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Using HSV-Thymidine Kinase for Safety in an Allogeneic Salivary Graft Cell Line*

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ABSTRACT

Extreme salivary hypofunction is a result of tissue damage caused by irradiation therapy for cancer in the head and neck region. Unfortunately, there is no currently satisfactory treatment for this condition that affects up to 40,000 people in the United States every year. As a novel approach to managing this problem, we are attempting to develop an orally implantable, fluid-secreting device (an artificial salivary gland). We are using the well-studied HSG salivary cell line as a potential allogeneic graft cell for this device. One drawback of using a cell line is the potential for malignant transformation. If such an untoward response occurred, the device could be removed. However, in the event that any HSG cells escaped, we wished to provide additional patient protection. Accordingly, we have engineered HSG cells with a hybrid adeno-retroviral vector, AdLTR.CMV-tk, to express the herpes simplex virus thymidine kinase (HSV-tk) suicide gene as a novel safety factor. Cells were grown on plastic plates or on poly-L-lactic acid disks and then transduced with different multiplicities of infection (MOIs) of the hybrid vector. Thereafter, various concentrations of ganciclovir (GCV) were added, and cell viability was tested. Transduced HSG cells expressed HSV-tk and were sensitive to GCV treatment. Maximal effects were seen at a MOI of 10 with 50 μM of GCV, achieving 95% cell killing on the poly-L-lactic acid substrate. These results suggest that engineering the expression of a suicide gene in an allogeneic graft cell may provide additional safety for use in an artificial salivary gland device.

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INTRODUCTION

Approximately 40,000 new cases of head and neck cancer occur each year in the United States, with about 500,000 new cases worldwide.[1] In industrialized countries, the vast majority of these patients receive irradiation treatment. The salivary glands are often included in the radiation field. If the irradiation treatment exceeds ~50 Gy, generally irreversible damage occurs to the salivary glands.[2] Indeed, it has been suggested (for the parotid gland) that a mean dose of ≤26 Gy should be a planning goal if substantial sparing of gland function is desired.[3] Irradiation results primarily in the destruction of the fluid secreting acinar cells, and in many individuals all salivary epithelial cells are replaced by nonsecretory tissue.[4] There is no effective conventional therapy for this condition.

We have initiated a project using tissue engineering principles to develop an orally implantable fluid secretory device for such patients.[5] This device will consist of a blind end tube fabricated from a biodegradable polymer, coated with a suitable extracellular matrix protein, and lined on its lumen by a monolayer of epithelial cells capable of unidirectional fluid transport.[6]

The use of autologous, amplified epithelial cells for the graft cell monolayer in such a device would be ideal, but the growth of acinar (fluid-secreting) mammalian epithelial cells *in vitro* has thus far been elusive. For this reason, we have chosen to use an allogeneic graft cell, the HSG cell line, as a model for feasibility studies.[5] These cells are derived from a human submandibular gland[7] and have been widely employed in physiological studies *in vitro*. HSG cells exhibit functionally coupled neurotransmitter receptors, intact Ca^{2+} signaling systems, and various ion channels and transporters important for fluid secretion.[8–11] Additionally, HSG cells respond to extracellular matrix signals that can direct morphological and tissue-specific differentiation.[12–14]

We recently showed that HSG cells seeded on poly-L-lactic acid coated with human fibronectin resulted in maximal cell growth and organization as a monolayer with a cobblestone, epithelioid appearance.[5] However, as a cell line, HSG cells possess unlimited growth potential. Although we recognize that an artificial gland device could be easily removed from patients in the event of any untoward host response, we wished to incorporate an additional level of patient safety to eliminate any residual cells after removal of the implant. For this reason we decided to incorporate a suicide gene into HSG cells. We chose the well-studied herpes simplex virus thymidine kinase (HSV-tk) gene initially for proof-of-concept studies.

The HSV-tk gene, in combination with the prodrug ganciclovir (GCV), has been widely employed in cancer gene therapy. GCV is a nucleoside analog that is converted to a monophosphate form in the cell by HSV-tk and then further phosphorylated by host cellular kinases to the triphosphate form. The latter will block DNA elongation and result in cell death.[15] We hypothesized that, if in the event that any HSG cells escaped from the artificial gland device, by expressing HSV-tk they could be eliminated through the metabolism of the administered GCV. The present report demonstrates that transduction of HSG cells with a hybrid recombinant adeno-retroviral vector leads to expression of HSV-tk and to sensitivity of these cells to GCV.

MATERIALS AND METHODS

Recombinant viral vector construction

We constructed a recombinant hybrid adeno-retroviral vector, capable of genomic integration, similar to one recently reported by us.[16] Adenoviral vectors are widely used in gene transfer.[6] These vectors can be produced at high titers, and can infect dividing and nondividing cells with considerable efficiency. However, adenoviral vectors exist in the nucleus in an epichromosomal location, making gene expression unstable in the long term. Adenoviral vectors also elicit a potent host immune response making it impossible to readminister vectors after transgene expression wanes. Retroviral vectors, such as those based on the Moloney murine leukemia virus, are able to integrate into the genome, albeit in dividing cells. Unfortunately, these vectors cannot be produced at high titers and have a low gene transfer efficiency. The hybrid adeno-retroviral vector combines the high titer and versatility of adenoviral vectors with the long term expression and genomic integration of retroviral vectors.[16]

We removed 2.7 kb of the 5′ long terminal repeat (LTR), which includes part of the envelope gene [1.5 kb], the 5′LTR [0.57 kb], and the packaging sequence [0.63 kb], and 1 kb of the 3′LTR (which contains about 0.5 kb of the envelope gene and the intact 3′LTR) of the Moloney murine leukemia virus from the plasmid pXT1 (Stratagene, La Jolla, CA) by digestion with *Eco*RI.[17] *Sal*I linkers were added to both ends of the 5′LTR fragment, and the 3′LTR fragment was filled in to form blunt ends. These two fragments were ligated into pACCMV-pLpA (a generous gift of Dr. C. Newgard, University of Texas–Southwestern) from which the CMV promoter/enhancer and the SV40 polyadenylation sequence had been deleted. Thereafter, the CMV promoter/enhancer and the SV40 polyadenylation sequence were reinserted between the 5′ and 3′LTRs using *Bgl*II and *Bam*HI sites. This created the plasmid pAC5′3′LTR.CMV. A unique *Bam*HI site remained that could be used to insert the gene of interest. This plasmid was linearized by *Bam*HI digestion and treated with Klenow fragment to form blunt ends. The 1149 bp HSV-tk cDNA was excised from the plasmid pbTK (a generous gift of Dr. F. Candotti, NHGRI, NIH) by *Bam*HI and *Eco*RI, and filled in with Klenow fragment to form blunt ends. The excised HSV-tk cDNA was then cloned into pAC5′3′LTR.CMV to create the plasmid pAC5′3′LTR.CMV-tk. Restriction analysis and DNA sequencing verified the plasmid construction. Next, pAC5′3′LTR.CMV-tk was cotransfected into 293 cells with the adenoviral (type 5; E1$^-$, E3$^-$) plasmid pBHG10 to create a replication-deficient recombinant adeno-retroviral vector by homologous recombination.[16,18] This hybrid vector, AdLTR.CMV-tk, contains the Moloney murine leukemia virus 5′ and 3′LTRs, and the HSV-tk cDNA with the CMV promoter/enhancer and SV40 polyadenylation sequence (Fig. 1).

Cells and cell culture

HSG cells were a generous gift from Prof. M. Sato (Tokushima University, Japan)[7] and were maintained *in vitro* as previously described.[19] For the experiments presented herein, HSG cells were dispersed from semiconfluent plates using a solution of Versene 1:5000 in Hanks' balanced salt solution without calcium and magnesium (BioFluids, Rockville MD). Cells were then resuspended in fresh media consisting of a 1:1

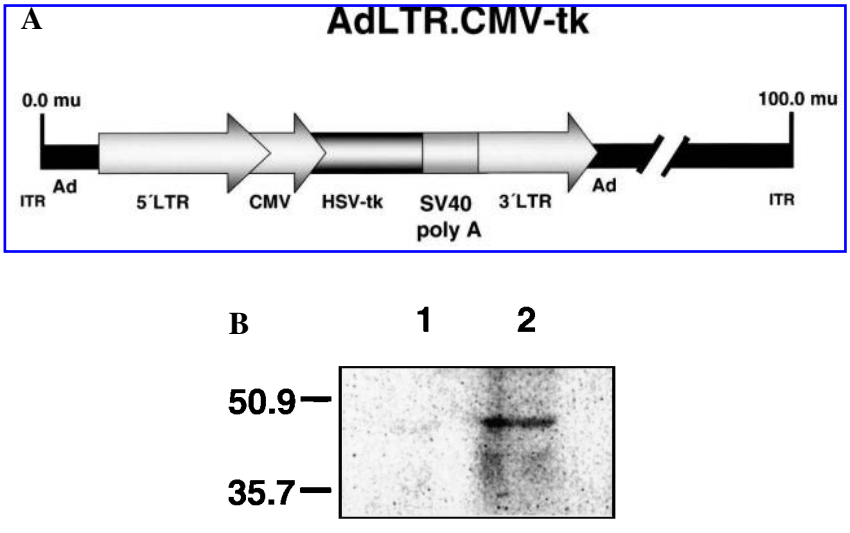

FIG. 1. (**A**) Schematic illustration of the hybrid adeno-retrovirus vector, AdLTR.CMV-tk. Shown are the Moloney murine leukemia virus 5′ and 3′ long terminal repeats (LTRs; 2.7 kb and 1 kb, respectively), the CMV promoter/enhancer, the SV40 polyadenylation sequence (poly A), and the HSV-tk cDNA (1149 bp). Adenoviral (Ad) sequences are shown as black bars. ITR, inverted terminal repeat. (**B**) Western blot analysis of HSG cell extracts. HSG cells were infected or not with AdLTR.CMV-tk at a MOI of 30 in 150-mm plates. After 72 h, cells from noninfected and infected cultures (lanes 1 and 2, respectively) were lysed, and 25 μg of total protein were electrophoresed, blotted onto nitrocellulose membranes, and incubated with mouse monoclonal antibody 10C11 anti-HSV-tk. Molecular mass markers are shown (in kDa).

mixture of Eagle's minimal essential medium (Dulbecco's modification) and Ham's F-12 supplemented with 2 mM glutamine (BioFluids), 10% fetal calf serum (Hyclone, Logan, UT), and 100 units/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL fungizone (BioFluids).

Infection and ganciclovir application

Cells were grown in 96-well culture plates at a density of 1×10^4 cells/well. After 24 h, cells were infected with AdLTR.CMV-tk at different MOIs (defined as plaque-forming units per cell). Six hours after infection, GCV (Cytovene®, Syntex Laboratories, Inc., Palo Alto, CA) was added to the media at concentrations of 0.5, 5, or 50 μM. After incubation for different time points, the sensitivity of cells to the GCV prodrug was evaluated using two separate methods, assaying either with the tetrazolium salt of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT assay, Chemicon International Inc., Temecula, CA) or direct cell counting after trypan blue staining (Bio-Whittaker, Walkersville, MD).

Polymer disk preparation

Two-dimensional films of poly-L-lactic acid (Boehringer Ingelheim Inc., Winchester, VA) were produced by melt processing polymer pellets between sheets of aluminum foil using a Carver press at 350°F to create disks with diameters of 20–25 mm. The disks were sterilized using γ-irradiation for $\sim$150 min with a dose of 20,000 Gy.

Cell growth on polymer disks

Disks were gently placed in each well of a six-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and immersed in phosphate-buffered saline (PBS; GibcoBRL, Grand Island, NY) for 18 h with 5 μg/mL fibronectin at 37°C.[5] Thereafter, the coated disks were blocked for 1 h with bovine serum albumin (Calbiochem-Novabiochem Corp., La Jolla, CA) at 37°C and then gently washed twice with PBS.[5] Cell suspensions (2×10^5 cells; 2 mL/well) were added in complete culture medium and incubated at 37°C for an additional 18 h. Thereafter, the medium was discarded, and 10 MOI of virus diluted in PBS was added for an additional 6 h. Next, GCV diluted in medium was added, and after an additional 125 h, the disks were washed twice in PBS, then stained with 0.2% crystal violet in 20% ethanol for 20 min.[5] The disks were then rinsed twice with PBS and examined under a light microscope using a $\times$10 objective. Three randomly selected photomicrographs were obtained from each specimen using 200 ASA color slide film (Elite Chrome, Kodak, Rochester, NY). The slides were scanned to convert them to digital images and, using Adobe Photoshop®, three 200 μm^2 regions were randomly chosen from each field, and cell number was determined visually.

Western blot analysis of thymidine kinase

Infected and noninfected HSG cells were grown for 72 h in 150-mm tissue culture plates. Thereafter, cells were scraped from the plates and hypotonically lysed with 1 mM $NaHCO_3$ and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, a serine protease inhibitor (ICN Biomedicals Inc., Aurora, OH). The cell lysate was frozen, thawed and vortexed for three cycles, and then centrifuged at 1,000*g* for 20 min. The resulting supernatant was precipitated with 80% ethanol for 17 h at -20°C and centrifuged at 16,000*g* for 20 min. The resulting pellet was solublized in double-distilled water, and the protein concentration was determined by the Bio-Rad/Bradford dye-binding protein assay (Bio-Rad Laboratories, Hercules, CA). Next, samples were mixed with an equal volume of 2 $\times$ sample loading buffer and heated at 95°C for 5 min before loading 25 μg of total protein on a polyacrylamide gel. The samples were resolved by electrophoresis using 12% Tris-HCl precast polyacrylamide gels (Bio-Rad), and the proteins were transferred onto nitrocellulose membranes. Membranes were incubated in 5% nonfat dry milk in supplemented TBS-T (Geno Technology, St. Louis, MO; Tris-buffered saline-Tween stock with additional 0.1% Tween 20), plus 1% bovine serum albumin, for 1 h. Thereafter, the membrane was incubated with a 1:250 dilution of mouse monoclonal antibody 10C11 anti-HSV-tk (obtained from Dr. W. Summers, Yale University) for 1 h at room temperature. The membrane was transferred to a solution containing a 1:500 dilution of anti-mouse–IgG

horseradish peroxidase–conjugated antibody solution in TBS-T (Amersham Life Science Inc., Arlington Heights, IL), and the incubation was continued for 1 h. Between each step, the membrane was washed twice for 7 min each in fresh supplemented TBS-T and then washed once with TBS-T stock alone. Finally, the membrane was incubated for 3 min in *femto*LUCENT Western detection reagent (Geno Technology) and exposed to X-OMAT film (Kodak).

Statistics

Mean values ± SEM for various groups were compared using a two-way analysis of variance with a 95% confidence interval.

RESULTS

Thymidine kinase production by infected HSG cells

In order to verify the production of the HSV-tk protein, HSG cells were infected with AdLTR.CMV-tk at a MOI of 30 for 72 h. No cytopathic effects were seen in the infected cells. Western blot analysis of cell extracts clearly showed a single, approximately 40-kDa immunoreactive protein band in samples from the infected cells corresponding to the expected molecular weight of the HSV-tk protein[20,21] (Fig. 1B). This protein band was not detectable in extracts from the control, noninfected cells.

Effects of ganciclovir on HSG cells grown on plastic

To assess the ability of the HSV-tk transgene to function in HSG cells, we initially studied cell viability on tissue culture plastic using the MTT assay. HSG cells were infected with different vector MOIs, and thereafter incubated with several concentrations of GCV previously shown to be useful.[22] In the absence of GCV, HSG cells infected with AdLTR.CMV-tk at a MOI of either 1 or 10 grew normally (Fig. 2). Cells infected with AdLTR.CMV-tk at a MOI of 100, however, exhibited substantially decreased viability even without GCV exposure. GCV was without effect on uninfected cells or on cells infected with AdLTR.CMV-tk at a MOI of 1. However, cells infected at a MOI of 10 were sensitive to GCV, showing >50% reduced viability at all prodrug concentrations used ($p < 0.02$, 0.006, and 0.004 for 0.5, 5, and 50 μM of GCV, respectively) compared to no GCV treatment.

To determine the surviving cell number under these growth conditions on a plastic substrate following GCV treatment, in separate experiments we used trypan blue staining and manual cell counting (Fig. 3). These results were consistent with the observations shown in Figure 2. Administration of GCV to cells infected with AdLTR.CMV-tk at a MOI of 10 demonstrated a substantial decrease (up to threefold) in number of viable cells compared with cells infected with 10 MOI alone ($p < 0.02$, 0.0004, and 0.001 for 0.5,

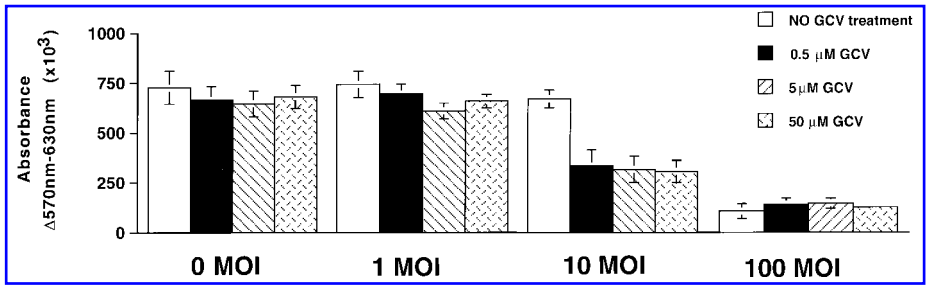

FIG. 2. Effect of ganciclovir on cell viability with the MTT assay. HSG (1×10^4) cells were plated in 96-well plates and, after 18 h, infected with AdLTR.CMV-tk at a MOI of 1, 10, or 100. After an additional 6 h, media were discarded and ganciclovir (GCV) diluted in media was added at the concentrations indicated. After 144 h, cell viability was determined using the MTT assay. Data shown are the mean ± SEM for seven individual determinations from two separate experiments.

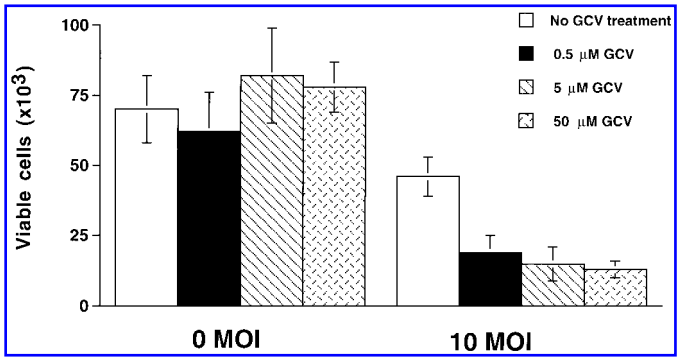

FIG. 3. Effect of ganciclovir on the number of viable cells on tissue culture plastic. HSG (1×10^4) cells were plated in 96-well plates and infected with AdLTR.CMV-tk at a MOI of 10. After an additional 6 h, media were discarded, and ganciclovir (GCV) diluted in media was added at the concentrations indicated. After 144 h, the numbers of viable cells were counted visually using trypan blue. Data shown are the mean ± SEM for eight individual determinations from two separate experiments.

5, and 50 μM GCV, respectively). In this set of experiments, note that a modest but significant reduction in cell viability was observed in cells infected with virus at a MOI of 10 in the absence of GCV ($p < 0.03$).

Effect of ganciclovir on HSG cells grown on poly-L-lactic acid disks

The envisioned artificial salivary gland device will have cells grown as a monolayer on a fibronectin-coated poly-L-lactic acid substratum,[5] rather than on tissue culture plastic. Therefore, we determined the ability of GCV to elicit HSG cell death when attached to poly-L-lactic acid disks precoated with 5 μg/mL of human fibronectin. As shown in Figure 4A, cells infected with AdLTR.CMV-tk (MOI of 10) were quite sensitive to exposure to GCV in a concentration-dependent manner. The GCV effect ranged from 40% cell death at 0.5 μM GCV to 95% cell death when the GCV concentration was 50 μM (Fig. 4A). HSG cells treated with 50 μM GCV alone appeared normal with a typical epithelioid cobblestone appearance of cell monolayers (Fig. 4B). Infection of cells with AdLTR.CMV-tk at a MOI of 10, followed by treatment with 50 μM of GCV, resulted in the loss of almost all of the cells from the disk (Fig. 4B).

DISCUSSION

The purpose of this study was to test the susceptibility to GCV of HSG cells engineered to express the HSV-tk suicide gene. We envision that inclusion of such a suicide gene could serve to provide added safety for future *in vivo* applications of HSG cells in an artificial salivary gland device. This study is part of our effort to develop an orally implantable fluid secretory device for patients suffering from irreversible damage to their salivary glands after irradiation. As noted above, the prototype design of such a device includes a blind-end tube made out of a biodegradable polymer as a scaffold, coated with matrix proteins, and lined on its lumen with a monolayer of graft cells.[6] This device would be implanted in the buccal mucosal tissue with an exit to the oral cavity, mimicking the orifices of the natural salivary duct system.[6]

For initial feasibility studies, we have chosen to use HSG cells because they possess many of the physiological signaling and transport features necessary for fluid secretion.[8–11] However, as a cell line, a significant drawback to their use is their transformation potential. We reasoned that the HSV-tk, or another, suicide gene may provide some protection against this drawback, well beyond that achieved merely by removal of the device. In cells expressing HSV-tk, the prodrug GCV is phosphorylated to its triphosphate metabolite. This derivative then competes with endogenous dGTP pools for incorporation into DNA and subsequently results in premature chain termination.[15] Thus, in the event of an untoward host response to cells, GCV can be administered and converted by the expressed HSV-tk to the cytotoxic triphosphate form

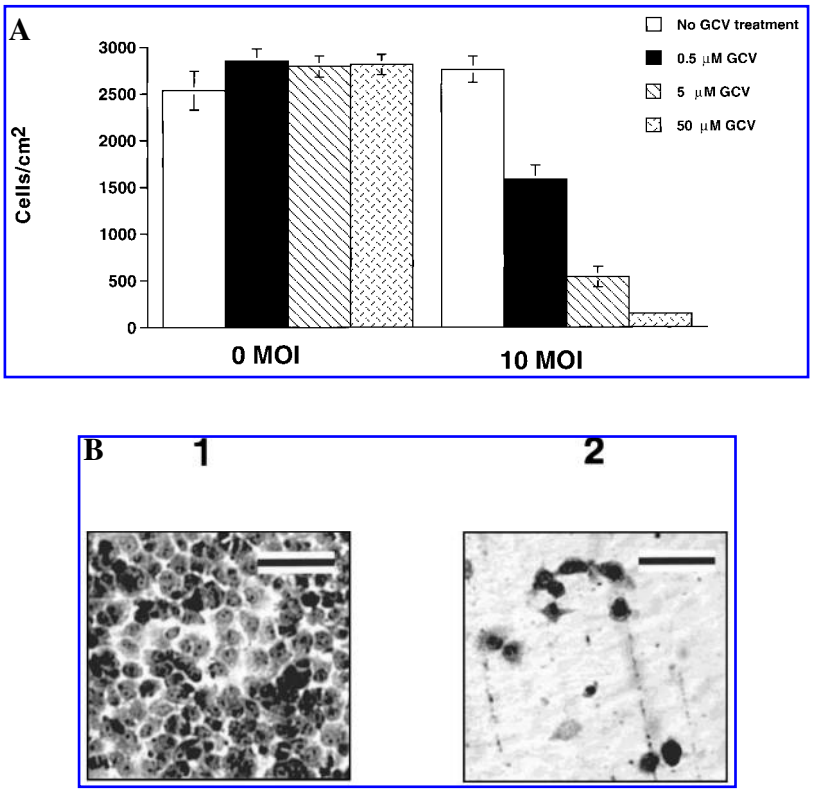

FIG. 4. (**A**) Effect of ganciclovir on HSG cells grown on poly-L-lactic acid disks. HSG cells (2×10^5/well) were plated on poly-L-lactic acid coated with human fibronectin. Thereafter, media were discarded, and AdLTR.CMV-tk, at a MOI of 10 diluted in PBS, was added. After 6 h, ganciclovir (GCV) was added in different concentrations to the media as indicated. After an additional 125 h, the disks were stained with 0.2% crystal violet in 20% ethanol; three randomly selected photomicrographs were obtained from each; and the number of attached cells was determined visually. The data shown are the mean ± SEM for 18 individual determinations from two separate experiments. All results with GCV treatment of infected cells were significantly different from control cells ($p < 0.0001$). (**B**) Appearance of HSG cells with or without infection by AdLTR.CMV-tk after GCV treatment. A selected 200 μm^2 region from representative cell cultures on poly-L-lactic acid is shown. Photomicrograph 1 represents HSG cells treated with 50 μM GCV alone. Photomicrograph 2 represents HSG cells infected with AdLTR-tk at a MOI of 10, in addition to exposure to 50 μM GCV. Bar = 50 μm.

leading to cell death, and thus host protection. This cytotoxicity is enhanced by a bystander effect in which cell death results from the phosphorylated GCV entering adjacent cells via gap junctions.[23]

We delivered the HSV-tk gene to HSG cells via a replication-deficient recombinant adeno-retroviral vector, AdLTR.CMV-tk. As we recently reported, the prototype for this hybrid vector is able to direct random genomic integration of the transgene and long-term gene expression into both dividing and nondividing cells *in vitro* and *in vivo*.[16] AdLTR.CMV-tk infection was without effect on HSG cell growth and viability at MOIs of ≤10 (Figs. 2 and 3). This is consistent with earlier reports showing maintenance of the functional integrity of HSG cells after infection with low doses of type 5 adenoviral vectors *in vitro*.[24,25] The encoded suicide gene is expressed in HSG cells, but it is essentially functionally latent until GCV administration. GCV, at doses up to 50 μM, is without effect on HSG cell growth and viability in the absence of AdLTR.CMV-tk infection (Figs. 2 and 3). However, we demonstrated that HSG cells expressing thymidine kinase after AdLTR.CMV-tk infection are quite sensitive to GCV, with cell viability reduced as much as 95%.

The HSV-tk suicide gene/GCV system was first described by Culver et al. for the treatment of experimental brain tumors.[26] Subsequently, this system has been widely used as a strategy for treating cancers in a variety of other tissues.[27–30] HSV-tk gene transfer has also been applied to prevent posterior capsule opaci-

fication after cataract surgery[31] and as a tool to reduce graft-versus-host disease.[32] Recently, during the course of this study, a report appeared using the HSV-tk gene to decrease the risk of immortalized human hepatocytes as mismatched allografts in life-saving metabolic support during acute liver failure.[33] To our knowledge, the present study is the first attempt to utilize the HSV-tk gene for safety with allogeneic graft cells for a bioartificial organ. Suicide gene technology may be a valuable way to circumvent certain problems associated with allografts or xenografts.[34]

In conclusion, this study shows that, after infection with a hybrid recombinant adeno-retrovirus capable of long-term expression and encoding HSV-tk, HSG cells are efficiently killed by GCV treatment. This suggests that the AdLTR.CMV-tk vector may be useful to provide an additional measure of safety for the use of allogeneic graft cells in an artificial tissue device.

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