

# Therapy of Colon Cancer with Oncolytic Adenovirus Is Enhanced by the Addition of Herpes Simplex Virus-*thymidine kinase*

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## ABSTRACT

A major obstacle to the successful application of suicide gene therapy strategies that rely on *in situ* transduction of tumor cells is the poor distribution of the vector throughout the tumor mass. To address this problem, we evaluated the use of Ad.TK<sup>RC</sup>, an E1b *M<sub>r</sub>* 55,000 deleted replicating adenoviral vector engineered to express the herpes simplex virus type 1 *thymidine kinase* gene (HSV-*tk*) in combination with ganciclovir (GCV) as a treatment for human colon cancer xenografts in nude mice. We compared the efficacy of this system with that of a standard replication-deficient adenovirus expressing HSV-*tk* (Ad.TK) in mice bearing LS180 tumors. In this system, Ad.TK<sup>RC</sup> alone was as effective as a traditional Ad.TK vector in combination with GCV. The addition of GCV significantly enhanced the antitumor effect of Ad.TK<sup>RC</sup>. Furthermore, we demonstrated that the survival of HT-29 human colon cancer xenografted mice treated with Ad.TK<sup>RC</sup> and GCV was prolonged compared with Ad.TK<sup>RC</sup> alone or with administration of a single cycle of topotecan. These data demonstrate that the addition of direct viral oncolysis to the HSV-*tk*/GCV suicide gene system resulted in a striking improvement in treatment efficacy and that it may offer advantages over the use of chemotherapeutic agents for treatment of localized disease.

## INTRODUCTION

With over 135,000 new cases and 57,000 deaths expected in 1998, colorectal carcinoma is the third most common cause of cancer death in the United States (1). Despite new insights into the molecular pathogenesis of colon cancer and improvements in surgery, radiotherapy, and chemotherapy, the median survival of patients with metastatic disease has remained unchanged for the last 40 years at ~15 months (2).

Suicide/prodrug gene therapy holds great promise to become an important addition to the therapy of some forms of cancer. This strategy involves the transfer and expression of nonmammalian genes encoding enzymes that convert nontoxic prodrugs into cellular toxins. The most widely used suicide gene is HSV-*tk*,<sup>2</sup> which confers sensitivity to GCV (3).

Initially, replication-incompetent retroviral vectors have been used for viral-mediated HSV-*tk* gene delivery (4). Subsequently, replication-deficient adenoviral vectors have been used as suicide gene transfer vectors (5, 6) because they offer, in contrast to retroviral vectors, higher transduction efficiency and transgene expression in a wide variety of cell types independently of their cell cycle status. Because these vectors are replication incompetent, their use for cancer gene therapy is limited by their inability to spread and infect additional cells subsequent to the initial infection event. The fundamental physics of diffusion of vector particles in tissue spaces and the high

density of viral receptors in tissue largely restrict distribution of these vectors only to the close proximity of the site of injection.

The presumed need to genetically modify every tumor cell may be partially obviated by the HSV-*tk*/GCV system because it is often associated with a “bystander effect” (3), *i.e.*, the extension of toxic effects to untransduced neighboring cells by diffusion of phosphorylated GCV through cellular gap junctions (7). Although the bystander effect increases tumor cell killing, it is nonetheless necessary to transduce a significant proportion of the tumor. In an effort to increase transduction efficiency, intratumoral replication of viral vectors has been attempted by cotransfection of replication-defective viral constructs expressing HSV-*tk* with wild-type virus (8, 9) or with viral genes critical for replication (10). Also, direct inoculation of xenogenic murine retroviral producer cells into brain tumors (11) has been performed in an effort to achieve continuous local vector production.

Exploiting the oncolytic effects of viral replication, Bischoff *et al.* (12) demonstrated that an E1b *M<sub>r</sub>* 55,000 deleted adenovirus preferentially replicates in p53 dysfunctional tumor cells (12). This virus, which does not express a therapeutic transgene, has entered clinical trials in combination with cisplatin in patients with advanced squamous cell carcinoma of the head and neck (13). To increase its efficacy and to be able to control the replication and spread of the virus, we and others have developed E1b *M<sub>r</sub>* 55,000 deleted adenoviral vectors expressing HSV-*tk* (14) or a *cytosine deaminase*/HSV-*tk* fusion gene (15).

Colorectal carcinoma has been evaluated as a potential target for HSV-*tk*/GCV suicide gene therapy (16–18) and appears to be an excellent tumor model for evaluation of the utility of an E1b attenuated replication competent vector because of the high prevalence of p53 abnormalities, especially in metastatic lesions (19, 20). In this report, we describe the use of an E1b *M<sub>r</sub>* 55,000-deleted, replication-competent adenovirus expressing HSV-*tk* in combination with GCV for the treatment of colorectal carcinoma. We compared its efficacy with those of a standard replication-deficient adenovirus expressing HSV-*tk* and the topoisomerase I inhibitor topotecan (reviewed in Ref. 21), which has shown significant antineoplastic activity against a variety of solid tumors including colorectal carcinoma.

## MATERIALS AND METHODS

**Generation of Adenoviral Vectors.** The construction and generation of the adenoviral vectors were described previously (14). Briefly, Ad.TK<sup>RC</sup> harbors in the E1 region an HSV-*tk*/Ad5 E1aE1b *M<sub>r</sub>* 19,000 expression cassette under the transcriptional control of the human CMV-IE promoter in combination with the adenovirus tripartite leader (22). This cassette was flanked upstream by the Ad5 packaging sequence and downstream by Ad5 pIX. The *E1* genes were transcriptionally linked to the HSV-*tk* gene *via* an encephalomyocarditis virus internal ribosome entry site (23) to reduce the likelihood of generating replication-competent deletion mutants lacking HSV-*tk*. In contrast to dl1520 (12, 24), Ad.TK<sup>RC</sup> expresses a suicide gene and the CMV-IE promoter as opposed to the endogenous *E1* promoter, which drives the *E1* genes. Furthermore, the E3 region of Ad.TK<sup>RC</sup> is removed, and the E1b deletion is larger than in dl1520 (1257 *versus* 827 bp).

For comparison, we generated the replication-deficient adenoviruses Ad.GFP and Ad.TK, which carry the GFP and the HSV-*tk*, respectively, driven by the CMV-IE promoter.

Received 8/9/98; accepted 11/11/98.

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<sup>2</sup> The abbreviations used are: HSV-*tk*, herpes simplex virus-1 *thymidine kinase*; GCV, ganciclovir; E1 and E3, adenovirus early regions 1 and 3, respectively; CMV-IE, cytomegalovirus immediate-early; GFP, green fluorescent protein; Ad5, wild-type human adenovirus type 5; MOI, multiplicity of infection.

**Tissue Culture.** The colon cancer cell lines SW-620, LS180, HCT 81, WiDr, HT-29, and DLD-1 were purchased from the American Type Culture Collection (Manassas, VA) and propagated in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) at 37°C and 5% CO<sub>2</sub>.

**Flow Cytometric Analysis of GFP Expression.** To determine the *in vitro* transduction efficiency of human colon carcinoma cell lines with human adenovirus type 5, subconfluent cell monolayers were exposed to Ad.GFP at a MOI of 10. Sixty h later, single-cell suspensions were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).

**HSV-*tk* Bystander Assay.** We evaluated the *in vitro* bystander effect of different colon cancer cell lines by coculturing mixtures of different ratios of Ad.TK transduced and untransduced cells as described previously (25).

The median fraction of affected cells ( $F_a$ ) was calculated according to the formula:

$$F_a = \frac{I_{MIX} - I_{100}}{I_{WT} - I_{100}}$$

Variables  $I_{WT}$ ,  $I_{100}$ , and  $I_{MIX}$  are the median [<sup>3</sup>H]thymidine incorporation of the unmodified wild-type cells, cells exposed to Ad.TK at an MOI of 25 for 12 h, and mixtures of the two, respectively. At this MOI, nearly all of the cells were susceptible to transduction *in vitro* with Ad.GFP (data not shown).

**Colony-forming Assay.** To evaluate the sensitivity of HT-29 to topotecan, 1 million subconfluent HT-29 cells were incubated with increasing concentrations of topotecan for 24 h. After drug exposure, the cells were washed, trypsinized, and counted. To assess the colony-forming ability of the cells after the treatment, 300 cells were dispersed in 100-mm tissue culture plates and incubated at 37°C for 10–14 days. Surviving colonies were fixed and stained with 1% crystal violet/formaldehyde, and colonies larger than 1 mm were counted. The fraction of unaffected cells ( $F_u$ ) was calculated by dividing the number of colonies present in drug-treated dishes by the number present in untreated control dishes. The concentration effect curve is represented as the fraction of affected cells ( $F_a = 1 - F_u$ ) versus topotecan concentration. Each experiment was performed in triplicate and was repeated twice.

**Animal Experiments.** Female athymic *nu/nu* mice (obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN) were s.c. injected with 10 million LS180 or HT-29 human colon cancer cells into the right dorsal lumbar region in 100  $\mu$ l of serum-free DMEM with 10% Matrigel (Collaborative Products, Bedford, MA). Blinded, bidimensional tumor measurements were performed twice a week with calipers, and tumor volume was determined using the simplified formula of a rotational ellipsoid ( $1 \times w^2 \times 0.5$ ; Ref. 26). Once tumors reached a volume of 100–150 mm<sup>3</sup>, animals were treated by intratumoral injection of 10<sup>9</sup> plaque-forming units of adenovirus suspended in 100  $\mu$ l of buffer or 100  $\mu$ l of buffer alone as a control. Animals with tumor volumes outside this range were excluded from the study prior to any treatment. GCV (Roche Laboratories, Nutley, NJ) 100 mg/kg of body weight was administered i.p. in 1 ml of saline twice daily for 5 consecutive days beginning 1 day after virus inoculation, the optimal timing for the replication defective Ad.TK, or after 3 days when the intratumoral Ad.TK<sup>RC</sup> replication reached its maximum (data not shown). Topotecan treatment (1.5 mg/kg i.p. in 1 ml of saline once daily for 5 days) alone or in combination with Ad.TK<sup>RC</sup> was initiated at the same time as GCV in the other treatment groups. Prior toxicity studies demonstrated that the topotecan dosage used was moderately toxic but not lethal. Tumors were resected by a combination of sharp and blunt dissection 3 weeks after implantation in all treatment groups and weighed on an analytical scale.

**Analytical and Statistical Methods.** The effect of the treatment on the tumor weight was analyzed with the Mann-Whitney *U* test and the survival of the animals with the Cox-Mantel test using STATISTICA release 5.1 for Windows (StatSoft, Inc., Tulsa, OK).

**RESULTS**

**Transduction Efficiency with Ad.GFP and Bystander Effect in Different Human Colon Cancer Cell Lines.** The human colon cancer cell lines SW-620, LS 180, HCT 81, WiDr, HT-29, and DLD-1 were tested for their susceptibility to transduction *in vitro* with a

replication-deficient adenovirus expressing GFP (Ad.GFP). At an MOI of 10, the median transduction efficiency obtained in SW-620 and HT-29 was ~40%, whereas a minimum of 85% was achieved in all other cell lines tested.

The same cell lines were also evaluated for their HSV-*tk*-associated bystander effect. After transduction with Ad.TK, mixtures consisting of 10% or 50% HSV-*tk*-positive cells were incubated with 10 mM GCV for 24 h. All cell lines, with the exception of SW-620, exhibited a significant bystander effect. These findings demonstrate that cell lines originating from the same tumor tissue can vary with respect to both their susceptibility to adenoviral gene transfer and their HSV-*tk*/GCV-associated bystander effect. We chose LS 180 and HT-29 cells for our *in vivo* studies. HT-29 cells transduce with Ad5 approximately one-half as well as LS 180, but they had a somewhat greater metabolic bystander effect.

**Antitumor Effect of Ad.TK or Ad.TK<sup>RC</sup> Treatment in Combination with GCV.** To assess the antitumor effect of Ad.TK and Ad.TK<sup>RC</sup> alone or in combination with GCV, we injected 10<sup>9</sup> plaque-forming units of virus directly into the LS 180 xenografts. GCV treatment was started 1 and 3 days after Ad.TK and Ad.TK<sup>RC</sup> injection, respectively. The tumors were resected *in toto* and weighed 3 days after completion of the GCV course in animals receiving Ad.TK and 1 day after completion in those receiving Ad.TK<sup>RC</sup>.

Analysis of the tumor masses revealed that the combination of either Ad.TK or Ad.TK<sup>RC</sup> with GCV increased the treatment efficacy ( $P \leq 0.002$ ; Fig. 1). The antitumor effect of Ad.TK<sup>RC</sup> alone was equal to that of Ad.TK followed by GCV. This demonstrates that Ad.TK<sup>RC</sup> has direct oncolytic activity in LS 180 cells and confirms our previous observations in a survival study using melanoma and cervical cancer xenografts (14). The antitumor effect in mice treated with Ad.TK<sup>RC</sup> plus GCV was significantly superior to that of Ad.TK followed by GCV ( $P \leq 0.001$ ).

**Treatment Efficacy of Ad.TK<sup>RC</sup>/GCV versus Topotecan.** By clonogenic inhibition assay, we found that the ED<sub>50</sub> of topotecan in HT-29 cells was 0.02  $\mu$ M (Fig. 2). Accordingly, topotecan administration to HT-29 xenografted mice resulted in a modest but significantly improved survival when compared with untreated tumor-bearing animals or GCV-treated mice with HSV-*tk*-negative tumors ( $P \leq 0.005$ ; Fig. 3). However, mice treated with Ad.TK<sup>RC</sup> alone or in combination with GCV or topotecan survived longer than those treated only with topotecan ( $P \leq 0.0005$ ). There was no statistically significant difference in survival whether Ad.TK<sup>RC</sup> was administered

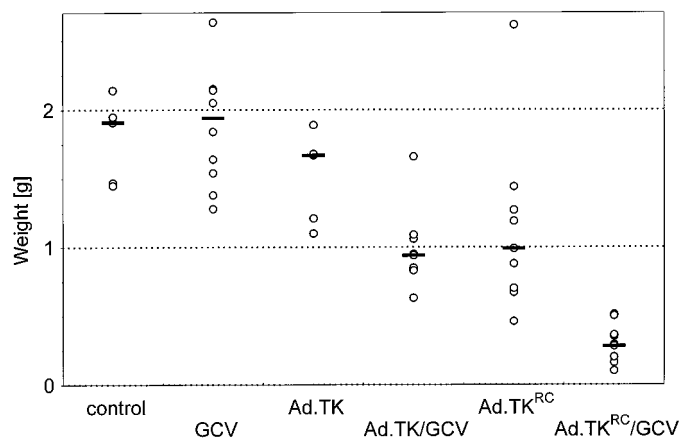


Fig. 1. The response of tumors to treatment was evaluated by resecting the LS180 xenografts and weighing them after completion of the GCV course. Each point represents an individual tumor. Horizontal lines, median weight for each treatment group. The control and Ad.TK groups consisted of 5 animals each, whereas all other treatment groups were composed of 10 animals each.

alone or in combination with topotecan. By contrast, when Ad.TK<sup>RC</sup> was followed by GCV, the survival of the animals was again significantly improved ( $P \leq 0.02$ ; Table 1).

**DISCUSSION**

To enhance tumor transduction by *in situ* amplification and spread of the initial viral inoculum within a tumor mass, we developed the recombinant adenovirus Ad.TK<sup>RC</sup> that combines viral replication and oncolysis with the HSV-*tk*/GCV suicide gene system to kill cancer cells. We compared its antitumor effect with that of a standard replication-deficient Ad.TK in the rapidly growing LS180 colon cancer xenograft model. In addition, we studied the antitumor response and survival of mice bearing xenografts of HT-29 colon adenocarcinoma cells, a tumor known to be resistant to most chemotherapeutic agents (27). Because of its slower growth (48-h doubling time), this tumor model more closely reflects clinical colon cancer, where only a small fraction of tumor cells are cycling at any given time. The HT-29 xenografted animals were treated with Ad.TK<sup>RC</sup> alone or in combination with GCV or topotecan.

Our studies in the LS180 xenograft model revealed that there was no significant difference in the antitumor effect whether animals received the replication-deficient Ad.TK in combination with GCV or Ad.TK<sup>RC</sup> alone, confirming that replicating human adenovirus has intrinsic oncolytic activity (12, 28). In addition, in both the LS180 and HT-29 colon cancer models, treatment with Ad.TK<sup>RC</sup> in combination with GCV starting 3 days after virus injection showed the best antineoplastic effects.

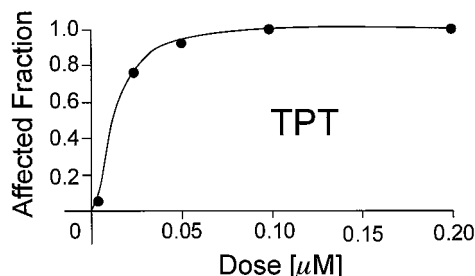


Fig. 2. The dose-response curve for HT-29 cells treated with topotecan (TPT) was determined by a clonogenic assay as described in "Materials and Methods."

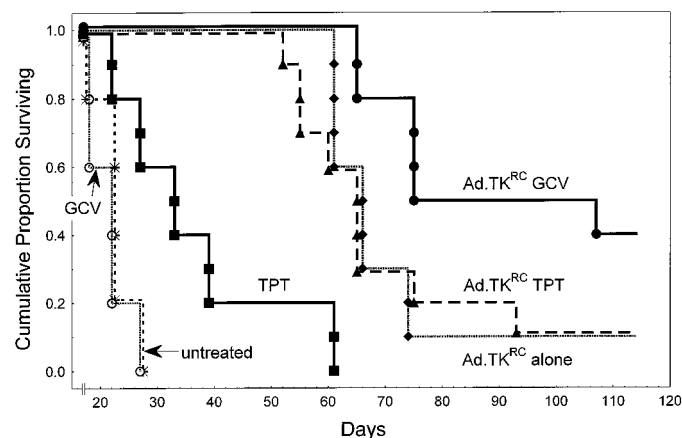


Fig. 3. Kaplan-Meier survival analysis of mice with s.c. HT-29 colon tumor xenografts treated with a replication competent Ad.TK<sup>RC</sup>, GCV, or topotecan (TPT). The vector was given alone or followed by GCV administration for 5 consecutive days starting 3 days after virus injection. One treatment group received Ad.TK<sup>RC</sup> in combination with topotecan.

Table 1 Comparison of animal survival between different treatment groups of nude mice bearing HT-29 xenografts

Group	Group size (n)	Long-term survivors (n)	Median survival (days)
Untreated	5	0	22
GCV alone	5	0	22
TPT alone	10	0	33
Ad. TK <sup>RC</sup>	10	1	65
Ad. TK <sup>RC</sup> GCV	10	4 <sup>a</sup>	91
Ad. TK <sup>RC</sup> TPT <sup>b</sup>	10	2	65

<sup>a</sup> Two of the four animals were tumor free at day 114; all other long-term survivors had tumors ( $V \leq 350 \text{ mm}^3$ ).

<sup>b</sup> TPT, topotecan.

Mice bearing HT-29 tumors treated with Ad.TK<sup>RC</sup> alone or in combination with GCV survived longer than animals treated with the topoisomerase I inhibitor topotecan. Topotecan is active against many colon carcinoma cell lines that are resistant to other frequently used antineoplastic agents. Preclinical drug screening models using athymic nude mice have shown that topotecan is the most active agent in HT-29 tumors (27).

The results of the experiments described here confirm our previous results obtained in human melanoma and cervical cancer models, demonstrating that the combination of direct viral oncolysis together with an increase in suicide gene distribution within the tumor results in a significant improvement of treatment efficacy (14). Furthermore, the addition of HSV-*tk* to the adenovirus also provides a safety mechanism that allows the viral infection to be aborted by GCV treatment should undesired spread of the infection occur during therapy.

Previous reports indicate that the binding of adenovirus E1a proteins to the cellular pRB and p300 proteins can induce quiescent cells to enter S-phase (29) and thereby conceivably increases susceptibility to S-phase-specific antineoplastic agents, like HSV-*tk*/GCV or topotecan (30). Furthermore, adenovirus E1a increases topoisomerase levels (31, 32) and thus potentially enhances the cytotoxicity of topoisomerase I inhibitors (33). However, we did not observe an increased survival of animals treated with Ad.TK<sup>RC</sup> followed by topotecan administration relative to those receiving Ad.TK<sup>RC</sup> alone, suggesting that the inhibition of viral replication and oncolysis by topotecan (34–36) might have counterbalanced the potential increased cytotoxicity of topotecan. In contrast, despite abolition of viral replication by GCV, we demonstrated that the combination of direct viral oncolysis together with HSV-*tk*/GCV mediated cell killing resulted in a striking improvement of treatment efficacy. Indeed, this combination proved to be more efficacious than a standard replication-deficient adenovirus expressing HSV-*tk*, as well as a single treatment cycle with one of few antineoplastic agents active against HT-29 colon cancer, topotecan. It is tempting to speculate that the efficacy of cancer treatment could be enhanced by the combined use of standard chemotherapy and gene therapy. Studies are presently in progress to verify this hypothesis.

**ACKNOWLEDGMENTS**

We are thankful to Dr. Fabio Candotti for helpful discussion and critical review of the manuscript and to J. Douglas Burke for technical assistance.

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