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Advances in Brief

Enzyme/Prodrug Gene Therapy: Comparison of Cytosine Deaminase/5-Fluorocytosine *Versus* Thymidine Kinase/Ganciclovir Enzyme/Prodrug Systems in a Human Colorectal Carcinoma Cell Line

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Abstract

We have been developing an enzyme/prodrug gene therapy approach for the treatment of primary and metastatic tumors in the liver. This system uses the cytosine deaminase/5-fluorocytosine (CD/5-FCyt) enzyme/prodrug combination. Another system that has received considerable attention is the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) enzyme/prodrug combination. The purpose of the present study was to compare these two enzyme/prodrug systems. The human colorectal tumor cell line, WiDr, was genetically modified to express either the *CD* gene (WiDr/CD) or the HSV-*TK* gene (WiDr/TK). The IC_{50} (concentration of drug producing 50% inhibition of cell growth) for GCV was approximately 3.4 μM in WiDr/TK cells, while the IC_{50} for 5-FCyt was approximately 27 μM in WiDr/CD cells. *In vivo* antitumor studies were conducted using high but nontoxic levels of GCV (50 mg/kg/day) or 5-FCyt (500 mg/kg/day). When tumor xenografts were composed of 100% of cells expressing either HSV-TK or CD, 100% tumor-free animals were observed after GCV or 5-FCyt treatment, respectively. However, when only 10% of the tumor cells expressed HSV-TK, no antitumor effect by GCV treatment could be observed. In contrast, when tumors were composed of 4% of the cells expressing CD, 60% of the animals were tumor-free after 5-FCyt treatment. Transmission electron microscopy of the WiDr solid tumors revealed the presence of desmosomes but no gap junctions.

Introduction

CRC[2] remains a significant medical challenge with an expected 350,000 new cases/year. Although the primary cancer can be successfully controlled by surgical resection, metastatic disease to the liver is the most common cause of death of the CRC patient. New innovative approaches must be developed for the treatment of CRC hepatic metastasis if the overall 2- and 5-year survival rates and quality of life assessments are to be improved (reviewed in Refs. 1–6).

We have been developing an approach for the treatment of solid tumors, including CRC, called VDEPT. In this approach, an artificial chimeric gene is created that consists of two components: (*a*) the transcriptional regulatory sequence of an appropriate tumor-associated marker gene; and (*b*) the protein coding domain of a nonmammalian enzyme. This artificial gene will express the nonmammalian enzyme only in tumor cells that express the tumor-associated marker. Expression of the nonmammalian enzyme, by itself, is nontoxic. However, the nonmammalian enzyme can metabolically convert a nontoxic prodrug to a toxic anabolite. Hence, the toxic anabolite will be selectively produced in the tumor cells to generate a selective antitumor effect (7–12). The artificial chimeric gene is delivered to the tumor cell via a viral gene delivery vehicle (*i.e.*, retrovirus, adenovirus, or adeno-associated virus) or perhaps a nonviral-based delivery vehicle, such as a liposome.

For the application of VDEPT to metastatic CRC, we have created an artificial, chimeric gene composed of the carcinoembryonic antigen transcriptional regulatory sequence (12) linked to the coding sequence of the *CD* gene (8–10). This chimeric gene results in the tumor-specific expression of *CD*, which can subsequently convert the nontoxic prodrug 5-FCyt to the toxic anabolite, 5-FUra. This approach may be significantly better than the direct administration of 5-FUra since VDEPT allows extremely high concentrations (C) of 5-FUra to be maintained for extended periods of time (T; $C \times T$) selectively at the tumor site. Most importantly, only a small percentage of tumor cells need express CD, since 5-FUra can diffuse by nonfacilitated diffusion (13) into adjacent cells to increase the efficiency of tumor cell kill (10). This property of 5-FUra may alleviate the potential problems of low gene transfer efficiency into a solid tumor mass and heterogeneity of carcinoembryonic antigen expression.

Another enzyme/prodrug system that has received considerable attention is the HSV-TK/GCV enzyme/prodrug combination (14–19). Several groups have shown that GCV treatment of cells transfected with HSV-TK results in conversion of GCV to GCV triphosphate, which acts as a chain terminator in DNA synthesis (14). A bystander killing effect on neighboring cells that do not express HSV-TK has been reported by several groups (14, 17, 20, 21). Since this bystander effect appears to be dependent upon close contact between cells, it has been hypothesized that gap junctions might be responsible for the transfer of phosphorylated GCV between cells (14, 17, 20, 21).

In this study, we compare the efficacy of the enzyme/prodrug systems CD/5-FCyt and HSV-TK/GCV. The sensitivity of transfected cells expressing either CD or HSV-TK to 5-FCyt or GCV, respectively, was determined. Both CD/5-FCyt and HSV-TK/GCV show effective antitumor activity *in vivo*. However, treatment of tumors composed of mixed cell populations in which only a percentage of the cells express the therapeutic gene showed a greater bystander effect with CD/5-FCyt compared to the HSV-TK/GCV.

Materials and Methods

Cell Lines and Cell Culture. The human colorectal carcinoma cell line WiDr (ATCC #CC1 218) was cultured in DMEM (GIBCO-BRL, Gaithersburg, MD) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. WiDr cells expressing the HSV-*TK* gene or the *CD* gene were designated WiDR/TK and WiDr/CD, respectively. The WiDr/CD cell line was described previously (8). *In vitro* cytotoxicity determinations using Hoechst 33342 dye were performed as described previously (7).

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[2] The abbreviations used are: CRC, colorectal carcinoma; VDEPT, virus-directed enzyme/prodrug therapy; CD, cytosine deaminase; 5-FCyt, 5-fluorocytosine; 5-FUra, 5-fluorouracil; HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir.

HSV-TK and CD Expression Vectors. The eukaryotic expression vector, pRC/CMV (Invitrogen, San Diego, CA), was used to construct vectors in which the CMV promoter controls expression of the HSV-*TK* gene or the *CD* gene. A 1.1-kb DNA fragment encoding the HSV-*TK* gene was amplified from pHSV-106 (BRL, Gaithersburg, MD) by standard PCR techniques (Fig. 1). Two oligonucleotide primers, TK1 and TK2, were designed to anneal to the 5′ and 3′ ends of the HSV-*TK* gene, respectively. The primer, TK1 (CGATGGAAGCTTACAATGGCTTCGTACCCCTGCCATCAAC), introduced a *Hin*dIII site upstream from the AUG start codon of the gene. TK2 (CGAGTCTCTAGATCAGTTAGCCTCCCCCATCTCCCGGG) annealed to the 3′ end and introduced an *Xba*I restriction site after the stop codon of the gene. The amplified HSV-*TK* fragment was subcloned into the pGEM-T vector (Promega, Madison, WI) for sequence verification. The HSV-*TK* fragment was excised from the pGEM-T vector by digestion with *Sac*II, followed by treatment with DNA polymerase I to create blunt ends and digestion with *Hin*dIII. The 1.1-kb HSV-*TK* fragment was then ligated into pRC/CMV that had been digested with *Bst*XI, treated with DNA polymerase I to create blunt ends, and digested with *Hin*dIII. This created the expression plasmid, pCMV/HSV-TK, which was used in all subsequent experiments (Fig. 1).

The plasmid, pCMV/CD, and the cell line, WiDr/CD, were described previously (8). In both the pCMV/CD and the pCMV/HSV-TK constructs, the *TK* or *CD* gene was transcriptionally controlled by the identical CMV promoter and identical 3′ flanking poly(A) signal. Expression of a neomycin resistance gene regulated by the SV40 promoter downstream of the *TK* or *CD* gene was used for selection of transfected cells. The expression vector, pCMV/HSV-TK, was transfected into WiDr cells by electroporation. Stable transfectants were selected using 500 g/ml geneticin (GIBCO-BRL).

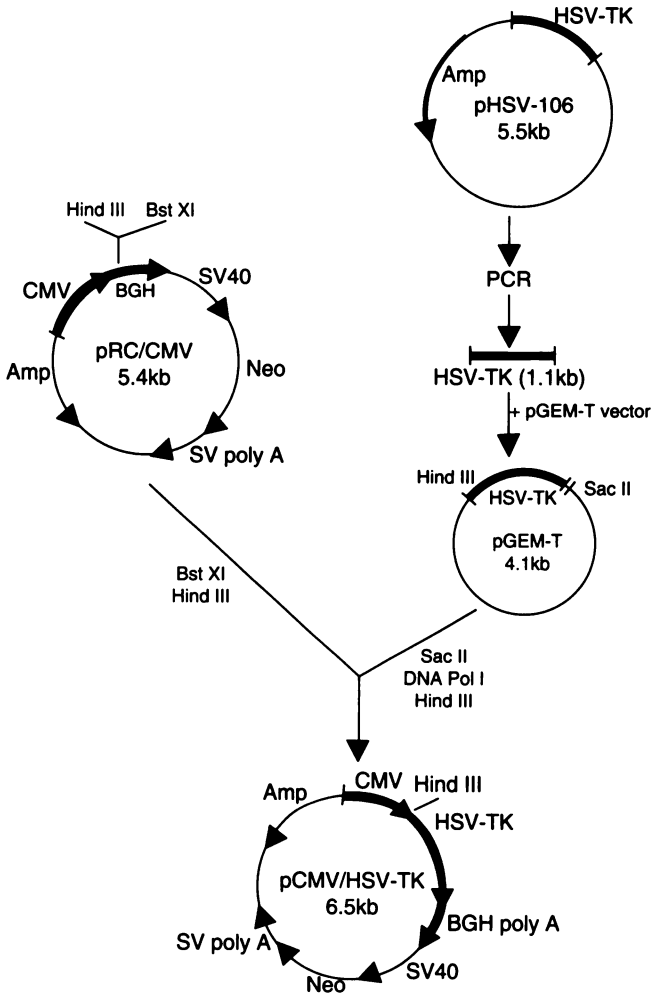

Fig. 1. Assembly of pCMV/TK plasmid. HSV-*TK* gene was amplified by PCR from the pHSV-106 plasmid. A new *Hin*dIII restriction site was created at the 5′ end to facilitate subcloning of the gene under the CMV promoter. The amplified fragment was cloned into the vector, pGEM-T. HSV-TK was then excised and cloned into pRC/CMV at the *Bst*XI and *Hin*dIII sites. The resulting plasmid contains a CMV promoter controlling TK expression and a SV40-regulated *Neo* gene used for selecting TK expression.

Table 1 *In vitro cytotoxicity (IC_{50}, μM) and therapeutic index*

	5-FCyt	GCV
	In vitro cytotoxicity (IC_{50},[a] μM)	
WiDr	26,000 ± 6,754	485 ± 53
WiDr/CD	27 ± 11	
WiDr/TK		3.5 ± 8
	Therapeutic index[b]	
WiDr:WiDr/CD	963	
WiDr:WiDr/TK		139

[a] IC_{50} is defined as the concentration that inhibits growth by 50% compared to untreated control cells.

[b] Therapeutic index is expressed as the toxicity (IC_{50}, μM) in parental cells/toxicity in genetically modified cells.

Animal Studies. Mixtures of WiDr/TK:WiDr cells were implanted s.c. into nude mice (CD-1 *nu/nu*; Charles River Laboratories) at ratios of 0:100, 10:90, 50:50, and 100:0, respectively. Each group had a total of 10 animals, consisting of 5 untreated controls and 5 treated animals. Each animal was implanted with 5×10^6 cells in 0.2 ml PBS. Tumor weights were determined as described previously (9). Dosing started 13 days after implantation with GCV (50 mg/kg body weight, which was the maximum nontoxic dose of GCV) and 5-FCyt (500 mg/kg, which was nontoxic as measured by weight gain). Animals were dosed with GCV and 5-FCyt daily for 21 days, then 3 times a week to day 63. The half-life of GCV and 5-FCyt is estimated to be approximately 1.7 h and 40 min, respectively. Procedures were performed with approved protocols and in accordance with recommendations for the proper care and use of laboratory animals (22).

Results and Discussion

***In Vitro* Cytotoxicity Assays.** WiDr cells expressing either the *CD* gene or the HSV-*TK* gene were assayed for sensitivity to 5-FCyt and GCV, respectively. The IC_{50} (the concentration that inhibits growth by 50% compared to untreated control cells) was determined to assess 5-FCyt and GCV sensitivity. For 5-FCyt, the IC_{50} shifted from 26,000 μM in WiDr cells to 27 μM in WiDr/CD cells (Table 1; Ref. 2). The IC_{50} for GCV shifted from 485 μM in WiDr to 3.5 μM in WiDr/TK cells (Table 1). Although the concentration of GCV needed to inhibit growth of TK-expressing cells is lower than the concentration of 5-FCyt required to inhibit growth of CD-expressing cells (3.5 μM *versus* 27 μM), the therapeutic index is larger for 5-FCyt. That is, the therapeutic index expressed as the toxicity in parental cells/toxicity in genetically modified cells is approximately 960 for 5-FCyt and 140 for GCV.

Tumor Mixing Studies. Nude mice were implanted s.c. with mixtures of WiDr:WiDr/TK at varying ratios of 100:0, 90:10, 50:50, and 0:100. Thirteen days after tumor implantation, when the average tumor weight was 100 mg, dosing with 50 mg GCV/kg body weight began. Treated animals were dosed daily for 21 days, then 3 times a week from day 34 until day 63. Drug-related toxicity as detected by weight loss was not observed.

The tumor growth in untreated animals was similar until day 31 (Fig. 2*A*). After day 31, animals in untreated groups had to be euthanized because of tumor burden. Following the death of an animal, the group was considered incomplete, and the line on the graph representing such a group was discontinued, although all animals were followed until termination. Because of tumor burden, all remaining untreated animals were euthanized by day 50. These data indicated that the growth rate for the tumors, regardless of the mixture, was similar in all untreated animals.

GCV treatment had no significant effect on tumors of parental WiDr cells (100% WiDr:0% WiDr/TK; Fig. 2*B*). In these animals, tumor size doubled in approximately 10.6 days for both GCV-treated

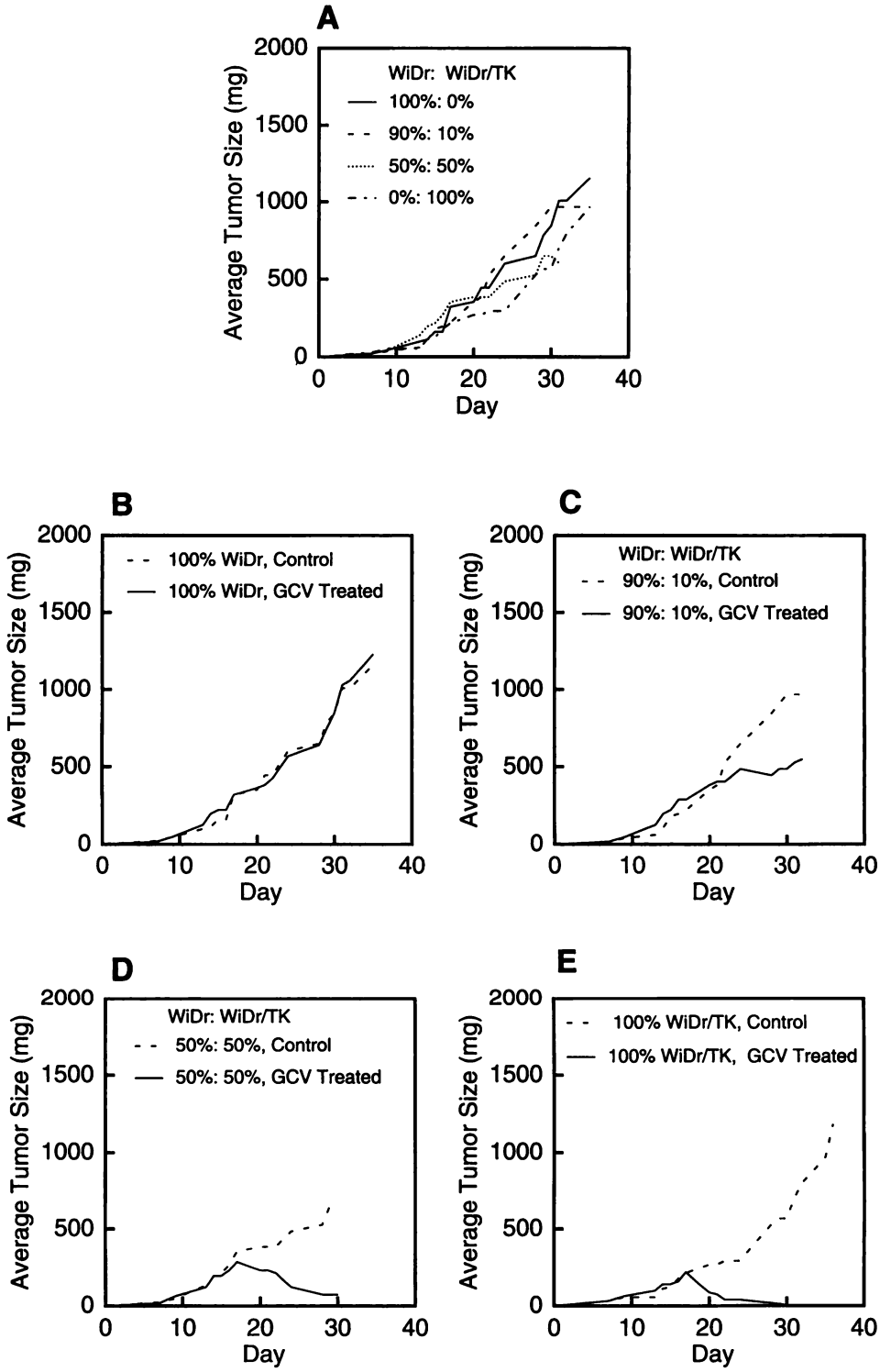

Fig. 2. *A*, untreated control tumors containing various percentages of HSV-TK-expressing cells grew at the same rate. The average tumor weight represents the average of 5 animals in each group. *B-E*, the average tumor weights in untreated controls (---) and GCV-treated animals (——).

and untreated control animals (Table 2). In animals with tumors composed of 90% WiDr:10% WiDr/TK cells (Fig. 2*C*), GCV treatment had a slight effect on tumor growth, with an average tumor size doubling of approximately 13 days in GVC-treated and 7 days in untreated animals. However, if the tumors consisted of 50% TK-expressing cells (50% WiDr:50% WiDr/TK), significant regressions of tumors were observed, with 60% being tumor-free at day 150 (Fig. 2*D*; Table 2). In animals with tumors that regressed but were not cured, there was a rebound in tumor growth after 65 days, possibly due to killing of WiDr/TK cells. In this group of animals, the doubling time for tumors was greater than 70 days in GCV-treated animals. One hundred % tumor-free animals were obtained for tumors consisting of 100% WiDR/TK cells (0% WiDR:100% WiDr/TK; Fig. 2*E*; Table 2).

When nude mice were implanted with different ratios of WiDr and WiDr/CD cells, significant antitumor effects were seen when only 4% of the cells expressed CD. Similar to above, the growth rate of the tumors was statistically identical in untreated control animals, regardless of the mixture of WiDr and WiDr/CD cells (Fig. 3). In animals

receiving 5-FCyt treatment, no antitumor effect was detected in tumor mixtures of 0% WiDr/CD and 100% WiDr. At a ratio of 4:96, 60% tumor-free animals were obtained with a 55-day delay in tumor doubling (Fig. 3; Table 2). One hundred % tumor-free animals were observed in animals with tumors composed of 100% CD-expressing cells (Fig. 3). A rebound was also detected in some tumors that regressed but were not eradicated, which may be the result of killing of the CD-expressing cells in the tumor. Thus, the antitumor effect of 5-FCyt on tumors composed of mixtures of WiDr/CD and WiDr cells in which only 4% of the cells expressed CD was comparable to the antitumor effect of GCV on tumors composed of 50% WiDr/TK cells.

Electron Microscopy Studies. It has been hypothesized that the bystander cell killing observed with HSV-TK may be the result of metabolic cooperation through gap junctions between cells (14, 17, 20, 21). WiDr tumor tissues were fixed in glutaraldehyde, sectioned, and examined by transmission electron microscopy for gap junctions or other junctional complexes necessary for macromolecular exchange between cells. No gap junctions were observed in WiDr solid tumors, but there was a relatively large number of desmosomes with associated tonofilaments observed between adjacent tumor cells (Fig. 4). Desmosomes are involved in the anchorage of intermediate filaments, which help form intercellular junctional complexes (23). Since tight junctions and desmosomes seal the intercellular spaces and presumably are not a common mode of intercellular communication, it is unlikely that phosphorylated GCV metabolites can enter adjacent cells via these structures.

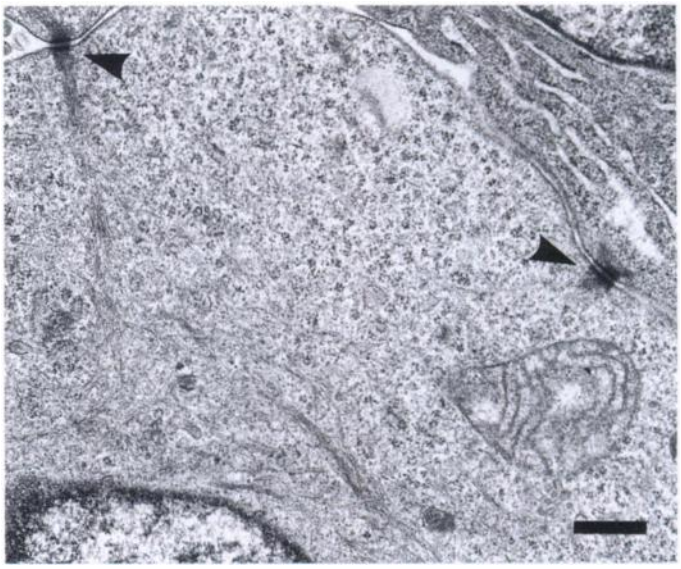

Fig. 4. A section of a WiDr tumor showing desmosomes (*arrowheads*) between adjacent cells, some of which have associated tonofilaments. ×19,090. *Bar*, 5 μm.

Table 2 *Effects of 5-FCyt and GCV treatments on WiDr/CD:WiDr and WiDr/TK:WiDr tumor mixtures*

Tumor mixtures	Time for two tumor doublings (days)	Tumor-free day 70
WiDr/CD:WiDr[a]		
0%:100%	16	0%
2%:98%	48	0%
4%:96%	>55	60%
8%:92%	>55	40%
100%:0%		100%
WiDr/TK:WiDr[b]		
0%:100%	10.6	0%
10%:90%	13	0%
50%:50%	>70	60%
100%:0%		100%

[a] All animals were treated with 500 mg/kg body weight of 5-FCyt.
[b] All animals were treated with 50 mg/kg body weight of GCV.

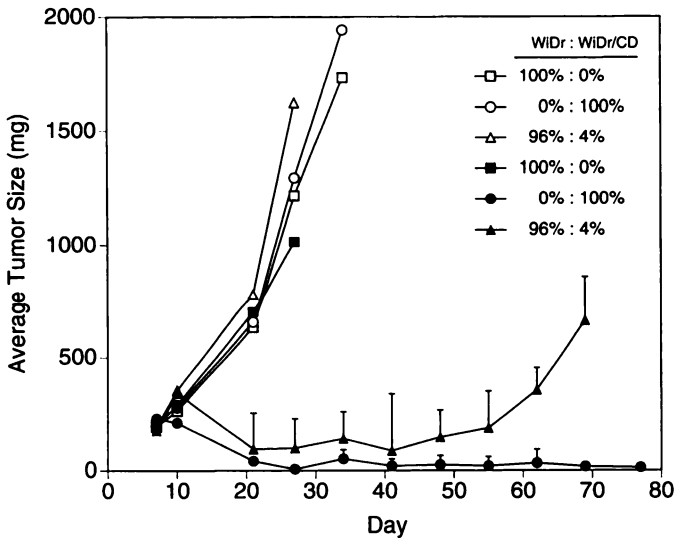

Fig. 3. The average tumor weights in untreated controls (□, ○, and △) and 5-FCyt-treated animals (■, ●, and ▲) at various percentages of CD-expressing cells. Each data point represents an average of 5 to 8 animals. For illustrative clarity, SDs (*bars*) are presented for only two groups.

Taken as a whole, the data from the *in vivo* tumor studies suggest that a high percentage of TK-expressing cells are required to achieve a profound antitumor effect with GCV on WiDr-derived s.c. tumors. In contrast, a significant antitumor effect can be generated if only a small percentage of WiDr-derived tumor cells express CD. This may be due, in part, to the fact that 5-FUra crosses biological membranes by nonfacilitated diffusion (13). Unlike 5-FUra, GCV metabolites are phosphorylated and may not be able to diffuse freely between cells. It has been hypothesized that gap junctions may be essential for bystander cell killing observed with HSV-TK/GCV (14, 17, 20, 21). In this study, we have demonstrated a significant bystander killing by GCV treatment if the tumor xenograft consisted of 50% of the cells expressing the HSV-*TK* gene. However, electron microscopy of tumor tissues did not show the presence of gap junctions. Rather, a large number of desmosomes were observed in these sections, suggesting that gap junctions may not be the sole means by which phosphorylated GCV moves intercellularly. A recent study examined the role of apoptotic vesicles in the bystander killing effect of GCV (24).

In summary, expression of HSV-*TK* in 50% of the cells in a WiDr-derived solid tumor mass is sufficient for successful eradication of 60% of the tumors. With current technology, it would be technically difficult to achieve 50% gene transfer efficiency. However, the HSV-TK/GCV combination may be more efficacious in other tumor types that routinely demonstrate gap junctions. GCV had a relatively narrow therapeutic window in *in vitro* cytotoxicity studies as compared to 5-FCyt. The lack of toxicity observed with the prodrug 5-FCyt, the diffusion of 5-FUra in and out of cells, and the relatively small percentage of CD-expressing cells required to achieve an antitumor effect following 5-FCyt treatment make the CD/5-FCyt enzyme/prodrug system a powerful tool for gene therapy.

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