Optimizing Prostate Cancer Suicide Gene Therapy Using Herpes Simplex Virus Thymidine Kinase Active Site Variants

ALLAN J. PANTUCK,[1] JAMIE MATHERLY,[1] AMNON ZISMAN,[1] DAVID NGUYEN,[1] FRANK BERGER,[2] SANJIV S. GAMBHIR,[2] MARGARET E. BLACK,[3,4] ARIE BELLDEGRUN,[1] and LILY WU[1]

ABSTRACT

The herpes simplex virus (HSV) thymidine kinase gene (tk) forms the basis of a widely used strategy for suicide gene therapy. A library of HSV thymidine kinase enzyme (TK) active site mutants having different affinities for guanosine analog prodrugs was developed. We sought to determine the optimal combination of tk variant and prodrug specifically for prostate cancer gene therapy, using *in vitro* and *in vivo* studies of adenovirally infected CL1, DU-145, and LNCaP tumor lines carrying wild-type tk, tk30, tk75, and sr39tk mutants expressed by a strong, constitutive cytomegalovirus promoter and treated with ganciclovir and acyclovir. *In vitro* experiments involving prostate cancer (CaP) cell line infection were carried out with a broad range of prodrug concentrations, and cell killing was determined by limiting dilution (colony-forming), MTT, and propidium iodide assays. *In vivo* studies based on CL1-GFP xenograft experiments were carried out to examine the ability of each TK variant to prevent tumor formation and to inhibit tumor growth and development of metastases in established orthotopic and subcutaneous tumors in SCID mice. Both *in vitro* and *in vivo* studies suggest improved killing with the sr39tk variant. Thus, the results suggest that the use of SR39 in future trials of prostate cancer tk suicide gene therapy may be beneficial.

OVERVIEW SUMMARY

Gene therapy holds promise as an alternative or adjuvant treatment for advanced, aggressive stages of cancer, such as prostatic carcinoma, for which no curative options are available. Herpes simplex virus 1 thymidine kinase enzyme (TK)-mediated suicide gene therapy is a widely used strategy in experimental models and clinical trials. One approach that could enhance the cytotoxic activity of TK is to use variant enzymes that have been reengineered with increased affinity for prodrugs as compared with thymidine. In this study, we evaluated the therapeutic efficacy of a panel of adenoviral vectors expressing the tk active site variants with the acyclovir and ganciclovir prodrugs in prostate cancer cell lines and tumor xenografts. Our ultimate goal is to improve the therapeutic efficacy of tk suicide gene therapy for prostate cancer.

INTRODUCTION

PATIENTS WITH advanced prostate cancer have a poor prognosis, and currently available treatment options for advanced prostate cancer are limited and lack curative potential. Gene therapy represents a powerful new alternative for cancer treatment. Several prototypes of gene therapy protocols have been investigated in preclinical studies for the treatment of prostate cancer, including gene replacement or antisense strategies to restore normal growth control (Eastham *et al.*, 1995; Dorai *et al.*, 1997; Morelli *et al.*, 1997), insertion of genes to stimulate the immune system (Vieweg *et al.*, 1994; Sokoloff *et al.*, 1996; Simons *et al.*, 1999; Belldegrun *et al.*, 2001), and delivery of genes that cause the activation of a prodrug that induces selective cytoxicity and destruction of tumor cells (Eastham *et al.*, 1996; Blackburn *et al.*, 1998).

Viral transduction of the herpes simplex virus type 1 (HSV-

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[1]Department of Urology, University of California School of Medicine, Los Angeles, CA 90095.
[2]Crump Institute for Molecular Imaging, University of California School of Medicine, Los Angeles, CA 90095.
[3]Department of Pharmaceutical Sciences, Washington State University, Pullman, WA 99164.
[4]Chiroscience R & D, Bothell, WA 98021.

1) thymidine kinase gene (tk), followed by systemic delivery of ganciclovir (GCV), is a cytotoxic gene therapy approach that is considered a potential strategy for prostate cancer treatment (Hall *et al.*, 1997; Herman *et al.*, 1999; Hassan *et al.*, 2000). In contrast to human thymidine kinase (TK), which phosphorylates acycloguanosines only minimally, HSV-1 TK has a relaxed substrate specificity for other nucleoside analogs, and can phosphorylate a variety of acycloguanosine and uracil derivatives, including the acyclic analogs of deoxyguanosine such as acyclovir (ACV) and GCV. The cytotoxic effect of GCV is due to its incorporation into DNA by a process involving several steps, beginning with its conversion by the TK enzyme to GCV monophosphate. The monophosphorylated GCV undergoes further phosphorylation by endogenous cellular kinases into the corresponding nucleoside triphosphate, which is incorporated into cellular DNA and prevents DNA synthesis, which ultimately results in cell death by several proposed mechanisms (Wallace *et al.*, 1996; Rubsam *et al.*, 1999; Mesnil and Yamasaki, 2000).

New tk variants have been developed to improve the potency of antitumor effects. These variants were generated by random mutagenesis of the binding site amino acids, and selected for increased affinity for the prodrugs GCV and ACV, as compared with thymidine (Black *et al.*, 1996, 2001). We compared the therapeutic efficacy of a panel of adenoviral (Ad) vectors expressing the tk active site variants with wild-type tk in terms of processing ACV and GCV prodrug in prostate cancer cell lines and tumor xenografts. Our goal is to improve the therapeutic efficacy of the tk suicide gene therapy scheme for prostate cancer.

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MATERIALS AND METHODS

Cell culture and viral vectors

The human prostate cancer cell line LNCaP was provided by the American Type Culture Collection (Rockville, MD). The human prostate cancer cell line CL1 was derived as previously described (Patel *et al.*, 2000). LNCaP cells were maintained in RPMI 1640 medium with 10% fetal bovine serum, glutamine, and antibiotics (penicillin [50 IU/ml] and streptomycin [50 μg/ml]). CL1 cells were maintained in RPMI 1640 medium with 10% charcoal-stripped serum, glutamine, and antibiotics. As previously described, CL1 cells were transfected with a green fluorescent protein (GFP) marker (Patel *et al.*, 2000). The established CL1-GFP cell line contained 99.9% GFP-expressing cells, as determined by flow cytometry.

First-generation E1-deleted Ad vectors containing wild-type thymidine kinase (Ad-CMV-HSV1-tk); the tk mutants sr39 (Ad-CMV-HSV1-sr39tk), tk30 (Ad-CMV-HSV1-tk30), and tk75 (Ad-CMV-HSV1-tk75); and control Ad containing either green fluorescent protein or β-galactosidase genes were generated, purified, and characterized as previously described (Gambhir *et al.*, 1999, 2000; Tan *et al.*, 1999).

Southern and Western blot analyses

A modified Hirt DNA isolation procedure (Tan *et al.* 1999) was used to obtain low molecular weight DNA from cells 24 hr after adenovector infection. Hirt DNA from 10^7 infected CL1 cells was analyzed by Southern blotting, using a nonradioac-

tive digoxigenin-labeled 1.9-kb HSV-TK DNA probe (Roche, Indianapolis, IN). Transferred DNA on nylon membranes (Hybond; Amersham, Arlington Heights, IL) was detected by colorimetric conversion of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). All procedures were performed according to the manufacturer protocols.

TK-expressing or control adenovirus-infected cells were lysed in a homogenization buffer containing pepstatin (1.45 mM), leupeptin (2.1 mM), dithiothreitol (DTT), TEA-HCl (50 mM), and EDTA/EGTA (0.1 mM). Total protein (5–20 μg) was loaded in Laemmli buffer onto a 7.5% polyacrylamide gel. The proteins were transferred to nitrocellulose membranes for 1 hr, using a Mini Trans blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked overnight in phosphate-buffered saline (PBS)–0.05% Tween 20–5% nonfat dry milk overnight at 4°C. After two washes in PBS–0.005% Tween 20, TK polyclonal serum (generously provided by M. Black, Department of Pharmaceutical Sciences, Washington State University, Pullman, WA) at a dilution of 1:5000 was added for 1.5 hr. After another three washes, secondary antibody was added for 1.5 hr. Using an enhanced chemiluminescence kit (Amersham Life Science, Piscataway, NJ), the membrane was developed on Kodak (Rochester, NY) film in a darkroom.

Cytotoxicity assays

Colony formation assay. Cells were infected at a multiplicity of infection (MOI) of 1, using tk variant or control Ad in 60-mm plates. Cells were trypsinized and replated into 12-well plates at a concentration of 100,000 cells/well at 24 hr postinfection. GCV or ACV was added to a final concentration of GCV ranging from 0.01 to 10 μM, and ACV ranging from 0.3 to 300 μM. After 4 days of prodrug exposure, the colony-forming efficiency of cell lines was measured by seeding cells at aliquots ranging from 1:10 to 1:500 in 60-mm petri culture dishes in prodrug-free media. After incubation for 10–14 days, cells were fixed with 80% alcohol for 30 min, and stained with 1% crystal violet solution in PBS. The visualized cell colonies were counted and compared.

MTT colorimetric assay. Subconfluent cells in 10-cm plates were infected with E1-deleted Ad containing the wild-type or mutant tk gene under the control of a cytomegalovirus (CMV) promoter at an MOI of 1. After 24 hr, cells were trypsinized, washed, centrifuged, and replated in triplicate at 10^4 cells/well in 24-well plates, with dose-escalating concentrations of prodrug added to each well. After 7 days, a 50-μl/well concentration of $10\times$ stock 3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and allowed to incubate for 4 hr. Cells were lysed with 0.04 M HCl in isopropanol, and shaken for 10 min to dislodge adherent cells and disrupt clumps of formazan blue crystals. Cells (200 μl from each well) were transferred to a 96-well plate, and absorbance was measured at 595 nm with a microplate reader. A standard curve was generated with varying concentrations of untreated cells.

Propidium iodide assay for apoptosis

Treated cells were harvested, fixed, and subjected to propidium iodide (PI) staining and flow cytometry analysis for apoptosis. The cells were treated as in the MTT assay (described above), and after 5–7 days of drug exposure were harvested into

fluorescence-activated cell sorting (FACS) tubes (Fisher Scientific, Pittsburgh, PA), using gentle repeated irrigation with Dulbecco's PBS (GIBCO-BRL Life Technologies, Frederick, MD) containing 0.05% EDTA (Biofluids, Rockville, MD). The cells were washed twice with PBS and fixed for 30 min in 70% ethanol (Gold Shield Chemical, Hayward, CA) at $-20°C$, and subjected to PI staining. PI solution (100 μl, 50 μg/ml; Roche) containing RNase A1 (50 μg/ml) was added to each tube and incubated for 30 min at 37°C in a darkroom, and then the volume brought to 1.0 ml by adding 0.9 ml of PBS. The cells were subjected to flow cytometric analysis, using the Coulter (Miami Lakes, FL) system II and Epics software version 3.0. The parameter settings were optimized at the beginning of the study and kept constant through subsequent analyses.

Animal studies

Male 6- to 8 week-old severely compromised immunodeficient (SCID; CB.17 *scid/scid*) mice were obtained from the breeding program at the University of California at Los Angeles (UCLA), under a protocol approved by the UCLA Chancellor's Animal Research Committee. All animals were anesthetized with ketamine and xylazine before survival surgery and inoculation with cancer cells. The flanks of five SCID mice per treatment group were injected subcutaneously by 27-gauge needle with either 5×10^4 cells resuspended in 5 μl of RPMI 1640 into the accessory sex gland, or with 5×10^6 cells resuspended in 100 μl of RPMI 1640. For orthotopic, intraprostatic injection, a small 1-cm vertical midline incision in the lower abdomen was dissected into the peritoneum. Using the seminal vesicles as an anatomic landmark, the prostate was exposed. Cells were injected into the dorsal prostate lobes under the prostatic capsule, and then the abdominal wall and skin were closed with fine surgical sutures. For tumor prevention studies, cells were grown in 10-cm plates to 80% confluency, medium was removed, and the cells were infected with each tk or control adenovirus at an MOI of 1. Cells were harvested and injected into animals 24 hr postinfection, and treatment commenced the following day with GCV at 20 mg/kg by daily intraperitoneal or tail vein injection for a total of 6 days. In the established tumor model, injection of tk-expressing Ad (a total of 3×10^9 infectious units [PFU] into three sites on three separate days) began when subcutaneous tumors reached a diameter of 5 mm. Three-dimensional tumor growth was measured twice weekly, using calipers.

Animals were killed when their performance status was noted to deteriorate or when tumor diameters reached 15 mm. Tumor invasion was examined grossly by necroscopy, and histologically by evaluation of formalin-fixed, paraffin-embedded sections obtained from the primary tumor and multiple organs, lymph nodes, and bones, which were mounted and stained with hematoxylin and eosin. Micrometastasis was detected by fluorescence microscopy of GFP-positive cells. Fresh tumors and organ samples were snap frozen in isopentane and dry ice, embedded in O.C.T. medium for cryostat sections of 5 μm, and examined with a fluorescence microscope (Carl Zeiss, Thornwood, NY) with an adjustable camera with 400ASA color film (Kodak).

Flow cytometry analysis

To quantify metastasis, a portion of liver from each mouse was enzymatically digested and subjected to FACS analysis to determine the percentage of GFP-positive cells. The right hepatic lobe was excised, diced into small fragments, and enzymatically digested by gentle shaking in sterile RPMI medium containing collagenase type I (200 U/ml), DNase type I (250 μg/ml) (Sigma, St. Louis, MO). After Ficoll-Hypaque density gradient centrifugation (LSM; Organon Teknica, Durham, NC), the single-cell suspension was washed twice and resuspended in 0.5 ml of assay buffer. Fluorescence was analyzed on a FAC-Scan II flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA), and 5000–30,000 events were acquired for each sample, using FACScan research software. GFP-expressing tumors were used as positive controls, and uninfected livers as negative controls, to delineate the unstained/autofluorescent population. Events registered outside this trace were scored as positive, and the percentage of positive events was calculated.

CL1 cells stably expressing the sr39tk gene

To better understand the efficacy and limitations of gene therapy, and to begin to understand whether lack of *in vivo* tumor regression is due to vector delivery, transgene expression, or drug delivery, we investigated the feasibility of using positron emission tomography (PET) to image sr39tk expression in our prostate cancer xenograft model. CL1 cells were stably transfected with the sr39tk gene for the purpose of PET scanning to demonstrate tumor imaging, based on TK conversion of a radiolabeled penciclovir molecule into a trapped, metabolic product. Transfection was performed using Effectene (Qiagen, Chatsworth, CA). CL1 cells were grown until they were 70% confluent, and then transfected with 5 μg of plasmid DNA (pcDNA3.1/pCMV-sr39tk) on a 100-mm dish. After transfection (48 hr), the cells were selected with G418 (400 μg/ml) for 4 weeks. Selection continued for another 4 weeks at a G418 concentration of 800 μg/ml, but by this time most of the cells were resistant to the antibiotic. Stable TK expression by CL1-sr39tk cells was confirmed by Western blot analysis for TK protein and TK enzyme activity assay.

TK-mediated micro-PET

Male 6- to 8-week-old SCID (CB.17 *scid/scid*) mice were obtained from the breeding program at UCLA, with a protocol approved by the UCLA Chancellor's Animal Research Committee. The right flanks of mice were injected subcutaneously by 27-guage needle with 5×10^6 CL1-sr39tk cells resuspended in 100 μl RPMI 1640, and an equivalent number of CL1 cells was injected into the left flanks of the SCID mice. Mice were anesthetized as described previously before injection of tracer. Mice with tumors were serially imaged with FDG (fluorodeoxyglucose) and FHBG (a side chain-fluorinated penciclovir) tracers. Once tumors were palpable, mice were injected with 200–300 μCi of radiolabeled tracer, placed in a spread supine position, and scanned with the micro-PET (Gambhir *et al.*, 2000). Scanning was performed 60–90 min after tracer injection, to allow for clearance of background activity before starting image acquisition. Scanning was performed with the long axis of the mouse parallel to the long axis of the scanner, with the mouse in a prone position. Scanning conditions were identical to those previously described (MacLaren *et al.*, 1999). Acquisition time was 56 min, 8 min per position, with seven bed positions. Images were reconstructed with filter-back pro-

A.

tk   sr39tk   tk75

B.

CL1   lacZ   tk   tk75   tksr39   M   + control
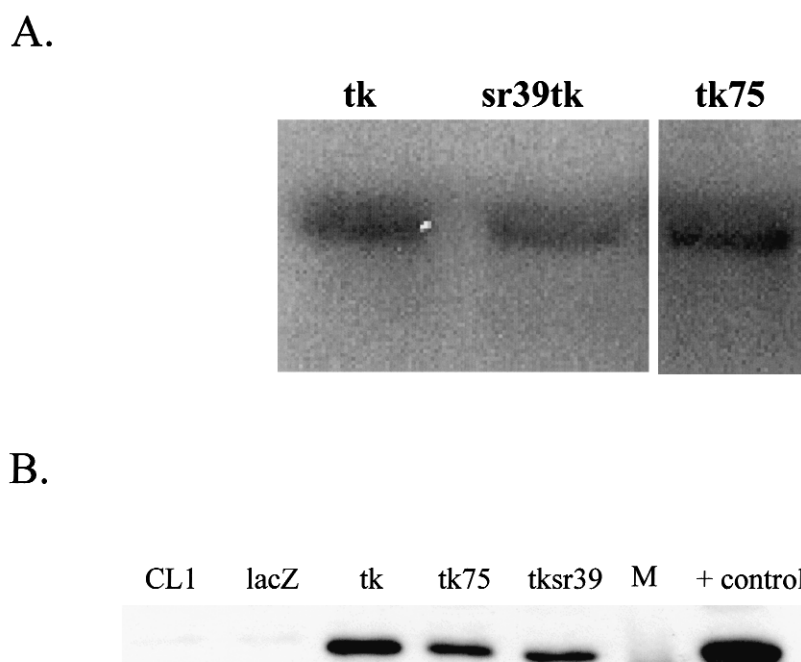
FIG. 1. Analysis of transduced viral gene and TK protein expression in infected cells. (**A**) Southern blot analysis of low molecular weight DNA from infected CL1 cells. Cells were infected at an MOI of 1 with the respective tk-expressing Ad. Hirt DNA isolated from 10^7 cells was subjected to restriction digestion, Southern blot transfer, and detection by the digoxigenin-labeled TK probe. Similar levels of tk gene were delivered to CL1 cells. (**B**) Western blot assay of infected CL1 cells. CL1 cells were infected at an MO1 of 1, and 10 μg of protein extract from uninfected or control Ad-infected or tk-expressing Ad-infected cells was loaded onto each lane. TK protein was visualized by a polyclonal antibody. "M" represents a marker lane, and "+ control" represents a sample from 293 cells infected with wild-type tk-expressing Ad.

jection and an iterative three-dimensional technique (Qi *et al.*, 1998), with an isotropic image resolution of 1.8 mm and a volumetric resolution of approximately 8 mm³.

RESULTS

Standardization of viral infections

Studies by others (Gambhir *et al.*, 2000) have suggested that the amount of TK expression is related to the degree of prodrug sensitivity. A valid comparison between the effects of different mutant tk genes therefore requires standardization of protein concentrations. To verify that any observed differences between the prodrug-mediated cell killing for each tk mutant were not due to differences in gene delivery or gene expression, accurate viral titration and Southern and Western blot assays were performed for each infectant to quantify enzyme expression at the DNA and protein levels. Southern blotting of intracellular low molecular weight DNA to detect tk DNA (Fig. 1A) and TK protein Western blotting (Fig. 1B) were performed. There were no major differences between the amount of HSV-TK DNA or protein for tumor cells infected with equivalent dosages of the vectors containing the wild-type and tk variants. Thus, any enhanced killing was not due to overexpression.

In vitro *prodrug sensitivity assays*

To evaluate the sensitivity of CL1 cells expressing the various TK variants to prodrug-mediated killing, tumor cells were cultured with increasing concentrations of GCV or ACV. The sensitivity of each TK variant to GCV or ACV was determined by three different cytoxicity assays, measuring both short- and long-term cell viability. Short-term cytotoxicity was evaluated by MTT and PI assays, whereas longer term cellular viability was measured by a colony formation assay. CL1 cells were infected with a viral vector expressing each tk variant, and were then assayed for their sensitivity to GCV or ACV. The results of a representative panel of cytotoxicity assays for the mutants and wild-type tk, along with a vector control for GCV and ACV, are shown in Figs. 2A–D and 3A–C, respectively.

Because of the intrinsic differences of the three cytotoxicity assays, the precise measurements of viability and cell survival are not exactly the same. The results of the three assays complement each other and illustrate the same order of treatment efficacy among the wild-type and variant tk-expressing Ad. A colony-forming assay evaluated long-term survival with a more stringent requirement for cell replication. Thus, a more drastic impact of treatment was observed with colony-forming assays than with the short-term assays. The short-term assays (MTT and PI) evaluated cell damage incurred at one time point 5 days after prodrug treatment. Moreover, PI flow cytometry appeared to be the least sensitive of the three assays to detect cell damage. As illustrated in Fig. 2, GCV is a potent prodrug, as the LD_{50} for wild-type or sr39tk-expressing CL1 cells is 0.1 μM (Fig. 2A–C). The degrees of sensitivity for GCV of wild-type tk and sr39tk were similar, and both were only slightly higher than tk75, but dramatically higher than tk30 (Fig. 2).

As evaluated by short-term MTT assay, the LD_{50} of sr39tk-

expressing CL1 cells is 3 μM ACV, as compared with wild-type tk, which is 10-fold higher at 30 μM, and tk75, which is intermediate in potency at $\sim$15 μM ACV (Fig. 3C). When comparing the results of ACV and GCV, however, equivalent killing was achieved with a 100-fold lower concentration of GCV compared with ACV. There was a strong correlation between the results of the three cytotoxicity assays. Assays were repeated several times, with similar results. These results were reproduced with a similar cytotoxicity profile in DU-145 cells, an androgen-independent prostate cancer line, and LNCaP cells, an androgen-responsive prostate cancer line (Fig. 4A and B). The same trend favoring sr39tk was also seen when the MTT assay was repeated with the HeLa cell line (data not shown). The results of early experimental data showing decreased cytotoxicity for tk30, compared with wild-type tk, precluded its use in further analysis.

In vivo assays of tumor xenografts

Tumor prevention. CL1 cells that were infected *ex vivo* with wild-type and variant tk-expressing Ad were injected orthotopically into SCID mice. The CL1 xenograft model is a well-established, reproducible model with a well-known time course for local tumor formation, time course and pattern of metastasis, and time to death or sacrifice (Patel *et al.*, 2000). Untreated CL1 cells and an Ad carrying the β-galactosidase gene were used as negative control groups in parallel. The time course to both palpable tumor formation and death were doubled in treatment versus control groups (data not shown). The results for wild-type tk- and tk75-treated cohorts were similar, whereas there was an approximately 10% reduction in time to death or sacrifice for sr39tk versus wild type (data not shown). At the time of sacrifice, primary tumors and organs were collected for evaluation of tumor metastatic spread. The harvested organs were evaluated by conventional and fluorescence microscopy to qualitatively detect CL1-GFP metastasis (Fig. 5A). An attempt was made to assess metastatic burden more quantitatively by FACS analysis of the percentage of GFP-expressing cells in single-cell suspensions of treated and control livers. When comparing the untreated CL1-GFP group with the vector control group, wild-type tk decreased the hepatic metastatic tumor burden of positive green fluorescent cells, in the tissue analyzed, from 30 to 15% (Fig. 5B). Mice injected with sr39tk Ad-infected CL1-GFP cells showed an additional decrease in tumor burden to as low as <1% of total hepatic cells measured.

Inhibition of established tumor progression. Subcutaneous CL1 tumors were established and allowed to grow to a tumor diameter of 5 mm before initiating treatment. Thereafter, mice were treated with two weekly intratumoral injections of Ad expressing wild-type or variant tk and six consecutive daily intraperitoneal injections of GCV (20 mg/kg). By 21 days after the treatment start date, the mean tumor diameter of the control, wild-type tk, tk75, and sr39tk groups was 11.2, 10.5, 9.5, and 6.5 mm, respectively. Although no tk mutant demonstrated complete eradication of tumor or improvement in overall survival, mice treated with sr39tk-expressing Ad showed a 63% reduction in tumor growth during the period of active treatment as compared with wild-type tk. We postulate that a limitation to more effective tumor eradication in this treatment strategy,

using tk-expressing Ad, might lie in the difficulty of achieving adequate or even distribution after intratumoral injection of Ad. A sensitive and noninvasive method is needed to monitor transgene expression *in vivo*. Thus, we proceeded to evaluate the utility of micro-PET in our prostate cancer therapy strategy.

Micro-PET imaging of sr39 tk-expressing prostate cancer xenograft

It is convenient that the therapy tk gene can also serve as a PET reporter gene. CL1 tumors are aggressive, anaplastic, highly proliferative prostate cancer tumors. Consequently, both CL1 and CL1-sr39tk tumors are easily imaged by micro-PET, using the 2-^{18}F-fluoro-2-deoxyglucose (FDG) tracer, which is an analog of glucose metabolism (Fig. 6A). The same mouse was imaged with FHBG, which is an ^{18}F-labeled penciclovir (PCV) derivative. Cells containing the tk gene can phosphorylate FHBG, converting it into a trapped metabolic product that can be imaged by micro-PET. CL1-sr39tk but not CL1 tumors were easily visualized by FHBG micro-PET (Fig. 6B).

DISCUSSION

Prostate cancer is currently the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths in American men (Landis *et al.*, 1999). Once disease advances to metastasis, few treatment options exist, and none have curative potential. Recombinant DNA technology and the ability to manipulate DNA sequences, combined with techniques to transfer these sequences into mammalian cells, offer an alternative treatment approach in the form of gene therapy. Although still limited by a variety of technical problems, gene therapy, with its ultimate goal of efficient and targeted delivery of gene products that have significant anticancer activity, provides a powerful new concept for cancer treatment.

Approximately one-quarter of ongoing clinical trials are based on cytoreductive "suicide gene therapy" approaches (Herman *et al.*, 1999). Thus far, the HSV-TK/GCV system is the most extensively tested in cell culture, animal models, and clinical trials. Current clinical trial protocols of tk suicide gene therapy for prostate cancer include one based on direct intraprostatic injection of an Ad containing the tk gene, followed by intravenous administration of GCV (Shalev *et al.*, 2000). Adenovirus-delivered HSV-tk + GCV is also being evaluated in a phase I clinical trial at the Mount Sinai School of Medicine (New York, NY) in patients with clinically localized prostate cancer before radical prostatectomy (Hassan *et al.*, 2000). Overall, the HSV-tk/GCV regimen appears to be safe, even for multiple and repeated injections. These early trials will provide valuable information about the toxicity, the optimal techniques for intraprostatic injection, the effects on the prostate and prostate cancer tissue, and patterns of serum PSA response. However, long-term efficacy remains to be elucidated.

Efforts to improve the results of the tk suicide gene system by enhancing tumor cell killing without increasing prodrug-mediated toxicity in normal cells have been reported, with the creation of novel HSV-tk gene mutants that have increased specificity for phosphorylating GCV and/or ACV. From a library of more than 1 million variant tk genes created (Black *et al.*, 1996), several of them have been reported to have alterations in sub-
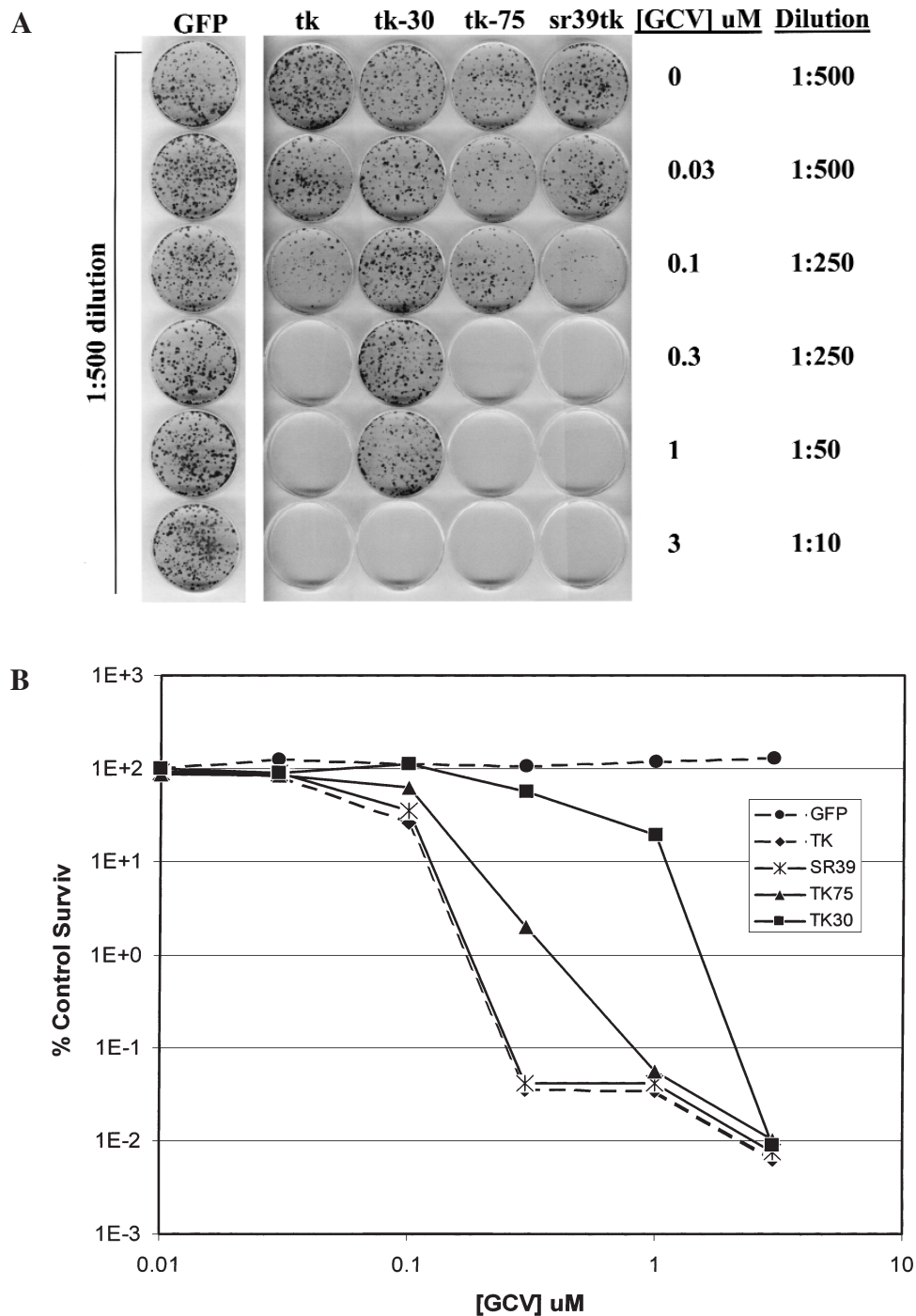
FIG. 2. *In vitro* cytotoxicity assays in infected CL1 cells mediated by GCV dose escalation. (**A** and **B**) Representative results and quantitation of a colony formation assay. Cells were infected as described in Fig. 1. Postinfection (24 hr), cells were aliquoted equivalently into wells containing various concentrations of GCV (0–3 μM, as specified). Four days after drug treatment, cells from each well were trypsinized and replated at low densities to allow formation of distinct colonies (**A**). (**B**) Graphic representation of the viable colonies (30–50 cells) formed approximately 10–14 days postplating. (**C**) MTT assay of infected CL1 cells. Cells were infected at an MOI of 1 with the specified virus. One day postinfection, cells were aliquoted into 24-well plates containing various concentrations of GCV prodrug. Seven days after drug exposure, cells were stained with MTT to score for cytotoxicity, as described in Materials and Methods. Percentages of cell survival were calculated in reference to control. (**D**) Propidium iodide assay to evaluate cell damage. Cell infection and prodrug treatment were as described in (**C**). Five to 7 days after drug exposure, cells were subjected to PI staining and flow cytometry analysis.
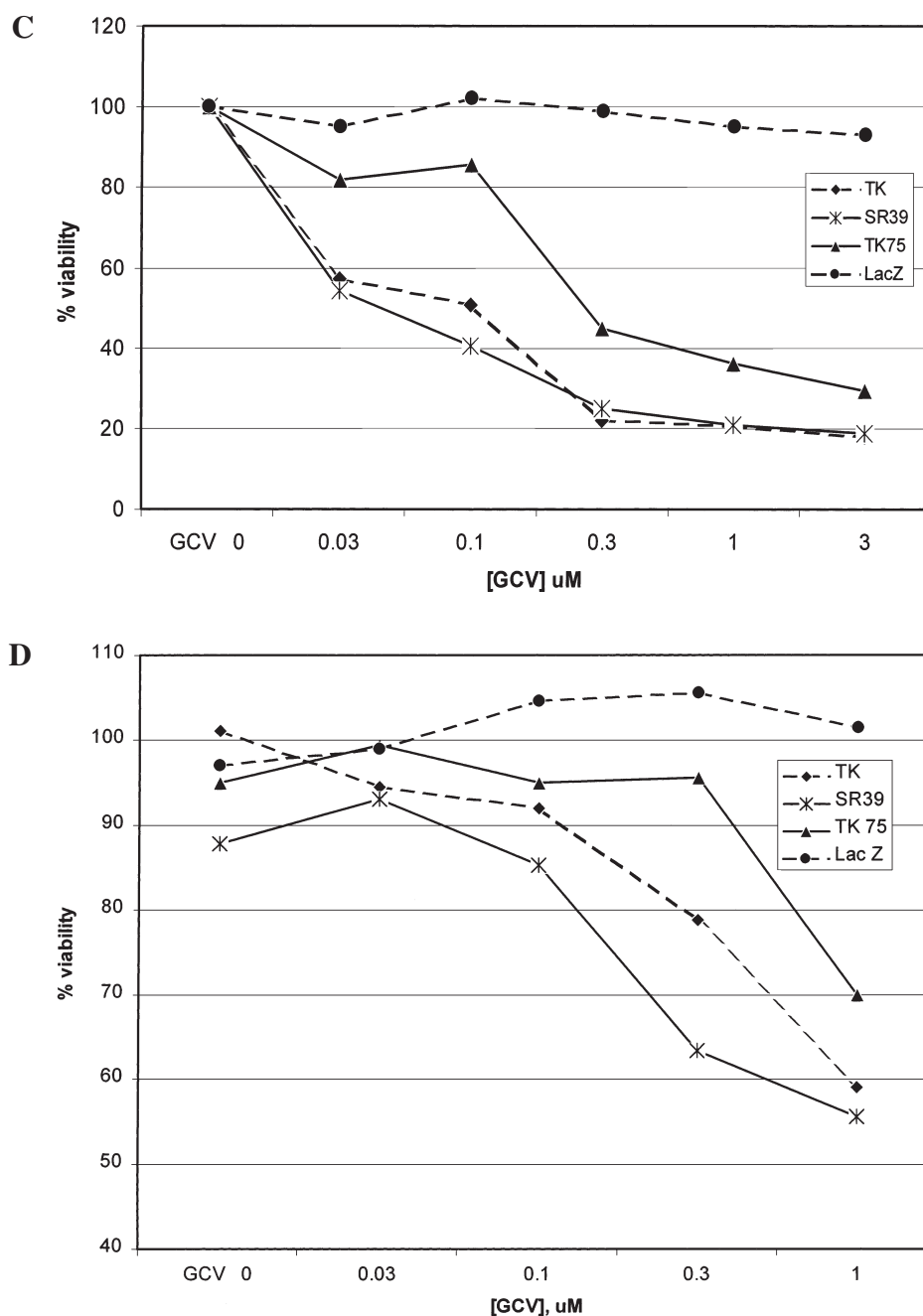
C

D

FIG. 2. *Continued.*

strate specificity. These mutants have increased sensitivity to and can preferentially phosphorylate GCV and/or ACV over thymidine, relative to the wild-type TK. Improvement in prodrug-mediated cell killing in transfected cell lines (Kokoris *et al.*, 1999; Black *et al.*, 2001), as well as enhancement of the bystander effect in human tumor cells transduced with a retroviral vector (Qiao *et al.*, 2000), have been demonstrated. Increased killing of human pancreatic tumor cells transfected with tk30 (Howard *et al.*, 2000) and improved radiosensitization of rat glioma cells expressing tk75 (Valerie *et al.*, 2000) have previously been reported.

Furthermore, sr39tk has been shown to improve sensitivity, compared with wild-type tk, when used as an imaging reporter gene for noninvasive PET imaging (Gambhir *et al.*, 2000).

To date, no published study has compared the effects and use of these tk mutants comprehensively in prostate cancer gene therapy. Previous *in vitro* studies of ovarian cancer cell lines have suggested differing sensitivities to ACV and GCV (Alvarez and Curiel, 1997). The current study demonstrates that both androgen-dependent and -independent cell lines show greatly increased sensitivity to ACV when infected with an
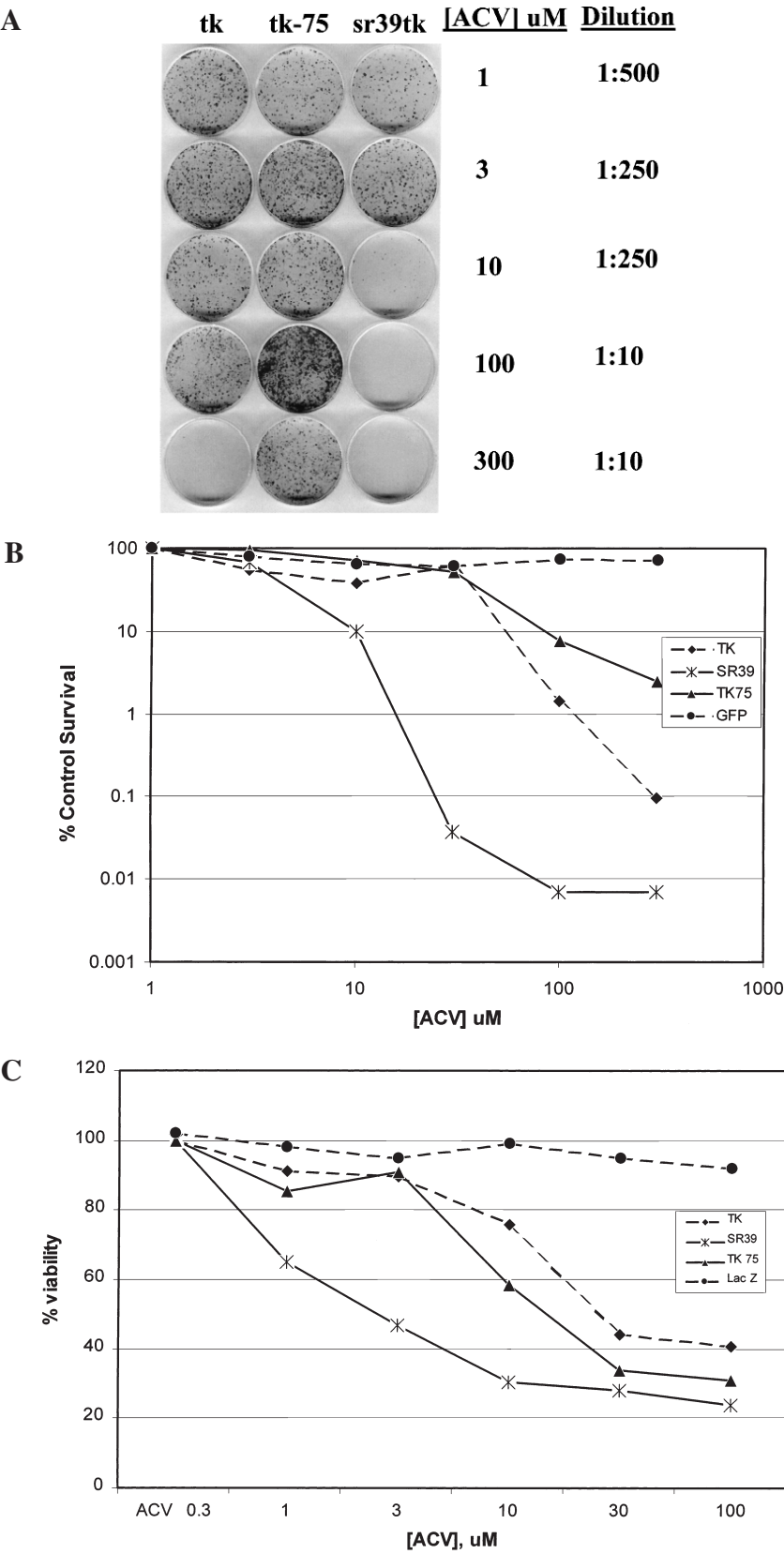
FIG. 3. *In vitro* cytotoxicity in infected CL1 cells mediated by ACV dose escalation. (**A** and **B**) Results of a colony formation assay. Experimental conditions were the same as described in Fig. 2, except that ACV was used instead of GCV at specified dosages. (**C**) MTT assay of infected CL1 cells after ACV treatment. Conditions were as previously described.
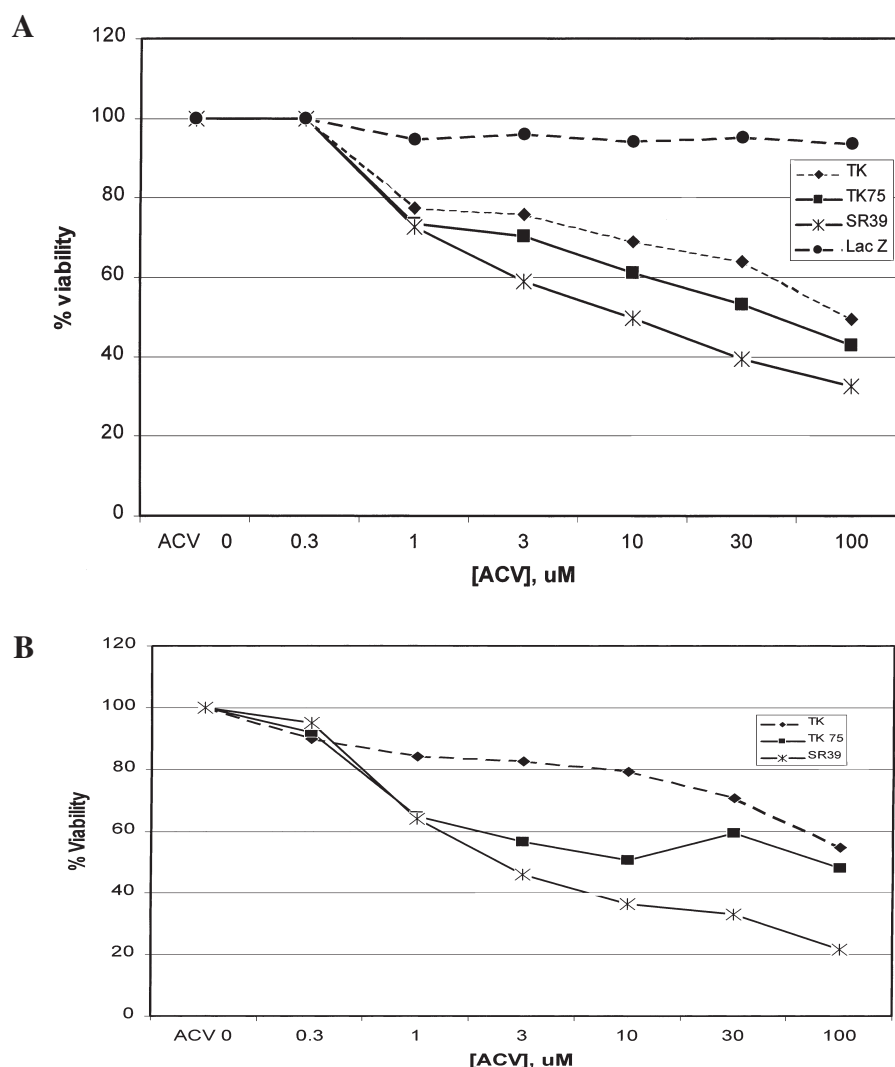
FIG. 4. Cytotoxicity profile of two prostate cancer cell lines treated with tk-expressing Ad and ACV. (**A**) MTT assay of infected DU-145 cells. (**B**) MTT assay of infected LNCaP cells. Conditions were as described in Figs. 2 and 3.

sr39tk-expressing Ad, as compared with wild-type tk, tk75, or tk30. When combined with GCV, wild-type tk and sr39tk display similar levels of *in vitro* killing that are slightly higher than for tk75. A distinct improvement of sr39tk-expressing Ad as compared with wild-type tk-expressing Ad, in conjunction with GCV treatment, is suppression of metastatic spread of the infected prostate cancer cells. This result in animals is unexpected because the cytotoxicity of GCV treatment in tissue culture systems showed no notable difference in sr39 tk-expressing CL1 cells over wild type. In the well-controlled tissue culture studies, prodrug delivery is not rate limiting and TK expression can be accurately assessed. However, in animals, the *in vivo* tissue environment can alter prodrug delivery or its accumulation within tumor cells. Under these limiting conditions, sr39tk could exhibit advantages over wild-type tk. Therefore, sensitive imaging or tracer studies are needed to monitor prodrug accumulation *in situ* in animals to better predict the therapeutic outcome of tk-mediated gene therapy.

Tumor cell eradication was limited in experiments conducted by treating subcutaneous tumors percutaneously injected with Ad carrying tk. Uneven distribution and low gene transduction may be two factors accounting for this limited cytotoxicity. To assess transduction efficiency, we performed percutaneous injections under the same conditions as described in Materials and Methods, with an Ad carrying the gene encoding green fluorescent protein. These experiments showed that distribution of Ad into the CL1 tumors was limited: less than 10% of tumor cells were infected, with a dispersion of only 1 mm beyond the path of the needle track (data not shown). CL1 cells form desmoplastic, densely packed solid tumors. This aggressive CL tumor model (Patel *et al.*, 2000; Tso *et al.*, 2000) is highly tumorigenic, and exhibits rapid growth, local invasion, and metastatic spread in the absence of androgen or other growth supplementation (e.g., Matrigel). In light of this combination of limited transduction efficacy and a rapidly growing tumor model, effective *in vivo* cytotoxicity will likely require the ability to augment the bystander

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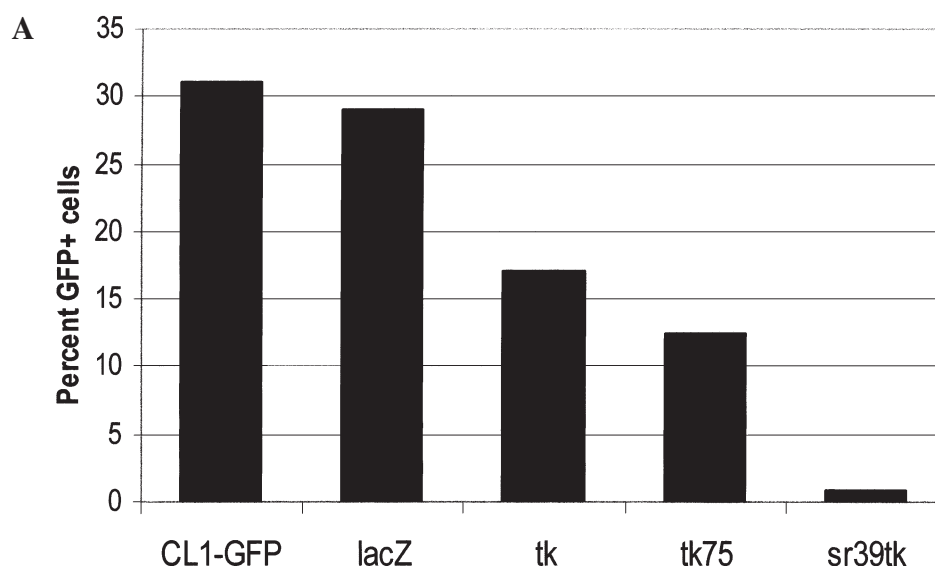
FIG. 5. Detection of metastatic CL1-GFP cells by fluorescence. (**A**) Liver sections were enzymatically digested and subjected to FACS analysis to determine the percentages of GFP-expressing prostate cancer cells (see Materials and Methods for a more detailed description). (**B**) Microscopy of liver sections obtained at sacrifice shows GFP-expressing, metastatic prostate cancer cells. CL1-GFP cells were infected with tk-expressing Ad at an MOI of 1. One day postinfection, 5×10^4 cells were injected orthotopically into the prostate (dorsal lobe) of SCID mice. Mice were treated with systemic GCV administration for 6 days. Three weeks after implantation of infected CL1-GFP cells (tumors were palpable), all animals were killed, and frozen sections of liver were visualized under a fluorescence microscope. Untreated CL1 tumors revealed macroscopic liver metastases. Mice receiving cells treated with wild-type tk- or tk75-expressing Ad revealed diffuse, microscopic metastases. Liver sections of mice receiving cells treated with sr39tk-expressing Ad showed minimal microscopic metastasis.

effect (Mesnil and Yamasaki, 2000) or intercellular spread of activating enzyme (Dilber *et al*., 1999).

Another issue that is unclear at this time is whether improvement in TK enzyme kinetics can be translated into improved cytotoxicity. Enhanced substrate uptake by SR39 TK enzyme as compared with wild-type TK has been documented with tritiated PCV and ^{18}F-labeled PCV (FPCV), which resulted in improved sensitivity of PET imaging (Gambhir *et al*., 2000). C6 rat glioma cells stably expressing SR39 were shown to be more sensitive to GCV-mediated tumor growth suppression compared with wild-type TK-expressing tumors (Black *et al*., 2001). Two factors could contribute to the difference between our results and those reported in the study by Black *et al*. As alluded to previously, our tk gene transduction is mediated by an Ad that does not confer stable tk expression. Thus, delivery and expression could be limiting factors *in vivo*. Another possibility that could explain why apparently improved TK enzyme kinetics fail to correlate with substantially improved cytotoxicity in our system is that the cytotoxic effect is more complicated than just one enzyme–ligand interaction. TK enzyme activity measures the conversion of the nucleoside into the monophosphate form, which is the first form that becomes trapped in the cell, and therefore can be used as a PET reporter probe (Gambhir *et al*., 1999). Studies have demonstrated that GCV incorporation in the DNA template was important for cytotoxicity (Rubsam *et al*., 1999; Thust *et al*., 2000). It is possible that different cell types could have differential abilities to convert the nucleoside monophosphates into the triphosphate forms or the form that causes cytotoxicity. These are issues that

need to be clarified for individual cell systems, in order to achieve optimal TK-mediated cytotoxicity. Further improvement in TK enzyme activity or novel prodrug substrates with enhanced toxicity should augment the efficacy of tk suicide gene-based cancer treatment strategies.

One approach to investigate treatment failure is to noninvasively assay transgene expression, such that correlation of the level of transgene expression and the observed cytotoxic effect can be established. As a first step to achieving this end, we stably transfected the CL1 tumor model with the sr39tk gene. These data suggest that micro-PET imaging can be used as a means to selectively image prostate cancer cells carrying the sr39tk gene, similar to other tumor models (Gambhir *et al*., 2000). The CL1-sr39tk tumor model can be repetitively imaged, and may prove to be useful for studies of the biology of metastasis, as well as for noninvasive evaluation of therapeutic interventions. In conclusion, *in vitro* and *in vivo* suicide gene therapy studies of an androgen-independent model of metastatic prostate cancer, using a panel of variant HSV1 tk genes in combination with ACV and GCV prodrugs, suggest the advantage of using sr39tk versus wild-type tk. The use of sr39tk for future clinical prostate cancer gene therapy trials might enhance cytotoxic effects and suppress metastatic spread.
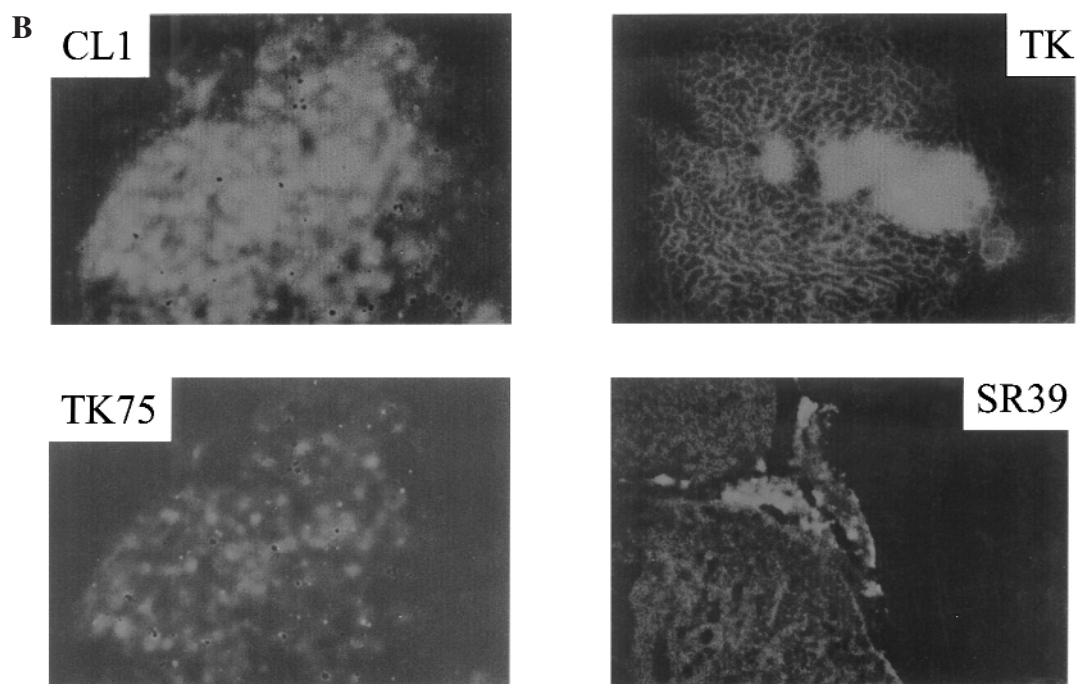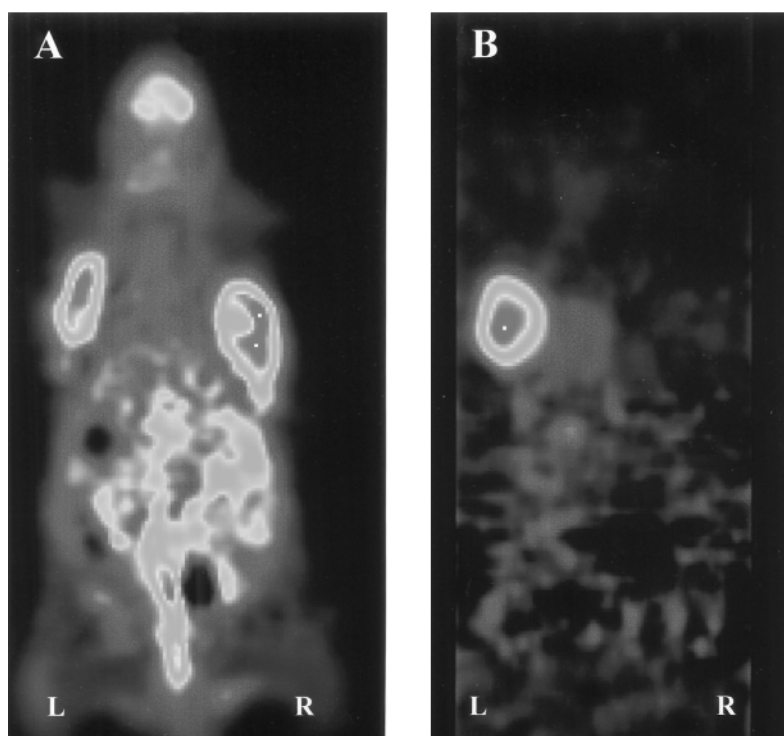
FIG. 5B.

FIG. 6. Micro-PET images of mice xenografted with CL1 cells or CL1 stably expressing the sr39tk gene. (**A**) Image visualized with FDG as PET reporter substrate. Both CL1 and CL1-sr39tk xenografts were visualized, indicating comparable glucose metabolism. (**B**) PET image of the same mouse, visualized with FHBG as substrate. Only the CL1-sr39tk tumor phosphorylates and retains FHBG in the cells. A robust signal was produced by the sr39tk-expressing tumor, whereas only background signal was seen from the site of the CL1 tumor.

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Address reprint requests to:
Dr. Lily Wu
Department of Urology
MRL 2210, Box 951738
University of California Los Angeles School of Medicine
Los Angeles, CA 90095-1738

E-mail: lwu@mednet.ucla.edu

AU 1
No Morelli *et al*. (1997) in reference list?

AU 2
Preceding sentence OK as edited? Or please amend to clarify meaning.