Long-term Follow-up of Patients with Malignant Pleural Mesothelioma Receiving High-Dose Adenovirus Herpes Simplex Thymidine Kinase/Ganciclovir Suicide Gene Therapy

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Abstract Purpose: Delineation of the long-term follow-up data on a series of patients with malignant mesothelioma, who received a single intrapleural dose of a nonreplicative adenoviral (Ad) vector encoding the herpes simplex virus thymidine kinase “suicide gene” (Ad.HSVtk) in combination with systemic ganciclovir.

Experimental Design: This report focuses on the 21 patients receiving “high-dose” therapy, defined by an intrapleural dose of vector (≥1.6 × 1013 viral particles), where transgene-encoded tk protein was reliably identified on immunohistochemical staining. In 13 patients, the vector was deleted in the E1 and E3 regions of the Ad; in the other eight patients, the vector had deletions in the Ad genes E1 and E4. Safety, immunologic responses, transgene expression, and clinical responses were evaluated.

Results: Both the E1/E3-deleted vector and the E1/E4-deleted vector were well tolerated and safe, although production of the E1/E4 vector was more difficult. Posttreatment antibody responses against the tumors were consistently seen. Interestingly, we observed a number of clinical responses in our patients, including two long-term (>6.5 year) survivors, both of whom were treated with the E1/E4-deleted vector.

Conclusions: Intrapleural Ad.HSVtk/ganciclovir is safe and well tolerated in mesothelioma patients and resulted in long-term durable responses in two patients. Given the limited amount of gene transfer observed, we postulate that Ad.HSVtk may have been effective due to induction of antitumor immune responses. We hypothesize that approaches aiming to augment the immune effects of Ad gene transfer (i.e., with the use of cytokines) may lead to increased numbers of therapeutic responses in otherwise untreatable pleural malignancies.

One of the most widely studied forms of cancer gene therapy, “suicide gene” transfer, involves the delivery of transgenes encoding enzymes that metabolize prodrugs into toxic metabolites capable of killing tumor cells (1, 2). The most commonly used suicide gene in human clinical trials has been the herpes simplex thymidine kinase (HSVtk) gene, whose protein product is an enzyme that can convert the nontoxic, clinically used, antiviral drug ganciclovir into a highly cytotoxic phosphorylated form (1, 2). One attractive feature of the HSVtk/ganciclovir system is the presence of a “bystander effect,” where tumor cell killing is elicited in a sizable proportion of neighboring, nontransfected, cells due to intracellular transfer of phosphorylated ganciclovir (3, 4) and induction of antitumor immune responses (5, 6). Demonstration of therapeutic efficacy in rat models of brain tumors using retrovirally transduced “producer cells” (7–9) and induction of antitumor immune responses (5, 6), as well as other animal studies showing proof of principle, culminated in the conduct of several clinical trials in patients with refractory gliomas. Phase I and II human clinical trials confirmed the safety of this gene therapy approach, with some evidence of efficacy, leading Novartis to sponsor one of the few randomized phase III trials ever conducted in the field of gene therapy (10). Unfortunately, this trial rather definitively showed no clinical benefit to the injection of retroviral-HSVtk producer cells in malignant gliomas.
Subsequent to the initial publication of the preclinical results using retrovirally mediated HSVtk/ganciclovir gene therapy, our group and others developed recombinant, replication-incompetent adenoviruses (Ad) expressing HSVtk and showed evidence of \textit{in vitro} and \textit{in vivo} efficacy in many different animal models of malignancy, including mesothelioma (11–14). Phase I trials have subsequently been conducted using Ad.HSVtk in a number of localized tumors, including ovarian carcinoma, brain tumors, and prostate cancer (15–21). All of these trials have shown that the approach is relatively safe, but efficacy has been minimal.

Our group has pursued this approach for the treatment of malignant pleural mesothelioma (MPM) for a number of reasons. First, the median survival for patients with mesothelioma from time of diagnosis ranges between 1 and 2 years, depending on comorbid disease, stage at presentation, and histologic subtype (22–24). Long-term survival (>5 years) with any treatment modality is exceedingly rare in MPM (25). Even the best combination chemotherapy using cisplatin and a new multitargeted antifolate agent, pemtrexed (Alimta), has been shown to improve the median survival of mesothelioma patients by only a few months (26). The best option for long-term survival in MPM involves aggressive surgical approaches (i.e., extrapleural pneumonectomy) coupled with radiation treatment and preoperative or postoperative chemotherapy, primarily for a small subgroup of patients with favorable histology and limited stage disease (27). Second, MPM’s location within the thoracic cavity makes the tumor uniquely accessible, facilitating directed administration of novel agents and subsequent analysis of treatment effects. Third, local persistence or recurrence of disease rather than the development of widespread distant metastases is responsible for the majority of the morbidity and mortality associated with this neoplasm. Eradication of local disease conceivably could lead to significant improvement in palliation or survival.

Accordingly, we conducted two prior phase I trials using Ad.HSVtk and published our initial results relating to safety, gene transfer, and immunologic response (28, 29). In the initial study, 21 patients were treated with increasing doses of the “first-generation” Ad.HSVtk vector, H5.010RSVtk carrying a partial deletion in the E1 early viral gene region (E1A), as well as a complete deletion of the E3 gene. E1A is an Ad gene necessary for viral replication; its deletion renders recombinant Ad vectors, such as H5.010RSVtk replication incompetent. In its place, we inserted the herpes simplex virus thymidine kinase gene (HSVtk) driven by a constitutive Rous sarcoma virus (RSV) promoter. In addition, as part of the initial E1/E3-deleted vector phase I studies, we conducted a separate pilot study in five patients investigating the use of systemic corticosteroids to mitigate antivector immune responses (30).

These trials showed that intrapleural delivery of Ad.HSVtk was safe and well tolerated, and no maximally tolerated dose was reached (28). Anti-Ad humoral and cellular immune responses were generated (29). An important feature of these initial phase I trials were post-vector delivery thoracoscopic biopsies obtained to assess for evidence of HSVtk gene transfer. Examination of these biopsies for HSVtk protein expression revealed the presence of intratumoral transgene in all patients who received Ad.HSVtk at or above a dose level of $1.6 \times 10^{13}$ viral particles. However, even when present, transgene expression proved relatively superficial (maximal depth of penetration: 50-100 cell layers).

To minimize the risk of vector contamination with replication-competent Ad, we initiated a phase I clinical trial with a modified Ad vector, H5.001RSVtk, containing deletions in the E1 and E4 early viral genes (with preservation of the E3 region) in June 1998 (UPPC 5597). Again, the HSVtk cassette was inserted in the E1A region driven by the RSV promoter. E1/ E4-deleted Ad vectors offer putative advantages over first generation vectors by virtue of diminished cytopathic effects and reduced cellular immune responses. (31, 32). In addition, because two replication-necessary genes are deleted, homologous recombination is unlikely to produce replication competent Ad in the vector production process.

In this article, we will describe the results of the eight patients treated using this modified E1/E4-deleted vector. In addition, we will also provide the long-term follow-up data on the 11 patients treated in our first trials with “high-dose” E1/E3-deleted Ad.HSVtk (defined as $\geq 1.6 \times 10^{13}$ viral particles, the dose where HSVtk protein expression was detected in all patients).

**Patients and Methods**

**Gene transfer vectors**

All vectors used in these studies were produced at the University of Pennsylvania Medical Center (Philadelphia, PA) under Good Manufacturing Practices conditions. The vector used in protocols 1 and 2 was an E1/E3-deleted, replication-incompetent serotype 5 Ad vector (H5.010RSVtk) and has been described in detail previously (28). The Ad vector (H5.001RSVtk) used in protocols 3 and 4 has also been described in detail elsewhere (32).

**Protocols**

All four protocols were phase 1 trials of intrapleural Ad-based gene transfer of the HSVtk gene followed by systemic administration of ganciclovir (see Table 1). Patients were eligible for these studies based upon (a) a pathologically confirmed diagnosis of malignant pleural mesothelioma; (b) an Eastern Cooperative Oncology Group performance status of 0, 1, or 2; and (c) an accessible pleural space for instillation of vector. Exclusion criteria included prior surgical resection, successful pleurodesis, recent chemotherapy or radiotherapy, or the presence of significant cardiac/hepatic/renal disease. All patients signed a detailed informed consent form. The study protocols and consent forms were approved by (a) the Institutional Review Board and the Biosafety Committee of the University of Pennsylvania Medical Center, (b) the Recombinant DNA Advisory Review Committee of the NIH, and (c) the Food and Drug Administration.

**Protocol 1 (initial E1/E3-deleted Ad.HSVtk/ganciclovir trial): patients 14 to 18 and 24 to 26.** This protocol has been previously described in detail (28, 33). In brief, on day 1 of the study, patients underwent videothoracoscopy for tissue acquisition, confirmation of diagnosis, and placement of a chest tube. They were then admitted to the General Clinical Research Center (GCRC) of the University of Pennsylvania Medical Center. For patients 16 to 18 and 24 to 26, a thoracostomy tube was inserted at the bedside without obtaining pretreatment biopsies. On day 2, the H5.010RSVtk viral vector, diluted in 50 to 100 mL normal saline, was instilled via the chest tube. Three days after vector instillation (on day 5), a videothoracoscopy was done for tumor specimen acquisition. The following morning (day 6), i.v. ganciclovir was initiated at 5 mg/kg twice daily (bid) for 14 days. The patients were then discharged from the GCRC for outpatient follow-up that included a chest computed tomography scan done on study day 30 and at 3-month intervals thereafter.
Patients 14 and 15 received $1.6 \times 10^{13}$ viral particles of H5.010RSVtk. Patients 16 to 18 also received $1.6 \times 10^{13}$ viral particles but did not undergo an initial thoracoscopy, as per an Institutional Review Board–approved study amendment. Patients 24 to 26 received $5 \times 10^{13}$ particles. The specific number of patients at a given dose level was a function of toxicity, discussions with the Food and Drug Administration, and (in one case) because of a protocol deviation.

Protocol 2 (E1/E3-deleted Ad.HSVtk/ganciclovir/steroid trial): patients 19 to 23. This protocol has been previously described in detail (30). After admission to the GCRC, patients underwent placement of a thoracostomy tube at the bedside. Intravenous corticosteroid administration (60 mg methylprednisolone) began on the night of admission and continued every 6 hours for a total of 12 doses. On day 2, $1.6 \times 10^{13}$ viral particles of the H5.010RSVtk viral vector, diluted in 100 mL of normal saline, were instilled into the pleural cavity via the thoracostomy tube. Three days later (on day 5), a videothoracoscopy was done for tumor specimen acquisition. The following morning (day 6), i.v. ganciclovir was initiated at 5 mg/kg bid for 14 days. Blood and pleural fluid samples were drawn periodically for analysis of the anti-Ad and anti-transgene immune responses. After the 14 days, patients were discharged from the GCRC for outpatient follow-up.

Protocol 3 (E1/E4-deleted Ad.HSVtk/ganciclovir trial): patients 27 to 31. As described above, an additional phase I dose escalation trial was designed to test the safety and efficacy of intrapleural E1/E4-deleted Ad.HSVtk vector (H5.001RSVtk; UPCC 5597) and systemic ganciclovir in patients with MPM. On day 1 of the study, patients 27 to 31 were admitted to the GCRC and then underwent placement of a thoracostomy tube at the bedside. On day 2, the H5.001RSVtk viral vector, diluted in 50 to 100 mL normal saline, was instilled via the chest tube. The first two

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UPCC 5597

| Protocol 3 | 27 | $1.5 \times 10^{13}$ | III | 1 | B, C | 7.0 | PD |
| Protocol 3 | 28 | $1.5 \times 10^{13}$ | III | 0 | E, C, R | 12.5 | PD |
| Protocol 3 | 29 | $5 \times 10^{13}$ | I | 0 | E | 80 | Alive |
| Protocol 3 | 30 | $5 \times 10^{13}$ | I | 0 | E | 79.5 | Alive |
| Protocol 3 | 31 | $5 \times 10^{13}$ | II | 1 | E, C | 9.0 | PD |

UPCC 5598

| Protocol 4 | 101 | $3 \times 10^{13}$ | II | 0 | E, C | 18.5 | PD/PE |
| Protocol 4 | 102 | $3 \times 10^{13}$ | I | 0 | E | 10.0 | PD |
| Protocol 4 | 103 | $3 \times 10^{13}$ | II | 0 | E, C, R | 28.0 | PD/PE |

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; E, epithelioid; B, biphasic; S, sarcomatoid; PD, progressive disease; PE, pulmonary embolism; C, chemotherapy; R, radiation therapy; E, experimental therapy; S, surgical debulking; P, photodynamic therapy.
patients received an intrapleural vector dose of $1.5 \times 10^{13}$ viral particles; the next three patients received $5.0 \times 10^{12}$ viral particles (Table 1). Three days after vector instillation (on day 5), a videothoracoscopy was done for tumor specimen acquisition. On day 6, i.v. ganciclovir was initiated at 5 mg/kg i.v. bid for 14 days. Patients were then discharged for outpatient follow-up that included a chest computed tomography scan done on study day 80 and at 3-month intervals thereafter. Patients without measurable tumor on baseline computed tomography scan also had pre- and post-HSVtk/ganciclovir protocol 18-fluorodeoxyglucose positron emission tomography (PET) scanning to assess for changes in tumor metabolic activity. This technique has shown capability in evaluating mesothelioma disease activity (34–36).

**Protocol 4 (E1/E4-deleted Ad.HSVtk with escalating ganciclovir trial): patients 32 to 34.** Because problems with vector production had made it difficult to continue the dose escalation trial above, we conducted an amended clinical study (UPCC 5598) to test the hypothesis (based on preclinical data) that increasing doses of ganciclovir would be more effective. Three patients were studied under this protocol using a similar intrapleural vector dose of $3 \times 10^{13}$ particles and an increased ganciclovir dose of 7.5 mg/kg i.v. bid for 14 days. This trial was halted after only three patients were entered because of a temporary moratorium on gene transfer trials at the University of Pennsylvania Medical Center resulting from the death of a patient in an unrelated Ad trial for ornithine transcarbamylase deficiency.

**Viral shedding: antibody fluorescent unit assays**

To detect viral shedding, antibody fluorescent unit assays (37) were done on stool, urine, and nasopharyngeal swabs obtained from study patients on days 1, 3, 5, 7, 14, and 19.

**Routine histology and immunohistochemical studies**

The baseline diagnosis of MPM was established based on standard pathologic criteria, immunohistochemical staining, and electron microscopic findings, if needed (38). Pre- and post-vector delivery biopsies, where obtained per protocol, were subdivided into portions that were snap-frozen for immunohistochemistry and one portion fixed in neutral-buffered formalin for routine histology. Frozen sections of biopsies were stained using standard techniques (39) with monoclonal antibodies directed against HSVtk protein (9G10 and 4C8) obtained from Dr. William Summers at Yale University (40) to assess for HSVtk protein expression in tissue samples.

**Immunoblotting studies**

To determine if treatment with Ad.HSVtk led to detectable humoral responses against mesothelioma proteins, a panel of seven human mesothelioma cell lines were grown in culture. When confluent, cells were lysed in ice-cold lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 0.5% Triton X-100, 0.5% SDS] and protease inhibitor cocktail (complete protease inhibitor, Roche Diagnostics Corporation/Roche Applied Science, Indianapolis, IN). The lysates were centrifuged at 12,000 rpm at 4°C in a microcentrifuge for 15 minutes, and protein extracts were assayed for protein concentration, using the Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL). Fifteen micrograms of extracted protein were separated by NuPAGE 4% to 12% gradient SDS Gels (Invitrogen, Carlsbad, CA) under reducing conditions and electroblotted onto PolyScreen polyvinylidene difluoride transfer membranes (NEN Life Science Products, Inc., Boston, MA). After blocking, immunoblotting was accomplished by adding patient serum samples (diluted either 1:1,500 or 1:3,000) from time points before treatment and 3 to 6 months after treatment to replicate gels. Bound human antibody was visualized by treating the washed membrane with peroxidase-conjugated donkey anti-human IgG (Jackson Immunology Research Labs, West Grove, PA) as a secondary antibody for 45 minutes at room temperature. The membrane was then developed using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA). Lanes from pretreatment and posttreatment serum were then directly compared for the presence of new bands.

**Follow-up data and statistical analysis**

Complete follow-up data was obtained on every patient. Survival time was recorded as the time from treatment with gene therapy until the time of death or until May 1, 2005 for the three patients still living. Wherever possible after the demise of patients enrolled in the protocols, autopsies were obtained at the University of Pennsylvania Medical Center. Clinical risk factors and treatment related variables were analyzed for significant association with long-term survival (defined as >24 months) using the χ² test or Fisher’s exact test.

**Results**

**Safety and toxicity.** The safety and toxicity results of protocols 1 and 2 using the E1/E3-deleted Ad.HSVtk vector have been previously published (28). In brief, the vector was extremely well tolerated, even at the highest dose levels used ($5 \times 10^{13}$ viral particles). There was a single grade 4 toxicity, an uncomplicated, transient lymphopenia without absolute leukopenia seen in patient 25. There was a single grade 3 toxicity noted, an asymptomatic elevation in GGT noted in patient 26. There were no requirements for de-escalation of ganciclovir or discontinuation of any aspect of the clinical protocol due to toxicities.

Similar results were seen with the intrapleural injection of high titers of the E1/E4-deleted Ad.RSVtk vector, H5.001RSVtk, combined with systemic ganciclovir in protocols 3 and 4. In June 1998, we started enrollment of patients at an initial dose of H5.001RSVtk of $1.5 \times 10^{13}$ viral particles, approximately one half log lower than the highest dose used with the E1/E3-deleted Ad vector, H5.010RSVtk, in the initial phase I clinical trials ($5 \times 10^{13}$ viral particles). Over the next 6 months, five mesothelioma patients were treated with intrapleural vector via chest tubes. Per protocol, 72 hours after vector instillation on day 5, repeat thoracoscopic tumor biopsies were obtained to evaluate for the presence of tk gene transfer; i.v. ganciclovir was given postoperatively on day 6 at a dose of 5 mg/kg bid for 14 days.

The first two patients in protocol 3 (Meso 27 and 28) received an intrapleural dose of $1.5 \times 10^{13}$ viral particles of the E1/E4-deleted Ad vector, H5.001RSVtk. At this dose, we saw minimal toxicity, primarily transitory fever (grade 1). The next three patients (Meso 29, 30, and 31) underwent intrapleural infusion of $5.0 \times 10^{13}$ viral particles with subsequent evidence of increased but non–dose-limiting toxicity. All three patients at the higher dose level experienced acute febrile responses (grade 1) after vector instillation, with rapid defervescence. One patient (29) developed hypotension (grade 2) and hypoxemia (grade 2) within hours after vector administration that resolved with supplemental oxygen and i.v. fluids. Patient 29 also developed elevated serum transaminases after vector delivery to levels approximately two to three times normal (grade 2), returning to baseline levels by the completion of the ganciclovir protocol. This patient had no associated elevations in serum bilirubin or prothrombin time, and no clinical evidence of hepatic dysfunction. The third patient to receive the higher dose level (patient 31) developed low-grade fever (grade 1) after intrapleural vector instillation, as well as contralateral pleural inflammation. Overall, there seemed a trend toward lower hepatotoxicity in the patients treated with the E1/E4-deleted vector compared with patients treated with equivalent doses of the E1/E3-deleted Ad but a similar pattern of increased systemic side effects at higher dose levels (data not shown).
All three patients in protocol 4 (patients 32-34) who received \(3 \times 10^{13}\) particles of the E1/E4-deleted vector also tolerated the treatment well. Toxicities were non–dose limiting and included mild to moderate fever after viral instillation, pruritus, constipation, anemia, lymphopenia, transient elevation of liver enzymes, and platelet elevation. One patient had transitory pleuritic pain after chest tube insertion and vector instillation. Another patient experienced tachycardia with occasional premature ventricular contractions related to concomitant hyponatremia and hypokalemia, which resolved with electrolyte repletion.

**Gene transfer and immune responses to adenovirus and mesothelioma tumor cell lines.** The gene transfer and immune response results of protocols 1 and 2 using the E1/E3-deleted Ad.HSVtk vector have been previously published (28). In brief, E1/E3-deleted Ad administered intrapleurally followed by i.v. ganciclovir induced both significant humoral and cellular Ad-specific immune responses. Although animal studies have indicated that significant neutralizing antibody titers would preclude transgene expression, four of the eight patients who exhibited transgene expression showed neutralizing anti-Ad antibody titers of \(>1:100\) (29). The preexisting levels of Ad-specific proliferation were also significant in almost all of the patients who had evidence of tk gene transfer. To detect gene transfer, we used immunohistochemistry with a monoclonal antibody directed against the HSVtk protein. Evaluation of selected post-gene delivery biopsy samples revealed evidence of tk gene transfer in 17 of 25 evaluable patients, including all patients treated above a dose level of \(1.6 \times 10^{13}\) viral particles (28).

In protocol 3, dose-related gene transfer was detected in all patients at both dose levels via immunohistochemistry using the anti-HSVtk monoclonal antibody. No gene transfer information is available from protocol 4, because post-vector instillation biopsies were not obtained. As in the initial phase I trial, significant humoral responses to the recombinant Ad vector were seen in all five patients, with the development of high serum titers of total and neutralizing anti-Ad antibodies within 15 to 20 days of vector instillation (Fig. 1).

Because leukocytes were not collected from the patients and cell lines were not generated from tumor tissue, we were not able to assay for cellular immune responses to mesothelioma tumor antigen in this study. However, the collection of serum samples before and after treatment did allow us to search for antibody responses to mesothelioma tumors. Immunoblotting was done on membranes containing protein that had been extracted from a panel of established human mesothelioma cell lines. Replicate membranes were treated with serum (diluted 1:1,500 or 1:3,000) collected before treatment and at a time point 3 to 6 months after treatment. Four examples (patients 14, 25, 27, and 28) are shown in Fig. 2. All pretreatment serum samples (left-hand gel lane in each comparison) reacted with a unique subset of proteins from the mesothelioma tumor cell lines. In virtually every case, however, antibodies in the posttreatment sera (right-hand gel lane in each comparison) identified new bands on the mesothelioma extracts (arrows), in addition to those identified by the pretreatment serum.

**Follow-up data.** Table 1 summarizes the follow-up data on all of the patients and includes vector dose level, disease stage, Eastern Cooperative Oncology Group performance status, cell type, survival time, and cause of death. For purposes of long-term follow-up, we have compared the responses in the 21 patients (eight patients from protocol 1, five patients from protocol 2, and all eight patients from protocols 3 and 4) who received a dose of vector where gene transfer was consistently seen by immunohistochemical staining (\(2 \times 1.6 \times 10^{13}\) viral particles) versus those 13 patients (all from protocol 1) who received doses of vector below this threshold value. Although our numbers are small, we have also compared those patients receiving the E1/E3-deleted vector (patients 14-26) versus those patients receiving the E1/E4-deleted vector (patients 27-34). Our data shows that long-term survival (defined as \(>24\) months) was significantly associated with lower stage (stage I or II versus stage III or IV; 42% versus 0%; \(P = 0.03\)) and with a better Eastern Cooperative Oncology Group performance status (0 versus 1 or 2; 53% versus 6%; \(P = 0.004\)). Although we saw trends (relative risks of 1.3) towards association between long-term survival and exposure to higher doses of vector (\(>1.6 \times 10^{13}\) particles) or with long-term survival and exposure to the E1/E4-deleted vector, these associations were not statistically significant.

![Fig. 1. Anti-Ad antibody titers. Significant humoral responses to the recombinant adenoviral vector were seen in all patients receiving the E1/E4-deleted Ad.HSVtk vector with the development of high serum titers of total anti-Ad antibodies within 15 to 20 days of vector instillation. Serum total anti-Ad antibody levels in the five patients receiving HSV001RSVtk and “standard dose” ganciclovir.](https://www.aacrjournals.org/clinicsignal/images/fig1.jpg)
One patient of the initial 13 patients treated at lower doses (without detectable protein expression) is alive at the time of this submission, ~9.5 years after vector instillation. This sole surviving patient from protocols 1 and 2 (E1/E3-deleted vector, steroids) developed evidence of disease progression 3 years after completion of the gene transfer protocol and then underwent surgical debulking followed by intrapleural photodynamic therapy. He continues to have evidence of minimal malignancy and has not required further therapy. The other 12 low-dose E1/E3 patients all died from progressive mesothelioma (see Table 1).

There were no long-term survivors (>5 years) among any of the patients in protocols 1 and 2 who received the E1/E3-deleted vector ± steroids) developed evidence of disease progression 3 years after completion of the gene transfer protocol and then underwent surgical debulking followed by intrapleural photodynamic therapy. He continues to have evidence of minimal residual malignancy and has not required further therapy. The other 12 low-dose E1/E3 patients all died from progressive mesothelioma (see Table 1).

Fig. 2. Fifteen micrograms of protein from a panel of human mesothelioma cell lines were run on replicate 4% to 12% reduced SDS-PAGE gels, and immunoblotting was done using patient serum (diluted 1:1,500 or 1:3,000) collected before treatment and at a time point 3 to 6 months after treatment. Pretreatment (Pre-Rx) serum samples (left-hand gel lanes in each comparison) are compared with the posttreatment (Post-Rx) sera (right-hand gel lane in each comparison). New bands (arrows).

Fig. 3. Serial PET scans of patient 29. Patient 29 had objective evidence of tumor response on pre- and post-gene therapy 18-fluorodeoxyglucose PET imaging, with near-complete absence of FDG uptake on serial 18-fluorodeoxyglucose PET scans done 48 months after completion of the protocol (Fig. 3). The objective metabolic response of patient 29 on 18-fluorodeoxyglucose PET scan correlated with her excellent clinical status and stability on serial chest computed tomography scans (Fig. 4). The patient has had no other antineoplastic therapy other than our gene transfer protocol. On repeat chest computed tomography and PET scans done in October 2003, there seemed a new area of tumor activity in the midportion of the diaphragmatic pleura within the same hemithorax as her original tumor. The patient was, and remains, asymptomatic and has declined biopsy of this new lesion as well as any additional therapy.
The other long-lived patient (patient 30) treated at the same dose level had evidence of stable disease clinically and radiographically on day 80 follow-up studies. The patient continues to show evidence of minimal residual disease, now 6.5 years after completion of the protocol despite refraining from other antineoplastic treatment. In October 2004, patient 30 developed a local chest wall recurrence measuring <2 cm in diameter at a prior thoracostomy site with minimal associated chest wall discomfort. A chest computed tomography scan done concomitantly showed no evidence of intrathoracic disease progression compared with computed tomography dating back 6 years. The chest wall tumor recurrence was resected under local anesthesia in November 2004, with the pathology resembling her original primary tumor. She is currently asymptomatic with no clinical evidence of further disease recurrence or progression.

The two patients treated in protocol 3 at the lower dose level and one other patient treated at the higher dose level showed evidence of progressive disease on both chest computed tomography and PET scan at 2-month follow-up and subsequently died from advanced mesothelioma.

No durable clinical responses were noted in any of the three patients treated in protocol 4 (using the E1/E4-deleted vector at a dose of $3 \times 10^{13}$ particles), although the initial patient (patient 101) treated in this cohort showed reduced 18-fluorodeoxyglucose uptake in the mediastinal and parietal pleural regions on his posttreatment scan (Fig. 5). Subsequent 18-fluorodeoxyglucose PET scanning at day 170, however, showed significant increase in tracer uptake consistent with increased tumor metabolic activity. This correlated with the patient’s increasing clinical symptoms and progressive pleural thickening and nodularity on repeat chest computed tomography scan. Patients 102 and 103 both showed increased 18-fluorodeoxyglucose uptake on their follow-up day 80 PET studies and also had clear evidence of progression on chest computed tomography. All three patients had evidence of progressive disease at the 6-month follow-up point and have since died related to complications of advanced mesothelioma. The median survival in the high-dose E1/E3 patients (protocols 3 and 4; including those receiving the increased ganciclovir dosage) was 15 months after gene delivery.

Overall, 17 of 34 patients treated in these initial phase I trials of Ad.tk/ganciclovir gene therapy subsequently underwent additional adjuvant therapy (Table 1). Eight patients underwent chemotherapy alone: four received chemoradiotherapy, two radiation alone, and one patient (as previously mentioned) underwent surgical debulking and photodynamic therapy for a...
late recurrence of disease. Four of these patients also underwent other experimental therapy for mesothelioma. Of the 21 patients included in the high-dose group, 11 were documented to have received additional adjuvant therapy (seven chemotherapy, two chemoradiotherapy, one radiation alone, and one experimental therapy alone). Three of these patients received additional experimental therapy after failing more standard adjuvant therapy. There did not seem to be any correlation between the type of additional therapy and survival in the high-dose group (data not shown). As described, two of the long-term survivors, patients 29 and 30, did not receive any additional therapy for their disease.

Discussion

In this report, we describe our long-term follow-up data on a series of 34 patients with malignant mesothelioma who received a single dose of a nonreplicative Ad vector encoding the herpes simplex virus thymidine kinase suicide gene in combination with systemic ganciclovir. We have focused on the 21 patients receiving high-dose therapy, defined by a dose of vector (≥1.6 × 10¹⁵ particles), where transgene-encoded protein was reliably seen by immunohistochemical staining. In 13 patients, the vector was deleted in the E1 and E3 regions of the Ad; in the other eight patients, the vector had deletions in the early Ad genes E1 and E4.

One conclusion from our experience was that intrapleural administration of Ad.HSVtk/ganciclovir continued to be safe and well tolerated. In the additional eight patients who were not previously reported upon (all of whom received the E1/E4-deleted vector), we saw no serious adverse events and did not reach a maximally tolerated dose. This data fits well with a large clinical experience showing the relative safety of Ad vectors (19–21, 41–43).

Given our previous findings showing that intrapleural gene transfer was detectable but only at the surface of the pleural tumors, it seemed unlikely that there would be enough cell killing to result in useful clinical responses in all but those patients with very small tumor burdens (small parietal pleural nodules or diffuse, ‘thin’ tumors). We were therefore somewhat surprised to observe that several patients had reductions in tumor, and that two patients had durable objective responses that have lasted for >6.5 years in both cases.

Do these responses mean anything? Only very small numbers of patients with mesothelioma have been reported to have had prolonged survivals (23, 24); however, these patients usually have stable or slowly progressive disease. To our knowledge, there is only one case report in the literature of a ‘spontaneous remission’ in pleural mesothelioma, but even this patient later recurred at a separate pleural location after only 7 months and died from her malignancy 20 months after diagnosis (25). The fact that we saw two documented long-term clinical responses associated with prolonged survival in a group of only 21 patients would thus be extremely unusual, although without a controlled trial we cannot make any definitive conclusions.

Although only 34 patients were studied, we were able to show statistically significant associations with survival and tumor stage and the performance status of the patients, confirming many other studies (22–24). There was no evidence of an association between adjuvant therapies and survival. As a phase I trial, this study was a priori underpowered to formally assess for treatment effects, especially given the strong confounding effects of stage and performance status. Because of these limitations, our observations should be considered as anecdotal. However, they are intriguing and raise a number of interesting issues about the use of gene therapy for mesothelioma or other local tumors. It is therefore important to try to answer the question, ‘why did these specific patients have significant tumor responses and prolonged survival?’

We postulate that the long-term efficacy of the Ad.HSVtk/ganciclovir protocol was due to induction of a potent antitumor immune response. First, our biopsy studies showed that the intrapleural instillation of the Ad.HSVtk vector resulted in successful gene transfer to tumors, however, at a relatively superficial level. Even with efficient intercellular transfer of triphosphorylated ganciclovir through gap junctions, it seems unlikely that we achieved sufficient tk gene transfer to eradicate the entire tumor. Second, the kinetics of the responses seen were quite slow, occurring over months rather than days or weeks. This is more consistent with the induction of an antitumor immune response than the rapid tumor cell killing via inhibition of DNA synthesis by ganciclovir triphosphate. Finally, although we were not able to look for cellular-mediated antitumor immune responses, we were able to show that posttreatment serum consistently contained antibodies against mesothelioma proteins that were not present in the pretreatment serum (Fig. 2, arrows). The identity of these newly identified proteins are not known (and would be very difficult to determine), but these data clearly show that the Ad.tk/ganciclovir gene therapy did induce a consistent humoral immune response to tumor antigens. We were not able to determine any obvious correlation between the number of new bands and dose of vector or in the number of new bands and the clinical response.

There are data in animal models to support the importance of immune responses in HSVtk/ganciclovir therapy. The induction of an antitumor immune response has been postulated to be an important part of the efficacy of Ad.HSVtk/ganciclovir when immunocompetent animal models have been studied (5, 6, 44). An important clue in this regard has been the observation that the combination of Ad.HSVtk/ganciclovir with cytokine therapy (such as Ad.II-2; see ref. 10) has enhanced efficacy in some models. Elegant work by the Vile group has suggested that HSVtk/ganciclovir-induced tumor necrosis (rather than apoptosis) with release of heat shock proteins in some tumors essential to the generation of an effective immune response (45).

If one accepts the hypothesis that our patients’ clinical responses were due to immunologic stimulation caused by the ability of Ad.HSVtk/ganciclovir to induce cell death and provide ‘danger signals,’ augmentation of this antitumor immune effect with cytokine-expressing vectors would be a rational next step. In fact, there are many studies in the literature showing enhanced efficacy when Ad.HSVtk was combined with cytokine genes such as IL-2 (46), IL-12 (47), GM-CSF (48), or a combination of both IL-2 and GM-CSF (49).

Accordingly, our group has decided to use the same principle but to pursue intrapleural gene transfer studies using an Ad encoding a single cytokine (IFN-β) that can both directly induce tumor cell death and augment natural killer and T-cell antitumor immune responses. Based on successful preclinical studies (50), we have recently completed a human clinical trial of intrapleural delivery of Ad.humanIFN-β done in collaboration with Biogen/
IDEC for the treatment of mesothelioma and other malignant pleural tumors. Remarkably, at our first dosing level, a patient with metastatic ovarian cancer showed a complete (albeit temporary) remission of disease, documented by 18-fluorodeoxyglucose positron emission scanning (data not shown).

One intriguing finding of our study is that two of our three long-term responses were seen in the patients that received the E1/E4-deleted vectors. There are some differences between the behavior of E3-deleted versus E4-deleted vectors, at least in animal models. Many of the Ad genes encoded in the E3 transcription unit function to prevent killing of infected cells by the host immune system (51). Loss of these E3 genes tends to result in increased inflammation after vector injection perhaps leading to shorter transgene expression times. The functions of the proteins in the E4 transcription unit are not completely understood but can affect viral protein production and apoptosis. Some investigators believe that transgene expression is more persistent in E1/E4-deleted vectors (31). This could potentially lead to more efficient generation of toxic ganciclovir metabolites and augmented cell death.

Interestingly, a recent article by Katabi et al. (52) found differences in the mechanisms of cell death induced by E1/E3-deleted and E1/E4-deleted vectors. It is thus possible that the treatment with the E1/E4 generation vector led to more necrotic cell death, resulting in a more effective immune response (45).

In our preclinical studies comparing the efficacy of an E1/E3-deleted Ad.HSVtk vector versus an E1/E4-deleted vector, we did note a small but significant difference in favor of the E1/E4-deleted vector (32). This difference was not seen in an immunodeficient mouse model of human mesothelioma. Additional experimentation will be needed to explore these possibilities.

In summary, our results suggest that Ad.HSVtk/ganciclovir combination treatment, in at least one type of malignancy (mesothelioma), was capable of inducing durable long-term responses in some patients. We postulate that these responses were due to generation of an antitumor immune reaction. These findings have a number of implications. First, perhaps future Ad-based immunogene therapy trials should be conducted with E1/E4-deleted vectors rather than E1/E3-deleted vectors. Second, we propose that approaches that aim to augment the immune effects of Ad gene transfer (i.e., with the use of cytokines) may lead to increased numbers of therapeutic responses in otherwise untreated diseases.

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References


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