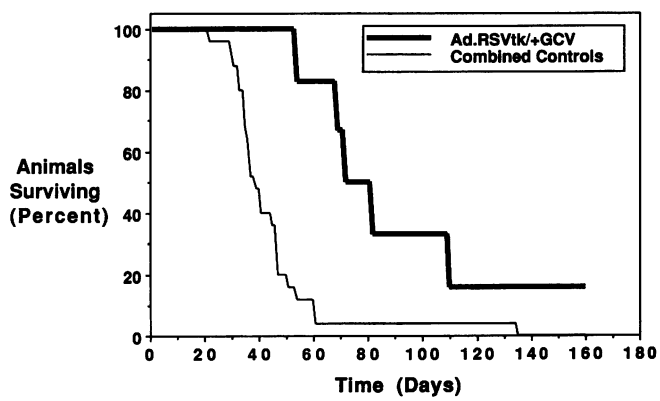

Figure 4. Survival benefit after treatment of intraperitoneal mesothelioma with Ad.RSV*tk*/GCV. Survival curves comparing SCID mice treated with Ad.RSV*tk*/GCV (heavy line) to all control groups combined (thin line). A significant treatment advantage was noted in Ad.RSV*tk*/GCV-treated animals ($p < 0.005$, log rank).

neoplasms that exert morbidity by growing within defined anatomic spaces. In addition to malignant mesothelioma, other examples include carcinoma of the ovary and bladder. Palliative treatment of metastatic disease, such as leptomeningeal carcinomatosis, malignant ascites, and pleural effusion arising from a variety of malignancies, are also potential therapeutic goals.

Author's Note

Two other studies recently have been published.[30,31]

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