Mechanisms for Ganciclovir Resistance in Gastrointestinal Tumor Cells Transduced with a Retroviral Vector Containing the Herpes Simplex Virus Thymidine Kinase Gene

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ABSTRACT

Transfer of the herpes simplex thymidine kinase (HSV-TK) gene into tumor cells confers sensitivity to the cells to the viral drug ganciclovir (GCV). Although the efficacy of the HSV-TK/GCV approach is well studied, the mechanisms for the resistance of HSV-TK-transduced tumor cells to GCV are poorly understood. Here, we examined the mechanisms for GCV resistance in HSV-TK-transduced gastrointestinal (GI) cell lines. Our results show that GCV sensitivities vary in vitro and in vivo among the different HSV-TK-transduced GI tumor cell lines. GCV-resistant colonies were isolated from several different HSV-TK-transduced GI tumor cell lines after 14 days of GCV treatment. Characterization of GCV-resistant colonies demonstrated that the HSV-TK gene was either partially or completely deleted from the resistant HSV-TK-transduced cells. In the HT-29 RM and MIAPACA-2 RM cells, a 220-bp deletion of the gene was found, whereas in the HT-29 R1-R5-resistant cells, the whole TK gene was found to be absent. Immunocytochemical studies using a polyclonal antibody to the TK protein demonstrated that the HSV-TK protein was absent in the GCV-resistant, HSV-TK-transduced cells. Transfection of the resistant cells with an adenoviral vector containing a HSV-TK gene restored sensitivity to GCV. The presence of GCV-resistant cells was only demonstrable in GI tumor cell lines that also demonstrated a poor bystander effect. Our results suggest that GCV resistance found in tumor cells transduced with a retroviral HSV-TK gene is due to the lack of a functional TK protein in the tumor cells rather than any intrinsic resistance of the cells to GCV. In tumor cells with a good bystander effect, the small percentage of TK-transduced cells that do not express the TK protein are probably killed by the bystander effect because GCV-resistant tumor cells were not found in these cell lines. GCV-resistant tumor cells were found only in tumor cell lines with a poor bystander effect, by which, presumably, the transduced tumor cells lacking a functional TK gene were not killed by the bystander killing effect.

INTRODUCTION

The transfer of suicide genes such as the HSV-TK<sup>2</sup> gene (1–5) into tumor cells renders the cells sensitive to nontoxic prodrugs such as GCV. The HSV-TK/GCV approach for the treatment of cancer is under clinical investigation in at least 11 trials (6). The expressed HSV-TK protein in transduced cells phosphorylates the prodrug GCV into monophosphorylated GCV, which is further converted into triphosphorylated GCV by cellular kinases. Triphosphorylated GCV, when incorporated into DNA, induces cell death. In mixed cocultures, experiments HSV-TK-transduced cells (TK<sup>+</sup>), as well as surrounding non-transduced cells (TK<sup>−</sup>), are killed by GCV treatment (7). This “bystander effect,” by which adjacent TK<sup>−</sup> cells are killed during killing of TK<sup>+</sup> cells, is thought to be mediated by transfer of toxic phosphorylated GCV from transduced tumor cells by intercellular gap junction communication to non-transduced tumor cells (8, 9). The bystander effect is essential for successful cancer gene therapy because the presence of this effect makes complete regression of the tumor possible when only a fraction of the tumor cells in a tumor mass are transduced (7).

The objective of cancer therapy is to eliminate all tumor cells in a tumor mass because experience with chemotherapy has shown that surviving tumor cells grow out to produce recurrent tumors. In in vitro and in vivo experiments, resistance of HSV-TK-transduced tumor cells to GCV has been described (10, 11). Development of GCV resistance in HSV-TK-transduced cells is of concern because this may limit the efficacy of the HSV-TK gene transfer approach for the treatment of cancer. The mechanisms for resistance of HSV-TK-transduced cells to GCV is unclear. Here, we investigated the mechanisms of GCV resistance in HSV-TK-transduced human GI tumor cell lines. Our results show that sensitivity to GCV treatment differed among the different GI tumor cell lines that are transduced with a retroviral HSV-TK gene. We found that TK<sup>−</sup> cell populations that are resistant to GCV have either a partial or complete

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2 The abbreviations used are: HSV-TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; GI, gastrointestinal; RT-PCR, reverse transcriptase-PCR; MTS, 3-(4,5-dimethylthiazol-2-yi)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; CMV, cytomegalovirus.
absence of the *HSV-TK* gene. In tumor cells with a good bystander effect, the small percentage of transduced cells that have lost the *HSV-TK* gene are killed by the bystander effect. GCV-resistant tumor cells were found only in tumor cell lines with poor bystander effects, by which, presumably, the transduced tumor cells lacking a functional TK protein were not killed by the bystander killing effect.

**MATERIALS AND METHODS**

**Cell Lines and Vectors.** BXPC-3, CAPAN-2, MIAPACA-2, PANC-1, PANC-3, and PANC-28 human pancreatic cancer cell lines, HT-29 colon cancer cells, and N87 gastric cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Colon cancer cells KM12C and KM12 SM were kindly provided by Dr. Fidler from M. D. Anderson Hospital (Houston, TX). We used retroviral vectors carrying the *HSV-TK* gene (G1Tk1SvNa.7) and a *Escherichia coli β-galactosidase* gene (G1BgSvNa). The G1Tk1SvNa.7 and G1BgSvNa vectors also carry a marker NeoR gene as a positive selectable marker gene. The producer cell lines for the vectors were obtained from Genetic Therapy Inc. (Gaithersburg, MD). The producer cell lines G1Tk1SvNa.7/PA317 and G1BgSvNa/PA317 were cultured in DMEM with 10% fetal bovine serum, and the supernatant containing the viral vectors was collected and frozen in aliquots at −70°C.

The adenoviral vectors Av1Tk1 and Av1LacZ4 were obtained from Genetic Therapy Inc. (Gaithersburg, MD). Av1Tk1 vector carries the *HSV-TK* gene, whereas the Av1LacZ4 carries the marker LacZ gene.

**Transduction of GI Tumor Cell Lines with Retroviral Vector G1Tk1SvNa.7.** GI tumor cells (5 × 10⁵ cells) were plated in 25-mm² tissue culture flasks for 24 h. The culture medium was then replaced with 3 ml of G1Tk1SvNa.7 retroviral vector supernatant with 8 μg/ml polybrene for 4 h. Forty-eight h following transduction, a neomycin analogue, G418 (0.3–2 mg/ml), was added to the culture medium to select for transduced BXPC-3, PANC-3, HT-29, and MIAPACA-2 cells. The G1Tk1SvNa retroviral vectors used in our studies all carry a neomycin resistance (NeoR) selectable marker gene; therefore, a selected population of HSV-TK-transduced cells is established after selection in G418. The cells were selected for 14 days in G418, and the surviving colonies were subcultured and established as permanent HSV-TK-transduced clones (TK+ cells).

**In Vitro and in Vivo Effects of GCV on HSV-TK+ Tumor Cells.** GCV killing curves were determined for both TK+ and TK− tumor cell lines by culturing the cells in different concentrations of GCV (Cytovene; Syntex Laboratories, Palo Alto, CA) for 12–14 days in 6- or 12-well tissue culture plates. The percentage of cells killed by GCV were estimated by examining the surviving cell populations under an inverted Nikon microscope after the resistant colonies were stained with a 1% methylene blue solution.

To examine for the *in vivo* effect of GCV on TK+ GI tumor cells, BXPC-3 cells (1 × 10⁵), or HT-29 cells (5 × 10⁵) were injected s.c. into nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Eleven to 13 days after tumor implantation, the mice received i.p. injection of GCV (100 mg/kg/day) for 14 days. The size of the tumors was measured every 3 days, and tumor volumes were calculated.

**Establishment of GCV-resistant Mixed and Clonal Cell Populations (TK+/GR) from HT-29 and MIAPACA-2 TK+ Cells.** To investigate the mechanisms of GCV resistance in HSV-TK-transduced tumor cells, GCV-resistant tumor cell colonies (TK+/GR) were subcultured out, and GCV-resistant cell clones were established. MIAPACA-2 and HT-29 TK+ cells were cultured in a medium containing 50 μg/ml GCV for 3 weeks. The surviving GCV-resistant colonies were harvested with trypsin-EDTA, and the cells from several different surviving colonies were collected together and repeatedly passaged to establish a GCV-resistant cell population (HT-29 RM and MIAPACA-2 RM cells).

To establish pure clonal populations of GCV-resistant TK+ cells, HT-29 TK+ cells were cultured in a medium containing 90 μg/ml GCV for 4 weeks, and the surviving HT-29 GCV-resistant colonies were picked up individually and subcultured. Thirty TK+ GCV-resistant HT-29 cell clones were established, and five resistant clones were randomly selected for further study (HT-29 R1–R5 cells).

**GCV Killing Curve of Resistant TK+ GI Tumor Cells.** HT-29 and MIAPACA-2 TK+, TK−, and TK+/GR8 cells (5 × 10⁵ cells/well) were plated in 12-well tissue cultures plates. After overnight culture, the culture medium was replaced with medium containing GCV (0–100 μg/ml), and the cultures were treated for 12 days. GCV in the culture medium was changed daily. At the end of the treatment period, the cells were fixed with 1% methylene blue solution, and the percentages of surviving cells in each treated well were estimated as a fraction of untreated control cells (no. of colonies in GCV-treated wells/no. of colonies in untreated wells × 100).

**Detection of HSV-TK Gene and Gene Expression in GCV-resistant Cell Populations by PCR and RT-PCR.** Genomic DNA was isolated from TK−, TK+, and TK+GR8 cell populations using an Elu-Quik DNA purification kit (Schleicher & Schuell Inc., Keene, NH). The PCR primers used to amplify TK gene were: TK1, forward primer, 5’-GTGTCCCTGCTCATGTCC-3’, and reverse primer, 5’-GGGCGATACCGAG-GTCAAG-3’, which amplify a 288-bp region of the TK gene. PCR primers used to amplify a 423-bp NeoR gene product were: forward primer, 5’-GCTGAGGAGCTATCCATG-3’, and reverse primer, 5’-AGGG TGCGGATGTGATGAG-3’, which amplify a 990-bp region of HSV-TK gene. The primers used to amplify a 423-bp NeoR gene product were: forward primer, 5’-GGTGGAGAGGCTATCCGCA-3’, and reverse primer, 5’-ATCCTGTGAAAGCCAGGT-3’, and a PCR amplification of TK and NeoR fragments was performed with a GeneAmp PCR core kit (Perkin-Elmer Corp., Branchburg, NJ).

For RT-PCR analysis, total RNA was isolated from the tumor cells to detect TK and NeoR gene expression in GCV-resistant HT-29 cell clones. RNA was isolated using a RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX). RT-PCR to amplify HSV-TK and NeoR mRNAs was performed using a Stratagene kit according to the manufacturer's specifications (Stratagene, La Jolla, CA). Briefly, 10 μg of total RNA were reverse-transcribed into DNA by using oligo(dT) primers. Three μl of a 50-μl cDNA mixture were further
amplified with TK or NeoR primers using Gene Amp PCR core reagents. All PCR products were visualized by electrophoresis on a 1% agarose gel with ethidium bromide.

**Analysis of HSV-TK Gene Expression by Northern Blot Analysis in GCV-resistant Cell Populations.** The full-length transcript of the HSV-TK gene was detected by Northern blot analysis. Total RNA was isolated using a RNA-STAT 60 kit (Tel-Test) and 30 μg were separated by electrophoresis and transferred to nylon transfer membrane (Hybond-N; Amersham) with a Megaprime DNA labeling system (Amersham). Hybridization of the blots was performed at 68°C (Amersham). Hybridization of the blots was performed at 68°C (Amersham). Hybridization of the blots was performed at 68°C (Amersham). Hybridization of the blots was performed at 68°C (Amersham).

**Detection of HSV-TK Protein by an Immunocytochemical Stain.** TK+, TK+, and TK+/GR tumor cells (3 × 10^6 well) were plated in Lab-Tek two-well chamber slides (Nunc, Inc., Naperville, IL) for 72 h. The chambers were then washed with PBS and fixed with ice-cold acetone for 10 min. The slides were air-dried and stained immediately or frozen at -70°C until use. Polyclonal rabbit antibody against HSV-TK protein was kindly obtained from Dr. W. P. Summers at Yale University (New Haven, CT). To detect the TK protein, the slides were blocked with 2% normal goat serum for 10 min and then washed in PBS. The primary antibody diluted at 1:50 was added onto the slides for 60 min. The slides were then washed three times with PBS, and a secondary antibody goat antirabbit IgG conjugated with peroxidase (1:100; Vector Laboratories, Burlingame, CA) was added to the slides for 30 min. The slides were washed five times with PBS and developed with a 3,3'-diaminobenzidine substrate kit (Vector Laboratories). After counterstaining with hematoxylin, the slides were examined and photographed under a Nikon microscope.

**Detection of the Bystander Effect in Vitro.** Presence of a bystander effect was examined in vitro using a MTS calorimetric cell proliferation assay that measures dehydrogenase activity in viable cells (Cell Titer-96 aqueous nonradioactive MTS cell proliferation assay; Promega, Madison, WI). HSV-TK-transduced and nontransduced tumor cells were mixed in different ratios and cocultured in 96-well tissue culture plates (3 × 10^3 cells/well). After a 24-h incubation period, fresh culture medium with 10 μg/ml GCV was added to the cells. The medium was changed to add a fresh lot of GCV each day to the cells for 14 days. MTS cell proliferation assays were performed every 3–4 days. Twenty μl of assay mix were added to the cell cultures, and A_490nm was measured on an ELISA reader. The absorbance values in this assay reflect viable cell densities.

**Treatment of TK+/GR Cells with Adenoviral HSV-TK Vectors.** To confirm our hypothesis that GCV resistance in TK-transduced tumor cell lines is due to the loss of production of the TK protein, we studied the effects of reintroducing the TK gene into the TK+/GR tumor cells with an adenoviral TK vector and treatment of the cells with GCV. An adenoviral vector was selected for these studies because the TK+/GR resistant cells were already transduced with a retroviral vector containing a NeoR gene and, therefore, selection is not necessary after transducing the cells with an adenoviral vector due to the high transduction efficiency of the vector.

**RESULTS**

**Detection of HSV-TK-transduced Tumor Cells That Are Resistant to GCV.** GCV treatment effects were studied in 10 human GI tumor cell lines in vitro by culturing the cells in 35-mm^2 dishes in the presence of logarithmic escalation of the concentration of GCV. We studied BXPC-3, CAPAN-2, MIAPACA-2, PANC-1, PANC-3, and PANC-28 pancreatic cancer cells, HT-29, KM12C, and KM12 SM colon cancer cells, HUTU duodenal cancer cells, and N87 gastric cancer cells. TK+ tumor cells all showed a marked increase in sensitivity to GCV treatment compared to TK- cells (Table 1). However, only HSV-TK-transduced BXPC-3 and PANC-3 cells were completely killed by GCV at nontoxic concentrations of GCV (non-toxic GCV doses were 10–20 μg/ml). In the other eight cell lines, TK+ cells were not completely killed by GCV, and

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<th>Table 1</th>
<th>GCV sensitivity of HSV-TK-transduced human GI tumor cell linesa</th>
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<tr>
<td>Cell line</td>
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<tr>
<td>BXPC-3</td>
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a This table shows the GCV sensitivity of four representative cell lines among 11 GI tumor cells examined. IC_{50} was measured by a MTS cell proliferation assay after the tumor cells received GCV treatment for 10 days.
Emergence of GCV-resistant colonies during treatment of HSV-TK-transduced GI tumor cells. HSV-TK+ and TK- BXPC-3, HT-29, and MIAPACA-2 cells (5 x 10^6 cells) were cultured in 12-well tissue culture plates. Culture medium was replaced 24 h later with a medium containing 0.5, 10, 30, 50, or 100 μg/ml GCV. After 14 days of GCV treatment, the surviving cells were fixed with a 1% methylene blue solution. The surviving colonies in each well represent GCV-resistant cell clones at each GCV concentration.

Fig. 1  GCV resistance and HSV-TK

Fig. 2  GCV sensitivity of HSV-TK+ and HSV-TK- tumor cells. TK+ and TK- tumor cells (4 x 10^3 cells) were cultured in 96-well tissue culture plates. Culture medium was replaced 24 h after plating with medium containing 10 μg/ml GCV, and the cells were treated for 12 days. The surviving cells were assayed by the MTS cell proliferation assay at days 0, 4, 8, and 12. The A_490 nm values reflect the number of viable cells in this assay. Similar results were obtained from three separate experiments (n = 8 per treatment group).
HSV-TK gene was investigated in the TK−/GR tumor cells, we investigated the integration of the HSV-TK gene in the transduced tumor cells. To understand the mechanisms for GCV resistance in TK+/GR tumor cells, we investigated the integration of the HSV-TK gene in the transduced tumor cells. To understand the mechanisms for GCV resistance, we investigated the integration of the HSV-TK gene in TK+/GR tumor cells.

Similar results were observed in vivo after s.c. implantation of TK+ tumor cells in nude mice (Fig. 3). After being treated with i.p. injections of GCV (100 mg/kg/day) for 14 days, tumor-bearing mice had a significant reduction in the size of their tumors. In the TK HT-29 and MIAPACA-2 tumors, the size of the treated HT-29 and MIAPACA-2 cells were different from that of the parent TK HT-29 and MIAPACA-2 cells (Fig. 4A).

GCV Killing Curve of TK+/GR Cells. The sensitivity of parent TK+ HT-29 and MIAPACA-2 cells is demonstrated by the killing of 95–99% of the cells by GCV (Fig. 4A). One and 5% of TK+ HT-29 and MIAPACA-2 cells, respectively, were resistant to GCV at concentrations of up to 100 μg/ml (Fig. 4A). The GCV killing curves of the resistant TK+/GR cell lines HT-29 R1, HT-29RM, and MIAPACA-2 RM cells were similar to those of the parent TK− HT-29 and MIAPACA-2 cells, suggesting that the TK+/GR cells have lost their sensitivity to GCV (Fig. 4).

Integration of the HSV-TK Gene in TK+ Parent and TK+/GR Cell Lines. To understand the mechanisms for GCV resistance in TK+/GR tumor cells, we investigated the integration of the HSV-TK gene in the transduced tumor cells. Presence of the HSV-TK gene was investigated in the TK+ parent cells and in the TK+/GR cell lines HT-29 RM and MIAPACA-2 RM and the HT-29-resistant clones R1–R5. The HSV-TK gene was amplified by PCR using TK1 and TK2 primers (Fig. 5A). We first analyzed the genomic DNA from the tumor cells by PCR amplification with TK1 primers that amplified a 288-bp fragment within the TK gene (Fig. 5A). A PCR product was not detected when the 288-bp fragment inside the HSV-TK gene was amplified with the TK1 primers in some of the TK+/GR cell lines, suggesting that the fragment of the TK gene that annealed with the TK1 primer sequence was deleted. Because the forward TK1 primer is between 410 and 429 bp and the reverse TK1 primer is between 678 and 698 bp of the TK gene, the deletion may be located either at the primer sequence regions (Fig. 5A).

The HSV-TK gene in the GITK15SvNa vector has a splice site located at the 328 bp. To examine whether the absence of PCR products after amplification with the TK1 primers was due to the presence of the spliced TK gene or whether the whole gene was lost from the cells, we also analyzed genomic DNA by PCR amplification with the TK2 primer sequences, which amplified 990 bp of the HSV-TK gene (Fig. 5A).

In the parent HT-29 and MIAPACA-2 TK+ cell populations, both the 288- and the 990-bp PCR products were amplified with TK1 and TK2 primer pairs (Fig. 5B), demonstrating integration of the HSV-TK retroviral vector in these cell lines. In contrast, in the HT-29 RM cells, both the 288- and 990-bp PCR products were absent; however, a 770-bp PCR product was found that amplified with the TK2 primer (Fig. 5B). In MIAPACA-2 RM cells, the 288- and 990-bp PCR products were found; however, a much stronger 770-bp band that amplified with the TK2 primers was also found (Fig. 5B). These results suggest that a 220-bp fragment of the HSV-TK gene is deleted in all or majority of the HT-29 RM and MIAPACA-2 RM cells.

In the HT-29-resistant clones R1–R5, no PCR products were detected after amplification of genomic DNA with both TK1 and TK2 primers (Fig. 5C). On the other hand, a 423-bp product that amplified with primers specific for the NeoR gene was present in all five HT-29-resistant cell clones (Fig. 5C). These results suggest that the HT-29 R1–R5 cells were transduced with the HSV-TK retroviral vector because the NeoR gene that conferred the cells' resistance to G418 was present in the genomic DNA. Absence of PCR products that amplified with TK1 and TK2 primers suggest that the TK gene is selectively lost from the HT-29 R1–R5 cells.

Expression of the HSV-TK Gene in TK+ Parent and TK+/GR Cell Lines. On Northern blot analysis, TK gene transcripts were not detected in the TK+ cells but were detected in the TK− HT-29 cells and MIAPACA-2 cells. In the HT-29 RM and MIAPACA-2 RM cells, the HSV-TK transcripts were overexpressed compared to the parent TK+ MIAPACA-2 and HT-29 cells. HT-29-resistant clones R1 and R3 did not express the 4-kb TK mRNA transcripts (Fig. 6A). Because TK transcripts were not detected in HT-29 R1–R5 cells, we examined for expression of NeoR transcripts in these cells by RT-PCR (Fig. 6B). Expression of the NeoR gene was demonstrable in four of the five HT-29-resistant clones (R1–R3 and R5); however, the HSV-TK gene was not expressed in any of the five HT-29-resistant clones on RT-PCR, confirming our findings on Northern blot analysis. Both the NeoR and the HSV-TK genes were expressed in the parent HT-29 TK+ and MIAPACA TK+ cell lines and were not expressed in the control TK− cells (Fig. 6B). Our findings of loss of TK gene expression in the HT-29 R1–R5 cells but presence of the NeoR gene expression, taken together with our findings from analysis of genomic DNA.
(described above), demonstrate that the HT-29 R1-R5 cells were transduced by the HSV-TK retroviral vector; however, the HSV-TK transgene is absent from these cells.

Detection of the HSV-TK Protein in TK⁺ and TK⁺/GR GI Tumor Cell Populations. HT-29 TK⁺ and MIAPACA-2 TK⁺ cells were treated with 50 µg/ml GCV for 12 days in two-well chamber slides, the surviving GCV-resistant cells were fixed and stained for the HSV-TK protein using a polyclonal antibody to the HSV-TK protein. Fig. 7A demonstrates that almost all of the GCV-resistant MIAPACA-2 and HT-29 cells do not express the HSV-TK protein because they stained negative with the HSV-TK antibody. The absence of the TK protein in the GCV-resistant cell colonies suggests that GCV treatment of TK⁺ HT-29 and MIAPACA cells selects out a resistant cell population that does not express the HSV-TK protein. Control untreated HT-29 TK⁺ and MIAPACA-2 TK⁺ cells strongly stained for the HSV-TK protein (Fig. 7A).

We also stained the TK⁻/GR cell lines HT-29 RM, MIAPACA-2 RM, and HT-29 R1-R5 for the presence of the HSV-TK protein and found that the HSV-TK protein was absent in the TK⁺/GR cell lines (Fig. 7A), providing further evidence that these cells are resistant to GCV due to loss of the TK protein in the cells. We stained untreated TK⁺/GR BXPC-3 cells with the TK antibody and found that about 10–20% of the TK⁺/GR BXPC-3 cell population did not stain or demonstrated a very weak stain with the TK antibody (Fig. 7B). Presumably, this is a population of BXPC-3 cells that does not express the TK protein and may potentially give rise to GCV-resistant cells. However, GCV-resistant cells were not detected during treatment of BXPC-3 cells with GCV, suggesting that TK⁺/GR BXPC-3 cells are killed during GCV treatment.

Killing of GCV-resistant Cells (TK⁺/GR Cells) by Adenoviral Vectors Carrying a HSV-TK Gene. About 86–100% of HT-29, HT-29 RM, and HT-29 R1 cells transduced with a control Av1LacZ4 vector were viable after GCV treatment. In contrast, only 22–32% of the HT-29 parent TK⁻ cells and the HT-29 RM and HT-29 R1 cells transduced with an Av1TK vector were alive following 9 days of GCV treatment (Fig. 8A). Similar results was obtained with MIAPACA-2 cells (Fig. 8B). Eighty % of the control MIAPACA-2 RM cells that were transduced with the Av1LacZ4 vector survived after treatment with GCV. In contrast, only 4% of MIAPACA-2 TK⁻ and MIAPACA-2 RM cells transduced by the Av1TK vector were viable after GCV treatment (all Av1TK1 versus Av1LacZ4 groups: P < 0.0001, n = 8 per group). There were no differences in the sensitivity of TK⁺ and TK⁺/GR cells to GCV in any of the groups (P > 0.05). These results demonstrate that TK⁺/GR cell lines (HT-29 RM and R4 and MIAPACA-2 RM) are sensitive to GCV if a functional HSV-TK protein is expressed by these cells.

Bystander Killing Effect in GI Tumor Cells. The degree of the bystander effects varied among the different GI
density of control wells as 100%. B, GCV killing curve of TK⁺/GR cells. The surviving cell clones in each well represent GCV-resistant cells after 12 days of GCV treatment. GCV-resistant cell lines HT29-RM and MIAPACA-2 RM cells and HT-29 R1 cells demonstrate similar killing curves to that of the parent HSV-TK⁺ cell lines.
Fig. 5  PCR amplification of genomic DNA for the HSV-TK gene in TK\(^-\) and TK\(^+/GR\) cell lines. A, two PCR primer pairs (TK1 and TK2) were used to amplify the HSV-TK gene. The TK1 primer pair amplified to a 288-bp fragment within the TK gene because the forward TK primer is between 410 and 429 bp and the reverse primer is between 678 and 698 bp. The TK2 primer amplifies to the 990 bp of the TK gene because the forward primer is between 100 and 120 bp and the reverse primer is between 1052 and 1072 bp. B, tumor DNA was isolated from TK\(^-\), TK\(^+\), and TK\(^+/GR\) cells and amplified with TK1 and TK2 primers. In the parent HT-29 and MIA PACA-2 TK\(^+\) cell populations, both 288- and 990-bp PCR products were amplified with TK1 and TK2 primer pairs. In the HT-29 RM cells, both the 288- and 990-bp PCR products were absent; however, a 770-bp PCR product was found that amplified with the TK2 primer. In MIA PACA-2 RM cells, the 288- and 990-bp PCR products were found; however, a much stronger 770-bp band that amplified with the TK2 primers was also found. C, the HSV-TK gene was not detected in any of the five HT-29-resistant cell clones when the genomic DNA was amplified with both TK1 and TK2 primers (top and bottom, respectively). A 423-bp Neo\(^+\) gene PCR product was detected when genomic DNA was amplified with primers specific for the Neo\(^+\) gene (top), suggesting that these cell clones were transduced with the HSV-TK retroviral vector.
Resistance and detected in the HT-29-resistant cell clones (HT-29 R1 and HT-29 R3). β-Actin was used as an internal loading control.

B. Demonstrating expression of the 4-kb HSV-TK transcripts in HSV-TK, HT-29 RM, and MIAPACA-2 RM cells. HSV-TK transcripts were not electrophoresed on a 1% agarose gel stained with ethidium bromide. PCR amplification products for HSV-TK and NeoR were detected in R1-R3 and R-5 cells.

Fig. 6. Expression of the HSV-TK gene detected by Northern blot analysis and RT-PCR in TK+ and TK−/GR cell lines. A, Northern blot analysis demonstrating expression of the 4-kb HSV-TK transcripts in HSV-TK+. HT-29 RM, and MIAPACA-2 RM cells. HSV-TK transcripts were not detected in the HT-29-resistant cell clones (HT-29 R1 and HT-29 R3). β-Actin was used as an internal loading control. B, RT-PCR products electrophoresed on a 1% agarose gel stained with ethidium bromide. PCR amplification products for HSV-TK and NeoR transcripts were 288 and 423 bp, respectively. The gel demonstrates that the TK and NeoR transcripts are detected in the parent BXPC-3, HT-29, and PANC-3 TK+ cell lines and the TK producer cell line (positive control) and are negative in the TK− cell lines. In the HT-29-resistant clones (R1–R5), the TK transcripts are absent but the NeoR transcripts are detected in R1–R3 and R-5 cells.

tumor cell lines (Fig. 9). BXPC-3 cells represents a cell line with excellent bystander effects. Presence of only 10% of TK+ BXPC-3 cells in a mixed cell population of TK+ and TK− cells lead to killing of over 90% of the cocultured cells. MIAPACA-2 and HT-29 cells demonstrated poor bystander effects. When 10–75% of TK+ MIAPACA-2 and HT-29 cells were cocultured with 25–90% of TK− and treated with GCV, little bystander killing of the TK− cells was seen, and the TK− cells grew out as GCV-resistant cells.

DISCUSSION

Near total killing of all of the tumor cells in a tumor mass is essential for the success of any drug based therapy of cancer because tumor cells that are resistant to a chemotherapeutic agent grow out as recurrent tumors. Observations from several laboratories have documented the development of resistance to GCV in HSV-TK-transduced tumor cells (10, 11). Moolten and colleagues (10) first demonstrated resistance to GCV in TK-transduced sarcoma and lymphoma cells in in vitro and in vivo studies. The resistant cells lacked TK activity, and the authors postulated that loss of the TK gene was responsible for the resistance to GCV (10). Similarly, Barba and colleagues (11) observed that, following 14 days of GCV treatment, a small fraction of HSV-TK-transduced rat glioma cells grew out as resistant colonies. Here, our own experience in 10 human GI tumor cell lines demonstrates that the presence of GCV resistance in HSV-TK-transduced tumor cells is a common phenomenon. Of the cell lines studied in vitro (five pancreas, three colon, one duodenum, and one gastric), complete killing of HSV-TK-transduced tumor cells at nontoxic concentrations of GCV was found in only two pancreas cancer cell lines, BXPC-3 and PANC-3. In the other cell lines, GCV-resistant TK+ cells were identified that demonstrated GCV sensitivity similar to that of TK− tumor cells.

The mechanisms for GCV resistance in HSV-TK-transduced tumor cells are unclear (10, 11). Elucidation of these mechanisms may have significant implications for clinical application of the HSV-TK/GCV gene therapy approach because development of therapeutic strategies to augment the efficacy of this approach may be possible. Two possible mechanisms may account for the development of GCV resistance in HSV-TK-transduced tumor cells: absence of a functional protein in the tumor cell; or development by the tumor cells of specific mechanisms to resist GCV analogous to the development of multidrug resistance to chemotherapeutic agents. For example, a specific mechanism has been identified for resistance to GCV by CMVs (12, 13). The UL97 gene in CMV codes for a phosphotransferase that phosphorylates GCV into its active metabolite (12, 13). Mutations in the UL97 gene have been associated with clinical resistance to GCV by CMVs (12, 13). To distinguish between the two mechanisms, we established HSV-TK-transduced cell lines that were resistant to GCV by selecting the TK+ transduced cells in GCV (50–90 µg/ml) for over 3 weeks. Our studies suggest that partial or complete deletion of the HSV-TK gene is responsible for GCV resistance in the TK+/GR cell lines because these changes in the TK transgene were accompanied by loss of expression of the TK protein in the transduced tumor cells. Our studies with the HSV-TK adenoviral vector in the TK+/GR cells demonstrate that GCV sensitivity is restored to the TK+/GR cells following transduction with an
Fig. 7  Detection of HSV-TK protein in TK⁺, TK⁻, and TK⁺/GR cell lines by immunostaining. Cells were cultured in two-well chamber slides and treated with GCV. At the end of the treatment period, the GCV-resistant cells were fixed with ice-cold acetone and stained with a polyclonal antibody to HSV-TK protein. A, HT-29 TK⁻ and MIAPACA-2 TK⁻ cells show the presence of the HSV-TK protein in greater than 90% of the cells, whereas TK⁻ cells stained negative with the HSV-TK antibody. The vast majority of TK⁻ cells (>99%) surviving treatment with 50 μg/ml GCV for 12 days do not stain positive with the TK antibody. Arrow, a few HSV-TK protein-positive cells survived GCV treatment are quiescent cells. Established TK⁺/GR cell lines (HT-29 RM and MIAPACA-2 RM) stained negative with the TK antibody. Original magnification. ×500. B, expression of HSV-TK protein in BXPC-3 TK⁺ cells. Only 20–30% of TK⁺ BXPC-3 cells showed a strong TK antibody staining. Some cells were weakly positive, whereas 10% of the cells did not have detectable levels of HSV-TK protein. Original magnification. ×500.
adenoviral TK vector. These findings further confirm our hypothesis that emergence of GCV resistance in TK\textsuperscript{+} tumor cells is due to the lack of a functional TK protein and not due to development of specific cellular mechanisms that resist the actions of GCV in the tumor cells.

The mechanisms by which tumor cells may silence the expression of transduced foreign genes are not fully understood. Challita and colleagues (14) have shown that methylation of the expression of transduced foreign genes are not fully understood. Due to development of specific cellular mechanisms that resist tumor cells is due to the lack of a functional TK protein and not treatment but with GCV treatment were defined as 100%.

\textbf{Fig. 8} Transduction of TK\textsuperscript{+}/G\textsuperscript{8} cell lines with an adenoviral TK vector. HSV-TK\textsuperscript{+}, TK\textsuperscript{+}, or TK\textsuperscript{+}/G\textsuperscript{8} HT-29 (A) or MIAPACA-2 cells (B) were plated in 96-well tissue culture plates and transduced with AV\textsubscript{TK} vector supernatant at multiplicity of infection of 20 (HT-29) or 200 (MIAPACA-2). Controls were transduced with an adenoviral vector containing a marker \(\beta\)-galactosidase gene (AV\textsubscript{LacZ}). The cells were treated with 10 \(\mu\)g/ml GCV for 9-10 days, and the numbers of surviving cells in each group were measured by the MTS cell proliferation assay \((n = 8)\). The absorbance values from the cells without AV vector treatment but with GCV treatment were defined as 100%.

The mechanisms by which tumor cells may silence the expression of transduced foreign genes are not fully understood. Challita and colleagues (14) have shown that methylation of the human glucocerebrosidase transgene in hematopoietic stem cells transduced with retroviral mediated gene transfer lead to reduced or loss of expression of the transgene. Our study documents mechanisms for loss of expression of the HSV-TK gene in transduced GI tumor cells. We have found partial deletion of the HSV-TK gene in the vector leads to loss of expression of the transgene in the HT-29 RM and MIAPACA-2 RM cells. In the HT-29 R1–R5 cells, complete loss of the HSV-TK gene was found. Because the RM cells were derived from harvesting a large number of colonies, whereas the HT-29 R1–R5 clonal cell lines were established from randomly picked colonies, it is possible that loss of the TK gene seen in the HT-29 R1–R5 cells is more representative than our findings in RM cells.

The mechanism for the deletion of the TK gene in the transduced GI cell lines is not clear from our studies. The 220-bp deletion in the TK gene may be a consequence of deletion of the TK gene after integration of the vector into the host DNA or may be due to the presence of a spliced gene in the original TK vector that transduced the cell. The HSV-TK gene in the G1TKSvNa vector has a splice site located at bp 328.\textsuperscript{3} Production and packaging of small amounts of retroviral vector particles with a 220-bp deletion of the HSV-TK gene has been demonstrated.\textsuperscript{3} Tumor cells transduced with vector particles containing the spliced gene lack TK activity and were not killed by GCV.\textsuperscript{3} Consistent with this observation, the 220-bp deletion that we have observed in the HT-29 and MIAPACA-2 RM cell lines is probably due to transduction of the tumor cells with retroviral particles that contain the spliced TK gene because we used the G1TKSvNa vector. It is unlikely that the 220-bp deletion in the TK gene that was observed in our study is due to cellular processes in the tumor cells because a uniform 220-bp deletion was observed in two different GI tumor cell lines. Similarly, the mechanism for the complete absence of the HSV-TK gene in the HT-29-resistant clones R1–R5 is also unclear from the present studies; it is unlikely, however, that the HSV-TK gene was selectively deleted from these tumor cells, and a likely explanation is that these cells were transduced with a retroviral vector containing only the Neo\textsuperscript{R} gene. Our studies suggest that, despite repeated propagation of TK\textsuperscript{+} cell lines in

\textsuperscript{3} S. Forry-Schaudies and Y. Chiang, unpublished observations.
culture, only about 1–6% of the cells lose expression of the transgene. This demonstrates remarkable stability of integration and expression of the transgene in the vast majority of the cells. Furthermore, despite prolonged propagation of TK+ cells under continuous exposure to GCV, resistance was not found in cells expressing the TK protein. These studies demonstrated the remarkable long-term efficacy of retrovirally mediated gene transfer.

Loss of all or part of the TK gene is probably not the major factor responsible for the development of GCV-resistant cells. Although the same retroviral TK vector supernatant was used to transduce all of our GI tumor cell lines, GCV-resistant cells were not found in TK+ BXPC-3 and PANCl-cell lines. These two cell lines also demonstrate significant bystander killing effects; for example, in BXPC-3 cells, admixture of only 10% of TK+ transduced cells with 90% TK− cells leads to killing of the 90% of the TK− cells during GCV treatment. Our studies suggest that only about 1–5% of the cells grew out as resistant colonies in the HT-29 and MIAPACA-2 experiments. If efficient bystander killing is present, then these transduced cells with an absent HSV-TK gene should have been killed by the bystander effect. Immunohistochemical studies confirmed that about 10% of TK+ BXPC-3 cells failed to stain with the TK antibody, presumably reflecting the population of tumor cells that were transduced with a vector incorporating a HSV-TK gene with a splice site. The finding of absence of any GCV-resistant colonies in TK+ BXPC-3 and PANCl-cell lines is probably due to the efficient bystander killing in these cells. GCV-resistant colonies were detected in TK− MIAPACA-2 and HT-29 cell lines; these cells also demonstrate poor bystander effects, and this may explain why the small percentage of HSV-TK-transduced cells lacking a functional TK protein were not killed and grew out as resistant colonies. Poor bystander killing may also explain the development of GCV-resistant cells in previously reported studies. GCV resistance was found in transduced rat glioma cells, a cell line with very poor gap junction communication in vitro. We and others (8, 9) have found that the magnitude of the bystander effect correlates with intercellular gap junction communication between tumor cells. Similarly, Moolten and colleagues (10) demonstrated recurrence of s.c. tumors derived from HSV-TK-transduced L18 lymphoma cells after GCV treatment. Lymphocytes lack gap junction communication and demonstrates poor bystander killing effects (15). These studies suggest that the efficiency of bystander killing in a cell line may determine the development of GCV-resistant colonies in HSV-TK-transduced cell lines.

Our findings emphasize that the bystander effect plays a critical role in the success of suicide gene therapy approaches. At present, there is a strong emphasis on achieving high transduction efficiencies for successful gene therapy of cancer. Our studies show that, even in a pure selected population of TK+ cells, the few cells that lack an intact TK transgene grow out as GCV-resistant colonies in the absence of a good bystander killing effect. Because, at present, 100% transduction of a tumor mass is not achievable, even with adenoviral vectors, future strategies aimed at improving both transduction efficiency and the bystander killing effect will be important for successful cancer gene therapy.

REFERENCES

Mechanisms for ganciclovir resistance in gastrointestinal tumor cells transduced with a retroviral vector containing the herpes simplex virus thymidine kinase gene.

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