ABSTRACT

In this study, we evaluated three herpes simplex virus-1 thymidine kinase (HSV-tk) carrying replication-competent adenoviral vectors with and without the Ad5 E1B 55 kDa gene to assess whether this gene product has an influence on their antitumor efficacy, replication kinetics, and potential hepatotoxicity. Furthermore, we assessed the efficacy of these vectors in combination with ganciclovir (GCV). When compared with wild-type adenovirus, the recombinant vectors, in particular the E1B 55 kDa-deleted vector Ad.TKΔ55(II), generated a more efficiently cytopathic effect in proliferating cells, independently of their p53 genotype. In a s.c. A549 lung cancer xenograft model, the cyto-reductive effect of Ad.TKΔ55(II) was enhanced when followed by GCV treatment. In contrast, the efficacy of both E1B 55 kDa-positive vectors could not be further improved by GCV. In an i.p. MDAH 2774 ovarian cancer xenograft tumor model, the survival of animals treated with a prototypical replication-deficient adenovirus expressing HSV-tk (Ad.TK) was improved compared to controls when followed by GCV. In contrast, the cytoreductive efficacy of the replication-competent vectors was diminished when combined with the virostatic GCV. However, the antitumor effect of all replication-competent vectors was superior to combination chemotherapy with paclitaxel and carboplatin. In both tumor models, the oncolytic effect of the replication-competent vectors was diminished when combined with the virostatic GCV. Consequently, the tumor penetration of replication-competent vectors was superior to combination chemotherapy with paclitaxel and carboplatin. In both tumor models, the oncolytic effect of the E1B 55 kDa-positive vectors was greater than that of Ad.TKΔ55(II). In an attempt to assess the toxicity of these vectors in a nonpermissive host, the viruses were administered systemically to immunocompetent and immunodeficient mice. Greater hepatotoxicity was seen with i.v. administration of the replication-competent viruses than with Ad.TK and in immunocompetent hosts, suggesting involvement of the immune system in the induction of tissue damage. The E1B 55 kDa gene had no significant influence on the liver toxicity of the vectors in this system. At therapeutic doses, intratumoral or i.p. injection of all vectors was well tolerated. Importantly, these replication-competent HSV-tk-expressing vectors were highly susceptible to GCV, representing an effective fail-safe mechanism to abolish viral replication in a clinical setting. Controllable intratumoral viral replication holds promise as a new treatment modality for cancer.

INTRODUCTION

Gene therapy for cancer is different from that of genetic diseases because an effective tumor therapy requires complete eradication of all tumor cells in the body, whereas successful therapy of genetic disease may require only a small percentage of cells to be genetically modified. The need to transduce all tumor cells might be partially overcome by the biological amplification of local and systemic anti-tumor effects by the transfer of immunostimulatory genes or by the bystander effects of certain suicide gene/prodrug combinations.

The most widely studied gene directed enzyme prodrug system is based on the selective phosphorylation of GCV by vector-encoded HSV-tk to GCV-monophosphate (1). Cellular kinases further phosphorylate GCV-monophosphate to di- and triphosphate metabolites (2). Incorporation of GCV into macromolecular DNA of dividing cells results in chain termination (3), chromosomal aberrations, and sister chromatid exchange (4, 5), ultimately leading to cell death. In many tumor models, the HSV-tk/GCV system is associated with a gap-junction-mediated bystander effect, the extension of cytotoxic effects to adjacent untransduced cells (1, 6). Furthermore, it has been demonstrated that in situ killing of tumors by HSV-tk/GCV can induce tumor-specific immunity that leads to rejection of successive attempts to implant untransduced tumor cells (7, 8).

Human adenovirus-based vectors have emerged as promising vehicles for in vivo gene transfer (9). The major emphasis on the safety of viral vectors for gene delivery has led to the generally accepted approach of disabling their ability to replicate and spread by successive cycles of infection. Consequently, the tumor penetration of intratumorally administered replication-deficient vectors is largely confined to cells adjacent to the needle tract. Responsible for this phenomenon are several factors, including the interconnection of tumor cells by tight junctions and high density of cellular receptors and binding of viral particles by these receptors effectively removing them from the intracellular space, thereby decreasing the driving gradient for particle diffusion (10). Vectors with the ability to replicate and spread within neoplastic tissue from the initial site of infection have the potential to overcome poor transduction efficiency. Attenuated replication-competent viruses have been used extensively in human vaccination programs (11). In addition, over the last 40 years, there have been several clinical trials in which replication-competent wild-type viruses, including adenoviruses, were intratumorally administered to patients with various malignancies (12–14). As a cancer treatment, virotherapy was abandoned because few responses were reported, its effects were unpredictable, and the development of more active chemotherapeutic agents supplant it. Partially responsible for this failure were insufficient methods for large-scale virus production, purification, and assessment of infectivity. Virotherapy is now being re-evaluated in the light of the findings by Bischoff et al. (15) demonstrating that the E1B 55 kDa-deleted adenovirus H5d1520 (16), also referred to as ONYX-015, replicates in and preferentially lyases p53-functional cells. This virus, which does not express a therapeutic or fail-safe gene, has been used in clinical trials in combination with cisplatin in patients with advanced carcinoma of the head and neck (17). To combine the advantages of the prodrug/suicide gene approach with that of lytic viruses, we and others have developed E1B 55 kDa-deficient adenoviral vectors expressing HSV-tk (18, 19) or carrying a cytosome deaminase-HSV-tk fusion gene (20). Preclinical s.c. tumor models with these E1B 55
kDa-deleted vectors demonstrated that this dual strategy is more efficacious than each component individually (18–20).

The rationale for undertaking this study was based on the finding that E1B 55 kDa-deleted mutants replicate significantly less well than Ad5wt (21). Responsible for this are most likely the lacking ability of the E1B 55 kDa gene product to relieve the restrictions on adenoviral replication imposed by the cell cycle (22), and to facilitate the preferential transport of viral mRNA during the late stage of a lytic adenoviral infection (16, 23).

In this study, we assessed a s.c. lung cancer and in an i.p. ovarian cancer model whether the expression of the Ad5 E1B 55 kDa gene product in replication-competent adenoviral vectors has an influence on their antitumor efficacy, replication, and potential hepatotoxicity. Furthermore, we examined whether the cytoreductive effect of these vectors could be further enhanced by the HSV-tk/GCV system.

MATERIALS AND METHODS

Construction of Adenoviral Vectors. The vectors used in this study are schematically depicted in Fig. 1.

**Ad.TK.** The E1 region of this replication-defective adenovirus was replaced with the HSV-tk (pHSV-106, Life Technologies, Grand Island, NY) gene under the transcriptional control of the human CMV-IE promoter (pCDM8, Invitrogen, Carlsbad, CA; Ref. 19).

**Ad.TK** (II). Ad.TK** (II) (19) is identical to Ad.TK** (II), except that the latter has a smaller E3 deletion (bp 27865–30995 versus bp 28593–30471; GenBank M73260). Both vectors harbor an HSV-tk-ires (24, 25)-Ad5 E1A 13s expression cassette driven by the human CMV-IE promoter in combination with the adenovirus TPL in the E1 region. This cassette is flanked upstream by the Ad5 packaging sequence and downstream by the Ad5 pIX. Ad.TK** (II) was generated by in vitro ligation (26) to Ad5/327 (kindly provided by T. Shenk, Princeton University, Princeton, NJ), using the unique Bst1107 I restriction site.

**Ad.OW34.** Ad.OW34 was generated by homologous recombination of the above-described HSV-tk Ad5 E1A expression cassette driven by the human CMV-IE promoter with pBHG10 (27). In this vector, the expression of E1A is transcriptionally coupled via an IRES to the HSV-tk gene. In contrast to Ad.TK** (II), this vector lacks the TPL upstream of the HSV-tk gene but carries the E1B 55 kDa gene.

**Ad.OW37.** The structure of this vector is identical to that of Ad.OW34, except the IRES was replaced with a linker (LRDPMARAAAT), and E1A consisted only of E1A 13s, creating an HSV-tk-E1A 13s fusion protein.

Amplification and Purification of Viruses. All viruses were propagated in 293 cells (Microbix, Toronto, ON), purified by two rounds of CsCl density centrifugation (28), dialyzed (Slide-A-Lyzer, Pierce, Rockford, IL) against 1500 ml of PBS with 1 mM MgCl2 and 10% glycerol four times (1 h each) at 4°C, and stored at −80°C. Virus concentration was determined by measuring absorbancy at 260 nm (29), and the titer was estimated by plaque assay on 293 cells (30). In all preparations, the ratio of infectious virus particles was 1:100. Under the same conditions, 2 ml of CsCl [1.34 g/ml, the density of Ad5wt virus (31)] were dialyzed and used as a control vehicle. All virus preparations were assessed for endotoxin by the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Falmouth, MA) according to the manufacturer’s instructions. The detection limit of this test was 0.05 endotoxin unit/1000 pfu/ml. The reference strain VR-5 (American Type Culture Collection, Manassas, VA) was used as wild-type adenovirus type 5 (Ad5wt).

**Tissue Culture.** The human cell lines A375 (CRL-1619) (32), A549 (CCL-185) (33), HeLa (CCL-2) (34), and MDAH 2774 (CRL-10303) (35) were purchased a few weeks before conducting the experiments from the American Type Culture Collection (Manassas, VA). The genotype of the lung cancer cell line A549 is wild type for p53 (36), and sequencing of the p53 DNA from the melanoma cell line A375 revealed no mutations (data not shown). The p53 gene of HeLa cells is wild type but dysfunctional because of the presence of human papillomavirus 18 E6 (37). The MDAH 2774 ovarian cancer cell line overexpressed wild-type p53 (38). All cell lines were propagated in D-10 medium, consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml of gentamicin. Tissue culture medium and supplements were purchased from Life Technologies. Cells were maintained in log-phase growth at 37°C in a humidified atmosphere of 95% air and 5% CO2.

**CPE.** Five days after infection, the cells were washed, paraformaldehyde-fixed, and stained with crystal violet when essentially complete lysis was observed in subconfluent cells infected with Ad5wt.

**Viral Replication Kinetics.** Five million HeLa cells were plated in T-75 cm2 flasks and infected at 80% confluency with Ad.TK** (II), Ad.TK** (II), Ad.OW34, Ad.OW37, or Ad5wt at a MOI of 10 pfu/cell. After 1 h, cell monolayers were extensively washed, and the medium was replaced with 50 ml of serum-free media. All cells were superinfected in duplicate at various time points with a real-time qPCR-based assay on an ABI Prism 7700 sequence detection system (PE Biosystems, Foster City, CA) using a fluorogenic probe and primers within the packaging sequence of Ad5wt (39). All samples were analyzed in duplicate. Control samples were spiked with vector DNA to exclude potential PCR inhibitors. The assay was linear over the entire detection range of vector copies in the supernatants, and the coefficient of variation, determined as the SD divided by the mean slopes of the standard curves times 100, was 1.8%. The ratio of infectious titers obtained by plaque titration/copies of adenoviral DNA determined by qPCR was ~1:100.

**Plaque Reduction Assay.** A plaque reduction assay was performed to determine the virostatic activity of GCV on Ad.OW34 and Ad5wt. Eighty % confluent monolayers of 293 cells in six-well plates were infected with 100 pfu/well of Ad.OW34 or Ad5wt. After static adsorption at 37°C, the monolayers were washed with PBS containing 1% of the human immunoglobulin preparation Sandoglobulin (Sandoz Pharmaceuticals Corp., East Hanover, NJ) and overlaid with MEM (Life Technologies) medium containing 2% SeaPlaque agarose (FMC Corp., Rockland, ME) and 0.1, 0.5, 1, or 5 μM GCV, respectively. The assay was performed in triplicate, and the plaques were counted at day 8. The IC50 and IC90 were determined by computer-fitted dose-response curves.

**Animal Studies.** All experimental protocols were approved by the Animal Care and Use Committee of the National Human Genome Research Institute in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23).

Six- to 8-week-old female BALB/c-nu/nu and C57BL/6 mice were obtained from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD). Animals were maintained under specific pathogen-free conditions and were euthanized with CO2 if their tumors exceeded 10% body weight or if the mice appeared to be in distress.

**s.c. Lung Cancer Xenograft Model.** Ten million viable A549 cells in 100 μl of serum-free DMEM with 10% Matrigel (Collaborative Products, Bedford, MA) were s.c.-injected into the right flank of nude mice. Two-dimensional tumor measurements were performed with calipers at least once a week, and tumor volume was determined using the simplified formula of a rotational ellipse ($l \times w^2 \times 0.5$; Ref. 40). The animals were randomly assigned to treatment groups when the tumors reached a volume of ~250 mm3. Five animals each were left untreated or received GCV alone (100 mg/kg i.p. b.i.d.) from days 2 to 6 in 1 ml of 0.9% NaCl solution. Ten animals in each group were subjected to a single intratumoral injection with 100 μl of 1 × 106 pfu Ad.TK** (II), Ad.OW34, or Ad.OW37 alone or followed by GCV (100 mg/kg i.p. b.i.d.) from days 8 to 12. End point of this study was reached if the tumor size of one animal in any treatment group reached 10% of its body weight.

**i.p. Ovarian Cancer Model.** Nude mice received 1 × 106 of viable MDAH 2774 cells in 1 ml of PBS i.p. One week later, animals were assigned to treatment groups, and therapy was initiated. Pilot experiments revealed that all animals had macroscopically detectable peritoneal carcinomatosis at this time point. Groups of five animals were left untreated or received i.p. injections of GCV alone (10 mg/kg i.p. b.i.d.) from days 2 to 8 in 1 ml of 0.9% NaCl solution. Other groups received a single i.p. injection of 1 × 106 pfu adenoviral vector in 1 ml of PBS alone or in combination with GCV (10 mg/kg i.p. b.i.d.) from days 2 to 8 after Ad.TK administration, and from day 8 to 14 for the replication-competent vectors. The i.p.-administered dose of GCV was similar to that used in clinical trials for the treatment of brain tumors (5 mg/kg twice daily for 14 days; Ref. 41). One group of animals received the combination of paclitaxel (Taxol® , Bristol-Meyers, Princeton, NY) at 24 mg/kg/day (days 1 to 30).
followed by carboplatin (Paraplatin, Bristol-Meyers) at a dose of 20 mg/kg/day (days 6 to 10; Ref. 42).

Toxicology Studies. Because human adenoviruses replicate only in human cells, toxicology studies with adenoviral vectors are hampered by the availability of animal models; we assessed this toxicity in a nonpermissive host. Hepatotoxicity is the principal side effect of systemically administered adenoviral vectors to an immunocompetent host. For this, C57BL/6 and nude mice received a single tail vein injection of \(1 \times 10^{10}\) or \(1 \times 10^{11}\) particles of Ad.TK, Ad.TK RC (II), Ad.OW34, or Ad5 wt. Forty-eight h after virus administration, blood samples were obtained by puncture of the retro-orbital plexus and the serum levels of AST, ALT, ALP, and GGT were determined by automated colorimetric assays.

Histology and TEM. For histological analysis, liver samples (left half of the large upper lobe) were fixed in 10% neutral buffered formalin for 24 h, processed by routine methods on a Tissue-Tek VIP Tissue Processor (Sakura Finetek U.S.A., Torrance, CA), and embedded in paraffin. Five-μm tissue sections were cut with a Leica rotary microtome (Leica Microsystems Wetzlar, Germany), followed by H&E staining.

For electron microscopy, liver samples (1 mm³) were fixed in phosphate-buffered mixture of 2.5% glutaraldehyde and 1.25% formaldehyde overnight, followed by a 1-h postfixation with 1% osmium tetroxide. The tissues were then rinsed in water, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon 812 resin (Shell Chemicals, Houston, TX). After examination of semi-thin sections, areas were selected and submitted to ultrathin sectioning. Sections collected on 200-mesh copper grids were contrasted with lead citrate and uranyl acetate, examined, and photographed with a JEOL 100CX (JEOL Ltd, Akishima, Japan) transmission electron microscope.

Statistical Methods. The software package STATISTICA (version 5.5 for Windows; StatSoft, Inc., Tulsa, OK) was used for Cox-Mantel survival analysis and tumor volume comparison with the Mann-Whitney U test.

RESULTS

**CPE Assay.** To examine whether the CPE of the Fig. 1-depicted replication-competent viruses is influenced by the p53 phenotype and/or cell cycle status, subconfluent and confluent monolayers of A549, A375, MDAH 2774, and HeLa cells were infected at a MOI of 1 pfu/cell with either Ad.TK, Ad5 wt, Ad.TK RC (II), Ad.OW34, or Ad.OW37. As shown in Fig. 2, there was no CPE detectable in subconfluent and confluent cell monolayers when left untreated or transduced with Ad.TK. Subconfluent A549 cell monolayers infected with the replication-competent viruses resulted in complete cell lysis on day 5. The oncolytic effect of all replication-competent vectors was similar to that of Ad5 wt. In contrast, all replication-competent adenoviral vectors expressing HSV-\( k \) caused at the same MOI only little CPE in confluent A549 cells. However, infection of confluent A549 cell monolayers with Ad5 wt caused lysis, although less efficient than in subconfluent monolayers. Subconfluent A375 or MDAH 2774 cell monolayers infected with the E1B 55 kDa-positive viruses resulted in complete lysis, and incubation with Ad.TK RC (II) caused only partial cell lysis. Infection of confluent A375 or MDAH 2774 cell monolayers with the replication-competent vectors caused almost no CPE. In contrast, infection of subconfluent or confluent HeLa monolayers with the E1B 55 kDa-positive replication-competent vectors caused complete lysis, this effect was somewhat less in monolayers infected with Ad.TK RC (II).

![Fig. 1. Organization of the vectors used in this study.](image-url)

![Fig. 2. CPE assay.](image-url)
Viral Replication Kinetics. To evaluate the replication kinetics of Ad.TK RC , Ad.TK RC (II), Ad.OW34, Ad.OW37, and Ad5 wt , HeLa cells were infected at a MOI of 10 pfu/cell, and the quantity of virus recovered from the media was assayed at serial time points by qPCR. As shown in Fig. 3, the replication kinetics of Ad.OW34 and Ad.OW37 in HeLa cells were similar to that of Ad5 wt . The E1B 55 kDa-deleted vectors grew slower than the E1B 55 kDa-positive counterparts, and the replication of Ad.TKRC (II) was more robust than that of Ad.TK RC .

Plaque Reduction Assay. The effect of GCV on the replication of Ad.OW34 was analyzed by plaque reduction assay, as shown in Fig. 4. The IC50 and IC90 of GCV were 0.11 μM and 0.587 μM, respectively. No viral plaques were observed at 5 μM GCV, a concentration well below the peak plasma concentrations of 45 μM achieved in clinical settings (43). The growth of Ad5 wt was not influenced by GCV at the concentrations tested (data not shown).

In Vivo Efficacy Studies. We tested in an A549 s.c. lung carcinoma and in a MDAH 2774 i.p. ovarian cancer model in nude mice whether the enhanced replication of Ad.OW34 and Ad.OW37, when compared to Ad.TK RC (II), would result in an improved oncolytic activity and whether the addition of GCV could further boost their antitumor efficacy.

In the first animal study, we examined whether the treatment efficacy of s.c. A549 xenografts with the HSV-tk-expressing replication-competent vectors could be improved when combined with GCV. As shown in Fig. 5, on day 33, the last day when all animals were alive, the median tumor size of untreated or GCV-treated A549 xenografted animals were 1800 mm³. A single intratumoral injection with Ad.TK RC (II) resulted in a median tumor volume of 530.5 mm³ on day 33. When combined with GCV, the median tumor size was 102.5 mm³, significantly smaller than those treated with Ad.TK RC (II) alone (P < 0.001). The median tumor size of animals treated with Ad.OW34 or Ad.OW37 was 142.2 and 141.4 mm³, respectively, and when combined with GCV, the median tumor volume was 86.9 and 284.1 mm³, respectively. There was no significant difference as to whether the animals received Ad.OW34 or Ad.OW37, and the addition of GCV did not enhance the efficacy of these vectors (P = NS). When compared to Ad.TK RC (II) alone, Ad.OW34 and Ad.OW37 resulted in a significantly greater reduction in tumor size (P < 0.001).
Hepatotoxicity Studies. Because the principle toxicity of high doses of i.v.-administered adenoviruses is hepatic injury, we compared the toxicity of Ad.TK, Ad.TKRC(II), Ad.OW34, and Ad5wt in C57BL/6 and athymic nude mice by serum liver chemistries, histology, and TEM 48 h after i.v. administration of $1 \times 10^{10}$ or $1 \times 10^{11}$ viral particles. All C57BL/6 mice receiving $1 \times 10^{11}$ particles of Ad.TKRC(II), Ad.OW34, or Ad5wt became ill and had to be euthanized within 3 days of virus inoculation. At necropsy, the livers of these animals were enlarged, pale, friable, and showed scattered petechiae. In contrast, none of seven mice receiving $1 \times 10^{10}$ virions of Ad.TKRC(II), Ad.OW34, or Ad5wt appeared ill, and all animals were alive at the end of the 21-day observation period. The injection of $1 \times 10^{11}$ particles of Ad.TK was well tolerated by the mice. As shown in Table 1, athymic nude mice exhibited less toxicity to i.v. administration of Ad.TKRC(II) and Ad.OW34. Injection of $1 \times 10^{10}$ particles of Ad.TK did not significantly alter the serum liver enzyme levels.

In the second animal study, the efficacy of the various vectors alone or in combination with GCV was evaluated in an i.p. ovarian cancer model and compared to animals treated with paclitaxel and carboplatin. As shown in Fig. 6, untreated or GCV-alone treated animals had a median survival of 29 days. Mice receiving an active combination chemotherapy regimen for ovarian cancer (42, 44) had a significantly improved survival with a median of 59 days (P < 0.001). The survival of animals treated with the replication-defective Ad.TK was enhanced when followed by GCV (P < 0.001). The median survival times were 33 and 45 days, respectively. Treatment with Ad.TKRC(II), Ad.OW34, or Ad.OW37 alone resulted in a median survival of 29 days. Mice receiving an active chemotherapy regimen and combination chemotherapy regimen for ovarian cancer (42, 44) had a significantly improved survival with a median of 59 days (P < 0.001). Both vectors were more efficacious than Ad.TKRC(II) (P < 0.05). In addition, inoculation of the animals with the replication-competent vectors Ad.TKRC(II), Ad.OW34, or Ad.OW37 without GCV was more active than the combined chemotherapy regimen (P < 0.03). At the end of the observation period (day 138), three animals survived in each of the groups treated with Ad.OW34 or Ad.OW37, and two survived in the Ad.OW37-plus-GCV treatment group.

### Table 1  Serum liver chemistries

Below is the hepatic function panel in mice 48 h after receiving $1 \times 10^{10}$ particles of viruses via tail vein injection. Each treatment group was composed of six (C57BL/6) or three (athymic nude mice) animals. Serum enzyme levels are presented as the mean unit/liter ($\bar{x}$) ± SEM.

<table>
<thead>
<tr>
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<th>Control</th>
<th>Ad.TK</th>
<th>Ad.TKRC(II)</th>
<th>Ad.OW34</th>
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<td>C57BL/6</td>
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<tr>
<td>AST</td>
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<td>63</td>
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### Control

In the first animal study, the toxicity of the various vectors alone or in combination with GCV was evaluated. All C57BL/6 mice receiving $1 \times 10^{11}$ particles of Ad.TKRC(II) and Ad.OW34 showed acute, focal, coagulative necrosis of the liver, which was accompanied by minimal focal perivascular lymphocytic cuffing. Ad.TKRC(II)-treated animals revealed moderate, acute, periportal degradation with areas of coagulative necrosis. There was minimal perivascular accumulation of lymphocytes, and the sinusoids were diffusely distended with erythrocytes. The findings in mice that received Ad.OW34 were similar to that of Ad.TKRC(II), but in addition, the liver exhibited a range of minimal to marked multifocal, perivascular lymphocytic cuffing affecting both the centrolobular and portal regions. The histological changes in mice that received Ad5wt were similar to that described in the previous groups, but the degree of degeneration was not as severe, and perivascular lymphocytic cuffing was rare.

As shown in Fig. 8, TEM of the liver of C57BL/6 mice that received $1 \times 10^{11}$ particles of Ad.TKRC(II) or Ad.OW34 demonstrated loss of normal hepatocellular architecture, with mitochondrial swelling and loss of cristae, reduction in the number of mitochondria, and amount of endoplasmic reticulum. In addition, the cytoplasm was filled with nonspecific degenerative material, vacuoles, secondary lysosomes, and lipids. Animals treated with Ad5wt showed similar changes as the previous treatment groups, but with less degenerative changes.

![Fig. 7. Histopathological responses in liver of C57BL/6 mice 48 h after a single i.v. injection of 1 × 10¹¹ particles of Ad5wt (A), Ad.TKRC(II) (B), Ad.TKRC(II) (D), and Ad.OW34 (E). A. Liver samples from mice inoculated with virus-free diluent served as the control. H&E; original magnification, ×10.](image-url)
material in the cytoplasm. Animals that received Ad.TK showed no evidence of mitochondrial swelling, but in some areas, the number of mitochondria appeared to be slightly reduced.

DISCUSSION

Almost any virus that has a lytic life cycle in human cells could provide the basis for a replicating gene transfer vector, but for the treatment of human cancer, the choice is constrained by issues of safety and efficacy. Safety considerations dictate the use of viruses with low pathogenicity that are already prevalent in the human population, whereas efficacy considerations point to viruses that replicate efficiently in the tissue to be targeted. The primary objective of the experiments reported here was to elucidate whether the expression of the Ad5 E1B 55 kDa gene in replication-competent adenoviral vectors enhances their antitumor effect and potential liver toxicity in mice after systemic administration. In addition, we assessed whether their antineoplastic properties could be further enhanced by combination with the HSV-tk/GCV system. To address the safety concerns associated with viral replication, the E1 genes were transcriptionally linked to the HSV-tk gene via an IRES or a linker creating a fusion gene. These strategies reduce the likelihood of generating replication-competent deletion mutants lacking GCV sensitivity.

In CPE assays on A549 and A375 cells, which are wild-type for p53, the E1B 55 kDa-deleted adenoviral vector Ad.TKRC(II) was able to lyse these cells when they were subconfluent but not when they were confluent. In MDAH 2774 cells, which are p53-mutated, a similar effect was seen, but it was not as pronounced. This demonstrates that the ability of the E1B 55 kDa-deleted adenoviral vector Ad.TKRC(II) to produce CPE is more dependent on the cell density and thus cell-cycle status than on the p53 phenotype of the cell line. In HeLa cells, there was almost no difference whether the cells were confluent or subconfluent at the time of infection with the replication-competent vectors; this was most likely attributable to the human papillomavirus 18 E6–E7 gene products driving quiescent cells into the S phase (45). These results are in agreement with previous reports demonstrating that E1B 55 kDa-deleted viruses are restricted by the cell cycle such that mutant virus growth is impaired in cells infected during G1 and least restricted in cells infected during the S phase (22). Furthermore, our findings provide additional evidence that deletion of the E1B 55 kDa gene does not restrict adenoviral replication and lysis to p53-dysfunctional cells (46–52). In addition, the ability of all E3-deleted replication-competent vectors to produce CPE in confluent cells was diminished when compared to Ad5wt; this was most likely attributable to the lack of the E3 11.6 kDa protein, which is required for efficient lysis and release of adenoviral progeny from infected cells (53). Thus, it is conceivable that in a clinical setting, these vectors and in particular Ad.TKRC(II) may replicate preferentially in proliferating tumor cells and less efficiently in the surrounding normal tissue.

In the s.c. A549 tumor model, the efficacy of Ad.TKRC(II) could be enhanced by GCV in contrast to the E1B 55 kDa-positive vectors. This suggests that the replication of the E1B 55 kDa-positive vectors is so robust that the addition of GCV, which is virostatic, did not enhance their intrinsic oncolytic effects. Because of its less robust replication and oncolysis, Ad.TKRC and its successor Ad.TKRC(II) benefited from the use of its enzyme prodrug system in solid tumors (18–20).

In the i.p. ovarian cancer model, GCV administration enhanced the survival of animals treated with the replication-deficient Ad.TK, as reported by others (54, 55). In contrast, the survival of animals receiving Ad.TKRC(II) or Ad.OW34 followed by GCV was significantly reduced when compared to the treatment with each respective vector alone. There was no significant difference in the survival of animals receiving Ad.OW37 alone or in combination with GCV, presumably because of the lower catalytic activity of the HSV-tk-E1A 13s fusion protein when compared to wild-type HSV-tk (data not...
shown). These results suggest that the inhibition of viral replication and oncolysis by GCV counterbalanced the increased cytotoxicity of the HSV-tk/GCV system, despite the significant bystander effect of MDAH 2774 cells (56). It is feasible that the more efficient diffusion of viral particles in the peritoneal cavity, when compared to the microenvironment of solid tumors, may have contributed to this finding. Furthermore, the survival of animals receiving effective chemotherapy for ovarian cancer was about half of that of Ad.OW34- or Ad.OW37-treated mice. Because previous reports indicate that the binding of adenovirus E1A proteins to the cellular retinoblastoma and p300 proteins can induce quiescent cells to enter the S phase (57), it is feasible that the combination of virotherapy and chemotherapy would have further improved the treatment efficacy (58).

In both tumor models, the inherent oncolytic activity of the EIB 55 kDa-positive vectors was greater than that of Ad.TK(II), probably because of the impaired intratumoral viral replication and spread of Ad.TK(II) because at any given time, only a small fraction of the tumor cells are in the S phase and only cells in the S phase efficiently support the growth of EIB 55 kDa-deleted adenovirus mutants. However, in the clinical setting, GCV administration might enhance the cytoselective effect of the vectors by immune-mediated mechanisms (8, 59). Whether GCV doses administered to animals can be given safely to humans to achieve this effect is presently being assessed in a clinical trial (60). Nevertheless, treatment of HSV-1 infections or CMV reinitis uses the nucleoside analogues acyclovir and its more toxic derivative GCV, which allow termination of viral replication of these HSV-tk-expressing vectors at clinical doses. Consequently, the HSV-tk/GCV system represents a safety feature for the use of these replication-competent vectors in human subjects. In contrast, therapeutic options for wild-type adenovirus infections are presently limited and nonspecific (61), thus giving Ad.TK(II) and Ad.OW34 a greater therapeutic index. Especially in the light of the recent concerns about adenoviral-mediated gene transfer (62), the importance of this point cannot be overstated.

The hepatocellular toxicity after i.v. inoculation is one of the significant elements in the emerging toxicological profile of adenoviral vectors. Four h after intratumoral injection of [35 S]-l-methionine-labeled adenovirus, 4.0% ± 3.7% of the total administered dose could be detected in the liver of nude mice bearing s.c. A549 xenografts (data not shown). To simulate the worst-case scenario in a clinical application, the vectors, which are replication-competent only in human cells, were administered i.v. to mice. We demonstrated that these vectors are more hepatotoxic than Ad.TK and Ad5(II), especially in an immunocompetent host. These findings are not surprising because E1-positive viruses have a greater accumulation of late gene products and more viral DNA replication than E1-negative vectors, resulting in an abortive but lytic infection of murine hepatocytes (63). In addition, the expression of viral late proteins elicits MHC-class-I-restricted CD8+ cytotoxic T cells, which mediate destruction of infected hepatocytes (64–67). In this system, the hepatotoxicity of Ad.TK(II) was indistinguishable from that of the EIB 55 kDa-positive counterparts. In contrast, pulmonary infection of Sigmodon hispidus cotton rats with an EIB 55 kDa-deleted virus resulted in markedly reduced inflammation when compared to wild-type Ad5 (68). For i.v. application or liver-directed gene therapy, the greater toxicity of the replication-competent, E3-deleted, HSV-tk-expressing vectors is disadvantageous, but for intratumoral injection, it is conceivable that these vectors might more efficiently induce the development of a systemic immune response than Ad.TK or Ad5(II). Furthermore, it needs to be taken into account that administered therapeutic doses of replication-competent vectors can be significantly lower than those of replication-defective vectors because of their intratumoral amplification and spread beyond initially infected cells. However, intratumoral or i.p. administration of the vectors did not cause obvious morbidity or death of the animals at therapeutic doses.

The replication of the EIB 55 kDa-deleted vector did not appear to be restricted by the p53 status, but it seems to preferentially replicate in mitotically active cells. This might aid in targeting therapy to tumor tissue irrespective of the p53 phenotype.

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The Role of the E1B 55 kDa Gene Product in Oncolytic Adenoviral Vectors Expressing Herpes Simplex Virus- tk: Assessment of Antitumor Efficacy and Toxicity

Oliver Wildner and John C. Morris


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