ANEXO II

PUBLICACIONES ASOCIADAS A ESTA TESIS

Distribution, persistency, toxicity, and lack of replication of an E1A-deficient adenoviral vector after intracardiac delivery in the cotton rat

Augusto Rojas-Martinez,[1,2,3] Philip R. Wyde,[4] Charles A. Montgomery,[5,6] Shu-Hsia Chen,[7] Savio L.C. Woo,[7,8] and Estuardo Aguilar-Cordova[2,3,9]

[1]Department of Human Genetics; [2]Gene Vector Laboratory; [3]Texas Children's Cancer Center; [4]Department of Microbiology and Immunology; [5]Department of Pathology; [6]Center for Comparative Medicine; [7]Department of Cell Biology; [8]Howard Hughes Medical Institute; and [9]Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030.

Adenoviral vectors were inoculated via intracardiac injection into 5- to 10-week-old cotton rats (*Sigmodon hispidus*) to evaluate the effects of systemic delivery. Cotton rats were chosen as a model because they are semipermissive to the replication of human adenoviruses. The vector used was AdV.RSV-tk, a replication-deficient adenovirus with a *herpes simplex virus thymidine kinase* gene inserted in the E1 region. Vector doses were 3×10^9, 3×10^9, and 3×10^{10} viral particles per animal with and without ganciclovir at 10 mg/kg twice a day. Animals were sacrificed and necropsied at 24 hours, 7 days, and 14 days postinoculation. Gross and microscopic pathologic observations in dosed groups were compared with an unmanipulated control group. From each animal, 10 different organ systems were analyzed for histopathology and vector distribution. The only significant microscopic lesions observed were epicardial inflammation and splenic hemosiderosis. Vector sequences persisted throughout the 14-day assay with preponderance in the heart, lung, and lymphoid organs. Infectious virions were detected for 24 hours, and these virions were only detected at the site of injection of two animals in the highest dose group. No viral replication was detected. Therefore, systemic delivery of up to 3×10^{11} viral particles/kg was well tolerated in this semipermissive host model and did not result in any significant pathology.

Key words: Cotton rat; replication-deficient adenovirus; systemic delivery; herpes simplex virus thymidine kinase.

The increased use of adenoviral vectors in clinical gene therapy highlights the importance of many aspects concerning the interaction of the recombinant vector with the host. Among these, distribution, persistence, pathogenicity, and viral replication are primary concerns that need to be addressed before intentional or incidental systemic inoculation in humans. Since some of the toxicity caused by adenoviral vectors may be a result of residual viral protein expression, analysis in a host which permits the expression of viral proteins may be necessary to fully appreciate potential toxicities. Cotton rats (*Sigmodon hispidus*) provide a unique animal model that can support the replication of human adenoviruses (AdVs) and develop a pathological picture similar to

that seen in humans.[1] [6] Unfortunately, there is currently only one commercial source of cotton rats in the U.S. (Virion Systems, Rockville, Md); thus, the availability of this model for the assessment of the gene therapy applications of adenoviral vectors has been limited.

Adenoviral vectors for current clinical studies have all been E1A deleted, with backbones based on human AdVs of serotype 2 or 5. For this study, a recombinant of serotype 5 with the *herpes simplex virus thymidine kinase* (*HSV-tk*) gene inserted in the E1A region (AdV.RSV-tk) was selected.[7] Cells that express the *HSV-tk* gene after vector transduction become sensitive to the cytotoxic effect of ganciclovir (GCV).[8] In the absence of GCV, AdV.RSV-tk will have only backbone effects and will represent a more generic version of adenoviral vectors. In addition, this vector was chosen because it has been approved for several human clinical trials Recombinant DNA Advisory Committee, National Institutes of Health #9412-098, #9601-144, #9602-147, and #9602-148) with the potential for inadvertent systemic distribution.

This study analyzed the distribution, replication, and pathogenicity of AdV.RSV-tk after intracardiac delivery in cotton rats. Despite its common name, the cotton rat belongs to the Cricetidae family and is more closely related to hamsters than rats. These animals do not have easily accessible peripheral vasculature; therefore, intracardiac injection was chosen as a route for systemic distribution in this study. A recent study[5] on the effects of intramuscular (i.m.) and intranasal (i.n.) delivery to cotton rats using 10^8 plaque-forming units (PFUs) per animal detected no vector sequences after 3 and 21 days, respectively. Detection after i.n. delivery was limited to the regional lymph nodes and lungs. The delivery of wild-type AdV i.n. resulted in a peak virus load at 3–5 days postinoculation, with no viral recovery after 14 days. Therefore, it appeared that the duration of adenoviral infections in cotton rats was limited to <14 days, and also that a dose of 10^8 PFUs was well tolerated after i.m. and i.n. delivery. The vector used in these studies had a viral particles (v.p.) to infections unit titer ratio of ≥ 30. Based on those data, a dose of 3×10^8 v.p. was estimated to be a no-effect dose, and 3×10^{10} v.p. was considered a potentially toxic dose for this study. The time points chosen were day 1 postinoculation as the most likely time to find vector sequences, day 7 as the end of GCV treatment, and day 14 as the end of the likely period for finding remaining vector sequences.

MATERIALS AND METHODS

Vector delivery and tissue preparation

The vector used for these studies was AdV.RSV-tk,[7] which was produced at the Baylor College of Medicine Gene Vector Laboratory (Houston, Texas) as previously described.[8] This vector is based on an E1A-deleted backbone of a human AdV type 5, containing a Rous sarcoma virus (RSV) promoter which drives the expression of an *HSV-tk* gene.[10] Infectious titers were analyzed by limiting dilution analysis on the 293 human embryonic kidney cell line.[11] The functionality of the vector lot was tested by *in vitro* transduction of GCV sensitivity to HeLa cells.

A total of 42 5- to 10-week-old cotton rats of either sex were randomly distributed into one control and 13 treatment groups of three animals each. Intracardiac delivery of 100 µL each was performed by percutaneous injection into the upper left quadrant of the thorax using a tuberculin syringe with a 30-gauge needle. Cardiac localization of the needle was determined by blood aspiration prior to vector injection.

Of the total animals, 3 received phosphate-buffered saline, 15 received 3×10^8 v.p. of the vector, 9 received 3×10^9 v.p., and 15 received 3×10^{10} v.p. At 24 hours postinjection, the control group and three animals from each dose group were sacrificed and necropsied. Half of the remaining animals from each dose group received GCV by intraperitoneal injection of 10 mg/kg twice a day for 6 days. At the end of treatment (day 7), three animals from each group were sacrificed and necropsied. One animal at 3×10^9 v.p. and another at 3×10^8 v.p. without GCV died for reasons unrelated to treatment. The remaining animals were sacrificed and necropsied on day 14. At necropsy, samples were taken from blood, gonads, mesenteric nodes, spleen, kidneys, liver, brain, thymus, lungs, and heart, in that order. The order was chosen, starting with those

organs least likely to contain vector sequences and continuing through those organs most likely to contain sequences, to minimize the potential for cross-contamination of the samples prior to polymerase chain reaction (PCR) analysis. Part of each tissue was fixed in 10% buffered formalin for histopathologic examination, and a portion was quick-frozen for DNA analysis.

Histopathology

Gross and microscopic observations in dosed groups were compared with those in the control group. Histopathologic assessment was performed in a single-blinded fashion on paraffin-embedded sections of kidney, heart, lung, striated muscle, liver, spleen, thymus, mesenteric lymph node, brain, and gonads that were all stained with hematoxylin-eosin.

DNA analysis

DNA isolation was performed by the overnight digestion of ~100 mg of tissue with 0.88 mg/mL proteinase K in TES buffer (50 mM tris(hydroxymethyl)aminomethane HCl (pH 8.0), 100 mM ethylenediaminetetraacetic acid, and 0.5% sodium dodecyl sulfate (SDS)) at 56°C, followed by treatment with DNAzol reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, Md). Purified DNA was resuspended in $1 \times$ TES buffer at 25 ng/µL. PCR amplification was performed as previously described.[12]

The primers for PCR amplification were directed toward the RSV-tk/HSV-tk junction of the vector insert sequence (primer RSV 372S: ACGCCATTTGACCATTCACC; primer HSV 244A: CAGTTGCGTGGTGGTGGTTTTC). The identity of the 319-base pair product was confirmed by an internal oligo-probe (AGCGCGTATGGCTTCGTACCCCGGC).

Amplification of the cK-*ras* gene was used as an internal control of DNA quality using primers that have been previously described (primer K1: TATTATAAGGCCTGCT GAAAATGACTGAAT; primer K2: TTACCTCTATTGTT GGATCATATTCGTCCA, product size: 135 base pairs).[13] Primers and probes were purchased from Genosys (The Woodlands, Tex).

Duplex PCR amplification was performed on 150 ng of genomic DNA (~50,000 cell equivalents) in a 15 µL reaction mix containing 2.5 mM MgCl₂, 50 mM KCl, 10 mM tris(hydroxymethyl)aminomethane HCl (pH 8.3), 0.33 µM of each primer, 33 µM of each deoxynucleotide triphosphate (USB, Cleveland, Ohio), and 1 U of *Taq* polymerase (Fisher Scientific, Pittsburgh, Penn). Forty rounds of amplification were performed in a PTC-100™ machine (MJ Research, Watertown, Mass) with a 94°C denaturation step (30 seconds), a 55°C annealing step (30 seconds), and a 72°C extension (1 minute). Each reaction (7 µL) was electrophoresed in 2% agarose (Bio-Rad Laboratories, Hercules, Calif) and visualized by ethidium bromide staining. Gels were blotted overnight onto 0.2-µm Nytran membranes (Schleicher and Schuell, Keene, NH) with 0.4 N NaOH. Next, 20 pmol of the oligoprobe were 5′ end-labeled in a 50-µL volume reaction with 3 U of T4 polynucleotide kinase (USB) and 12.5 µCi [γ-³²P]adenosine triphosphate (Amersham, Arlington Heights, Ill). Prehybridizations were performed at 65°C in 15 mL of hybridization buffer (0.5 M Na₂HPO₄ (pH 7.2), 1 mM ethylenediaminetetraacetic acid, 7% SDS, and 100 µg/mL of fragmented salmon DNA) for 30 minutes. Hybridizations were completed by adding the probe to the prehybridization solution and incubating overnight at 65°C. Filters were washed two times for 20 minutes at room temperature with wash solution (40 mM Na₂HPO₄ and 1% SDS), and autoradiography was completed

Table 1. Vector Sequence Distribution

Day	Dose	GCV	n	Blood	Brain	Gonads	Heart	Kidney	Liver	Lungs	Mesenteric nodes	Spleen	Thymus
1	3×10^8	No	3	—	—	—	1	—	—	—	—	1	1
	3×10^9	No	3	1	—	—	3	—	1	3	1	1	3
	3×10^{10}	No	3	—	1	1	2	—	—	3	—	1	2
7	3×10^8	No	3	1	—	1	1	—	—	2	1	2	—
	3×10^8	Yes	3	2	—	—	—	—	—	1	2	1	—
	3×10^9	No	2	—	—	—	2	—	—	2	—	1	1
	3×10^9	Yes	3	—	—	—	3	—	1	3	—	1	3
	3×10^{10}	No	3	2	—	—	3	—	2	2	—	2	2
	3×10^{10}	Yes	3	—	—	—	3	—	1	3	2	1	3
14	3×10^8	No	2	—	—	—	—	1	—	1	1	—	—
	3×10^8	Yes	3	—	—	1	—	—	1	3	2	—	3
	3×10^{10}	No	3	—	—	—	2	1	1	3	1	2	2
	3×10^{10}	Yes	3	—	—	—	3	—	1	3	1	1	2

Numbers indicate the number of tissue samples that were positive from the number of samples tested (*n*).

overnight on X-Omat AR films (Kodak, Rochester, NY) at $-70°C$.[14]

Plaque assays

Sections of frozen tissues that were found positive for vector sequences by PCR were thawed, ground, and suspended in 1 mL of phosphate-buffered saline. One-tenth of the suspension (100 μL) was plaqued on a confluent monolayer of 293 human embryonic kidney cells in a 24-well plate. These cells are stably transfected with the adenoviral E1 region; thus, they are able to form plaques from E1-deleted vectors and wild-type virus.[9] The detection limit varied according to the organ tested and ranged from 100 to 1000 PFUs/organ ($0.1 \times$ fraction of the organ tested).

RESULTS

Vector distribution and persistence

Distribution results are summarized in Table 1. The distribution of the vector was assessed by PCR amplification of the junction sequence of the RSV promoter and the *HSV-tk* gene followed by hybridization using an end-labeled oligonucleotide probe for the HSV-tk region. The sensitivity of this methodology was found to be 1–10 vector particles in the context of 150 ng of cotton rat cellular DNA (Fig 1).

The vector was most often detected in thoracic organs (Table 1; lungs, 29 of 37; heart, 23 of 37; and thymus, 22 of 37) followed by other lymphoid organs (spleen, 14 of 37; and mesenteric lymph nodes, 11 of 37), liver (8 of 37), blood (6 of 37), gonads (3 of 37), kidney (2 of 37), and brain (1 of 37). There was no significant difference in vector distribution correlated with GCV treatment (51 of 150 tissue samples versus 42 of 130 tissue samples, with and without GCV, respectively). The majority of positive blood samples (5 of 6) were found at day 7, with no positives detected at day 14. No other time-correlated differences were observed. Of the tissues from animals that received 3×10^8 v.p., 21% (30 of 140) were positive for vector sequences compared with 38% (30 of 80) and 39% (59 of 150) of tissues from 3×10^9 and 3×10^{10} v.p., respectively.

Gross and histopathology

No gross lesions were observed at the time of necropsy. Histopathologic findings are summarized in Table 2 (only organs with treatment-related abnormalities or a high incidence of background lesions are included).

Treatment-related lesions were observed in the epicardium of the heart and in the spleen. The epicardium was thickened and contained focal to diffuse infiltrations of plasma cells, lymphocytes, macrophages, and occasional polymorphonuclear leukocytes (Fig 2). Epicarditis was limited to the top two vector doses. No epicardial lesions were observed at 3×10^8 v.p. In the spleen, an increased number of hemosiderin-laden macrophages was observed in the red pulp of treated animals at 7 days, but this number returned to normal levels at day 14. A slight difference in these lesions was observed between the lower dose groups without GCV (0 of 5) and those with GCV (4 of 6). The effects of GCV were not apparent at the 3×10^{10} v.p. dose, where both groups had an equal incidence of the lesions.

Background microscopic lesions were observed in the kidney and heart of control and experimental animals.
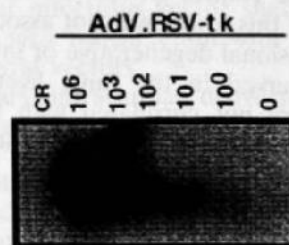
AdV.RSV-tk

Figure 1. Vector detection sensitivity assay. DNA was extracted from a control cotton rat and quantified by ultraviolet adsorption at 260 nm. AdV.RSV-tk particle concentration was determined by ultraviolet adsorption using a conversion factor of 1.1×10^{12} particles per optical density of a 1:20 dilution. The PCR reactions were all performed in the context of 150 ng of cotton rat DNA and were analyzed by Southern blot. CR, cotton rat DNA alone; 10^6 to 10^0, the calculated number of vector particles in the context of cotton rat DNA; 0, no DNA or vector.

Table 2. Histopathology Results of Adenoviral Vector Toxicity Study in Cotton Rats

	24-Hour necropsy			
	Control	3×10^8 AdV	3×10^9 AdV	3×10^{10} AdV
	Inc/Avg Sev	Inc/Avg Sev	Inc/Avg Sev	Inc/Avg Sev
Lesions				
Kidney				
Nephropathy	3/3 (1.3)	3/3 (1.0)	3/3 (1.7)	2/3 (1.0)
Hemosiderin pigment, tubules				
Heart				
Degenerative cardiomyopathy	2/3 (1.5)	2/3 (2.0)	3/3 (1.7)	1/3 (1.0)
Chronic inflammation, epicardium	0/3	0/3	0/3	1/3 (2.0)
Acute inflammation, epicardium				
Acute inflammation, myocardium	0/3	0/3	1/3 (3.0)	0/3
Spleen				
Acute inflammation, red pulp				
Hemosiderin pigment, red pulp				

The renal lesions consisted of tubular degeneration, tubular regeneration, tubular dilatation, protein casts within tubular lumina, mineralization, and infiltration of mononuclear cells in the interstitium. This nephropathy was similar to that described previously in the rat and mouse.[15,16] The incidence and degree of severity for nephropathy was similar for all experimental and control groups; thus, it was not deemed to be treatment-related. Spontaneous lesions in the heart were limited to the myocardium and consisted of myofiber degeneration, mineralization, and infiltration of macrophages and lymphocytes. This degenerative cardiomyopathy was similar to that described with vitamin E deficiency[17] and has been observed before in the cotton rat colony at our institution (data not shown). The cotton rat appears to be more sensitive to vitamin E deficiency than mice and rats.[18] Again, there was no significant difference in the incidence or degree of severity of this lesion between experimental and control groups. Consequently, it was determined that this lesion was not associated with the treatment. Occasional degenerative or inflammatory lesions were observed in the lung, liver, spleen, and thymus but were not correlated with treatment. No microscopic lesions were observed in striated muscle, lymph nodes, gonads, or brain.

Assays for infectious adenoviral vectors

All of the tissues that were found positive by PCR were analyzed for PFUs to detect the persistence of infectious particles or replication-competent recombinants. PFUs were only detected in samples from the site of injection of two animals at the highest dose, and were only seen at the first time point. In the two PFU-positive animals, 40 and 120 PFUs were detected from undiluted samples.

DISCUSSION

In this study, the distribution and toxicity after the systemic delivery of adenoviral vectors were analyzed in a semipermissive host. The only significant microscopic lesions observed in a time-course analysis of three vector doses (3×10^8, 3×10^9, and 3×10^{10} v.p. per animal) were in the epicardium of the heart and in the spleen. The inflammatory changes in the outer portion of the heart appear to be related to the vector and may be specific to the intracardiac route of administration. Other routes of administration may not have the same effect. Splenic hemosiderosis may have resulted from a hemolytic crisis, visceral hemorrhage, or a defect in iron metabolism. Since this lesion decreased by day 14 of the experiment, the most likely explanation is that the vector caused minimal hemolysis, and that the resulting hemosiderin was cleared by the macrophage system within a week. Thus, systemic delivery of up to 3×10^{11} v.p./kg of AdV.RSV-tk was not significantly toxic in this animal model, even with concomitant administration of GCV.

In contrast to high liver presence after tail vein injections in mice and other animal models, vector sequences were primarily detected in thoracic organs near the site of injection in this study. Although this was surprising, a similar result as observed in another report of systemic delivery to cotton rats.[6] In that study, vectors were delivered i.m. or intravenously. Even though expression from the vectors was found to be high in the liver, v.p. were mostly detected in lung and spleen tissue. The implication of this result is that there may be a differential between vector DNA presence and the level of expression for the vector. Therefore, distribution may be influenced by whether it is associated with the vector product or vector particles. In another report, Yei et al[19]

Table 2. (Continued)

	7-Day necropsy						14-Day necropsy			
	3×10^8 AdV w/o GCV Inc/Avg Sev	3×10^9 AdV w/o GCV Inc/Avg Sev	3×10^{10} AdV w/o GCV Inc/Avg Sev	3×10^8 AdV w/GCV Inc/Avg Sev	3×10^9 AdV w/GCV Inc/Avg Sev	3×10^{10} AdV w/GCV Inc/Avg Sev	3×10^8 AdV w/o GCV Inc/Avg Sev	3×10^{10} AdV w/o GCV Inc/Avg Sev	3×10^8 AdV w/GCV Inc/Avg Sev	3×10^{10} AdV w/GCV Inc/Avg Sev
	2/3 (1.0)	2/2 (1.5)	3/3 (1.3)	3/3 (2.0)	3/3 (2.0)	2/3 (1.0)	1/2 (1.0)	3/3 (1.7)	3/3 (1.3)	3/3 (1.3)
				2/3 (1.5)	0/3	0/3	0/2	1/3 (1.0)	1/3 (2.0)	1/3 (1.0)
	1/3 (1.0)	2/2 (1.5)	3/3 (1.3)	2/3 (1.0)	2/3 (1.0)	3/3 (1.7)	1/2 (1.0)	3/3 (2.0)	3/3 (1.3)	2/3 (1.5)
	0/3	1/2 (2.0)	2/3 (3.0)	0/3	3/3 (2.7)	3/3 (2.0)	0/2	1/3 (2.0)	0/3	2/3 (1.5)
							0/2	0/3	0/3	1/3 (2.0)
							0/2	0/3	1/3 (4.0)	0/3
	0/3	0/2	3/3 (2.0)	2/3 (2.0)	2/3 (2.5)	2/3 (2.0)	0/2	0/3	0/3	1/3 (2.0)

Inc = Incidence of lesion.
Avg Sev = Average degree of severity of lesion.
Severity code (in parentheses): 0 = no lesion; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

delivered adenoviral vectors to cotton rats via an i.n. route. In that study, the vector was only detected in the lung; it was not detected in any other organs, including liver. The results from those two reports and the distribution pattern observed here may be characteristic of the cotton rat model. Another hypothesis is that the distribution observed may reflect a rapid adsorption of the vector near the site of injection, especially in the lungs, leading to minimal distribution to the liver. Alternatively, the distribution observed may reflect poor systemic dissemination by intracardiac delivery, perhaps due to cardiac muscle uptake. It is unlikely that the latter possibility is the sole explanation, since the second most prominent site for vector sequences was lymphoid organs; in addition, there was an apparent peak of vector presence in the blood at day 7, suggesting some systemic distribution. Since primary vector distribution may be limited by vector sequestration from cells that are proximal to the site of injection, selection of the site of injection may be critical to the design of clinical protocols. A comparative analysis of delivery sites will be required to address this question.

Surprisingly, there were no discernible differences in the presence and persistence of the vector between animals with or without GCV. This may reflect the preponderance of quiescent cells in vivo and the relatively innocuous effect of HSV-tk and GCV treatment on nondividing cells. The only exception to this lack of apparent GCV effect was seen at day 7 in the two lower dose groups. Splenic hemosiderosis was not observed in any of the animals without GCV, while 4 of 6 animals with GCV had these lesions. This difference was no longer evident at day 14, possibly reflecting a temporal toxic effect on hematologic tissues that is mostly resolved by day 14. However, the data suggest that at the dose levels tested here, the tk/GCV treatment is not toxic to normal tissues.

Vector sequences were detected in various organs for the duration of the study. No significant correlation was observed between the vector dose used or GCV treatment and the distribution or incidence of detection. This lack of correlation may reflect a stochastic distribution within the organs treated as well as the limited proportion of each organ represented by the DNA used for PCR analysis. However, this possibility would equally affect the various organs and consequently is unlikely to have an impact on the comparative results of the study. Infectious particles were only detected from two of nine animals and were only seen at the site of injection at the first time point (24 hours postinjection). The two animals from which PFUs were detected were from the highest dose group. The absence of PFUs in the majority of animals and at all later time points suggests that the injected infectious virions were not maintained, and that there was no vector replication in the in vivo environment. Thus, it is unlikely that infectious vector shedding occurs after systemic in vivo transduction with replication-defective adenoviral vectors.

In summary, intracardiac delivery of an E1-deleted adenoviral vector at 3×10^9 to 3×10^{11} v.p./kg was well tolerated in a semipermissive host. There was a dose- and time-related inflammatory response at the site of injection and dose- and GCV-associated splenic hemosiderosis, but this hemosiderosis was resolved by day 14. No major pathology or viral replication was observed in any of the animals.
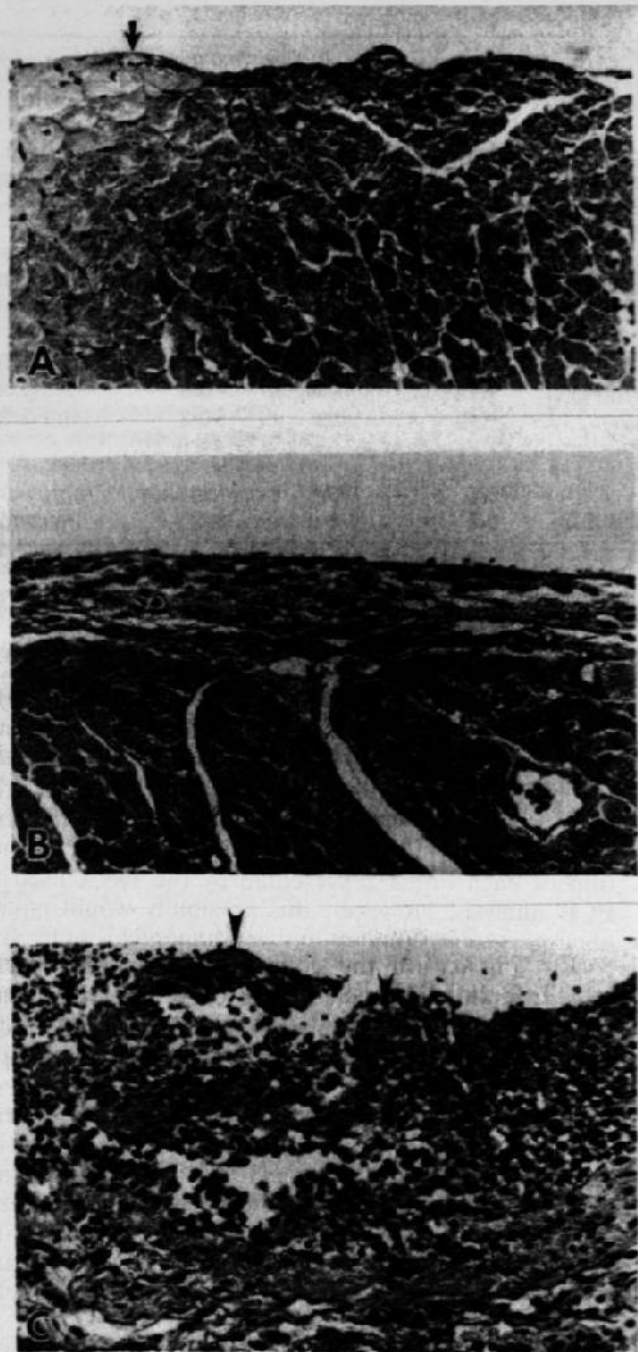
ACKNOWLEDGMENTS

Figure 2. Treatment-related cardiac lesions (×200). **A** shows the normal myocardium (✻) and epicardium (arrow) from a control cotton rat. **B** indicates moderate thickening of the epicardium with diffuse infiltration of lymphocytes and macrophages. **C** shows diffuse thickening of the epicardium with infiltration of lymphocytes, macrophages, and fibrin deposition (arrowheads). **B** and **C** received 3×10^{10} AdV.RSV-tk without GCV and were necropsied 7 days postinoculation.

REFERENCES

1. Pacini DL, Dubovi EJ, Clyde WA. A new animal model for human respiratory tract disease due to adenovirus. *J Infect Dis.* 1984;150:92–97.

2. Ginsberg HS, Horswood RL, Chanock RM, Prince GA. Role of early genes in pathogenesis of adenovirus pneumonia. *Proc Natl Acad Sci USA.* 1990;87:6191–6195.

3. Ginsberg HS, Moldawer LL, Pravinkumar BS, et al. A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. *Proc Natl Acad Sci USA.* 1991; 88:1651–1655.

4. Prince GA, Porter DD, Jenson AB, et al. Pathogenesis of adenovirus type 5 pneumonia in cotton rats (*Sigmodon hispidus*). *J Virol.* 1993;67:101–111.

5. Oualikene W, Gonin P, Eloit M. Short and long term dissemination of deletion mutants of adenovirus in permissive (cotton rat) and non-permissive (mouse) species. *J Gen Virol.* 1994;75:2765–2768.

6. Oualikene W, Gonin P, Eloit M. Lack of evidence of phenotypic complementation of E1A/E1B-deleted adenovirus type 5 upon superinfection by wild-type virus in the cotton rat. *J Virol.* 1995;69:6518–6524.

7. Chen S-H, Shine HD, Goodman JC, et al. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo. Proc Natl Acad Sci USA.* 1994;91:3054–3057.

8. Fyfe JA, Keller PM, Furman PA, et al. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxy-methyl)guanine. *J Biol Chem.* 1978;253:8721–8727.

9. Graham FL, Prevec L. Manipulation of adenovirus vectors. In: Murray EJ, ed. *Methods in Molecular Biology, Vol 7: Gene Transfer and Expression Protocols.* Clifton, NJ: The Humana Press; 1991:109–128.

10. Gingras MC, Arevalo P, Aguilar-Cordova E. Potential salmon sperm origin of the E3 region insert of the adenovirus 5 d1309 mutant. *Cancer Gene Ther.* 1996;3:151–154.

11. Nyberg-Hoffman C, Shabram P, Li W, Giroux D, Aguilar-Cordova E. Sensitivity and reproducibility in adenoviral infectious titer determination. *Nat Med.* 1997;3: 808–811.

12. Saiki RF. Amplification of genomic DNA. In: Innis MA, Gelfland DH, Sninsky JJ, White TJ, eds. *PCR Protocols.* San Diego: Academic Press; 1990:13–20.

13. Rogers BB, Alpert LC, Hine EAS, Buffone GJ. Analysis of DNA in fresh and fixed tissue by the polymerase chain reaction. *Am J Pathol.* 1990;136:541–548.

14. Brown T. Analysis of DNA sequences by blotting and hybridization. In: Ausubel FM, Brent R, Kingston RF, et al, eds. *Current Protocols in Molecular Biology.* New York: John Wiley and Sons; 1995:2.9.1–2.10.16.

15. Montgomery C, Seely J. Kidney. In: Boorman G, Eustis S, Elwell M, et al, eds. *Pathology of the Fischer Rat.* Orlando, Florida: Academic Press; 1990:127–153.

16. Montgomery CA. Interstitial Nephritis, Mouse. In: Jones TC, Mohr U, Hunt RD, eds. *Urinary System, Monographs on Pathology of Laboratory Animal.* New York: Springer-Verlag; 1986:210–215.

17. Van Vleet JF, Ferrans VJ. Nutritional cardiomyopathy: selenium-vitamin E deficiency, mouse and rat. In: Jones TC, Mohr U, Hunt RD, eds. *Cardiovascular and Musculoskeletal System, Monographs on Pathology of Laboratory Animals.* Berlin: Springer-Verlag; 1991:3–9.

18. Telford IR. The Florida cotton rat. In: *Experimental Muscular Dystrophies in Animals: A Comparative Study.* Springfield, Illinois: Charles C. Thomas; 1971:110–123.

19. Yei S, Mittereder N, Wert S, Whitsett J, et al. In vivo evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. *Hum Gene Ther.* 1994;5:731–744.

In Situ Gene Therapy for Adenocarcinoma of the Prostate: A Phase I Clinical Trial

JAMES R. HERMAN,[1] HOWARD L. ADLER,[1] ESTUARDO AGUILAR-CORDOVA,[2–4]
AUGUSTO ROJAS-MARTINEZ,[3–5] SAVIO WOO,[6,7] TERRY L. TIMME,[1] THOMAS M. WHEELER,[1,8]
TIMOTHY C. THOMPSON,[1,6] and PETER T. SCARDINO[1]

ABSTRACT

For patients with local recurrence of prostate cancer after definitive irradiation therapy there is no treatment widely considered safe and effective. After extensive preclinical testing of prodrug gene therapy *in vitro* and *in vivo*, we conducted a phase I dose escalation clinical trial of intraprostatic injection of a replication-deficient adenovirus (ADV) containing the herpes simplex virus thymidine kinase gene (HSV-*tk*) injected directly into the prostate, followed by intravenous administration of the prodrug ganciclovir (GCV). Our goal was to determine safe dose levels of the vector for future trials of efficacy. Patients with a rising serum prostate-specific antigen (PSA) level and biopsy confirmation of local recurrence of prostate cancer without evidence of metastases one or more years after definitive irradiation therapy were eligible for the trial. After giving informed consent, patients received injections of increasing concentrations of ADV/HSA-*tk* in 1 ml into the prostate under ultrasound guidance. Ganciclovir was then given intravenously for 14 days (5 mg/kg every 12 hr). Patients were monitored closely for evidence of toxicity and for response to therapy. Eighteen patients were treated at 4 escalating doses: group 1 ($n = 4$) received 1×10^8 infectious units (IU); group 2 ($n = 5$) received 1×10^9 IU; group 3 ($n = 4$) received 1×10^{10} IU; group 4 ($n = 5$) received 1×10^{11} IU. Vector was detected by PCR of urine samples after treatment, increasing in frequency and duration (up to 32 days) as the dose increased. All cultures of blood and urine specimens were negative for growth of adenovirus. Minimal toxicity (grade 1–2) was encountered in four patients. One patient at the highest dose level developed spontaneously reversible grade 4 thrombocytopenia and grade 3 hepatotoxicity. Three patients achieved an objective response, one each at the three highest dose levels, documented by a fall in serum PSA levels by 50% or more, sustained for 6 weeks to 1 year. This study is the first to demonstrate the safety of ADV/HSV-*tk* plus GCV gene therapy in human prostate cancer and the first to demonstrate anticancer activity of gene therapy in patients with prostate cancer. Further trials are underway to identify the optimal distribution of vector within the prostate and to explore the safety of repeat courses of gene therapy.

OVERVIEW SUMMARY

We conducted a phase I dose escalation clinical trial of intraprostatic injection of a replication-deficient adenovirus (ADV) containing the herpes simplex virus thymidine kinase gene (HSV-*tk*) injected directly into the prostate, followed by intravenous administration of the prodrug ganciclovir (GCV). Eighteen patients were treated at 4 escalating doses from 1×10^8 to 1×10^{11}. No or minimal toxicity (grade 1–2) was encountered in most patients. One patient

[1]Scott Department of Urology, Matsunaga-Conte Prostate Cancer Research Center, Baylor College of Medicine, Houston, TX 77030.
[2]Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030.
[3]Gene Vector Laboratory, Baylor College of Medicine, Houston, TX 77030.
[4]Texas Children's Cancer Center, Texas Children's Hospital, Houston, TX 77030.
[5]Department of Human Genetics, Baylor College of Medicine, Houston, TX 77030.
[6]Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.
[7]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030.
[8]Department of Pathology, Baylor College of Medicine, Houston, TX 77030; and The Methodist Hospital, Houston, TX 77030.

at the highest dose level developed spontaneously reversible grade 4 thrombocytopenia and grade 3 hepatotoxicity. Three patients achieved an objective response, one each at the three highest dose levels, documented by a fall in serum PSA levels by 50% or more sustained for 6 weeks to 1 year. This study is the first to demonstrate the safety of ADV/HSV-*tk* plus GCV gene therapy in human prostate cancer and the first to demonstrate anticancer activity of gene therapy for patients with prostate cancer.

INTRODUCTION

EARLY STAGE PROSTATE CANCER, localized within the prostate, may be managed conservatively or treated actively with radical prostatectomy or irradiation therapy. Of the 184,500 new cases expected this year, more than 80% will be early stage and a third of these will be treated with definitive irradiation therapy (Landis *et al.*, 1998). While modern radiotherapy usually controls the local tumor, some patients will develop local recurrence within the prostate that becomes evident through a rising serum prostate-specific antigen (PSA) level and a positive needle biopsy result in the absence of evidence of metastases. For the estimated 10,000–15,000 patients with local recurrence each year, there is no accepted, standard treatment. Androgen ablation, whether early or delayed, is palliative and radical prostatectomy, although sometimes curative, carries substantial risks (Rogers *et al.*, 1995). Transfer or insertion of genes that (1) result in the activation of prodrugs to produce cytotoxicity (Moolten, 1986; Moolten and Wells, 1990), (2) replace inactivated tumor suppressor genes (Bacchietti and Graham, 1993; Wills *et al.*, 1994; Zhang *et al.*, 1994), or (3) stimulate the immune system (Vieweg *et al.*, 1994), offers new, potentially effective therapeutic approaches to the treatment of prostate cancer. One method of cytotoxic gene therapy is viral transduction of the herpes simplex virus thymidine kinase (HSV-*tk*) gene followed by systemic administration of the nucleoside analog ganciclovir (GCV). The HSV-*tk* gene product phosphorylates GCV, which is incorporated into DNA during cell division and leads to cell death. Moreover, the number of cells killed significantly exceeds the number of cells transduced with the HSV-*tk* gene (Freeman *et al.*, 1992). This phenomenon, the "bystander effect," may be caused by the passage of toxic metabolites across gap junctions, the uptake of the products of apoptotic bodies, a local immune response, and/or inhibition of endothelial cells (Freeman *et al.*, 1993; Vile *et al.*, 1994; Elshami *et al.*, 1996; Hamel *et al.*, 1996; Ramesh *et al.*, 1996a,b). Adenoviral HSV-*tk*/GCV gene therapy has been reported in clinical trials of patients with brain tumors (Alavi *et al.*, 1998), mesotheliomas (Sterman *et al.*, 1998), and ovarian carcinomas (Alvarez and Curiel, 1997).

We demonstrated significant cytotoxicity induced by HSV-*tk*/GCV in both mouse and human prostate cancer cells *in vitro* and in mouse prostate carcinomas *in vivo* (Eastham *et al.*, 1996). HSV-*tk*/GCV treatment-induced cytotoxicity *in vivo* was accompanied by increased areas of necrosis, increased numbers of apoptotic bodies, and extensive immunocyte infiltration relative to controls. In addition, we produced significant growth inhibition of local prostate cancer together with suppression of spontaneous and induced metastases, in an orthotopic *in vivo*

mouse model of prostate cancer when the vector was injected directly (*in situ*) into local tumor within the prostate gland (Hall *et al.*, 1997). Further, we have reported no systemic spread and minimal local spread of the adenoviral vector within the genitourinary system in our mouse model (Timme *et al.*, 1998). To determine the efficacy of this form of suicide gene therapy against human prostate cancer, we initiated a phase I dose escalation trial of a replication-deficient adenoviral vector carrying the HSV-*tk* gene followed by the intravenous administration of GCV in men with local recurrence of prostate cancer after irradiation therapy (IND 6636). Herein, we report the results of this trial.

MATERIALS AND METHODS

Treatment protocol

The protocol was approved by the Baylor Institutional Review Board (IRB) (H-3826) on April 3, 1995 and by the Food and Drug Administration (BB-IND 6636) on May 1, 1996. Patients reviewed the informed consent document and were individually counseled before agreeing to participate in this study. Counseling included a thorough discussion of alternative treatments including watchful waiting, salvage radical prostatectomy, cryosurgery, and androgen ablation.

Eligibility criteria

Patients were eligible for this phase I clinical trial if they had evidence of local recurrence of cancer within the prostate, proven by biopsy, at least 1 year after completion of irradiation therapy. They must have had evidence of a biologically active tumor with a rising serum PSA level on at least three occasions at least 2 weeks apart. Patients must have had no evidence of metastases (a normal serum acid phosphatase, pelvic and abdominal computerized tomography [CT] scan, and normal bone scan). Any area causing suspicion of metastatic disease on bone scan was evaluated by appropriate plain radiographs and/or magnetic resonance imaging (MRI) studies of the area. Patients must have had adequate baseline organ function and coagulation factors. Patients were excluded if they ever received hormonal therapy (androgen deprivation) or had depressed hematopoietic function (platelet count, $<100,000/cm^3$; hemoglobin, <8.5 mg/dl; absolute neutrophil count, $<1000/cm^3$).

Vector

The vector used was an adenovirus of serotype AD5 that contains the herpes simplex virus-thymidine kinase gene and a Rous sarcoma virus long terminal repeat promoter in the region of the excised E1/E2 wild-type adenoviral genes. The adenoviral vector was constructed as described previously by Chen *et al.* (1994). It was produced in the Baylor College of Medicine gene vector laboratory, in accordance with good manufacturing practice (21 CFR210 and 211). The vector was characterized for purity and potency for clinical use. It was found to be free of adventitious contaminants, including replication-competent adenoviruses at up to 1×10^{10} viral particles (VPs). The infectious unit (IU) titer was calculated at 1×10^{11} IU/ml with a vector particle content of 2×10^{12} VPs/ml; thus, it had

an approximate VP:IU ratio of 20:1 (Nyberg-Hoffman *et al.*, 1997). Once produced, it was stored at $-80°C$ and was diluted to the dose specified for each patient cohort.

The prodrug ganciclovir (Cytovene-Syntex, Palo Alto, CA) is Food and Drug Administration (FDA) approved for treatment of cytomegalovirus (CMV) retinitis in the immunocompromised patient and for prevention of CMV disease in transplant patients at risk. The planned dose used in this study was 5 mg/kg every 12 hr for a total of 28 doses.

Course of treatment

The predominant focus of cancer within the prostate of each patient was identified from the digital rectal examination, transrectal sonogram, and systematic needle biopsy results. Patients were admitted to the hospital on the day of vector injection. After preparation with oral antibiotics (ciprofloxacin 500 mg orally every 12 hr, 24 hr in advance), and a phosphate enema the morning of the injection, all patients were given a single 1-ml injection of vector into the prostate gland in the region judged to have the greatest concentration of tumor-bearing tissue. The dose of ADV/HSV-*tk* viral gene vector was increased for each group of patients. Group 1 (patients 8-1 through 8-4) received an injection of 1×10^8 IU of vector; group 2 (patients 9-1 through 9-5) received 1×10^9 IU of vector; group 3 (patients 10-1 through 10-4) received 1×10^{10} IU of vector; group 4 (patients 11-1 through 11-5) received 1×10^{11} IU of vector. See Table 1 for patient disease status at diagnosis and at study entry.

Parenteral ganciclovir infusions were started 24 hr after vector injection. The protocol included daily urinalysis and cultures for adenovirus in the serum, sputum, and urine, as well as serial hematologic and hepatologic studies. Vector shedding in urine was determined by DNA isolation followed by poly-merase chain reaction (PCR) amplification with vector-specific primers as previously described (Timme *et al.*, 1998).

Tumor progression was evaluated by serum PSA immediately prior to the injection on day 0, and on days 2, 7, and 14; at 6 and 12 weeks; at 6, 9, and 12 months; and every 6 months thereafter. Digital rectal examinations and transrectal sonograms of the prostate were performed at 2, 6, and 12 weeks; at 6, 9, and 12 months; and every 6 months thereafter. Transrectal ultrasound-guided systematic needle biopsies of the prostate were performed at the end of the 2-week course of ganciclovir, at 3 months, and at 1 year. Pathology slides before and after treatment were interpreted by the study pathologist (T.M.W.).

Toxicity was graded according to the Cancer Therapy Evaluation Program (CTEP) common toxicity criteria published by the National Cancer Institute. All patients who experienced grade 3 or greater toxicity were immediately reported to the Institutional Review Board and to the FDA.

RESULTS

Eighteen patients were enrolled in the protocol between August 1996 and August 1997 (see patient profiles in Table 1). No patient has been lost to follow-up. Seven additional patients were evaluated but were ineligible or declined participation: three patients had metastases, one patient chose cryotherapy, two patients did not have evidence of local cancer recurrence by systematic biopsy (each had two biopsy sessions), and one patient had radiation proctitis and was unable to tolerate the transrectal ultrasound probe. The first 11 treated patients were hospitalized for the entire course of treatment. The next five patients were discharged with home health care-assisted ganciclovir infusions after they met the following criteria: (1) no ev-

TABLE 1. PATIENT DISEASE STATUS AT DIAGNOSIS AND AT STUDY ENTRY

| | | Diagnosis | | | | Study entry | | | | |
	Patient	Date (mo/yr)	Clinical stage[a]	Gleason grade	PSA	Age (years)	Date	Clinical stage[a]	Gleason grade	PSA
Group 1:	8-1	12/90	T2	N/A	N/A	72	8/28/96	T2	7	4.2
Dose	8-2	1/89	T2	5	11.7	76	9/25/96	T2	7	17.8
1×10^8	8-3	1/95	T2	7	11.8	57	10/15/96	T2	7	5.7
	8-4	3/93	T2	6	6.6	71	10/15/96	T2	6	4.2
Group 2:	9-1	7/91	T2	N/A	10.7	75	11/12/96	T2	7	5.3
Dose	9-2	8/92	T2	N/A	32.4	61	11/12/96	T1c	7	7.9
1×10^9	9-3	7/93	T2	N/A	8.1	71	11/12/96	T2	7	10.5
	9-4	7/93	T2	8	23	70	12/5/96	T2	8	9.4
	9-5	10/91	T2	N/A	N/A	71	12/5/96	T2	6	3.3
Group 3:	10-1	1/90	T2	6	24.1	76	1/14/97	T2	7	6.1
Dose	10-2	10/89	T2	4	17.6	62	1/28/97	T2	7	9.2
1×10^{10}	10-3	8/92	T2	7	11.1	63	3/31/97	T2	8	3.3
	10-4	2/90	T2	5	30.3	73	4/30/97	T1c	8	10.0
Group 4:	11-1	2/91	T3	7	15.2	73	5/22/97	T2	7	12.5
Dose	11-2	5/94	T2	8	94	57	6/2/97	T2	7	13.8
1×10^{11}	11-3	6/95	T2	6	11.2	72	6/11/97	T3	7	6.5
	11-4	1/94	T3	8	19.3	69	6/26/97	T2	8	4.4
	11-5	7/87	T3	6	N/A	69	8/14/97	T2	7	13.9

[a]Stage is UICC clinical TNM stage.

TABLE 2. SUMMARY OF TOXICITY DURING PHASE I GENE THERAPY CLINICAL TRIAL
FOR RECURRENT PROSTATE CANCER

Patient	Toxicity	Grade	Outcome
9-1	GGT (<1.5 times normal)	1	Resolved following completion of ganciclovir
10-1 and 10-2	Shaking chills	1	Resolved within 24 hr
10-4	Cellulitis	2	Resolved with intravenous antibiotics
11-1 and 11-2	Mild ALT/AST elevation	1	Resolved spontaneously following completion of ganciclovir treatments
11-3	Fever	2	Resolved with intravenous antibiotics
11-5	Fever, GGT, and LDH elevation	2	Resolved with intravenous antibiotics; ganciclovir started within 48 hr
	Total bilirubin, ALT/AST, alkaline phosphatase elevations	3	Resolved with early termination of ganciclovir (7 days)
	Thrombocytopenia	4	Resolved; required platelet transfusions for 5 days

Abbreviations: GGT, γ-Glutamyltransaminase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

idence of adenoviral vector as determined by PCR in two consecutive urine samples and (2) no evidence of toxicity.

Toxicity

No patient in group 1 experienced any toxicity; toxicity levels for the first 17 patients were grade 2 or lower (see Table 2). One patient experienced a temperature of 103.3°F (grade 2 toxicity) the night of vector injection. The patient was placed on parenteral gentamicin, ampicillin, and Flagyl (metronidazole); he promptly defervesced and completed a 1-week course of oral ciprofloxacin. All blood, urine, and nasal cultures were negative for bacterial, fungal, and viral organisms. Parenteral ganciclovir was started 72 hr after vector injection, and the patient completed the full 28-dose course without further sequelae. Another patient developed cellulitis (grade 2 toxicity) at an intravenous site that required readmission and treatment with parenteral antibiotics.

One patient (11-5) experienced grade 4 toxicity. The patient was injected with 1×10^{11} IU of adenoviral vector and developed a fever of 101.5°F early the following morning. He was placed on intravenous ampicillin, gentamicin, and Flagyl for 48 hr and promptly defervesced. A 1-week course of oral ciprofloxacin was then prescribed. All nasal, urine, and blood cultures were negative for bacterial, viral, and fungal infection, and all hematologic parameters were normal at this time. Parenteral ganciclovir infusions were started 72 hr after vector injection. On the second day of ganciclovir, the patient complained of myalgias and developed a low-grade fever of 100.2°F. Physical examination and laboratory tests remained within normal limits. On the seventh day of ganciclovir infusion, however, the patient was again febrile to 100.8°F and complained of myalgias. Tests showed thrombocytopenia of 52,000 platelets per microliter, leukocytosis of 22,000 lecukocytes per microliter, and abnormal liver function tests (alanine aminotransferase [ALT], 94 mg/dl; aspartate aminotransferase [AST],
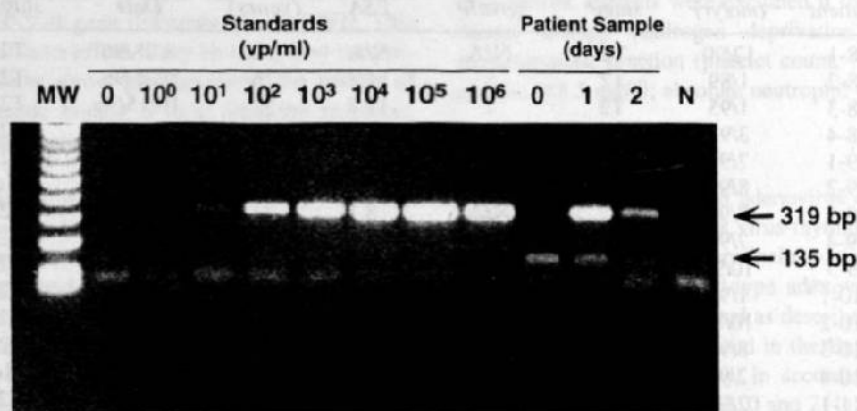

FIG. 1. Ethidium bromide-stained gel for visualization of polymerase chain reaction (PCR) products to determine extent of viral shedding in patient urine (patient 10-3). Patient sample days correspond to the day before vector delivery (day 0) and the first and second days after vector delivery. 319 bp, the size of the RSV/HSV-*tk* amplified junction for detection of vector targets; 135 bp, size of K-*ras* amplified product (internal control); N, negative control (water); vp/ml, vector particles per milliliter; MW, molecular weight standard.

117 mg/dl; alkaline phosphatase, 129 mg/dl; and lactate dehydrogenase [LDH], 1059 units per liter). The ganciclovir was discontinued, as were all other medications (gemfibrozil). The platelet count subsequently fell to 10,000 per microliter. Platelet levels were supported with five platelet transfusions for what appeared to be peripheral consumption. By day 16, the leukocyte count had returned to normal, the patient's somatic symptoms had resolved, and liver function tests and platelet count had improved.

The patient never appeared toxic and had no evidence of bleeding associated with his low platelet count. A full evaluation for sepsis and disseminated intravascular coagulopathy were negative. A CT scan of the abdomen and pelvis revealed no evidence of abdominal or pelvic pathology, and a bone marrow biopsy was normal. These findings are not typical of ganciclovir toxicity.

Vector shedding

Daily viral cultures for wild-type adenovirus in serum, ear, and nasal swabs were consistently negative in all patients.

Four or more consecutive daily urine samples for each patient were examined for the presence of adenoviral vector by PCR for the thymidine kinase gene (see Fig. 1). Vector detection in urine was dose dependent. Urine tests (test sensitivity of 10 to 100 viral particles per milliliter) were negative for the viral vector in the lowest dose group (1×10^8 IU). At 1×10^9 IU, two of five patients were positive on day 1 only (Table 3). At 1×10^{10} IU, three of four were positive, one until day 11. And at 1×10^{11} IU all five patients were positive, one until day 32.

One patient of the 18, from group 4, was able to produce a semen sample for analysis. The sample was positive for vector by PCR at 14 days postinjection.

Efficacy

Serum PSA levels (normal 0–4 ng/ml) were obtained on the day of study entry and on days 2, 5, 7, and 14; at 6 and 12 weeks; and at 6, 9, and 12 months following entry into the study. Figure 2 plots the serum PSA levels in each patient from 100 days before study entry until 1 year after, or until subsequent anticancer treatment was initiated. All patients had a rising serum PSA level before study entry. There was no immediate rise in serum PSA in response to vector injection into the prostate. No changes in PSA levels were noted at the lowest vector dose level (group 1, 1×10^8) (Fig. 2A). At 1×10^9, 1 patient (9-3, Fig. 2B) exhibited a fall in PSA levels from 10.5 ng/ml at study entry to 4.6 ng/ml 12 weeks later. Subsequently, the levels steadily rose. One patient at 1×10^{10} (patient 10-1) showed a precipitous fall from 6.1 ng/ml to a nadir of 3.0 ng/ml at 6 weeks. At the highest dose level (group 4) the first patient (11-1) showed an immediate decline from 12.5 ng/ml to a nadir of 4.8 at 12 weeks. The PSA remained less than 50% of the level at entry 1 year later (Fig. 2D). In summary, three patients (1 each from groups 2, 3, and 4) had a greater than 50% decrease in PSA levels lasting from 6 weeks to more than 1 year.

Digital rectal examination and transrectal ultrasound were performed on all patients at routine intervals. No consistent changes were noted in the size of the prostate over time. Of note was the appearance of a cystic cavity at the site of vector injection detected in patient 11-1 at 6 months postinjection (Fig.

TABLE 3. LAST DAY OF VECTOR-POSITIVE URINE AS DETERMINED BY PCR

Patient	Post-injection day
8-1	Never positive
8-2	Never positive
8-3	Never positive
8-4	Never positive
9-1	Never positive
9-2	Never positive
9-3	1
9-4	Never positive
9-5	1
10-1	11
10-2	Never positive
10-3	4
10-4	6
11-1	14
11-2	9
11-3	11
11-4	32
11-5	4

3A and B). Systematic needle biopsies of the prostate were performed at 2 weeks, 12 weeks, and 1 year. No patient had complete histologic regression of cancer. In one patient at the highest dose level (patient 11-1) the biopsy at 2 weeks showed extensive necrosis (Fig. 3D). However, the subsequent biopsy at 12 weeks showed persistent cancer.

Several patients have been treated for progressive prostate cancer subsequent to this phase I trial. Patients 8-3 and 8-4 had a salvage radical prostatectomy for pathologic T3N0M0 prostate cancer. The surgical margins were negative. Hormonal manipulation (androgen ablation) was initiated in three patients for rising serum PSA level (9-4), lymph node metastases on CT scan (10-2), and visceral metastases on ProstaScint (Cytogen Corporation, Princeton, NJ) scan (11-2).

DISCUSSION

This clinical trial was the result of a systematic plan to develop gene therapy for prostate cancer by our research group. Woo and colleagues modified HSV-*tk* plus ganciclovir for use with an adenoviral vector with activity when injected *in situ* into a variety of tumors in animal models (Chen *et al.*, 1994). Thompson and colleagues demonstrated activity of this form of HSV-*tk*/GCV gene therapy against mouse and human prostate cancer cell lines *in vitro* (Eastham *et al.*, 1996). Using cell lines from the mouse prostate reconstitution (MPR) model of prostate cancer, this group next demonstrated growth suppression of the local tumor and prolongation of survival in an orthotopic model system (Hall *et al.*, 1997). These effects were enhanced by concomitant androgen ablation (Hall *et al.*, 1999). Remarkably, a single course of gene therapy administered *in situ* into the tumor in the prostate also led to systemic activity against spontaneous and induced metastases (Hall *et al.*, 1997). There was no systemic toxicity or evidence of toxic effects in other genitourinary organs and no evidence of vector in sperm in these mice (Timme *et al.*, 1998).
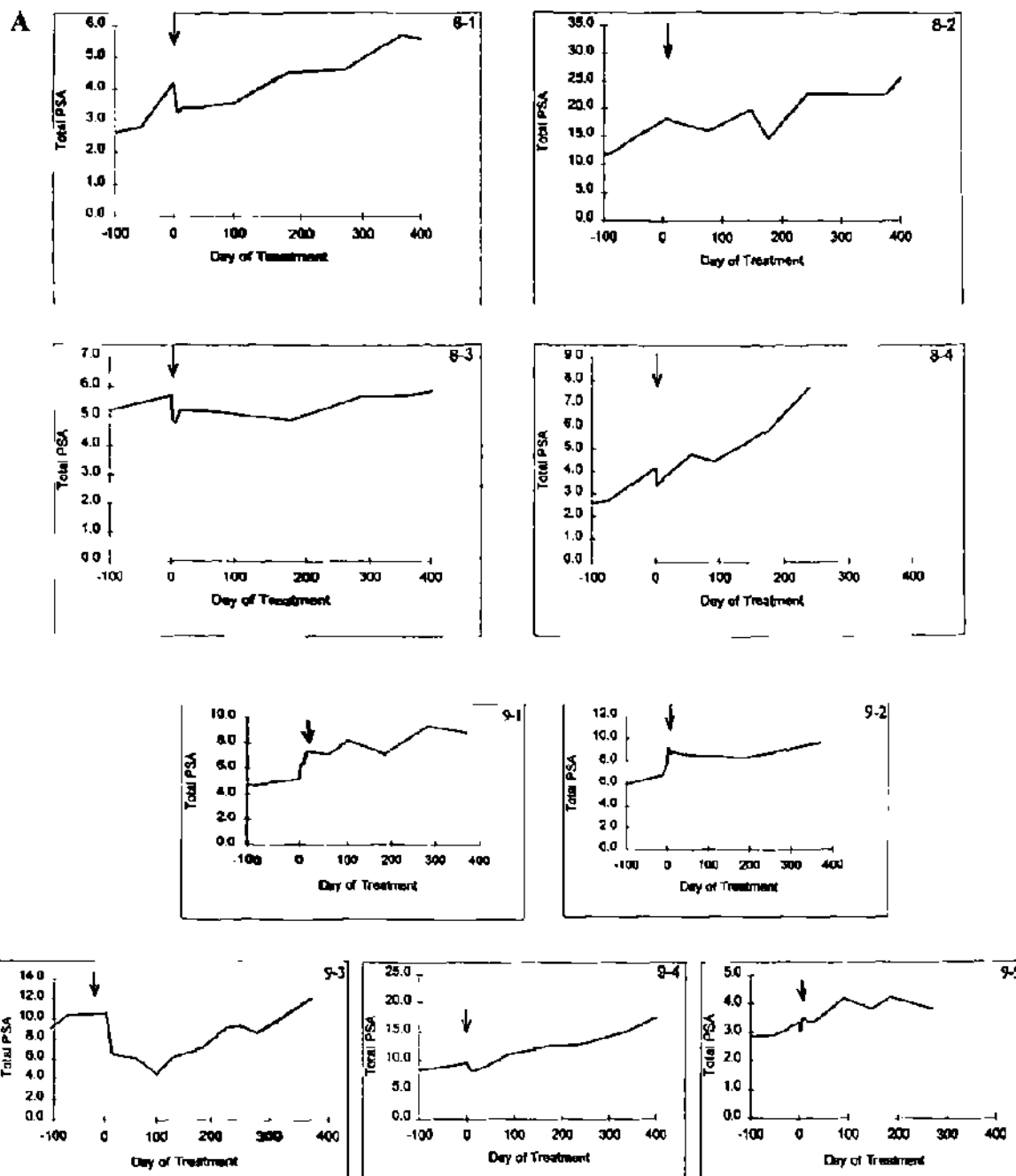
FIG. 2. Prostate-specific antigen (PSA) values of the study patients, beginning 100 days prior to study entry and continuing through the last evaluation. All values are in nanograms per milliliter. Downward arrows indicate date of injection of vector. Patient data are reported by vector dose group. All patients received a single injection of vector. A. Patients 8-1 through 8-4 received 1 × 10⁸ infectious units (IU) vector. B. Patients 9-1 through 9-5 received 1 × 10⁹ IU vector.

As a result of this favorable preclinical experience, we initiated a phase I dose escalation trial of ADV/HSV-*tk* with the intravenous administration of ganciclovir, selecting men with local recurrence of prostate cancer after definitive irradiation therapy for whom no widely accepted standard form of therapy is available. Alternatives include salvage radical prostatectomy or cryotherapy, which carry substantial risks, and watchful waiting or androgen ablation, which are palliative (Rogers *et al.*, 1995). The biological activity of prostate cancer in this setting

can be demonstrated by a consistently rising serum PSA level and biopsy evidence of viable cancer. Local rather than distant recurrence can be verified by bone scintigraphy, abdominal and pelvic CT or MRI imaging, and in selected cases by a monoclonal antibody scan (ProstaScint). Since ADV/HSV-*tk*/GCV therapy cannot be administered systemically, we wanted to investigate the safety and efficacy of this form of gene therapy given intralesionally in men without clinically apparent metastases. With no previous information about intraprostatic injec-
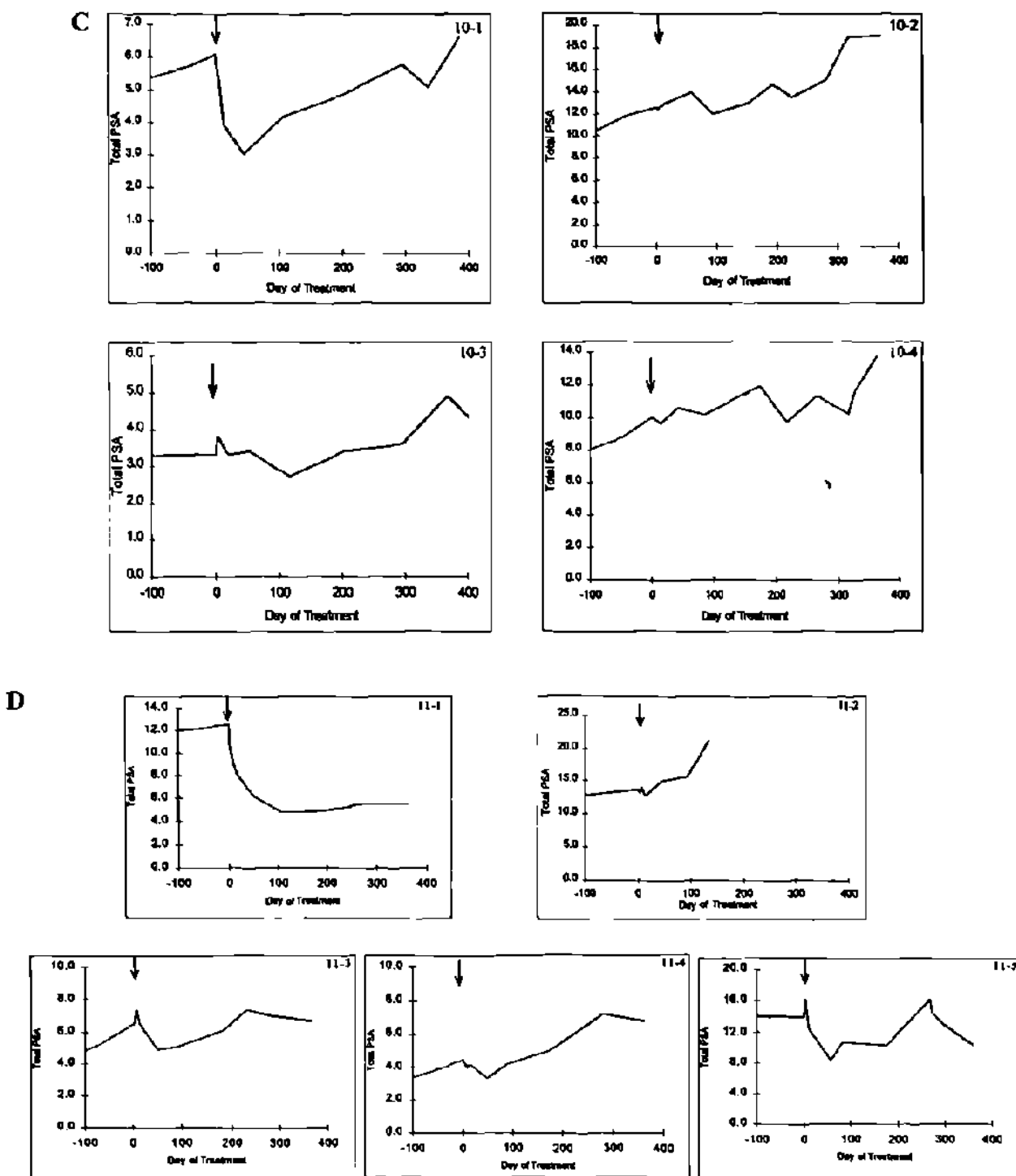
FIG. 2. *Continued.* **C.** Patients 10-1 through 10-4 received 1×10^{10} IU vector. **D.** Patients 11-1 through 11-5 received 1×10^{11} IU vector.

tions of ADV/HSV-*tk*, we did not want to treat men with newly diagnosed prostate cancers, for whom several well-defined management strategies are available (Scardino and Grayhack, 1994).

We completed the trial in 12 months, enrolling 18 patients. The regimen proved safe, with minimal toxicity in the first 17 of 18 planned patients (Table 2). One patient at the highest dose

level (1×10^{11} IU) experienced grade 4 toxicity with thrombocytopenia and hepatotoxicity, possibly related to extravasation of the vector intravascularly during the injection. These complications resolved and this patient had no further sequelae. One of these patients (11-1) developed a cystic cavity within the prostate, evident sonographically in the area of the intralesional injection, that persisted during 9 months of follow-
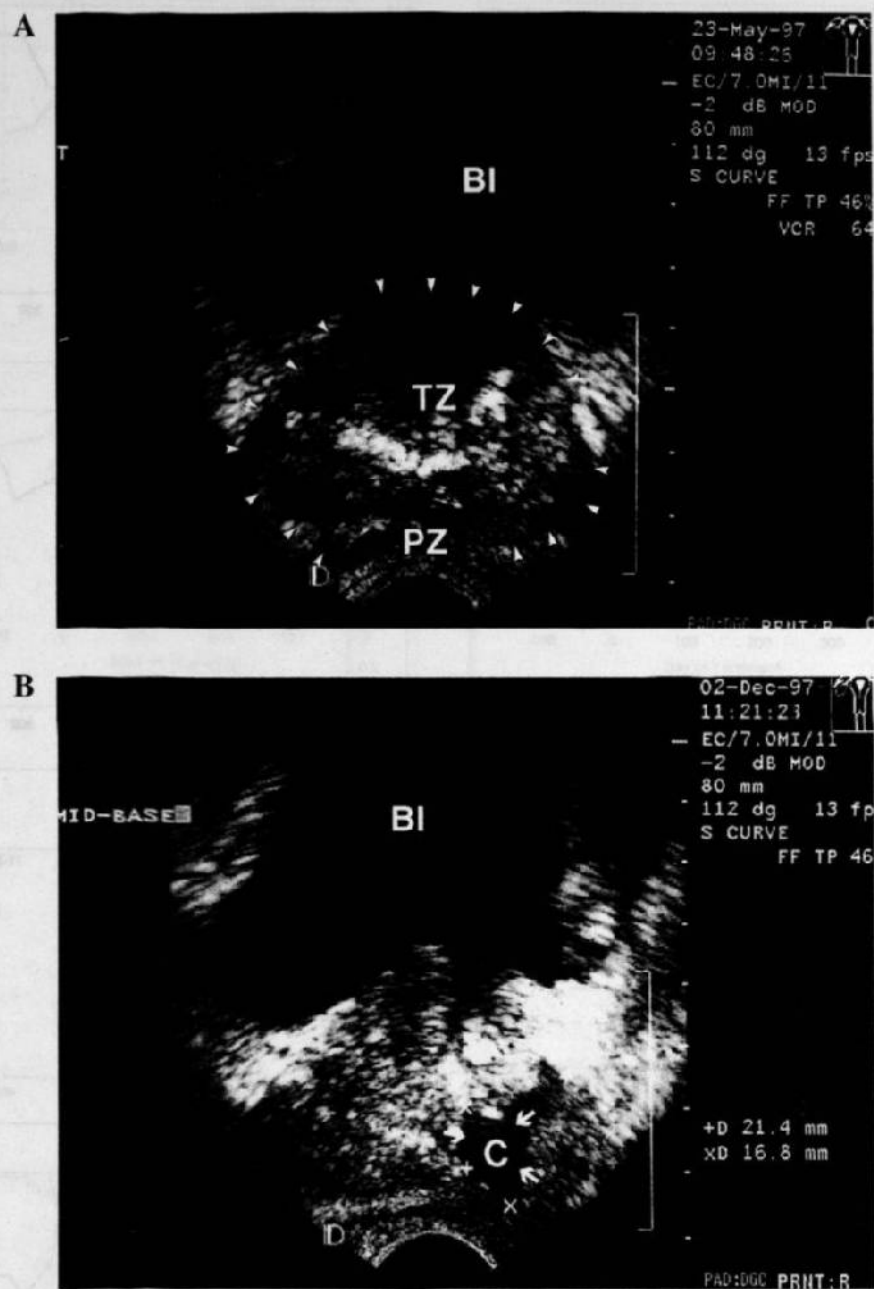
FIG. 3. Transrectal ultrasound and biopsy of patient 11-1, who had an objective response to the therapy. (Bl, Bladder; TZ, transition zone of the prostate; PZ, peripheral zone of the prostate.) **(A)** Transverse view of prostate following vector injection (arrowheads outline border of prostate). **(B)** Transverse view of prostate 6 months after therapy shows cystic cavity (C) at the site of vector injection (arrows, + and × symbols indicate cystic area).

up. Since only one of five patients experienced grade 3 or 4 toxicity at 1×10^{11} IU, we believe that dose is safe when HSV-*tk* is given as a single injection into the prostate.

HSV-*tk* plus ganciclovir has been shown to be safe in clinical trials in patients with brain tumors, ovarian cancer, and mesothelioma. We are not aware, however, of other published trials of gene therapy that show evidence of activity against human prostate cancer (Scardino *et al.*, 1998). Three patients (9-3, 10-1, and 11-1) had a decrease in serum PSA levels by

more than 50% for periods ranging from 45 to 330 days despite steadily increasing PSA levels for more than 100 days before treatment (Fig. 2). No patient received any other anticancer therapy during the trial.

A decline in the serum marker PSA by more than 50% over three consecutive measurements two or more weeks apart has been associated with prolonged survival in several clinical trials of systemic therapy for hormone refractory prostate cancer (Kelly *et al.*, 1996), and seems a reasonable indicator of re-
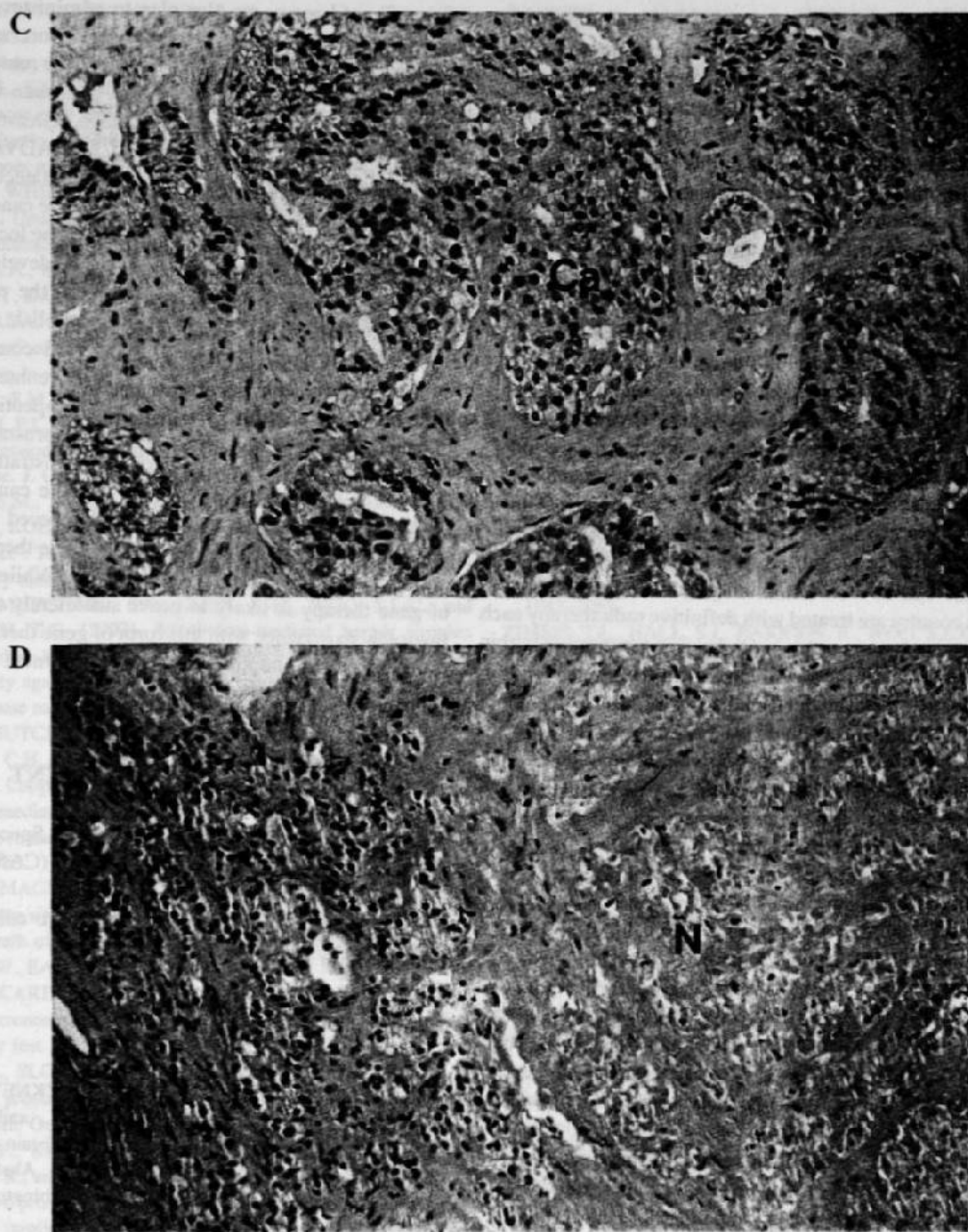
FIG. 3. *Continued.* (C) Photomicrograph of pretreatment biopsy of the left midportion of the prostate shows viable cancer (Ca). (D) Necrotic tumor tissue (N) on biopsy of the same region 2 weeks following vector injection. Original magnification: ×100.

sponse in this trial. PSA is less valid as a marker of response when antiandrogen therapy is first introduced, since this hormonal manipulation may block expression of PSA as well as reduce tumor mass (Arai *et al.*, 1990). While serial transrectal sonograms showed no consistent changes in overall prostate size over time, even among those who responded with a decrease in PSA levels, ultrasound was not sensitive enough to evaluate changes in tumor size in these patients (Egawa *et al.*, 1992). We also measured serial changes in the length of the tumor in the systematic biopsy specimens. Again, this parameter did not provide consistent results.

Despite the apparent success of this trial, there are several

caveats. We did not eradicate prostate cancer from any patient. Other, well-described forms of therapy can effectively remove the local tumor in similar patients, albeit with appreciable risk of morbidity (Rogers *et al.*, 1995). We were not able to evaluate whether ADV/HSV-*tk* gene therapy had any antimetastatic effect in these patients, and it is not clear whether ADV/HSV-*tk* gene therapy will also be active against hormone-refractory prostate cancer. Also, we did not investigate the mechanism of activity in this trial. A direct, mechanical effect of intralesional injection of 1 ml seems unlikely. In our animal studies, intralesional injection of vector alone in a volume comparable to the size of the orthotopic tumor was not effective in controlled trials.

Also in this article, we do not include an evaluation of the efficacy of gene transfer or of immunological response to HSV-*tk* plus GCV therapy. We had previously reported these activities in preclinical studies using *in vivo* mouse prostate cancer models (Eastham *et al.*, 1996; Hall *et al.*, 1997). We intend to compare the efficacy of gene transfer in this selected subgroup of patients with others as well as to evaluate an extended immunological response to this gene therapy protocol and report these data at a later time. Therefore, to what extent any antitumor effect can be attributed to the direct cytotoxicity or to immunological mechanisms remains under investigation. However, it seems unlikely that the sustained response seen in patient 11-1 would have been due to direct cytotoxicity alone.

We selected for this investigational protocol patients with local recurrence of prostate cancer after definitive irradiation therapy because no standard therapy is widely considered safe and effective in this situation. About one-third of men treated for clinically localized prostate cancer each year are treated with definitive radiotherapy. In 1998, 184,500 new cases of prostate cancer are expected to be diagnosed (Landis *et al.*, 1998); about 80% of these will be localized at diagnosis. More than 50,000 men in this country are treated with definitive radiotherapy each year. Of these, at least one-third will develop progression within 5 years, and about half of these will have local recurrence only with no evidence of metastases (Kupelian *et al.*, 1997). Thus, we estimate that some 9000 patients per year face the dilemma of local recurrence of cancer after definitive radiotherapy.

Prostate cancer, among the solid tumors, may be particularly suitable for gene therapy. The organ is expendable after reproductive needs are met, the primary tumor is readily accessible both for intralesional injections and for repeat biopsies, and a well-characterized circulating marker of response (PSA) is readily available. Gene therapy appears to cause little morbidity and could be applied repeatedly to palliate these slow-growing cancers long enough for patients to live out their natural lives even if the cancer is not completely eradicated. In addition, limited, occult micrometastases—the major cause of treatment failure—may be particularly susceptible to gene therapy strategies that augment the immunogenicity of tumor cells and thereby elicit systemic anti-tumor immunity (Hall *et al.*, 1997, 1999). Intraprostatic injections can be administered without anesthesia in an outpatient sonography room, ganciclovir can be administered intravenously in an outpatient infusion center, and the patients can be monitored in the outpatient clinic; thus the expense of this therapeutic regimen is minimal. If this form of gene therapy can be shown to be effective in larger phase II trials, other possibilities for its use might include treatment in conjunction with androgen ablation before radiation therapy, and primary treatment for low-risk cancers in young men or for high-risk cancers in older men or those with serious comorbid conditions.

While we saw some evidence of acitivity in this phase I trial, intralesional injection at a single site is unlikely to provide adequate control of many prostate cancers. Since cancer of the porstate is multifocal, and the location of cancer within the prostate cannot be determined accurately, optimal therapy will likely require a uniform distribution of the vector throughout the gland. Consequently, we have extended the clinical trial to explore the safety of multiple sites of injection and repeat injections. Since the distribution of the vector around a line of in-

jection is not known, we also plan to administer two different doses of the vector alone several days before radical prostatectomy to examine its distribution within the removed prostate. In an additional trial, we also plan to treat men with high-risk cancers (defined by their clinical stage, Gleason grade, and serum PSA level) (Kattan *et al.*, 1998), with ADV/HSV-*tk*/GCV gene therapy 4–6 weeks prior to radical prostatectomy. The distribution of necrosis and apoptosis within the cancer will be determined along with a detailed analysis of the local immune response. These studies should allow us to develop an optimal strategy for delivery of the vector within the prostate. Additional preclinical studies should also establish a rationale for the use of ADV/HSV-*tk*/GCV therapy in combination with other gene therapy approaches designed to enhance and/or sustain the local and possible systematic therapeutic effects.

In conclusion, gene therapy via an intraprostatic injection of ADV/HSV-*tk* with the intravenous administration of the prodrug GCV is safe in patients with prostate cancer. Early evidence of efficacy suggests we should proceed with trials that will evaluate the utility of this form of gene therapy as primary or adjuvant treatment for prostate cancer. While no single form of gene therapy is likely to prove sufficiently efficacious, our favorable experience with this form of gene therapy for prostate cancer may have implications for the treatment of other human malignancies.

ACKNOWLEDGMENT

REFERENCES

ALAVI, J.B., JUDY, K., ALAVI, A., HACKNEY, D., PHILLIPS, P., SMITH, J., RECIO, A., WILSON, J., and ECK, S. (1998). Phase I trial of gene therapy in primary brain tumors. American Society of Gene Therapy/Program and Abstracts, 1st Annual Meeting, May 28–31, 1998, Seattle, Washington, 112a. [Abstract 444]

ALVAREZ, R.D., and CURIEL, D.T. (1997). A phase I study of recombinant adenovirus vector-mediated intraperitoneal delivery of herpes simplex virus thymidine kinase (HSV-*tk*) gene and intravenous ganciclovir for previously treated ovarian and extraovarian cancer patients. Hum. Gene Ther. 8, 597–613.

ARAI, Y., YOSHIKI, T., and YOSHIDA, O. (1990). Prognostic significance of prostate specific antigen in endocrine treatment for prostatic cancer. J. Urol. 144, 1415–1418.

BACCHETTI, S., and GRAHAM, F. (1993). Inhibition of cell proliferation by an adenovirus vector expressing the human wild type p53 protein. Int. J. Oncol. 3, 781–788.

CHEN, S.-H., SHINE, H.D., GOODMAN, J.C., GROSSMAN, R.G., and WOO, S.L.C. (1994). Gene therapy for brain tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. Proc. Natl. Acad. Sci. U.S.A. 91, 3054–3057.

EASTHAN, J.A., HALL, S.J., SEHGAL, I., WANG, J., TIMME, T.L., YANG, G., CONNEL-CROWLEY, L., ELLEGE, S.J., ZHANG, W.-W., HARPER, J.W., and THOMPSON, T.C. (1995). *In vivo* gene

therapy with p53 and p21 adenovirus for prostate cancer. Cancer Res. 55, 5151–5155.

EASTHAM, J.A., CHEN, S.-H., SEHGAL, I., YANG, G., TIMME, T.L., HALL, S.J., WOO, S.L.C., and THOMPSON, T.C. (1996). Prostate cancer gene therapy: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. Hum. Gene Ther. 7, 515–523.

EGAWA, S., WHEELER, T.M., GREENE, D.R., and SCARDINO, P.T. (1992). Unusual hyperechoic appearance of prostate cancer on transrectal ultrasonography. Br. J. Urol. 69, 169–174.

ELSHAMI, A.A., SAAVEDRA, A., ZHANG, H., KUCHARCZUK, J.C., SPRAY, D.C., FISHMAN, G.I., AMIN, K.M., KAISER, L.R., and ALBELDA, S.M. (1996). Gap junctions play a role in the "bystander effect" of the herpes simplex virus thymidine kinase/ganciclovir system *in vitro*. Gene Ther. 3, 85–92.

FREEMAN, S.M., WHARTENBY, K.A., KOEPLIN, D.S., MOOLTEN, F.L., ABBOUD, C.N., and ABRAHAM, G.N. (1992). Tumor regression when a fraction of the tumor mass contains the HSV-*tk* gene. J. Cell Biochem. 16F, 47.

FREEMAN, S.M., ABBOUD, C.N., WHARTENBY, K.A., PACKMAN, C.H., KOEPLIN, D.S., and MOOLTEN, F.L. (1993). The "bystander effect": Tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res. 53, 5274–5283.

HALL, S.J., MUTCHNIK, S.E., CHEN, S.-H., WOO, S.L.C., and THOMPSON, T.C. (1997). Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. Int. J. Cancer 70, 183–187.

HALL, S.J., MUTCHNIK, S.E., YANG, G., TIMME, T.L., NASU, Y., BANGMA, C.H., WOO, S.L.C., SHAKER, M., and THOMPSON, T.C. (1999). Cooperative therapeutic effects of androgen ablation and adenovirus-mediated herpes simplex virus-thymidine kinase gene and ganciclovir therapy in experimental prostate cancer. Cancer Gene Ther. 6, 54–63.

HAMEL, W., MAGNELLI, L., CHIARUGI, V.P., and ISRAEL, M.A. (1996). Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. Cancer Res. 56, 2697–2702.

KATTAN, M.W., EASTHAM, J.A., STAPLETON, A.M.F., WHEELER, T.M., and SCARDINO, P.T. (1998). A preoperative nomogram predicting recurrence after radical prostatectomy for prostate cancer. J. Natl. Cancer Inst. 90, 766–771.

KELLY, W.K., SLOVIN, S., and SHER, H.I. (1996). Clinical use of posttherapy prostate-specific antigen changes in advanced prostate cancer. Semin. Oncol. 23, 8–14.

KUPELIAN, P., KATCHER, J., LEVIN, H., ZIPPE, C., SUH, J., MACKLIS, R., and KLEIN, E. (1997). External beam radiotherapy versus radical prostatectomy for clinical stage T1–2 prostate cancer: Therapeutic implications of stratification by pretreatment PSA levels and biopsy Gleason scores. Cancer J. Sci. Am. 3, 78–87.

LANDIS, S.H., MURRAY, T., BOLDEN, S., and WINGO, P.A. (1998). Cancer statistics, 1998. CA Cancer J. Clin. 48, 6–29.

MOOLTEN, F.L. (1986). Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. Cancer Res. 46, 5276–5281.

MOOLTEN, F.L., and WELLS, J.M. (1990). Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. J. Natl. Cancer Inst. 82, 297–300.

NYBERG-HOFFMAN, C., SHABRAM, P., LI, W., GIROUX, D., and AGUILAR-CORDOVA, E. (1997). Sensitivity and reproducibility in adenoviral infectious titer determination. Nature Med. 3, 808–811.

RAMESH, R., MARROJI, A.J., MUNSHI, A., ABBOUD, C., and FREEMAN, S.M. (1996a). *In vivo* analysis of the "bystander effect": A cytokine cascade. Exp. Hematol. 24, 829–838.

RAMESH, R., MARROJI, A.J., MUNSHI, A., ABBOUD, C., and FREEMAN, S.M. (1996b). Expression of costimulatory molecules: B7 and ICAM up-regulation after treatment with a suicide gene. Cancer Gene Ther. 3, 373–384.

ROGERS, E., OHORI, M., KASSABIAN, V.S., WHEELER, T.M., and SCARDINO, P.T. (1995). Salvage radical prostatectomy: Outcome measured by serum prostate specific antigen levels. J. Urol. 153, 104–110.

SCARDINO, P.T., and GRAYHACK, J.T. (guest eds.). (1994). Detection and treatment of early stage prostate cancer. J. Urol. 152(Suppl.), 1677–1940.

SCARDINO, P.T., ADLER, H.L., HERMAN, J.R., WOO, S.L.C., AGUILAR-CORDOVA, E., TIMME, T.L., and THOMPSON, T.C. (1998). Herpes simplex virus-thymidine kinase (HSV-*tk*) mediated gene therapy is safe and active against locally recurrent prostate cancer after irradiation therapy. ASCO Program/Proc. 17, 308a. [Abstract 1186]

STERMAN, D.H., TREAT, J., LITZKY, L.A., AMIN, K.M., COONROD, L., MOLNAR-KIMBER, K., RECIO, A., KNOX, L., WILSON, J.M., ALBELDA, S.M., and KAISER, L.R. (1998). Adenovirus-mediated herpes simplex virus thymidine kianse/ganciclovir gene therapy in patients with localized malignancy: Results of a phase I clinical trial in malignant mesothelioma. Hum. Gene Ther. 9, 1083–1092.

TIMME, T.L., HALL, S.J., BARRIOS, R., WOO, S.L.C., AGUILAR-CORDOVA, E., and THOMPSON, T.C. (1998). Local inflammatory response and vector spread after direct intraprostatic injection of a recombinant adenovirus containing the herpes simplex virus thymidine kinase gene and ganciclovir therapy in mice. Cancer Gene Ther. 5, 74–82.

VIEWEG, J., ROSENTHAL, F.M., BANNERJI, R., HESTON, W.D.W., FAIR, W.R., GANSBACHER, B., and GILBOA, E. (1994). Immunotherapy of prostate cancer in the Dunning rat model: Use of cytokine gene modified tumor vaccines. Cancer Res. 54, 1760–1765.

VILE, R.G., NELSON, J.A., CASTLEDEN, S., CHONG, H., and HART, I.R. (1994). Systemic gene therapy of murine melanoma using tissue specific expression of the HSV-*tk* gene involves an immune component. Cancer Res. 54, 6228–6234.

WILLS, K.N., MANEVAL, D.C., MENZEL, P., HARRIS, M.P., SUTJIPTO, S., VAILLANCOURT, M.-T., HUANG, W.-M., JOHNSON, D.E., ANDERSON, S.C., WEN, S.F., BOOKSTEIN, R., SHEPARD, H.M., and GREGORY, R.J. (1994). Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. Hum. Gene Ther. 5, 1079–1088.

ZHANG, W.-W., FANG, X., MAZUR, W., FRENCH, B.A., GEORGES, R.N., and ROTH, J.A. (1994). High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. Cancer Gene Ther. 1, 5–13.

Address reprint requests to:
Dr. Peter T. Scardino
c/o Irene Albright, Medical Editor
Scott Department of Urology
6560 Fannin Street, Suite 2100
Houston, TX 77030
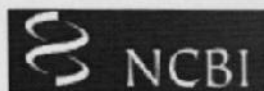
Search PubMed ▼ for

☐ Limits   Preview/Index   History   Clipboard
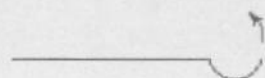
Abstract ▼

☐ **1** : *Anticancer Res* 1999 May-Jun;19(3B):2163-5

Thymidine kinase (TK) gene therapy of solid tumors: valacyclovir facilitates outpatient treatment.

Hasenburg A, Tong XW, Rojas-Martinez A, Nyberg-Hoffman C, Kieback CC, Kaplan AL, Kaufman RH, Ramzy I, Aguilar-Cordova E, Kieback DG

Department of Obstetrics and Gynecology, Freiburg University Medical Center, Germany.

BACKGROUND: In a Phase I study replication-deficient adenovirus containing the herpes simplex virus (HSV) thymidine kinase (TK) gene (AdV-HSV-TK) was instilled intraperitoneally in patients with recurrent ovarian cancer. Patients were treated with Acyclovir (ACV) or Valacyclovir (VCV) as enzymatic substrates. The purpose of this study was to compare serum levels of ACV and VCV. PATIENTS AND METHODS: The antiherpetic prodrug and Topotecan (1.0 mg/m2 over 30 minutes each day for 5 days) were started 24 hours after vector application. Eight patients received ACV (15 mg/kg i.v. over one hour every 8 hours for 42 doses), two patients were started on ACV for 5 days and then switched to oral VCV (2 g every 8 hours for a total of 42 doses). Blood samples were obtained 20 minutes prior to each drug. RESULTS: Serum levels of ACV and VCV (converted to ACV) were comparable. CONCLUSIONS: Suicide gene therapy with TK is under investigation in a

variety of solid tumors. Replacing ACV by VCV will offer a cost-effective alternative and will significantly reduce duration of hospital stay improving quality of life and facilitating an outpatient gene therapy concept.

Publication Types:

- Clinical trial
- Clinical trial, phase i
- Controlled clinical trial

PMID: 10472325, UI: 99401545

Abstract

Phase I Study of Adenoviral Delivery of the HSV-tk Gene and Ganciclovir Administration in Patients with Recurrent Malignant Brain Tumors

Todd W. Trask,[*,1] Rebecca P. Trask,[*] Estuardo Aguilar-Cordova,[†,‡]
H. David Shine,[*,§,¶] Philip R. Wyde,[‖] J. Clay Goodman,[*,**,††]
Winifred J. Hamilton,[*] Augusto Rojas-Martinez,[†,‡] Shu-Hsia Chen,[‡‡]
Savio L. C. Woo,[‡‡] and Robert G. Grossman[*]

*Department of Neurosurgery, †Department of Pediatrics, **Department of Neurology, §Department of Molecular and Cellular Biology,
‖Department of Microbiology and Immunology, ††Department of Pathology, ¶Division of Neuroscience, and
‡Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas 77030
‡‡Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, New York 10029

Between December 1996 and September 1998, 13 patients with advanced recurrent malignant brain tumors (9 with glioblastoma multiforme, 1 with gliosarcoma, and 3 with anaplastic astrocytoma) were treated with a single intratumoral injection of 2×10^9, 2×10^{10}, 2×10^{11}, or 2×10^{12} vector particles (VP) of a replication-defective adenoviral vector bearing the herpes simplex virus thymidine kinase gene driven by the Rous sarcoma virus promoter (Adv.RSVtk), followed by ganciclovir (GCV) treatment. The VP to infectious unit ratio was 20:1. Our primary objective was to determine the safety of this treatment. Injection of Adv.RSVtk in doses ≤2×10^{11} VP, followed by GCV, was safely tolerated. Patients treated with the highest dose, 2×10^{12} VP, exhibited central nervous system toxicity with confusion, hyponatremia, and seizures. One patient is living and stable 29.2 months after treatment. Two patients survived >25 months before succumbing to tumor progression. Ten patients died within 10 months of treatment, 9 from tumor progression and 1 with sepsis and endocarditis. Neuropathologic examination of postmortem tissue demonstrated cavitation at the injection site, intratumoral foci of coagulative necrosis, and variable infiltration of the residual tumor with macrophages and lymphocytes.

Key Words: gene therapy; HSV-tk; ganciclovir; glioblastoma; astrocytoma; adenovirus; stereotaxic technique.

INTRODUCTION

The American Cancer Society estimates that 16,800 individuals will develop and 13,100 individuals will die from primary tumors of the nervous system in 1999, with the majority of these cases being malignant gliomas (1). Although there have been significant advances in neurosurgery, neuroradiology, radiation therapy, and medical oncology, malignant brain tumors remain resistant to all current therapies, and survival is often less than 1 year (2–4). Even when conventional treatments extend survival, malignant brain tumors almost invariably recur because of surviving tumor cells that have invaded the surrounding brain. All treatments must balance effective tumor killing against toxicity to surrounding neural tissue. The prognosis after recurrence of a malignant brain tumor is particularly poor (3, 5).

Transduction of tumor cells with the herpes simplex virus thymidine kinase (HSV-tk) gene, which activates the nucleoside analog prodrug ganciclovir (GCV), has been one of the most effective approaches in treating experimental brain tumors (6–12). This therapy selectively kills dividing cells, an advantageous feature in the brain where most normal cells are not actively dividing. Treatment efficacy may be enhanced by the "bystander effect," whereby surrounding nontransduced tumor cells are also killed through a variety of mechanisms which are believed to include transfer of toxic metabolites via gap

[1] To whom correspondence should be addressed at Department of Neurosurgery, Baylor College of Medicine, 6560 Fannin, Suite 944, Houston, TX 77030. Fax: 713.798.3739. E-mail: ttrask@bcm.tmc.edu.

junctions (13-15), induction of apoptosis (16), and activation of a host immune response (17-19).

Toxicity studies of intracerebral injection of an adenoviral vector, followed by GCV treatment, in cotton rats (20), which are permissive to adenoviral infection, and in other rodent models (7, 10, 21) have shown no clinical or systemic pathological sequalae. Our and other centers have also conducted preclinical toxicity studies in primates (21-23). Although clinical symptoms have not been observed following intracerebral injection of the vector at lower doses, dose-dependent toxicity has been observed in animals receiving higher doses of the vector and GCV (21, 22). The goal of the present study was to determine the safety of Adv.RSVtk + GCV treatment for recurrent advanced malignant brain tumors in humans and to determine the maximal safely tolerated vector dose for intratumoral injection at a single site.

MATERIALS AND METHODS

Permission for this Phase I clinical trial was granted by the Baylor College of Medicine Institutional Review Board (IRB), the Food and Drug Administration (FDA), and the National Institutes of Health Recombinant DNA Advisory Committee (RAC) on the basis of the efficacy and toxicity studies we performed in animal models (7-10, 20, 22) and after production and testing of clinical grade adenoviral vector. Adverse events and patient deaths were reported to the IRB, FDA, and RAC according to the recommended guidelines.

Patient Selection

Adults (≥18 years of age) with recurrent and clinically progressive malignant glial or metastatic tumors were eligible for enrollment. Histological confirmation of the diagnosis was required, and tumors had to be identifiable on neuroimaging studies. Subjects eligible for the study had received and failed conventional treatments, including surgery and radiation therapy. A Karnofsky performance score ≥60 was required. In addition, adequate systemic organ function was required as determined by the following criteria: serum creatinine of ≤1.5 mg/dl or creatinine clearance ≥45 ml/min/m², platelet count ≥100,000/mm³, absolute neutrophil count ≥8.5/mm³, normal PT and aPTT, and bilirubin ≤2.5 mg/dl.

Specific exclusion criteria included acute infection (including HIV infection), progressive systemic malignancy, or unacceptable anesthesia risk. Patients with tumors with cavities likely to communicate with either a subarachnoid space or the ventricular system were excluded. Patients with tumors causing significant mass effect or increases in intracranial pressure and requiring surgical debulking were not eligible. Pregnant patients were excluded, and female subjects of child-bearing age were required to practice birth control for the duration of the study. Patients were informed that the treatment was experimental, and detailed informed consent was obtained at enrollment.

Prior to the gene therapy treatment, each patient underwent a complete history and physical examination, laboratory testing, magnetic resonance imaging (MRI) of the brain, and neuropsychological testing. The neuropsychological test battery was tailored to each patient's level of functioning, area of brain involvement, and endurance, with the goal being detection of significant changes from the baseline examination. Tumor histology was reviewed by the study neuropathologist (J.C.G.) to reconfirm tumor diagnosis. Pretreatment presence of serum-neutralizing antibodies to wild-type adenovirus and of adenovirus in serum, urine, and nasal mucosa was determined for each patient.

Creation of the Adv.RSVtk Vector

Construction of the replication-deficient viral vector has been described in detail previously (10, 24). The adenovirus E1A region was replaced with the HSV-tk gene under the control of the Rous sarcoma virus (RSV) long-

terminal repeat and grown in 293 cells. The vector was produced at the Baylor College of Medicine Gene Vector Laboratory using Good Laboratory Practices. In addition to standard quality control procedures used in vector production, we verified that the vector preparation did not contain measurable wild-type contamination or E1A recombination from 293 cells by polymerase chain reaction (PCR) for the wild-type E1A sequence. The vector preparation was certified to be free of replication-competent adenovirus at <1 per 10¹⁰ VP. Additionally, the failure of cotton rats to sustain productive adenoviral infections after intracerebral delivery of the vector and the failure of adenoviral proliferation on HeLa cells in culture established that there was no significant replication-competent viral contamination. The sensitivity of the HeLa culture assay was one wild-type genome per 75,000 vector genome equivalents.

Adv.RSVtk Vector Dose

Based on our previous toxicity trials (22), we designed a dose-escalation protocol to test the safety of four Adv.RSVtk vector doses: 2×10^9, 2×10^{10}, 2×10^{11}, and 2×10^{12} VP. The VP to infectious unit (IU) ratio, measured using the cytopathic effect (CPE) assay (25), was determined to be 20:1. A minimum of two patients were injected with each dose, beginning with the lowest dose, 2×10^9 VP (1×10^8 IU); if no toxicity was observed over the next month, subsequent patients received the next higher dose.

Injection of the Adv.RSVtk Vector

A Leksell stereotactic frame was applied to the patient's skull using local anesthesia and monitored anesthesia care. Tumor coordinates were generated after computerized tomography (CT) or MRI with the Leksell frame in place. Patients were returned to the operating room, and an 18-gauge stainless steel injection cannula was directed to the target through a twist-drill craniostomy or burr hole. One milliliter of vector solution was injected through the cannula over 5 min. The cannula was left in place for an additional 5 min and then withdrawn. A central venous catheter was placed in most patients to facilitate subsequent intravenous access.

Patients were monitored in the neurosurgical intensive care unit and then transferred to a private room on the neurosurgical unit. A postoperative MRI was obtained within the first 24 h to confirm injection placement and to observe if swelling or hemorrhage had occurred. Standard perioperative medications were administered, including antibiotics, dexamethasone, anticonvulsants, and analgesics.

Ganciclovir Treatment

Twenty-four hours after vector injection, intravenous GCV administration was begun, using a dose of 5 mg/kg over 1 hour, every 12 h, for a total of 28 doses over 14 days. During GCV treatment, patients underwent daily physical and neurological examinations and frequent laboratory studies including complete blood counts, electrolyte levels, and liver function tests. Serum, urine, and nasal swab samples were monitored for vector shedding, and serum was tested for neutralizing antibodies.

Toxicity following vector injection and during GCV treatment was graded using the Common Toxicity Criteria published by the Cancer Therapy Evaluation Program of the National Cancer Institute (26). Permanent Grade 3 or recurrent Grade 4 toxicity was the criterion for cessation of treatment.

The GCV (Cytovene) used in this trial was manufactured by Syntex Corp. (Palo Alto, CA). The drug is approved for the treatment of cytomegalovirus (CMV) retinitis in immunocompromised patients and for the prevention of CMV disease in transplant patients at risk for CMV disease.

Posttreatment Assessment

Patients underwent a neuropsychological evaluation approximately 2 weeks after vector injection. After discharge from the hospital, patients were seen as outpatients every 2 weeks for the first 2 months and then monthly for the first year. Physical examination and laboratory studies were obtained at each visit. A second neuropsychological evaluation was performed at 2 months postinjection. MRI scans were obtained in the first week after completion of HSV-tk/GCV treatment and every 12 weeks

TABLE 1

Patient Pretreatment Characteristics

PT	Age at DX (years)	Age at GT (years)	DX-GT period (months)	Sex	Tumor path	Side	Loc	KFS %	No. Pretreatment resect	XRT	Chem
1	33.2	34.8	19.3	M	GBM	L	Fr	60	1	Y	Y
2	46.2	52.4	74.6	M	AA	R	P	60	3 + 1P	Y	Y
3	57.2	57.8	7.3	M	GBM	L	O	70	2	Y*	Y
4	42.4	53.8	136.7	M	AA	R	FrT	80	1	Y*	Y
5	67.3	68.6	15.7	M	GBM	R	Fr	60	1	Y	YY
6†	41.0	41.8	9.8	F	GBM	L	Fr	60	2	Y*	YY
7	38.3	39.3	12.2	M	GBM	L	P	90	2 + 1P	Y + 1P	Y
8	64.1	65.3	14.9	M	GS	B	Fr	80	2	Y	N
9	64.3	64.9	7.5	M	GBM	R	T	60	1	Y	Y
10	47.6	48.5	10.7	M	GBM	R	PO	80	3	Y	N
11	46.2	50.0	44.6	M	GBM	L	T	80	2	Y	YY
12	37.3	38.3	12.3	F	GBM	R	P	80	1	Y	Y
13	43.9	45.0	13.6	M	AA	L	Fr	60	2	Y	Y
	Mean 48.4 ± 11.2	Mean 50.8 ± 11.0	Mean 29.2 ± 34.4	11 M 2 F	9 GBM 1 GS 3 AA	7 L 6 R	5 Fr 3 P 2 T 1 O 1 FrT 1 PO	6 60% 1 70% 5 80% 1 90%	2 3r 6 2r 5 1r	13 Y	11 Y 2 N

Note. Abbreviations used: AA, anaplastic astrocytoma; B, bilateral; Chem, chemotherapy; DX, diagnosis; F, female; Fr, frontal; FrT, frontotemporal; GBM, glioblastoma multiform; GS, gliosarcoma; GT, gene therapy; KFS, Karnofsky scale; L, left; Loc, tumor location; M, male; N, no; O, occipital; P, parietal; PO, parietooccipital; PT, patient; DX-GT period, time between tumor diagnosis and GT; r, resect, resections; R, right; T, temporal; XRT, X-radiation treatment; Y, yes; YY, two courses of chemotherapy; ±, standard deviation; *, subsequent XRT boost to tumor; +1P, also underwent a post-GT resection or XRT; †, patient diagnosed and treated (resection, radiation, and chemotherapy) for oligodendroglioma 4 years before GBM, for which she also underwent resection, radiation (focused), and chemotherapy.

thereafter. The MRIs were read independently by a neuroradiologist and by a neurosurgeon. Tumor response was monitored by clinical criteria and by MRI. Palliative and therapeutic options were discussed and made available to patients who deteriorated due to tumor progression.

Neutralizing serum antibodies to adenovirus type 5 (AV5) were determined before and at 14, 28, 42, 56, and 84 days after vector injection in most patients and thereafter at outpatient follow-up visits in patients with extended survival. The assay used to measure AV5-specific neutralizing serum antibodies has been described in detail previously (20). Neutralizing antibody titers were expressed as the $\log_2$ of the reciprocal of the last dilution of antiserum that inhibited virus-induced cytopathic effects by 100%.

Serum, urine, and nasal swabs were obtained at 1, 2, and 4 weeks after vector injection and examined by plaque assay for evidence of adenovirus shedding. A detailed description of the plaque assay has been published previously (20). Minimum detection in these assays was 20 plaque-forming units (pfu).

The presence of viral vector DNA in urine was determined by PCR and Southern blot hybridization of the PCR product. The sensitivity was 10 VP/ml of urine. Urine was analyzed daily for vector DNA during hospitalization and at 2 and 6 weeks after vector injection. The procedure used has been previously described (22).

RESULTS

Thirteen patients were enrolled into the study between December 1996 and September 1998 (Table 1). The mean age was 50.8 years (range 34.8 to 68.6 years). There were 11 males and 2 females. Pretreatment Karnofsky scores ranged from 60 to 90. All patients had primary central nervous system neoplasms. Tumor histologies were nine

glioblastoma multiformes (GBM), one gliosarcoma, and three anaplastic astrocytomas (AA). The mean intervals between initial diagnosis and adenoviral treatment for patients with a GBM or GS were 15.5 (±SD 11.6) and 14.9 months, respectively; for patients with an AA the mean interval between initial diagnosis and Adv.RSVtk treatment was 74.9 (±SD 61.5) months.

All patients had undergone previous tumor resection and had received conventional external beam radiation therapy. Eleven of the 13 patients had received chemotherapy, and 2 patients had received additional chemotherapy after tumor recurrence. Three patients had received conformal radiation boosts, with 2 of the 3 receiving the boost after tumor recurrence. Eight patients had undergone reoperation for tumor debulking.

Tumor characteristics on imaging were typical of advanced recurrent gliomas, with complex configurations, cystic areas, and brain invasion. Quantitative descriptions are difficult in this setting, but the cross-sectional diameter was greater than 3 cm in the majority of patients.

Clinical Course during Treatment

Table 2 summarizes the clinical courses of all patients enrolled. The sequence of dose escalation was as follows: 2 patients each received 2×10^9 and 2×10^{10} VP; 3 patients received 2×10^{11} VP; and 2 patients received $2 \times$

TABLE 2

Gene Therapy and Outcome

PT	VP	Cog (2 weeks)	GT Tox	Postoperative adverse events (days 1–14)	Post-DX survival (months)	Post-GT survival (months)
1	2×10^9	—	N	Perioperative focal seizures, small intratumoral hemorrhage	47.6	28.4
2	2×10^9	↓	N	Slight rash	78.2	3.5
3	2×10^{10}	—	N		11.3	4.0
4	2×10^{10}	—	N		162.6	25.9
5†	2×10^{11}	↓	N	Increased left hemiparesis, thrombocytopenia	16.8	1.1
6	2×10^{11}	—	N	Seizure day 6	40.0**	29.2**
7	2×10^{11}	↑	N	Thrombocytopenia	21.5	9.3
8	2×10^{12}	↓↓*	Y	Lethargy, confusion, mild hyponatremia, fever, leukocytosis, intratumoral hemorrhage day 6	16.9	2.0
9^	2×10^{12}	↓↓*	Y	Air in ventricle, lethargy, confusion, fever, hyponatremia, leukocytosis, increased CSF protein, hydrocephalus	10.1	2.6
10	2×10^{11}	↓	N		16.2	5.5
11	2×10^{11}	↓	N	Increased right hemiparesis, lethargy	45.7	1.1
12	2×10^{11}	—	N	Thrombocytopenia, increased liver enzymes	14.3	2.0
13	2×10^{11}	—	N	Increased right hemiparesis, hyponatremia	20.9	7.4
					M 38.4 ± 42.1 (median 20.9)	M 9.2 ± 10.5 (median 4.0)

Note. Abbreviations used: Cog, change in cognition from baseline at 2 weeks after Adv.RSVtk injection; DX, diagnosis; GT, gene therapy; M, mean; N, no; Tox, toxicity; VP, vector particles (20:1 VP:IU ratio); Y, yes; ↑, slightly better; —, same; ↓, slightly worse; ↓↓, worse; ^, completed 4 of 28 GCV treatments; †, received 25 of 28 GCV treatments; *, unable to undergo formal testing due to neurological decline; **, patient is alive at time of publication.

10^{12} VP. After evidence of toxicity at 2×10^{12} VP, the 4 subsequent patients were treated with 2×10^{11} VP. Eleven of the patients completed the full 2-week course of treatment with GCV. Patient 9 received 4 of the 28 GCV doses. Patient 5 received 25 of the 28 GCV doses.

No patient who received 2×10^9 VP ($N = 2$) or 2×10^{10} VP ($N = 2$) experienced any significant treatment-related toxicity other than brief perioperative focal seizures in patient 1 who had a known seizure disorder at the time of enrollment. This patient also developed a small intratumoral hemorrhage that resolved without treatment. Patient 2 developed a slight rash that may have been caused by the GCV. The rash resolved without treatment and without interruption of the GCV treatment.

In the 2×10^{11} VP group ($N = 7$), three patients (patients 5, 11, and 13) experienced a mild increase in preexisting hemiparesis during GCV treatment. Patient 13 also developed transient hyponatremia (131 meq/liter on day 7 after vector injection). This patient had required Na tablets for hyponatremia prior to gene therapy. Patient 12 developed thrombocytopenia (platelet count = 132,000/mm^3 on day 13), and the platelet counts of two patients (patients 5 and 7) with low baseline platelet counts (132,000 and 144,000/mm^3, respectively) declined during GCV treatment (101,000/mm^3 on day 11 and 78,000/mm^3 on day 12, respectively). Platelet counts returned to baseline following GCV treatment; platelet transfusion was not required.

Three (patients 5, 11, and 12) of the seven patients who received 2×10^{11} VP had rapidly deteriorating clinical courses (patient 5 withdrew from the study near the completion of GCV treatment) and died within 2 months of gene therapy. There was no evidence of treatment-related toxicity in these three patients. Patients 5 and 11 had evidence of rapid tumor progression that continued during treatment. Patient 12 developed pneumonia 5 weeks after injection, followed by disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), sepsis, and multiorgan failure. She died 2 months after vector injection; postmortem examination revealed marantic endocarditis.

Both patients treated with 2×10^{12} VP of the Adv.RSVtk vector demonstrated clinically significant toxicity during the treatment period. The first patient (patient 8) had been treated for a left frontal gliosarcoma that had recurred in the right frontal lobe after two previous resections. Twenty-four hours after injection of the vector into the right frontal tumor mass, the patient exhibited confusion and lethargy. He improved and GCV was started on day 2. Six days after vector injection, the patient had a generalized seizure, and a CT scan demonstrated a right frontal hematoma. The patient improved after the postictal period, but did not return to his neurological baseline. He had hyponatremia (Na = 129 meq/liter on day 12), leukocytosis, and low-grade fever (38.7°C on day 11). He improved, completed GCV treatment, and was discharged from the hospital. He remained stable after discharge until 6 weeks after vector injection when he presented with status epilepticus. He did not regain consciousness. A CT scan demonstrated severe bifrontal edema suggestive of tumor progression. The patient's family asked that care be withdrawn. He died 2 months after vector injection. Post-

mortem examination of the brain showed cavitation in the right frontal lobe along the injection tract, surrounding edema, and mass effect. Gross residual tumor was seen in both frontal lobes. The residual right frontal tumor was heavily infiltrated with lymphocytes, and foci of coagulative necrosis were present. Scattered perivascular lymphocytes were seen in the white matter of the injected hemisphere and in the pons, and there was also patchy denudation of the ependyma on the injected side. No demyelination or macrophage infiltration was seen in association with the perivascular lymphocytes. There was no evidence of brain herniation.

The second patient (patient 9) who was treated with 2×10^{12} VP had a recurrent right temporal GBM. He complained of a severe headache in the immediate postinjection period, and a CT scan demonstrated air within the ventricular system, raising the possibility of vector entry into the cerebrospinal fluid. Twelve hours after the injection the patient became obtunded, was unable to follow commands, and developed a fever of 38.9°C. He subsequently recovered rapidly to his neurological baseline, and GCV treatment was initiated. Three days postinjection the patient developed hyponatremia (Na = 126 meq/liter) and obtundation. Ganciclovir treatment was stopped. He initially improved after correction of the hyponatremia, but later deteriorated. A CT scan demonstrated hydrocephalus, and a ventriculoperitoneal shunt was placed on day 14. CSF protein was found to be markedly elevated (624 mg/dl). The patient subsequently gradually improved until tumor progression supervened. The patient died 2.6 months after vector injection. An autopsy was not performed.

One patient (patient 6) with a GBM is alive 29.2 months after vector injection. She is clinically stable and her left frontal tumor has changed little over the 2-year period (Fig. 1). Two other patients, one with a GBM (patient 1) and one with an AA (patient 4), survived for 28.4 and 25.9 months, respectively. A transient reduction in tumor dimensions was observed in patient 4, and both patients had a posttreatment interval of reduced steroid requirements. Survival times to date range from 1 to 29.2 months (median 4.0, mean 9.4 ± SD 10.8 months) (Fig. 2).

Neuropathologic examination of six brains from patients who received 2×10^{10} VP (patients 3 and 4), 2×10^{11} VP (patients 7, 10, and 12), or 2×10^{12} VP (patient 8) disclosed recurrent viable malignant glioma in all specimens. Minimal to moderate intratumoral inflammation and foci of coagulative necrosis were observed in all specimens. As noted earlier, minimal extratumoral inflammation was seen in the brain of patient 8, who received the highest vector dose. Patient 7, who received 2×10^{11} VP, underwent tumor debulking at 2 months and died 9 months after vector injection. Neuropathologic examination of the resected tumor at 2 months showed heavy macrophage and lymphocyte infiltration and areas of coagulative necrosis within the GBM. No inflammation was observed in more viable areas of the tumor. At autopsy, considerable coagulative necrosis was found in the recurrent tumor but minimal intratumoral inflammation. No

inflammation was seen in the brain outside of the tumor bed. Detailed neuropathologic studies will be reported separately.

No adenovirus was detected by plaque assay in any of the serum, urine, or nasal swab samples obtained after vector injection or in any of the urine samples examined by PCR.

Posttreatment elevations in adenoviral antibody titers, relative to pretreatment levels, were detected in 10 of the 12 patients studied (Fig. 3A). In 3 patients with extended survival whose antibody titers were followed for more than 100 days after vector injection (Fig. 3B), 2 patients (patients 4 and 6) maintained significantly elevated titer levels. The third patient (patient 1) had titer levels around baseline throughout his treatment.

Neuropsychological testing was performed shortly before vector injection in all patients, at 2 weeks (at the completion of GCV treatment) in 11 of the 13 patients (testing could not be performed for patients 8 and 9), and at 2 months after vector injection in 6 of the 9 patients alive at 2 months. Because of significant differences in the patients' cognitive abilities and because of the deteriorating clinical course of most of the patients, meaningful neuropsychological data for the group as a whole could not be obtained. Comparison of each patient's performance before and at the end of GCV treatment (Table 2) identified no obvious treatment-associated effect on neuropsychological performance.

DISCUSSION

Genetic approaches to treating neoplasia have included the transduction of tumor cells with genes or gene products that activate cytotoxic prodrugs (27), upregulation of tumor suppressor genes such as p53 (28, 29), stimulation of immune response (30–32), and enhancement of cellular response to other therapies such as radiation (33, 34).

A number of viral and nonviral vectors are available for gene delivery. Experimental brain tumors have been successfully treated with retroviral (35–39) and adenoviral (6, 10–12, 28) vectors, and clinical trials using these approaches to treat malignant brain tumors have been and are being conducted (40–45). Adenoviral vectors are attractive for use in gene therapy because of their efficiency in transducing many cell types, ease of high titer production, episomal genome location, and ability to transduce nondividing cells.

Our Phase I trial examined the safety of the injection of Adv.RSVtk into a single intratumoral site, followed by intravenous GCV, in patients with advanced malignant brain tumors. Pre- or posttreatment tumor resection was not included as a part of this protocol although two patients underwent postinjection tumor debulking because of tumor growth. Multiple or repeat vector injections were not carried out. The trial was designed in this manner for the following reasons: (i) the simple design offered the best opportunity to establish the safe/toxic dose levels, (ii) any treatment effect would not be confounded with other treatments, and (iii) the design com-

FIG. 1. Forty-two-year-old female (patient 6) with a recurrent left frontal glioblastoma multiforme who received 2×10^{11} VP (1×10^{10} IU) of the Adv.RSVtk vector, followed by intravenous GCV. (A) Pretreatment (1 day before injection of Adv.RSVtk). (B) Two months after vector injection. (C) One year after vector injection. (D) Two years and 2 months after vector injection.
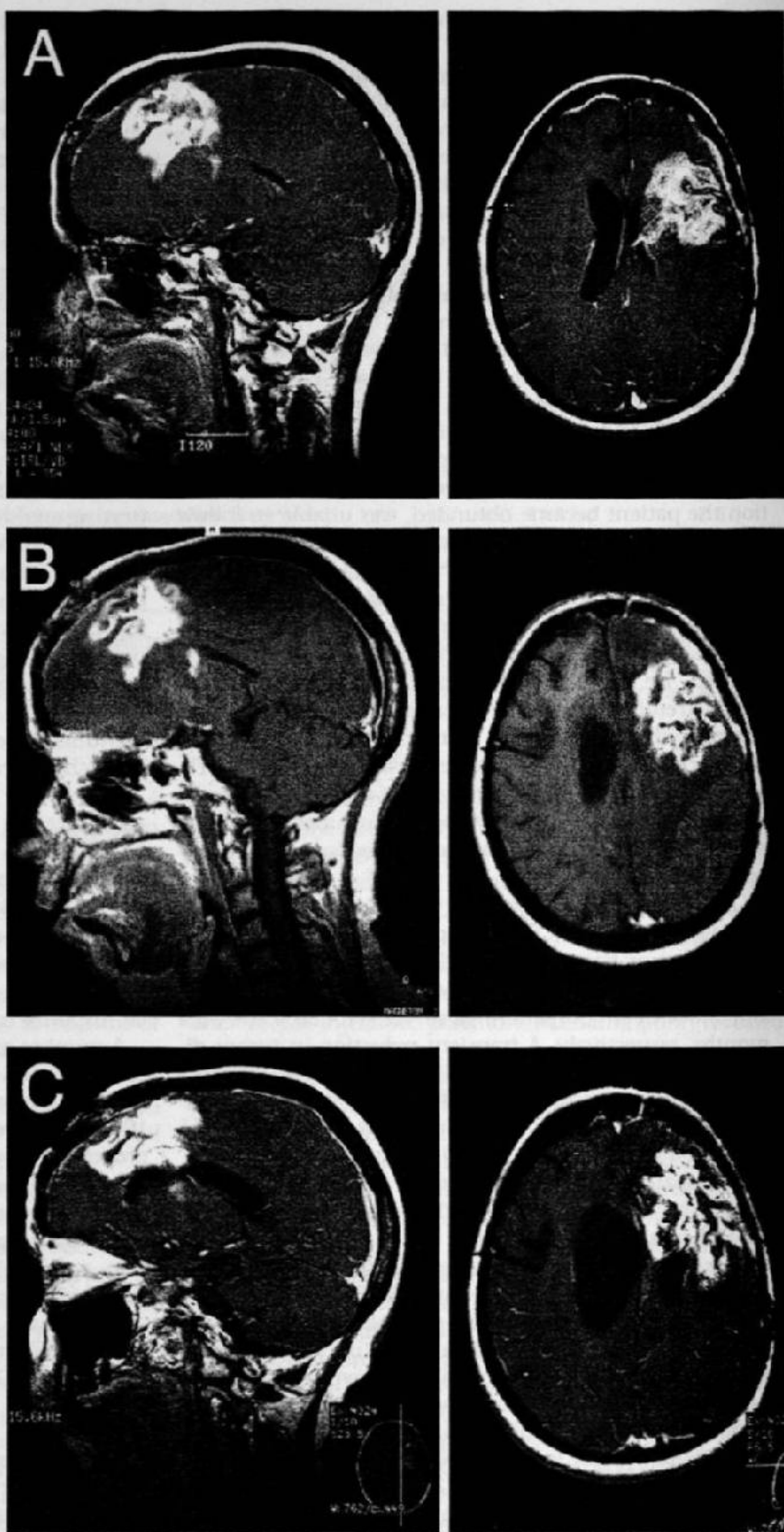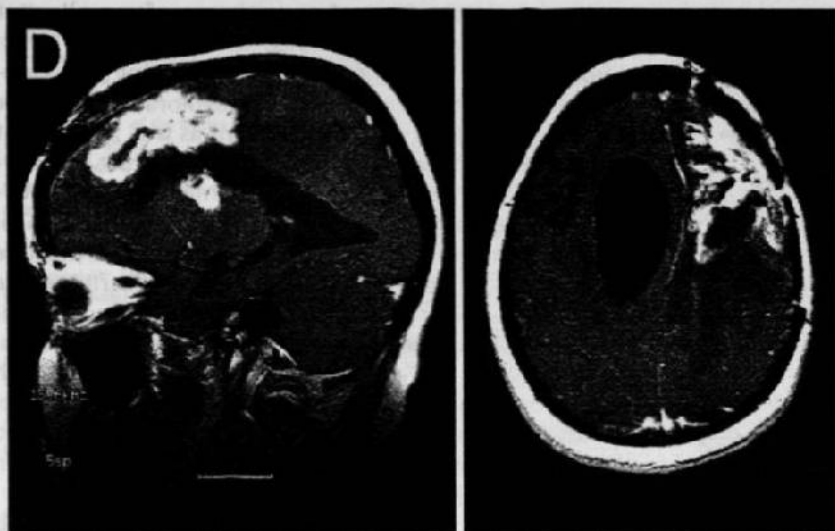
FIG. 1—Continued

plemented another Phase I trial of patients with advanced brain tumors in which resection and multiple adenoviral vector injections were being used (44).

We conclude that the form of gene therapy used in our Phase I trial is safe when the vector dose does not exceed 2×10^{11} VP (1×10^{10} IU). There was significant toxicity in patients 8 and 9 who were treated with 2×10^{12} VP. Factors that may have potentiated toxicity in these two patients include older age (age 65), bilateral tumor and hemorrhage in patient 8, and possible intraventricular injection in patient 9.

Toxicity of the nature we observed was not seen in another Phase I brain tumor protocol using an adenoviral vector which is being carried out at the University of Pennsylvania (personal communication, Stephen L. Eck, University of Pennsylvania Medical Center, October 1999). In that trial, patients received two intratumoral injections, 1 week apart, using an adenoviral vector dose of 1×10^{11} pfu. Whether the differences in toxicity observed reflect dissimilarities between the vectors, the doses administered, the patients, or the protocols is currently not known. Although the highest dose we used [2×10^{12} VP (1×10^{11} IU as determined by CPE)] appears similar to that used in the University of Pennsylvania study, comparison of vector doses used in different studies is difficult. Nyberg-Hoffman and associates have demonstrated that the various assays and methodologies used to titer adenoviral vectors can produce markedly different estimates of the number of infectious units in a vector sample (25). In addition, toxicity following injection of an adenoviral vector may relate not only to the infectious dose employed, but also to the physical number of vector particles injected. Standardization of the methods used to determine adenoviral titers is needed in order to compare vector doses and to better assess toxicity.

The toxicity associated with adenoviral vectors appears to be the result of a multifactorial cellular and humoral immune response, which may also play an important role in the destruction of malignant neoplasms. In our Phase I

study, neuropathological examination of tumor after vector injection demonstrated marked intratumoral infiltration with inflammatory cells, suggesting that recruitment of an antitumor response of the immune system may play a role in killing tumor cells. Dewey and associates have recently reported long-term inflammation in normal brain in a rat tumor model in which the rats were injected intratumorally with an adenoviral vector (46). We did not observe such severe or widespread inflammatory changes in normal brain tissue in any of the specimens examined.

Three patients in our study, 2 with a GBM (patients 1 and 6) and 1 with an AA (patient 4), survived >25 months, considerably beyond the expected survival for patients with recurrent malignant gliomas. A recent survival study followed a group of 130 patients with glioblastoma multiforme, some of whom underwent chemotherapy (74%) or stereotactic radiation (7%) but not resection, following tumor recurrence (3). These patients had, upon initial diagnosis, undergone resection of the tumor and had received external beam radiation therapy, usually with concomitant chemotherapy. The median survival
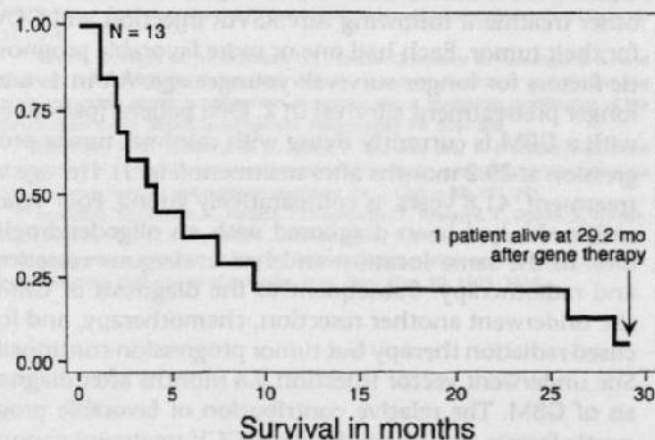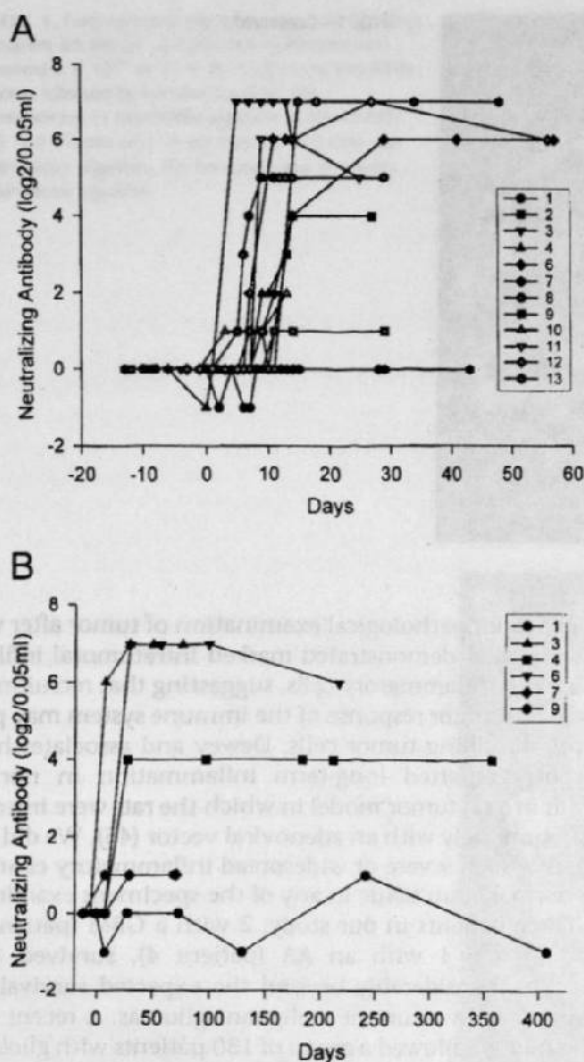

FIG. 2. Patient survival.

A

B

FIG. 3. Adenovirus antibody titers relative to levels immediately before gene therapy (day 0). (A) Titers for 12 patients over the first 2 months after Adv.RSVtk injection. (B) Titers for 6 patients with extended survival after Adv.RSVtk injection.

time after tumor recurrence for these 130 patients was 23 weeks (5.75 months). None of our 3 patients received any other treatment following Adv.RSVtk injection and GCV for their tumor. Each had one or more favorable prognostic factors for longer survival: younger age, AA in 1, and longer pretreatment survival in 2. One patient (patient 6) with a GBM is currently living with minimal tumor progression at 29.2 months after treatment (Fig. 1). Her age at treatment, 41.8 years, is comparatively young. Four years earlier she had been diagnosed with an oligodendroglioma in the same location and had undergone resection and radiotherapy. Subsequent to the diagnosis of GBM, she underwent another resection, chemotherapy, and focused radiation therapy but tumor progression continued. She underwent vector injection 9.8 months after diagnosis of GBM. The relative contribution of favorable prognostic factors and of Adv.RSVtk + GCV treatment cannot be determined in this case.

Current development of gene therapy for malignant brain tumors is being addressed in a number of areas: improving vector efficiency and minimizing immunologic response to the vector (47, 48), exploration of vector constructs utilizing different deletions as well as different promoters, and improvements in the construct or delivery of GCV or similar prodrugs that may extend the bystander effect and reduce GCV-mediated toxicity (14, 15, 49). In addition, the optimal delivery technique must be determined in order to more uniformly distribute the vector throughout the tumor. Possibilities include multiple stereotactic injections, injection of postresection tumor margins, and vector delivery in conjunction with blood–brain barrier disruption. Several centers have recently developed models that improve vector delivery to brain tumors by selectively modifying the blood–brain barrier (50, 51).

Finally, the best results may be obtained when this treatment is combined with other modalities such as radiation therapy, chemotherapy, and other gene therapy approaches. Improved tumor killing has been observed in several cancer models by combining HSV-tk/GCV treatment with radiotherapy (52, 53).

REFERENCES

[1] American Cancer Society (1999). 1999 New Facts and Figures: Estimated New Cancer Cases and Deaths. http://www.cancer.org/statistics/cff99/data/data_newCaseSex.html.

[2] Shrieve, D. C., Alexander, E., III, Black, P. M., Wen, P. Y., Fine, H. A., Kooy, H. M., and Loeffler, J. S. (1999). Treatment of patients with primary glioblastoma multiforme with standard postoperative radiotherapy and radiosurgical boost: Prognostic factors and long-term outcome. *J. Neurosurg.* **90:** 72–77.

[3] Barker, F. G., II, Chang, S. M., Gutin, P. H., Malec, M. K., McDermott, M. W., Prados, M. D., and Wilson, C. B. (1998). Survival and functional status after resection of recurrent glioblastoma multiforme. *Neurosurgery* **42:** 709–723.

[4] Durmaz, R., Erken, S., Arslantas, A., Atasoy, M. A., Bal, C., and Tel, E. (1997). Management of glioblastoma multiforme: With special reference to recurrence. *Clin. Neurol. Neurosurg.* **99:** 117–123.

[5] Black, P. (1998). Management of malignant glioma: Role of surgery in relation to multimodality therapy. *J. Neurovirol.* **4:** 227–236.

[6] Mizuno, M., Yoshida, J., Colosi, P., and Kurtzman, G. (1998). Adeno-associated virus vector containing the herpes simplex virus thymidine kinase gene causes complete regression of intracerebrally implanted human gliomas in mice, in conjunction with ganciclovir administration. *Jpn. J. Cancer Res.* **89:** 76–80.

[7] Perez-Cruet, M. J., Trask, T. W., Chen, S. H., Goodman, J. C., Woo, S. L., Grossman, R. G., and Shine, H. D. (1994). Adenovirus-mediated gene therapy of experimental gliomas. *J. Neurosci. Res.* **39:** 506–511.

[8] Colak, A., Goodman, J. C., Chen, S. H., Woo, S. L., Grossman, R. G., and Shine, H. D. (1995). Adenovirus-mediated gene therapy in an experimental model of breast cancer metastatic to the brain. *Hum. Gene Ther.* **6:** 1317–1322.

[9] Colak, A., Goodman, J. C., Chen, S. H., Woo, S. L., Grossman, R. G., and Shine, H. D. (1995). Adenovirus-mediated gene therapy for experimental spinal cord tumors: Tumoricidal efficacy and functional outcome. *Brain Res.* **691:** 76–82.

[10] Chen, S. H., Shine, H. D., Goodman, J. C., Grossman, R. G., and Woo, S. L. (1994). Gene therapy for brain tumors: Regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc. Natl. Acad. Sci. USA* **91:** 3054–3057.

[11] Lanuti, M., Gao, G. P., Force, S. D., Chang, M. Y., El Kouri, C., Amin, K. M., Hughes, J. V., Wilson, J. M., Kaiser, L. R., and Albelda, S. M. (1999). Evaluation of an E1-E4-deleted adenovirus expressing the herpes simplex thymidine kinase suicide gene in cancer gene therapy. *Hum. Gene Ther.* **10:** 463–475.

[12] Viola, J. J., Ram, Z., Walbridge, S., Oshiro, E. M., Trapnell, B., Tao-Cheng, J. H., and Oldfield, E. H. (1995) Adenovirally mediated gene transfer into experimental solid brain tumors and leptomeningeal cancer cells. J. Neurosurg. 82: 70-76.

[13] Elshami, A. A., Saavedra, A., Zhang, H., Kucharczuk, J. C., Spray, D. C., Fishman, G. I., Amin, K. M., Kaiser, L. R., and Albelda, S. M. (1996). Gap junctions play a role in the 'bystander effect' of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. Gene Ther. 3: 85-92.

[14] Carystinos, G. D., Katabi, M. M., Laird, D. W., Galipeau, J., Chan, H., Alaoui-Jamali, M. A., and Batist, G. (1999). Cyclic-AMP induction of gap junctional intercellular communication increases bystander effect in suicide gene therapy. Clin. Cancer Res. 5: 61-68.

[15] Estin, D., Li, M., Spray, D., and Wu, J. K. (1999). Connexins are expressed in primary brain tumors and enhance the bystander effect in gene therapy. Neurosurgery 44: 361-369.

[16] Hamel, W., Magnelli, L., Chiarugi, V. P., and Israel, M. A. (1996). Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. Cancer Res. 56: 2697-2702.

[17] Ramesh, R., Munshi, A., Marrogi, A. J., and Freeman, S. M. (1999). Enhancement of tumor killing using a combination of tumor immunization and HSV-tk suicide gene therapy. Int. J. Cancer 80: 380-386.

[18] Ramesh, R., Munshi, A., Abboud, C. N., Marrogi, A. J., and Freeman, S. M. (1996). Expression of costimulatory molecules: B7 and ICAM up-regulation after treatment with a suicide gene. Cancer Gene Ther. 3: 373-384.

[19] Vile, R. G., Nelson, J. A., Castleden, S., Chong, H., and Hart, I. R. (1994). Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. Cancer Res. 54: 6228-6234.

[20] Shine, H. D., Wyde, P. R., Aguilar-Cordova, E., Chen, S. H., Woo, S. L., Grossman, R. G., and Goodman, J. C. (1997). Neurotoxicity of intracerebral injection of a replication-defective adenoviral vector in a semipermissive species (cotton rat). Gene Ther. 4: 275-279.

[21] Smith, J. G., Raper, S. E., Wheeldon, E. B., Hackney, D., Judy, K., Wilson, J. M., and Eck, S. L. (1997). Intracranial administration of adenovirus expressing HSV-TK in combination with ganciclovir produces a dose-dependent, self-limiting inflammatory response. Hum. Gene Ther. 8: 943-954.

[22] Goodman, J. C., Trask, T. W., Chen, S. H., Woo, S. L., Grossman, R. G., Carey, K. D., Hubbard, G. B., Carrier, D. A., Rajagopalan, S., Aguilar-Cordova, E., and Shine, H. D. (1996). Adenoviral-mediated thymidine kinase gene transfer into the primate brain followed by systemic ganciclovir: Pathologic, radiologic, and molecular studies. Hum. Gene Ther. 7: 1241-1250.

[23] Driesse, M. J., Vincent, A. J., Sillevis Smitt, P. A., Kros, J. M., Hoogerbrugge, P. M., Avezaat, C. J., Valerio, D., and Bout, A. (1998). Intracerebral injection of adenovirus harboring the HSVtk gene combined with ganciclovir administration: Toxicity study in nonhuman primates. Gene Ther. 5: 1122-1129.

[24] Graham, F., and Prevec, L. (1991). Manipulation of adenovirus vectors. In Gene Transfer and Expression Protocols (E. J. Murray, Ed.), Vol. 7, pp. 109-127, Humana Press, Clifton, NJ.

[25] Nyberg-Hoffman, C., Shabram, P., Li, W., Giroux, D., and Aguilar-Cordova, E. (1997). Sensitivity and reproducibility in adenoviral infectious titer determination. Nat. Med. 3: 808-811.

[26] National Cancer Institute Cancer Therapy Evaluation Program (1999). Common Toxicity Criteria (CTC). Version 2.0, April 30, 1999. http://ctep.info.nih.gov/CTC3/ctc.htm.

[27] Singhal, S., and Kaiser, L. R. (1998). Cancer chemotherapy using suicide genes. Surg. Oncol. Clin. N. Am. 7: 505-536.

[28] Li, H., Alonso-Vanegas, M., Colicos, M. A., Jung, S. S., Lochmuller, H., Sadikot, A. F., Snipes, G. J., Seth, P., Karpati, G., and Nalbantoglu, J. (1999). Intracerebral adenovirus-mediated p53 tumor suppressor gene therapy for experimental human glioma. Clin. Cancer Res. 5: 637-642.

[29] Gomez-Manzano, C., Fueyo, J., Alameda, F., Kyritsis, A. P., and Yung, W. K. (1999). Gene therapy for gliomas: p53 and E2F-1 proteins and the target of apoptosis. Int. J. Mol. Med. 3: 81-85.

[30] Visse, E., Siesjo, P., Widegren, B., and Sjogren, H. O. (1999). Regression of intracerebral rat glioma isografts by therapeutic subcutaneous immunization with interferon-gamma, interleukin-7, or B7-1-transfected tumor cells. Cancer Gene Ther. 6: 37-44.

[31] Liau, L. M., Fakhrai, H., and Black, K. L. (1998). Prolonged survival of rats with intracranial C6 gliomas by treatment with TGF beta antisense gene. Neurol. Res. 20: 742-747.

[32] Ambar, B. B., Frei, K., Malipiero, U., Morelli, A. E., Castro, M. G., Lowenstein, P. R., and Fontana, A. (1999). Treatment of experimental glioma by administration of adenoviral vectors expressing Fas ligand. Hum. Gene Ther. 10: 1641-1648.

[33] Lang, F. F., Yung, W. K., Raju, U., Libunao, F., Terry, N. H., and Tofilon, P. J. (1998). Enhancement of radiosensitivity of wild-type p53 human glioma cells by adenovirus-mediated delivery of the p53 gene. J. Neurosurg. 89: 125-132.

[34] Badie, B., Kramar, M. H., Lau, R., Boothman, D. A., Economou, J. S., and Black, K. L. (1998). Adenovirus-mediated p53 gene delivery potentiates the radiation-induced growth inhibition of experimental brain tumors. J. Neurooncol. 37: 217-222.

[35] Ram, Z., Walbridge, S., Shawker, T., Culver, K. W., Blaese, R. M., and Oldfield, E. H. (1994). The effect of thymidine kinase transduction and ganciclovir therapy on tumor vasculature and growth of 9L gliomas in rats. J. Neurosurg. 81: 256-260.

[36] Galipeau, J., Li, H., Paquin, A., Sicilia, F., Karpati, G., and Nalbantoglu, J. (1999). Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. Cancer Res. 59: 2384-2394.

[37] Benedetti, S., Bruzzone, M. G., Pollo, B., DiMeco, F., Magrassi, L., Pirola, B., Cirenei, N., Colombo, M. P., and Finocchiaro, G. (1999). Eradication of rat malignant gliomas by retroviral-mediated, in vivo delivery of the interleukin 4 gene. Cancer Res. 59: 645-652.

[38] Tamura, M., Ikenaka, K., Tamura, K., Yoshimatsu, T., Miyao, Y., Kishima, H., Mabuchi, E., and Shimizu, K. (1998). Transduction of glioma cells using a high-titer retroviral vector system and their subsequent migration in brain tumors. Gene Ther. 5: 1698-1704.

[39] Kondo, S., Tanaka, Y., Kondo, Y., Ishizaka, Y., Hitomi, M., Haqqi, T., Liu, J., Barnett, G. H., Alnemri, E. S., and Barna, B. P. (1998). Retroviral transfer of CPP32beta gene into malignant gliomas in vitro and in vivo. Cancer Res. 58: 962-967.

[40] Ram, Z., Culver, K. W., Oshiro, E. M., Viola, J. J., DeVroom, H. L., Otto, E., Long, Z., Chiang, Y., McGarrity, G. J., Muul, L. M., Katz, D., Blaese, R. M., and Oldfield, E. H. (1997). Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. Nat. Med. 3: 1354-1361.

[41] Klatzmann, D., Valery, C. A., Bensimon, G., Marro, B., Boyer, O., Mokhtari, K., Diquet, B., Salzmann, J. L., Philippon, J., and Study Group on Gene Therapy for Glioblastoma (1998). A phase I/II study of herpes simplex virus type I thymidine kinase "suicide" gene therapy for recurrent glioblastoma. Hum. Gene Ther. 9: 2595-2604.

[42] Long, Z., Lu, P., Grooms, T., Mychkovsky, I., Westley, T., Fitzgerald, T., Sharma-Chibber, S., Shand, N., McGarrity, G., and Otto, E. (1999). Molecular evaluation of biopsy and autopsy specimens from patients receiving in vivo retroviral gene therapy. Hum. Gene Ther. 10: 733-740.

[43] Puumalainen, A. M., Vapalahti, M., Agrawal, R. S., Kossila, M., Laukkanen, J., Lehtolainen, P., Viita, H., Paljarvi, L., Vanninen, R., and Yla-Herttuala, S. (1998). Beta-galactosidase gene transfer to human malignant glioma in vivo using replication-deficient retroviruses and adenoviruses. Hum. Gene Ther. 9: 1769-1774.

[44] Eck, S. L., Alavi, J. B., Alavi, A., Davis, A., Hackney, D., Judy, K., Mollman, J., Phillips, P. C., Wheeldon, E. B., and Wilson, J. M. (1996). Treatment of advanced CNS malignancies with the recombinant adenovirus H5.010RSVTK: A phase I trial. Hum. Gene Ther. 7: 1465-1482.

[45] Shand, N., Weber, F., Mariani, L., Bernstein, M., Gianella-Borradori, A., Long, Z., Sorensen, A. G., and Barbier, N. (1999). A phase 1-2 clinical trial of gene therapy for recurrent glioblastoma multiforme by tumor transduction with the herpes simplex thymidine kinase gene followed by ganciclovir. GLI328 European-Canadian Study Group. Hum. Gene Ther. 10: 2325-2335.

[46] Dewey, R. A., Morrissey, G., Cowsill, C. M., Stone, D., Bolognani, F., Dodd, N. J., Southgate, T. D., Klatzmann, D., Lassmann, H., Castro, M. G., and Lowenstein, P. R. (1999). Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: Implications for clinical trials. Nat. Med. 5: 1256-1263.

[47] Morral, N., O'Neal, W., Rice, K., Leland, M., Kaplan, J., Piedra, P. A., Zhou, H., Parks, R. J., Velji, R., Aguilar-Cordova, E., Wadsworth, S., Graham, F. L., Kochanek, S., Carey, K. D., and Beaudet, A. L. (1999). Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. Proc. Natl. Acad. Sci. USA 96: 12816-12821.

[48] Shinoura, N., Yoshida, Y., Tsunoda, R., Ohashi, M., Zhang, W., Asai, A., Kirino, T., and Hamada, H. (1999). Highly augmented cytopathic effect of a fiber-mutant E1B-defective adenovirus for gene therapy of gliomas. Cancer Res. 59: 3411-3416.

[49] Kokoris, M., Sabo, P., Adman, E., and Black, M. (1999). Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. Gene Ther. 6: 1415-1426.

[50] Rainov, N. G., Ikeda, K., Qureshi, N. H., Grover, S., Herrlinger, U., Pechan, P., Chiocca, E. A., Breakefield, X. O., and Barnett, F. H. (1999). Intraarterial delivery of adenovirus vectors and liposome-DNA complexes to experimental brain neoplasms. Hum. Gene Ther. 10: 311-318.

[51] Kroll, R. A., Pagel, M. A., Muldoon, L. L., Roman-Goldstein, S., Fiamengo, S. A., and Neuwelt, E. A. (1998). Improving drug delivery to intracerebral tumor and surrounding brain in a rodent model: A comparison of osmotic versus bradykinin modification of the blood-brain and/or blood-tumor barriers. Neurosurgery 43: 879-889.

[52] Blackburn, R. V., Galoforo, S. S., Corry, P. M., and Lee, Y. J. (1999). Adenoviral transduction of a cytosine deaminase/thymidine kinase fusion gene into prostate carcinoma cells enhances prodrug and radiation sensitivity. Int. J. Cancer 82: 293-297.

[53] Kawashita, Y., Ohtsuru, A., Kaneda, Y., Nagayama, Y., Kawazoe, Y., Eguchi, S., Kuroda, H., Fujioka, H., Ito, M., Kanematsu, T., and Yamashita, S. (1999). Regression of hepatocellular carcinoma in vitro and in vivo by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation. Hum. Gene Ther. 10: 1509-1519.