[CANCER RESEARCH 61, 8743–8750, December 15, 2001]

Treatment of Malignant Gliomas with a Replicating Adenoviral Vector Expressing Herpes Simplex Virus-*Thymidine Kinase*[1]

Dharmin Nanda, Ronald Vogels, Menzo Havenga, Cees J. Avezaat, Abraham Bout, and Peter Sillevis Smitt[2]

Department of Neuro-oncology [D. N., P. S. S.], Daniel den Hoed Cancer Center, University Hospital Rotterdam, 3008 AE Rotterdam; Department of Neurosurgery [C. J. A.], University Hospital Rotterdam, 3015 GD Rotterdam; and Crucell Holland B. V. [R. V., M. H., A. B.], 2301 CA Leiden, the Netherlands

ABSTRACT

We evaluated the interaction between oncolytic, replication-competent adenoviral vectors and the herpes simplex virus-1 *thymidine kinase* (HSV1-*tk*) gene/ganciclovir (GCV) suicide system for the treatment of malignant gliomas. We constructed a panel of replication-competent adenoviral vectors in which the luciferase (*IG.Ad5E1*$^+$*. E3Luc*) or HSV1-*tk* gene (*IG.Ad5E1*$^+$*.E3TK*) replace the M_r 19,000 glycoprotein (gp19K) coding sequence in the E3 region. IG.Ad5E1. IG.Ad5.ClipLuc and IG.AdApt.TK are E1-deleted viruses that contain the *luciferase* or *the* HSV1-*tk* gene in the former E1 region driven by the human cytomegalovirus promoter. IG.Ad5.Sarcoma 1800HSA.E3Luc contains an irrelevant gene in the E1 region, whereas the gp19K coding sequence in the E3 region is replaced by the *luciferase* gene as in the replicating virus IG.Ad5E1$^+$.E3Luc. For *in vitro* experiments, we used a panel of human glioma cell lines (U87 MG, T98G, A172, LW5, and U251), a rat gliosarcoma cell line (9 L), and human lung (A549) and prostate carcinoma (P3) cell lines. *In vitro*, GCV sensitivity (10 μg/ml) was studied in U87 MG cells after infection at a multiplicity of infection of 1 and 10. A s.c. U87 MG glioma xenograft model was established in NIH-bg-nu-xid mice. Tumors of 100–150 mm^3 were treated with a single injection of adenovirus 10^9 IU suspended in 100 μl of PBS, and GCV 100 mg/kg was administered i.p. twice daily for 7 days. The cytopathic effect of all three replication-competent adenoviral vectors was similar to the cytopathic effect of wild-type adenovirus 5 on all human cell lines tested, indicating that deletion of the E3 gp19K sequences did not affect the oncolytic effect of the vectors. *In vitro*, luciferase expression was the same for both E1-deleted vectors (IG.Ad5.ClipLuc and IG.Ad5.Sarcoma 1800HSA.E3Luc), demonstrating the strength of the internal E3 promoter even in the absence of E1A. However, *in vitro* expression levels obtained with replication-competent IG.Ad5E1$^+$. E3Luc were 3 log higher (allowing infection with a 2–3-log lower multiplicity of infection) in the human cell lines. In U87 MG glioma cells, the oncolytic effect of replication-competent IG.Ad5E1$^+$.E3TK was significantly enhanced by the addition of GCV and greatly exceeded the cytotoxicity of replication-incompetent IG.AdApt.TK combined with GCV. In established s.c. U87 MG glioma xenografts, a single injection of IG.Ad5E1$^+$.E3TK resulted in a significant slowing of tumor growth and prolonged survival compared with injection of IG.AdApt.TK. Addition of GCV slowed tumor growth, further adding to survival. In conclusion, the oncolytic effect of replicating adenoviral vectors and HSV1-tk/GCV have potent antitumor effects in gliomas. When combined, these two approaches are complementary, resulting in a significantly improved treatment outcome. In addition, replication-competent adenoviral vectors missing the E3 gp19K coding sequences, have oncolytic efficacy comparable with wild type. In combination with high expression levels obtained with the natural E3 promoter, such vectors are promising new anticancer agents.

INTRODUCTION

The incidence of primary brain tumors in the Western world is 9–10/100,000 persons and 7/100,000 die of brain tumors each year (1, 2). In children, brain tumors constitute a quarter of all cancer deaths, second only after leukemia (3). In adults, malignant gliomas are the most common primary brain tumors, and despite advances in neurosurgical techniques, radiation treatment, and chemotherapy, the prognosis of these tumors remains dismal, with a median survival of <1 year; and $<5\%$ survive for 5 years or more (4–6). Limitations of surgery, radiotherapy, and chemotherapy make the development of new treatment strategies necessary (7). Despite the fact that individual tumor cells spread through the brain at great distances from the primary site (8, 9), ~80% of malignant gliomas recur within a 2-cm margin of the contrast-enhancing rim on computed tomography (10, 11). This high rate of local recurrence within the region of the original tumor, combined with the very low incidence of distant metastases, warrants the additional pursuit of locoregional treatment strategies including gene therapy.

Gene therapy for brain tumors has demonstrated efficacy in a variety of animal models using many different vector systems, including retrovirus (12), adenovirus (13) adeno-associated virus (14), herpes virus (15), and reovirus vectors (16).

Despite promising results in experimental studies, clinical gene therapy trials in brain tumor patients have generally been disappointing. A Phase III study of adjuvant gene therapy in 248 patients with glioblastoma multiforme could not demonstrate any benefit of the injection of HSV-*tk*[3] retrovirus vector-producing cells (17). A much smaller, Phase I, study of adeno.*tk* injection into malignant gliomas demonstrated that the approach was safe but not very effective (18). A small, uncontrolled trial of HSV-*tk* gene therapy in malignant gliomas demonstrated better efficiency of replication-deficient adenovirus vector compared with a retrovirus vector (19).

Better efficacy is crucial for adenoviral cancer gene therapy to become clinically relevant. Most of the adenoviruses used in the clinical studies thus far carry a deletion in the E1 region which renders the virus replication defective or helper-dependent. The efficacy of adenoviral vectors could be greatly increased by using replication-competent vectors (20). The theoretical advantages for the use of replication-competent recombinant adenovirus vector are: (*a*) the cytolytic effect on infected cells; (*b*) the subsequent spread of virus to neighboring cells, resulting in additional tumor/tissue penetration; (*c*) an enhanced immune response; and (*d*) higher levels of expression of the therapeutic gene are achieved as a result of the replication of adenovirus DNA.

For safety reasons, all replication competent adenoviral vectors are conditionally replicating, usually by small deletions in the *E1A* or *E1B* gene. These modifications may, however, decrease efficiency of viral replication, whereas, in the past, intratumoral injection of wild-type adenoviruses has not led to unwanted effects (21). To enhance further the tumoricidal effect of the replication-competent vectors and to provide a "fail-safe" in case of replication outside the tumor, we left E1 intact and placed the HSV1-*tk* suicide gene in E3 under control of the natural E3 promoter. We then examined the antitumor efficacy of

Received 4/16/01; accepted 10/16/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by European Union Biomed Grant PL96-1436.

[2] To whom requests for reprints should be addressed, at Department of Neuro-oncology, Daniel den Hoed Cancer Center, University Hospital Rotterdam, P. O. Box 5201, 3008 AE Rotterdam, the Netherlands. E-mail: sillevis@neuh.azr.nl.

[3] The abbreviations used are: HSV-tk, herpes simplex virus thymidine kinase; GCV, ganciclovir; gp19K, M_r 19,000 glycoprotein; CMV, cytomegalovirus; HSA, heat-stable antigen; IU, infectious unit(s); m.o.i., multiplicity(ies) of infection: CPE, cytopathic effect; wtAd5, wild-type adenovirus 5; ITR, inverted terminal repeat; Nucl., nucleotide.

the nonconditional replication-competent adenoviral vectors in a panel of glioma cell lines and in an animal model. We could demonstrate a direct lytic effect of the vectors in human cell lines and *in vivo* that was enhanced further by the suicide HSV1-tk/GCVsystem.

MATERIALS AND METHODS

Cell Lines

The human glioma cell lines U87 MG, T98G, and A172 were obtained from the American Type Culture Collection (Manassas, VA). The LW5 and U251 human glioma cell lines were obtained from Dr. Langeveld (Department of Pharmacology, Free University Hospital Amsterdam, Amsterdam, the Netherlands; Ref. 13). The 9L-rat gliosarcoma brain tumor was a gift from Dr. Hebeda (Department of Experimental Neurosurgery, Free University Hospital Amsterdam; Ref. 13). The A549 human lung carcinoma cell line was purchased from Biowhittaker (Brussels, Belgium; Ref. 13). PC3, a human prostate cell line (22), and human embryonic kidney 293 cells (HEK-293) were purchased from the American Type Culture Collection. All cell lines were grown in DMEM (Life Technologies, Inc., Breda, the Netherlands) containing 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) and cultured at 37°C in a 5%-CO_2 atmosphere.

Virus Constructions

Fig. 1 shows the structure of the different viruses used in this study.

Nonconditional Replicating Viruses. IG.Ad5E1$^+$. E3Luc and IG.Ad5E1$^+$.-E3TK are replication-competent Ad5-based viruses in which the *luciferase* gene and the HSV1-*tk* gene, respectively, replace the gp19K coding sequence in the E3 region. IG.Ad5E1$^+$. E3$-$ is a replicating vector in which the *gp19K* gene in the E3 region is deleted. Coding sequences of the adenovirus death protein (E3–11.6K) were not disrupted (data not shown). The IG.Ad5E1+.E3TK vector was generated as follows: a 2.7 kb *Eco*RI fragment from wtAd5 containing the 5′ part of the E3 region was cloned into the *Eco*RI site of pBluescript (KS$^-$; Stratagene). Next, the *Hin*dIII site in the polylinker was removed by digestion with *Eco*RV and *Hin*cII and subsequent religation. The resulting clone, pBS.Eco-Eco/ad5ΔHIII, was used to delete the gp19K coding region. Primers 1 (5′-GGG TAT TAG GCC AA AGG CGC A-3′) and 2 (5′-GAT CCC ATG GAA GCT TGG GTG GCG ACC CCA GCG-3′) were used to amplify a sequence from pBS.Eco-Eco/ad5ΔHIII corresponding to sequences 28511–28734 in wtAd5 DNA. Primers 3 (5′-GAT CCC ATG GGG ATC CTT TAC TAA GTT ACA AAG CTA-3′) and 4 (5′-GTC GCT GTA GTT GGA CTG G-3′) were used on the same DNA to amplify Ad5 sequences from 29217 to 29476. The two resulting PCR fragments were ligated together by virtue of the new introduced *Nco*I site and subsequently digested with *Xba*I and MunI. This fragment was then ligated into the pBS.Eco-Eco/ad5ΔHIII vector that was digested with *Xba*I (partially) and MunI-generating pBS.Eco-Eco/ad5ΔHIII.Δgp19K. To allow insertion of foreign genes into the *Hin*dIII and *Bam*HI site, an *Xba*I deletion was made in pBS.Eco-Eco/ad5ΔHIII.Δgp19K to remove the *Bam*HI site in the pBS polylinker. The resulting plasmid pBS.Eco-Eco/ad5ΔHIIIΔgp19KΔ*Xba*I, contains unique *Hin*dIII and *Bam*HI sites corresponding to sequences 28733 (*Hin*dIII) and 29218 (*Bam*HI) in Ad5. The *HSV1-TK* gene was then introduced as a *Hin*dIII-*Bam*HI fragment into these sites, generating pBS.Eco-Eco/Ad5.Δ*Hin*dIII.Δgp19K.ΔXba.TK. After digestion of this construct with MunI and *Hin*dIII, the TK-containing fragment was then introduced into pBS.Eco-Eco/ad5ΔHIII.Δgp19K, generating pBS.Eco-Eco/Ad5.Δ*Hin*dIIIΔgp19k/TK. The unique SrfI and *Not*I sites in this plasmid were used to transfer the region comprising the *TK* gene into the corresponding region of pBr/Ad.Bam-rITRsp, yielding construct pBr/Ad.Bam-rITR.gpTKsp. pBr/Ad.Bam-rITRsp is a pBr322-based vector containing adenoviral sequences from Ad nucl.21562 (*Bam*HI site) to the end of the right ITR, the latter being flanked by a unique *Pac*I site. The last step entailed the subcloning of the *Spe*I-*Pac*I fragment from pBr/Ad.Bam-rITR.gpTKsp into the cosmid vector pWE.Ad5.*Afl*II-rITRsp. pWE.Ad5.*Afl*II-rITRsp is an pWE15- (CLONTECH) based cosmid clone containing Ad nucl.3534 to the right ITR flanked by *Pac*I sites. The final construct, pWE.Ad5.*Afl*II-rITRgpTKsp, was made by ligating the *Pac*I-digested cosmid backbone with a *Pac*I-*Spe*I fragment corresponding to the Ad nucl.3534–27082 from pWE.Ad5.*Afl*II-rITRsp and the *Spe*I-*Pac*I fragment from pBr/Ad.Bam-rITR.gpTKsp. The ligation mixture was packaged in λ-phage packaging extracts (Stratagene) according to the manufacturer's protocol and introduced into DH5α cells to isolate cosmid DNA.

To generate the IG.AdE1+.E3TK virus, one additional construct was made that contains the 5′ end of the Ad5, including the E1 region. Construct pBr.AdLITR-Sal(9.4) is a pBR322-based construct that contains adenovirus sequences from nucl. 1 to 9462 (*Sal*I site). Transfection of pBr.AdLITR-Sal(9.4) digested with *Sal*I and pWE.Ad5.*Afl*II-rITRgpTKsp digested with *Pac*I into PER.C6 packaging cells generates viruses through recombination of the overlapping sequences in both constructs.

E1-deleted Viruses. IG.Ad5.ClipLuc and IG.AdApt.TK are E1-deleted Ad5-based viruses containing the *luciferase* or the *HSV-1 TK* gene, respectively, driven by the human CMV promoter in the former E1 region. The CMV promoter in IG.AdApt.TK spans nt -735 to nt $+95$ (numbering according to Ref. 23), whereas in IG.Ad5.ClipLuc, a shorter version of the CMV promoter is present (nt -601 to nt -14). Furthermore, IG.AdApt.TK lacks the SV40 intron sequences that are present in IG.Ad5.ClipLuc. Both expression cassettes are terminated by the SV40 polyadenylation sequence.

IG.Ad5.Sarcoma 1800HSA.E3Luc contains the mouse HSA gene (24) driven by a Molony murine leukemia virus long terminal repeat fragment (including the retroviral splice donor and splice acceptor site) in the former E1 region. In addition, the gp19K coding sequence in the E3 region is replaced by the *luciferase* gene as in the replicating virus IG.Ad5E1$^+$.E3Luc.

All viruses were produced on PER.C6 cells (25). After one round of plaque purification, viruses were amplified further on PER.C6 cells. Cells and viruses were harvested 2–3 days after the final amplification in triple-layer flasks, and

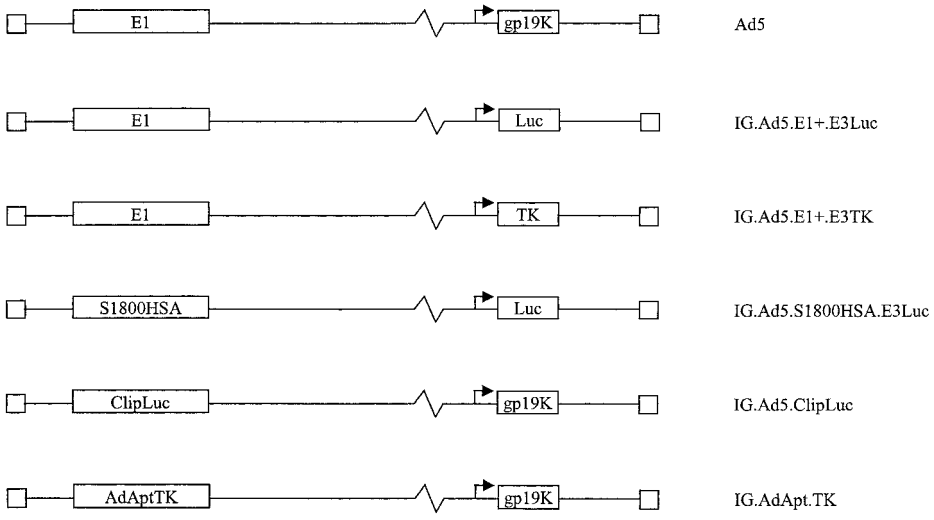

Fig. 1. Scheme of nonconditional, replication-competent adenoviral vectors and replication-incompetent control adenoviral vectors.

viral particles were purified by a two-step CsCl gradient. Virus particles in purified virus batches were determined by high-performance liquid chromatography (26), and IU were determined by end-point titration on 911 cells (27). Virus particles:IU ratios were in all cases <30. m.o.i. is expressed as IU/cell.

In Vitro Replication

To assess replication, we determined the CPE in cultured cell lines after infection with the various vectors. Cells were plated in 6-well plates at a density of 10^5 cells/well. Twenty-four h after plating, the cells were infected with the vectors at m.o.i. of 0, 1, or 10. The number of days from infection to full CPE or 100% cell death was scored. As positive controls for replication, we used the constitutively Ad5 E1-expressing cell line 293 (28) and wtAd5. The nonpermissive rat gliosarcoma cell line 9L was used as negative control. All experiments were performed in triplicate.

In a separate experiment, we tested the potential of HSV1-*tk* in combination with GCV as a "fail-safe" for adenoviral replication. A549 cells were seeded in 24-well plates at a density of 10^5 cells/well. Twenty-four h after plating, the cells were incubated with IG.Ad5E1$^+$.E3TK or IG.Ad5E1$^+$. E3− for 2 h at m.o.i. of 0, 0.1, or 1. Cells were treated immediately with GCV (Roche, Mijdrecht, Netherlands) or PBS. The medium was refreshed every day, and GCV was kept at a concentration of 10 μg/ml. CPE was scored daily, and cell viability was assessed quantitatively at days 3, 6, and 8 using the XTT-assay following the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO).

In Vitro Luciferase Expression

Cells were plated in 6-well plates at a density of 10^5 cells/well. After 24 h, the cells were infected with the vectors at a m.o.i. of 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, or 100. Forty-eight h after infection, the cells were harvested using Trypsin-EDTA (Life Technologies, Inc.). The cells were washed twice with PBS (Life Technologies, Inc.). After the last wash, the cells were resuspended in 100 μl Reporter lysis buffer (Promega, Madison, WI). After centrifugation at 14,000 rpm for 5 min, the luciferase activity was measured in the supernatant. The protein content of the supernatant was measured, and the luciferase activity was expressed as relative light units/μg of protein. All experiments were performed in triplicate.

In Vitro GCV Sensitivity

U87 MG cells were plated in 6-well plates at a density of 10^5 cells/well. After 24 h, the cells were infected with the replication-competent and -incompetent vectors carrying the HSV1-*tk* transgene at m.o.i. of 1 and 10. Forty-eight h after infection, the cells were treated with GCV (Roche) or PBS for 7 days. Day 0 is the start of treatment with GCV or PBS. The medium was refreshed every day, and GCV was kept at a concentration of 10 μg/ml. At days 1–6, the living cells were counted using trypan blue exclusion (29). To calculate the percentage of living cells, we divided the number of cells in the experimental wells by the number of cells in the control wells (no vector, no GCV; ×100%). All experiments were performed in triplicate.

Animal Experiments

All experimental protocols were approved by the Institutional Animal Care and Use Committee, in compliance with the Guide for the Care and Use of Laboratory Animals, University Hospital and Erasmus University Rotterdam, The Netherlands. Female Hsd:NIH-bg-nu-xid mice 5–6 weeks of age were purchased (Ref. 30; Harlan Sprague Dawley, Inc., Oxon, England). Mice were hosted 3–4/cage and allowed access to food and water *ad libitum*. After 1 week, 10^7 U87 MG cells were inoculated s.c. into both flanks in 500 μl of HBSS (Life Technologies, Inc.). The tumor growth was assessed by measuring bidimensional diameters three times a week with calipers. The tumor volume was determined by using the simplified formula of a rotational ellipse ($l \times \text{width}^2 \times 0.5$; Ref. 31). When the tumor reached a volume of 100–150 mm^3, animals were randomly assigned to treatment groups. Animals were treated with a single intratumoral injection of adenovirus 10^9 IU suspended in 100 μl of PBS or 100 μl of PBS alone as a control. GCV 100 mg/kg (or PBS) was administered i.p. twice daily for 7 days beginning 48 h after vector inoculation. The animals were killed by isoflurane when their tumors reached a volume of >4000 mm^2 or after 60 days. Each treatment group consisted of a minimum of three animals (six tumors).

Statistical Analysis

Data were analyzed using GraphPad Prism version 3.0 software (GraphPad Software, Inc., San Diego, CA, 1999). The CPE induced by replication-competent IG.Ad5E1$^+$. E3Luc and IG.Ad5E1$^+$.E3TK and wtAd5 in the human cell lines were compared and analyzed by repeated-measures ANOVA, and posttest analysis was performed using Dunn's multiple comparison test. The various treatment strategies testing *in vitro* U87 MG survival were compared side by side and analyzed for significance on days 2 and 6 by Student's *t* test. Statistical significance of various vector and treatment combinations *in vivo* were analyzed by Student's *t* test for normally distributed values and by nonparametric Mann-Whitney test in the case of non-normal distribution. All tests were performed two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Replication of E1+ Adenoviral Vectors. Full CPE was scored on a variety of cell lines (Table 1). Application of all vectors containing the wild-type E1 sequences resulted in full CPE at m.o.i. of 1 and 10. This indicates that deletion of the gpigK coding sequences in the E3 region had not affected the oncolytic effect of the vectors. As expected, no CPE occurred on the nonpermissive rat gliosarcoma 9L cell line. On 293 cells, all vectors, replication competent and deficient, gave full CPE within 2 days after infection. The efficacy of replication of the E1-containing vectors is similar to wtAd5, as measured by the time after infection to full CPE at m.o.i. 1 (ANOVA; $P = 0.19$) and m.o.i. 10 (ANOVA; $P = 0.49$). The duration to full CPE seems to be dose dependent (ANOVA; $P < 0.002$). In the posttest analysis, only infection with IG.Ad5E1$^+$. E3Luc resulted in significantly shorter time to CPE at m.o.i. 10 than at m.o.i. 1 ($P < 0.05$). The shorter time to CPE after infection with IG.Ad5E1$^+$.E3TK and wtAd5 at m.o.i. 10 *versus* 1 did not reach statistical significance ($P > 0.05$). The time to full CPE differed largely between cell lines. This may indicate more efficient infection or replication in certain cell lines.

In the fail-safe experiment, the A549 cells that were treated with GCV, immediately after infection with IG.Ad5E1$^+$.E3TK remained 96 (m.o.i., 0.1) to 98% (m.o.i., 1) viable whereas the cells treated with PBS were only 10 (m.o.i ,1; full CPE) to 38% (partial CPE) viable at day 8 (Fig. 2). The A549 cells infected with the replicating empty control vector IG.Ad5E1$^+$. E3− had significantly reduced viability at day 8 (full CPE at m.o.i. 1 and partial CPE at m.o.i. 0.1) irrespective

Table 1 *Cytopathogenic effect of replication-competent adenoviral vectors*

Cytopathogenic effect of replication-competent adenoviral vectors (IG.Ad5E1$^+$.E3Luc and IGAd5E1$^+$.E3TK) is compared with wtAd5 and nonreplicating E1-deleted adenoviral vectors (IG.Ad5.S1800HAS.E3Luc and IGAdApt.TK) on human glioma cells (U87MG, T98G, A172, and LW5), human lung carcinoma cells (A549), human prostate cancer cells (PC3), and a rat glioma cell line (9L). The cells were infected with the adenoviral vectors at a m.o.i. of 10 or 1. The days from infection to full CPE are scored. − indicates that no full CPE was reached at the end of the experiment, 14 days after infection. Time to full CPE is comparable between wild-type and replication-competent adenoviral vectors. As expected, no CPE occurred on the nonpermissive rat 9L glioma cell line. On the constitutively E1-expressing 293 cell line, all vectors, including the E1-deleted vectors, reached full CPE.

	U87MG	T98G	A172	LW5	A549	PC3	293	9L
m.o.i. = 10								
IG.Ad5E1$^+$.E3Luc	7	10	3	6	3	5	2	−
IGAd5E1$^+$.E3TK	6	10	4	7	3	7	2	−
wtAd5	6	10	4	7	3	5	2	−
IG.Ad5.S1800HAS.E3Luc	−	−	−	−	−	−	2	−
IGAdApt.TK	−	−	−	−	−	−	2	−
m.o.i. = 1								
IG.Ad5E1$^+$.E3Luc	8	11	4	10	6	6	2	−
IGAd5E1$^+$.E3TK	7	11	6	10	4	8	2	−
wtAd5	7	11	4	7	4	6	2	−
IG.Ad5.S1800HAS.E3Luc	−	−	−	−	−	−	2	−
IGAdApt.TK	−	−	−	−	−	−	2	−

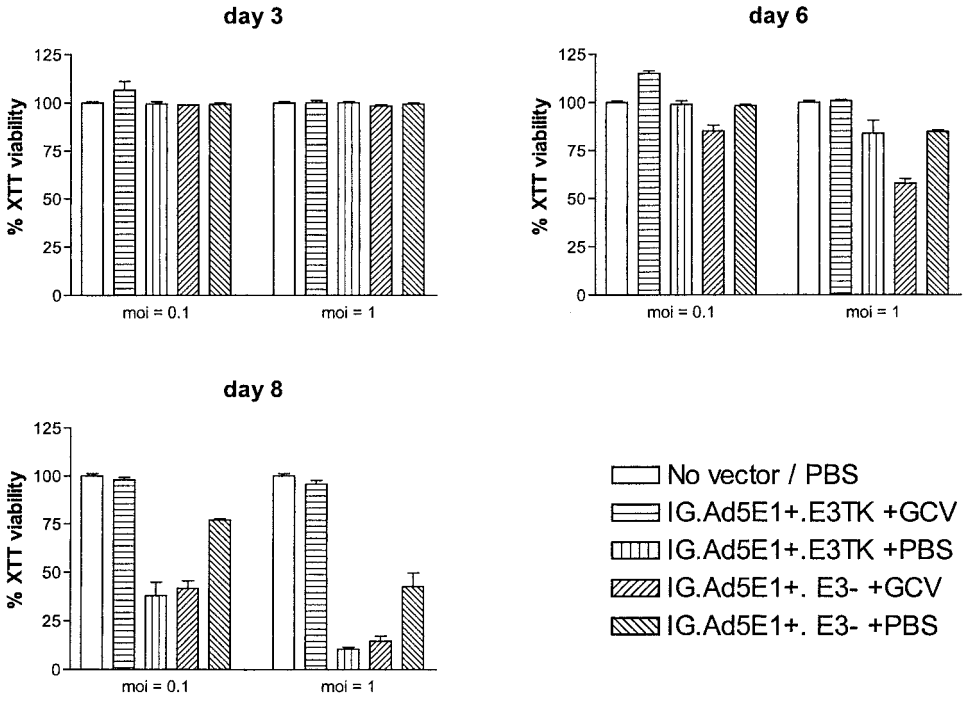

Fig. 2. Replication of adenovirus vectors containing the HSV1-*tk* gene is effectively blocked by GCV. A549 cells were infected at a m.o.i. of 0.1 and 1 with the replication-competent vectors IG.Ad5E1$^+$.E3TK or IG.Ad5E1$^+$. The cells infected with IG.Ad5E1$^+$.E3TK and immediately treated with GCV remain almost 100% viable. Cells transfected with IG.Ad5E1$^+$.E3TK and subsequently treated with PBS and the cells infected with IG.Ad5E1$^+$ (and then either GCV or PBS) are significantly less viable.

of treatment with GCV; Fig. 2). These results show that GCV can inhibit the replication and subsequent spread of IG.Ad5E1$^+$.E3TK, and that the inhibition of adenoviral replication by GCV is HSV1-*tk*-dependent.

***In Vitro* Luciferase Expression.** Luciferase expression using the E1$^+$.E3luc replication-competent vector was compared with two nonreplicating vectors containing the *luciferase* transgene in either E1 (driven by a modified CMV-promoter) or in E3 (driven by the E3 promoter, as in the replication-competent vector). The two nonreplicating vectors gave similar luciferase expression levels in most cell lines, indicating that the internal E3-promoter is of comparable strength with the modified CMV-promoter (Fig. 3). However, at the same m.o.i., the replication-competent vector yielded luciferase expression levels that were ~3 log higher than with both nonreplicating vectors in all human glioma cell lines tested (U87 MG, T98G, A172, and LW5). Alternatively, to obtain a similar expression level, 2–3 log

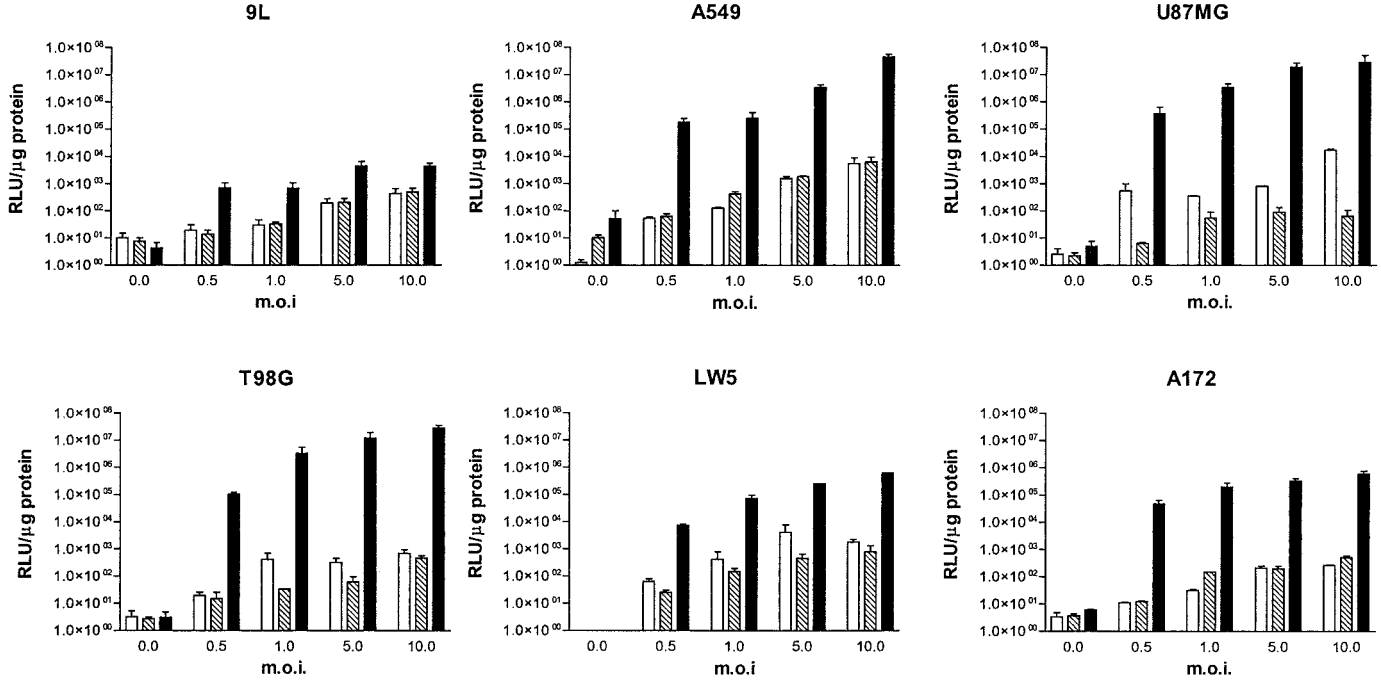

Fig. 3. Forty-eight h after infection, the luciferase expression was measured in the human glioma cell lines U87 MG, T98G, LW5, and A172; the human lung cancer cell line A549; and the nonpermissive rat glioma cell line 9L. Expression levels with the replication-competent vector IG.Ad5E1$^+$. E3Luc are in the permissive cell lines 3 log higher than with the replication-incompetent vectors. The expression levels obtained with nonreplicating IG.Ad5.ClipLuc (luciferase in E1 driven by a modified CMV promoter) and IG.Ad5.Sarcoma 1800HSA.E3Luc (luciferase in E3 driven by the internal E3 promoter) are comparable with each other in most cell lines. □, IG.Ad5.ClipLuc; ▨, IG.Ad5.S1800HSA.E3Luc; ■, IG.AdE1$^+$.E3Luc.

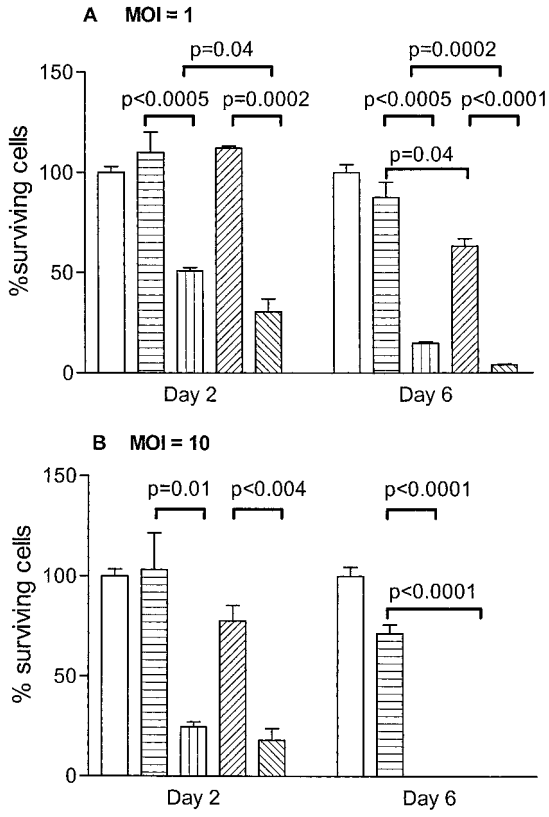

Fig. 4. *In vitro* GCV sensitivity of U87 MG glioma cells. Forty-eight h after infection with m.o.i. of 1 (*A*) or 10 (*B*), cells were exposed to PBS or GCV (10 μg/ml). Surviving cells were counted on days 2 and 6 using trypan blue exclusion and expressed as a percentage of cells in the control wells. Compared with controls, infection with replication-competent vector expressing the HSV1-*tk* gene (IG.Ad5E1$^+$.E3TK) is more toxic than the nonreplicating vector expressing HSV1-*tk* (IG.AdApt.TK) in combination with PBS. The addition of GCV significantly increases the cytotoxicity of both IG.Ad5E1$^+$.E3TK and IG.AdApt.TK. At the lower m.o.i., IG.Ad5E1$^+$.E3TK in combination with GCV is more toxic than IG.AdApt.TK. At m.o.i. = 10, both vectors are highly effective, killing almost all cells. □, no vector/PBS; ▤, IG.AdApt.TK/PBS; ▥, IG.AdApt.TK/GCV; ▨, IG.AD5E1+.E3TK/PBS; ▧, IG.AD5E1+.E3TK/GCV.

less IU of the replication-competent *versus* the nonreplicating vectors could be used. The A549 cell line showed the same pattern as the human glioma cell line. In the nonpermissive rat 9L cell line, expression levels with the replication-competent vector were ~1 log higher than with the nonreplicating vectors, indicating that some low-level replication may occur.

***In Vitro* GCV Sensitivity of U87 MG Cells.** Results of GCV sensitivity of U87 MG cells, after infection with m.o.i. 10 and 1, are summarized in Fig. 4. The addition of GCV at a concentration of 10 μg/ml in the absence of adenoviral vectors was not cytotoxic to U87 MG cells (data not shown). Infection with nonreplicating control vector IG.AdApt.TK and then the addition of PBS caused no significant cytotoxicity on days 2–6 at both m.o.i. ($P > 0.1$). However, infection with replication-competent IG.Ad5E1$^+$.E3TK and then PBS resulted in a significant reduction of the percentage of surviving cells compared with IG.AdApt.TK at day 6 [m.o.i. of 1, $P = 0.04$ (Fig. 4*A*); m.o.i. of 10, $P < 0.0001$ (Fig. 4*B*)]. Addition of GCV further increased the cytotoxicity of IG.Ad5E1$^+$.E3TK. After infection with IG.Ad5E1$^+$.E3TK at a m.o.i. of 1, the percentage of surviving cells subsequent to GCV treatment was significantly lower than after PBS, on both day 2 ($P = 0.0002$) and day 6 ($P < 0.0001$; Fig. 4*A*). After infection with the replicating vector at m.o.i. 10, the number of surviving cells was significantly lower with GCV than with PBS on day 2 ($P = 0.004$), whereas no surviving cells remained on day 6 in both groups (Fig. 4*B*).

We then compared the percentage of surviving cells after infection with either the replication-incompetent or replicating HSV1-tk-containing vectors in combination with GCV administration. After infection with m.o.i. 1, the percentage of surviving cells was significantly lower with the replication-competent IG.Ad5E1$^+$.E3TK compared with the nonreplicating vector at both day 2 ($P = 0.04$) and day 6 ($P = 0.0002$; Fig. 4A). After infection with m.o.i. 10, the percentage of surviving cells was already low in both groups at day 2 ($P = 0.3$), and at day 6, no cells had survived either treatment (Fig. 4*B*).

***In Vivo* Treatment of U87 MG Xenografts in bg-nu-xid Mice.** Treatment results of s.c. U87 MG xenografts in bg-nu-xid mice are summarized in Fig. 5 and Table 2, A–C. The growth curve of tumors treated with PBS and then i.p. PBS was identical to the growth curve of tumors treated with IG.AdApt.TK and then PBS. Compared with these control curves, the *intratumoral* injection of replication-competent IG.Ad5E1$^+$.E3TK gave slowing of tumor growth, resulting in significantly reduced tumor size at days 6, 11, 18, and 27 (Table 2A and Fig. 5). The oncolytic effect of IG.Ad5E1$^+$.E3TK was strongly enhanced by the administration of GCV, resulting in additional slowing of tumor growth and smaller tumor size at days 6, 11, 18, and 27 (Table 2B and Fig. 5). Compared with the combination treatment of IG.AdApt.TK and then GCV, replication-competent IG.Ad5E1$^+$.E3TK and then GCV was significantly more effective (Table 2C and Fig. 5).

Survival curves demonstrate prolonged survival of mice treated with IG.Ad5E1$^+$.E3TK in combination with GCV (Fig. 6).

DISCUSSION

To improve the efficacy of E1-deleted adenoviral vectors, we constructed several replication-competent adenoviral vectors carrying both E1A and E1B sequences and the HSV1-*tk* suicide gene. The HSV1-*tk* and *luciferase* genes replaced the coding sequence of the E3 region gp19K that binds to class I MHC in the endoplasmic reticulum, preventing antigen presentation on the cell surface (32). The transgenes were placed under control of the natural E3 promoter, because

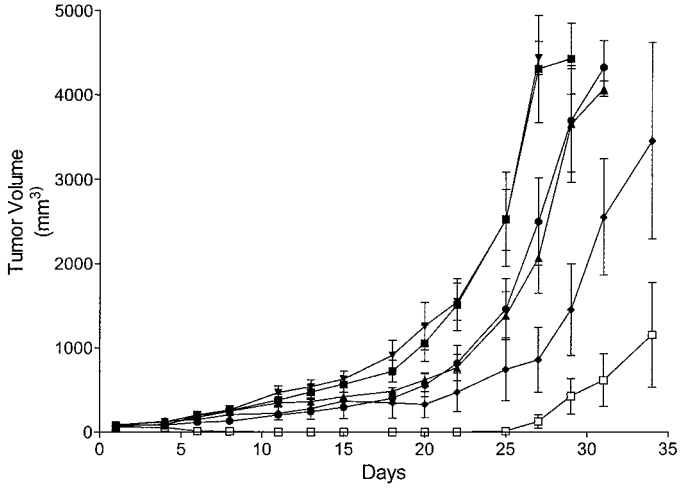

Fig. 5. Growth of U87 MG glioma xenografts in nude mice. U87 MG cells (10^7) were inoculated s.c. into both flanks in 500 μl of HBSS. When the tumor reached a volume of 100–150 mm^3, animals were treated with a single intratumoral injection with 10^9 IU of replication-competent IG.Ad5E1$^+$.E3TK, 10^9 IU of nonreplicating IG.AdApt.TK, or PBS. GCV (or PBS) was administered i.p. twice daily for 7 days (days 2–9). No difference was observed in tumor growth in animals treated with PBS/PBS IG.AdApt.TK/PBS; addition of GCV to animals treated with IG.Ad5E1$^+$.E3TK resulted in a significant slowing of tumor growth; addition of GCV to animals treated with IG.AdApt.TK also resulted in a slowing of tumor growth, although to a lesser extent than the combined treatment with replication-competent IG.Ad5E1$^+$.E3TK and GCV. —■—, PBS/PBS; —▼—, IG.AdApt.TK/PBS; —●—, IG.Ad5E1$^+$.E3TK/PBS; —▲—, PBS/GCV; —♦—, IG.AdApt.TK/GCV; —□—, IG.Ad5E1$^+$.E3TK/GCV.

Table 2 *U87MG xenograft tumor growth after adenoviral vector treatment*

Nude mice carrying s.c. U87MG glioma xenografts were treated with a single injection of 10^9 IU adenoviral vector or PBS and then by either GCV or PBS (days 2–9). Administration of replication-competent IG.Ad5E1+.E3TK and then PBS results in significant reduction in tumor growth, indicating a direct oncolytic effect of the vector (A). Administration of GCV significantly enhances the growth suppression by the replication-competent vector (B). The replication-competent vector is more effective than the nonreplicating vector in combination with GCV. Tumor volumes (mm^3) are expressed as mean ± SE, and each treatment group was composed of at least six tumors.

A. Replication-competent IG.Ad5E1+.E3TK and then PBS *versus* control

Day	PBS/PBS	IG.Ad5E1+.E3TK/PBS	*P*
1	85.3 ± 10.2	85.3 ± 10.2	1.0
6	198.3 ± 51.3	381.9 ± 160.0	0.3
11	381.6 ± 69.2	204.2 ± 28.7	0.04
18	723.8 ± 130.0	403.1 ± 71.1	0.05
27	4299 ± 635.0	2495 ± 517.4	0.05

B. Replication-competent IG.Ad5E1+.E3TK and then PBS or GCV

Day	IG.Ad5E1+.E3TK/PBS	IG.Ad5E1+.E3TK/GCV	*P*
1	85.3 ± 10.2	62.5 ± 0.6	0.2
6	381.9 ± 160.0	12.8 ± 4.9	0.002
11	204.2 ± 28.7	1.3 ± 0.8	0.002
18	403.1 ± 71.1	0.03 ± 0.02	0.002
27	2495 ± 517.4	124.7 ± 77.7	0.002

C. Replication-competent IG.Ad5E1+.E3TK *versus* nonreplicating IG.AdApt.TK and then GCV

Day	IG.AdApt.TK/GCV	IG.Ad5E1+.E3TK/GCV	*P*
1	85.4 ± 12.7	62.5 ± 0.6	0.2
6	217.0 ± 61.1	12.8 ± 4.9	0.002
11	223.0 ± 78.2	1.3 ± 0.8	0.002
18	345.7 ± 181.9	0.03 ± 0.02	0.002
27	855.3 ± 385.7	124.7 ± 77.7	0.06
31	2547 ± 689.1	614.2 ± 314.9	0.06

heterologous promoters were found to be silent when inserted in this area (33–35). To examine the transgene expression levels obtained from the internal E3 promoter, we also constructed an E1-deleted vector carrying the *luciferase* gene in E3. The luciferase expression obtained with this construct was similar to a first-generation, E1-deleted vector carrying the *luciferase* gene in E1 driven by a modified CMV-promoter, indicating the strength of the natural E3 promoter in these constructs, even in the absence of E1.

Another concern was the potential disruption of the adenovirus death protein (E3–11.6K) by our cloning strategy. Adenovirus death protein is produced in large amounts at the late stage of infection and is required for effective cell lysis and virus release (36). However, the oncolytic effect of the gp19K-deleted, replicating vectors was similar to wtAd5, as measured by the time after infection to full CPE. The time to full CPE with both wtAd5 and the replication-competent vectors differed largely between the tested cell lines. The time to CPE seemed not to correlate with the level of luciferase expression, indicating that the efficacy of replication may not only depend on the efficacy of cell entry. Elucidating mechanisms affecting the efficiency of adenoviral replication in different cell lines requires additional study to enhance replication potentially (37). As expected, the replication-competent vectors and wtAd5 did not cause CPE in the nonpermissive 9L rat gliosarcoma cell line.

We then compared the luciferase expression levels of the E1+ luciferase vector with both E1-deleted vectors and found that luciferase expression was ~3 logs higher with the E1+ vector in all permissive (human) cell lines tested. Similar expression levels were obtained with ~2 log lower m.o.i. of the E1+ vector. Interestingly, infection of the nonpermissive 9L cell line with the E1+ vector resulted in significantly higher luciferase expression than with the E1-deleted vectors. This may indicate either low-level replication of the E1+ vector in the rodent cell line or transactivation of the E3 promoter by E1 proteins.

Apart from the direct oncolytic effect on tumor cells (38), replication-competent vectors may enhance further the antitumor efficacy by increased expression levels of a tumoricidal transgene. To test this hypothesis, we examined the antitumor efficacy of E1+ and E1-deleted vectors carrying the HSV1-*tk* suicide gene. *In vitro*, the E1+ HSV1-tk vector demonstrated cytotoxicity to the U87 MG glioma cell line that was greatly enhanced by the addition of GCV. The latter combination was also significantly more toxic than infection with the E1-deleted HSV1-tk control vector and then GCV. *In vivo*, a single injection of the replicative E1+ HSV1-tk vector in established s.c. U87 MG xenografts resulted in a significant slowing of tumor growth. The addition of GCV resulted in additional slowing of tumor growth and significantly prolonged survival. Also, the E1+ HSV1-tk vector in combination with GCV was significantly more effective than the E1-deleted HSV1-tk vector with GCV.

Replication-competent adenoviral vectors are a new and rapidly evolving platform for gene therapy (39). In the past, intratumoral injection of wild-type adenoviruses has not led to unwanted effects (21). However, "virotherapy" for cancer was abandoned because of only a few documented responses and because of the advent of more effective chemotherapeutic agents (20). Bischoff *et al.* (38) reintroduced the oncolytic effect of adenoviruses with vectors carrying mutations in the early region of the encoding E1B-M_r 55,000. These vectors only replicate in cells with a deficient p53 pathway and cause specific cytolysis after intratumoral and i.v. administration (40). Subsequently, an additive effect of HSV1-*tk*/GCV and radiotherapy to the oncolytic effect of E1B-deleted vectors was demonstrated (41, 42). The use of conditional replicating vectors that contain deletions in E1B (or E1A) carries the risk of reduced replication as compared with wtAd5. The present study demonstrates that even vectors replicating as well as wtAd5 don't cause tumor eradication in some models. Therefore, addition of a therapeutic transgene is probably mandatory, and genes with improved bystander and cytotoxic effects may add

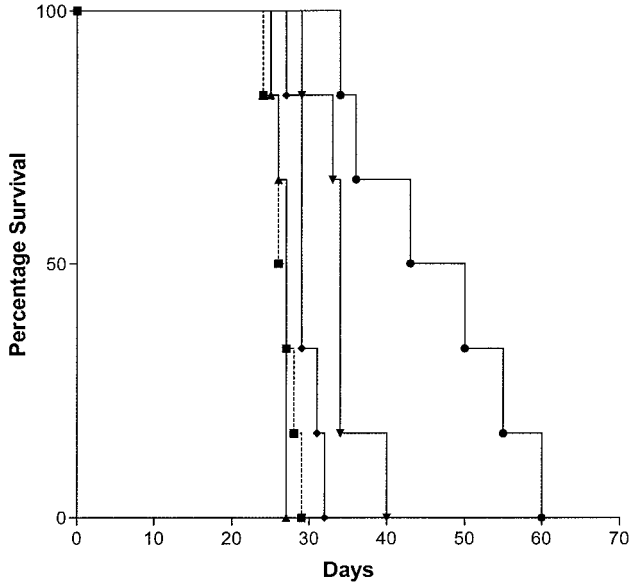

Fig. 6. Kaplan-Meier survival analysis of nude mice with s.c. U87 MG xenografts. To establish s.c. tumors, mice were inoculated s.c. in both flanks with U87 MG cells (10^7) in 500 μl of HBSS. The tumor growth was assessed by measuring bidimensional diameters, and when the tumor reached a volume of 100–150 mm^3, animals were assigned randomly to a single intratumoral injection with 10^9 IU of replication-competent IG.Ad5E1$^+$.E3TK, 10^9 IU of nonreplicating IG.AdApt.TK, or PBS. GCV 100 mg/kg (or PBS) was administered i.p. twice daily for 7 days beginning 48 h after vector inoculation. The animals were killed by isoflurane when the tumors reached a volume of >4000 mm^2 or after 60 days. Animals treated with replication-competent IG.Ad5E1$^+$.E3TK and GCV lived significantly longer than animals treated with IG.Ad5E1$^+$.E3TK alone or with nonreplicating IG.AdApt.TK combined with GCV.

further to the efficacy of replicating vectors (43). The toxic genes, such as the HSV1-*tk* gene, can also function as a fail-safe mechanism when replication outside the tumor occurs (41). Here, we demonstrated that adenoviral replication and subsequent spread can be effectively blocked by the immediate administration of GCV. The complex interactions between viral replication and GCV require additional *in vivo* studies to determine the optimal interval between vector injection and first GCV administration. In clinical studies, conditional replication will be required for patient- and biosafety. An alternative for selective replication is to drive the *E1B* or/and *E1A* genes by a tissue or tumor-specific promoter. Such vectors may replicate in target tissue (and tumor) even better than wtAd5. Examples include the use of enhancer/promoter sequences of the *prostate specific antigen* gene to drive E1A in prostate cancer (44), the α-fetoprotein promoter in hepatocellular carcinoma (45), and the DF3/MUC1 promoter in breast cancer (46).

In the present study, we have demonstrated that the addition of the HSV1-tk/GCV suicide system enhances the tumoricidal efficacy of replication competent adenoviral vectors in glioma cell lines and after intratumoral injection in a glioma xenograft model. The use of a suicide gene has the additional advantage of fail-safe in case of spread of the vector outside the tumor. Safety will be increased further by conditional replication, for instance by using a glia-specific promoter such as the gfa2 promoter, to drive the *E1A* gene (47).

ACKNOWLEDGMENTS

We gratefully acknowledge Eric Brouwer for technical assistance.

REFERENCES

1. Central Brain Tumor Registry of the United States. 1997 Annual Report. Chicago, IL: CBTRUS, 1998.
2. Visser, O., Coebergh, J. W. W., Schouten, L. J., and van Dijck, J. A. A. M. Incidence of Cancer in the Netherlands 1995. Utrecht, the Netherlands: Vereniging van Integrale Kankercentra, 1998.
3. Grovas, A., Fremgen, A., Rauck, A., Ruymann, F. B., Hutchinson, C. L., Winchester, D. P., and Menck, H. R. The National Cancer Data Base report on patterns of childhood cancers in the United States. Cancer (Phila.), *80:* 2321–2332, 1997.
4. Surawicz, T. S., Davis, F., Freels, S., Laws, E. R., Jr., and Menck, H. R. Brain tumor survival: results from the National Cancer Data Base. J Neurooncol., *40:* 151–160, 1998.
5. Davis, F. G., Freels, S., Grutsch, J., Barlas, S., and Brem, S. Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: an analysis based on Surveillance, Epidemiology, and End Results (SEER) data, 1973–1991. J. Neurosurg., *88:* 1–10, 1998.
6. Davis, F. G., McCarthy, B. J., Freels, S., Kupelian, V., and Bondy, M. L. The conditional probability of survival of patients with primary malignant brain tumors: surveillance, epidemiology, and end results (SEER) data. Cancer (Phila.), *85:* 485–491, 1999.
7. Perry, J. R., DeAngelis, L. M., Schold, S. C., Jr., Burger, P. C., Brem, H., Brown, M. T., Curran, W. J., Scott, C. B., Prados, M. D., Kaplan, R., and Cairncross, J. G. Challenges in the design and conduct of Phase III brain tumor therapy trials. Neurology, *49:* 912–917, 1997.
8. Burger, P. C. Pathologic anatomy and CT correlations in the glioblastoma multiforme. Appl. Neurophysiol., *46:* 180–187, 1983.
9. Kelly, P. J., Daumas-Duport, C., Kispert, D. B., Kall, B. A., Scheithauer, B. W., and Illig, J. J. Imaging-based stereotaxic serial biopsies in untreated intracranial glial neoplasms. J. Neurosurg., *66:* 865–874, 1987.
10. Massey, V., and Wallner, K. E. Patterns of second recurrence of malignant astrocytomas. Int. J. Radiat. Oncol. Biol. Phys., *18:* 395–398, 1990.
11. Wallner, K. E., Galicich, J. H., Krol, G., Arbit, E., and Malkin, M. G. Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma. Int. J. Radiat. Oncol. Biol. Phys., *16:* 1405–1409, 1989.
12. Culver, K. W., Ram, Z., Walbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. *In vivo* gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. Science (Wash. DC), *256:* 1550–1552, 1992.
13. Vincent, A. J. P. E., Vogels, R., Van Someren, G., Esandi, M. C., Noteboom, J. L., Avezaat, C. J. J., Vecht, C., Van Bekkum, D. W., Valerio, D., Bout, A., and Hoogerbrugge, P. M. Herpes simplex virus thymidine kinase gene therapy for rat malignant brain tumors. Human Gene Ther., *7:* 197–205, 1996.
14. Okada, H., Miyamura, K., Itoh, T., Hagiwara, M., Wakabayashi, T., Mizuno, M., Colosi, P., Kurtzman, G., and Yoshida, J. Gene therapy against an experimental glioma using adeno-associated virus vectors. Gene Ther., *3:* 957–964, 1996.
15. Kramm, C. M., Chase, M., Herrlinger, U., Jacobs, A., Pechan, P. A., Rainov, N. G., Sena-Esteves, M., Aghi, M., Barnett, F. H., Chiocca, E. A., and Breakefield, X. O. Therapeutic efficiency and safety of a second-generation replication-conditional HSV1 vector for brain tumor gene therapy. Hum. Gene Ther., *8:* 2057–2068, 1997.
16. Coffey, M. C., Strong, J. E., Forsyth, P. A., and Lee, P. W. Reovirus therapy of tumors with activated Ras pathway. Science (Wash. DC), *282:* 1332–1334, 1998.
17. Rainov, N. G. A Phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. Hum. Gene Ther., *11:* 2389–2401, 2000.
18. Trask, T. W., Trask, R. P., Aguilar-Cordova, E., Shine, H. D., Wyde, P. R., Goodman, J. C., Hamilton, W. J., Rojas-Martinez, A., Chen, S. H., Woo, S. L., and Grossman, R. G. Phase I study of adenoviral delivery of the *HSV-tk* gene and ganciclovir administration in patients with current malignant brain tumors. Mol. Ther., *1:* 195–203, 2000.
19. Sandmair, A. M., Loimas, S., Puranen, P., Immonen, A., Kossila, M., Puranen, M., Hurskainen, H., Tyynela, K., Turunen, M., Vanninen, R., Lehtolainen, P., Paljarvi, L., Johansson, R., Vapalahti, M., and Yla-Herttuala, S. *Thymidine kinase* gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. Hum. Gene Ther., *11:* 2197–2205, 2000.
20. Wildner, O., Morris, J. C., Vahanian, N. N., Ford, H., Ramsey, W. J., and Blaese, R. M. Adenoviral vectors capable of replication improve the efficacy of *HSVtk/GCV* suicide gene therapy of cancer. Gene Ther., *6:* 57–62, 1999.
21. Smith, R. R., *et al.* Studies on the use of viruses in the treatment of carcinoma of the cervix. Cancer (Phila.), *9:* 1211–1218, 1956.
22. Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Investig. Urol., *17:* 16–23, 1979.
23. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell, *41:* 521–530, 1985.
24. Kay, R., Takei, F., and Humphries, R. K. Expression cloning of a cDNA encoding M1/69-J11d heat-stable antigens. J. Immunol., *145:* 1952–1959, 1990.
25. Fallaux, F. J., Bout, A., van der Velde, I., van den Wollenberg, D. J., Hehir, K. M., Keegan, J., Auger, C., Cramer, S. J., van Ormondt, H., van der Eb, A. J., Valerio, D., and Hoeben, R. C. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. Hum. Gene Ther., *9:* 1909–1917, 1998.
26. Shabram, P. W., Giroux, D. D., Goudreau, A. M., Gregory, R. J., Horn, M. T., Huyghe, B. G., Liu, X., Nunnally, M. H., Sugarman, B. J., and Sutjipto, S. Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. Hum. Gene Ther., *8:* 453–465, 1997.
27. Fallaux, F. J., Kranenburg, O., Cramer, S. J., Houweling, A., Van Ormondt, H., Hoeben, R. C., and Van Der Eb, A. J. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum. Gene Ther., *7:* 215–222, 1996.
28. Allard, A., and Wadell, G. Physical organization of the enteric adenovirus type 41 early region 1A. Virology, *164:* 220–229, 1988.
29. Vincent, A. J., Esandi, M. C., Avezaat, C. J., Vecht, C. J., Sillevis Smitt, P., van Bekkum, D. W., Valerio, D., Hoogerbrugge, P. M., and Bout, A. Preclinical testing of recombinant adenoviral herpes simplex virus thymidine kinase gene therapy for central nervous system malignancies. Neurosurgery, *41:* 442–451, 1997.
30. Kamel-Reid, S., and Dick, J. E. Engraftment of immune-deficient mice with human hematopoietic stem cells. Science (Wash. DC), *242:* 1706–1709, 1988.
31. Dethlefsen, L. A., Prewitt, J. M., and Mendelsohn, M. L. Analysis of tumor growth curves. J. Natl. Cancer Inst. (Bethesda), *40:* 389–405, 1968.
32. Rawle, F. C., Tollefson, A. E., Wold, W. S., and Gooding, L. R. Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. J. Immunol., *143:* 2031–2037, 1989.
33. Berkner, K. L. Expression of heterologous sequences in adenoviral vectors. Curr. Top. Microbiol. Immunol., *158:* 39–66, 1992.
34. Johnson, D. C., Ghosh-Choudhury, G., Smiley, J. R., Fallis, L., and Graham, F. L. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology, *164:* 1–14, 1988.
35. Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R., and Hung, P. P. Recombinant adenovirus induces antibody response to hepatitis B virus surface antigens. Proc. Natl. Acad. Sci. USA, *84:* 4626–4630, 1987.
36. Tollefson, A. E., Scaria, A., Hermiston, T. W., Ryerse, J. S., Wold, L. J., and Wold, W. S. The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. J. Virol., *70:* 2296–2306, 1996.
37. Heise, C., Hermiston, T., Johnson, L., Brooks, G., Sampson-Johannes, A., Williams, A., Hawkins, L., and Kirn, D. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. Nat. Med., *6:* 1134–1139, 2000.
38. Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A., and McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science (Wash. DC), *274:* 373–377, 1996.
39. Kirn, D. Selectively replicating viruses as therapeutic agents against cancer. *In:* E. C. Lattime and S. L. Gerson (eds.), Gene Therapy of Cancer, pp. 235–248. San Diego, CA: Academic Press, Inc., 1999.
40. Heise, C. C., Williams, A., Olesch, J., and Kirn, D. H. Efficacy of a replication-competent adenovirus (ONYX-015) following intratumoral injection: intratumoral spread and distribution effects. Cancer Gene Ther., *6:* 499–504, 1999.

41. Wildner, O., Blaese, R. M., and Morris, J. C. Synergy between the herpes simplex virus tk/ganciclovir prodrug suicide system and the topoisomerase I inhibitor topotecan. Hum. Gene Ther., *10:* 2679–2687, 1999.
42. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. Hum. Gene Ther., *11:* 67–76, 2000.
43. Erbs, P., Regulier, E., Kintz, J., Leroy, P., Poitevin, Y., Exinger, F., Jund, R., and Mehtali, M. *In vivo* cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene. Cancer Res., *60:* 3813–3822, 2000.
44. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res., *57:* 2559–2563, 1997.
45. Hallenbeck, P. L., Chang, Y. N., Hay, C., Golightly, D., Stewart, D., Lin, J., Phipps, S., and Chiang, Y. L. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum. Gene Ther., *10:* 1721–1733, 1999.
46. Kurihara, T., Brough, D. E., Kovesdi, I., and Kufe, D. W. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. J. Clin. Investig., *106:* 763–771, 2000.
47. Brenner, M., Kisseberth, W. C., Su, Y., Besnard, F., and Messing, A. GFAP promoter directs astrocyte-specific expression in transgenic mice. J. Neurosci., *14:* 1030–1037, 1994.

Treatment of Malignant Gliomas with a Replicating Adenoviral Vector Expressing Herpes Simplex Virus- *Thymidine Kinase*

Dharmin Nanda, Ronald Vogels, Menzo Havenga, et al.

Cancer Res 2001;61:8743-8750.