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Lipofection of early passages of cell cultures derived from murine adenocarcinomas: *In vitro* and *ex vivo* testing of the thymidine kinase/ganciclovir system

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Early passages of cultured cells derived from four spontaneous Balb/c murine adenocarcinomas were used to explore the feasibility of a nonviral HSV*tk*-based suicide gene therapy system. After lipofection with pCMV*tk*, the transiently HSV*tk* expressing P07 (lung), M3, M05, and M38 (mammary gland) cells were, respectively, about 130-, 30-, 120-, and 170-fold more sensitive to ganciclovir (GCV) *in vitro* than their respective controls. Eighty percent of Balb/c mice subcutaneously inoculated with *ex vivo* pCMV*tk*-lipofected P07 cells, followed by intraperitoneal GCV injection for 7 days, displayed a complete inhibition of tumor growth for over 70 days. Control animals started to display tumors 13 days after inoculation. We present evidence showing that early passages of cultured tumor cells can efficiently express lipofected genes and that they are sensitive to the lipoplex-mediated HSV*tk*/GCV system. *Cancer Gene Therapy* (2002) **9,** 96–99 DOI: 10.1038/sj/cgt/7700417

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Most of cancer gene therapy studies carried out on animal models use established tumor cell lines that were kept in culture for many generations, making them very different from the original tumors. Some of these lines were reported to be sensitive to guanosine analogues after both *in vitro* and *in vivo* HSV*tk* gene transfer.[1] In this work, we used early passages of cultured cells derived from four spontaneously arising Balb/c murine adenocarcinomas, isolated and characterized in our institute and transplanted into a syngeneic host,[2] rather than a xenograft in an immunoincompetent host. We explored the feasibility of a nonviral HSV*tk*-based suicide gene therapy system, finding evidence that early cultured tumor cells can efficiently express genes transferred as lipoplexes and, when *in vitro* lipofected with HSV*tk*, they are sensitive to ganciclovir (GCV) both *in vitro* and *in vivo*. However, this *in vitro* sensitivity to GCV does not necessarily correlate with *in vitro* lipofection efficiency.

Materials and methods

Preparation of early passages of cell cultures

Primary cultured cells, derived from P07 (lung), M3, M05, and M38 (mammary gland) murine adenocarcinomas were obtained by enzymatic digestion of tumor fragments with 0.01% Pronase (Sigma, St. Louis, MO) and 0.035% Dnase (Sigma) in serum-free culture medium. They were then cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 with IMDM/F12 medium (Gibco-BRL, Carlsbad, CA) containing 15% FBS (Gibco-BRL), 10 mM HEPES (pH 7.4) and antibiotics. Serial passages were done by trypsinization (0.25% trypsin and 0.02% EDTA in PBS) of subconfluent monolayers.

Plasmids

$pCMV_0$ was obtained from pCMVβ (Clontech, Palo Alto, CA) by removing the β-gal gene. The *tk* gene from HSV-1 was cloned downstream of the cytomegalovirus (CMV) promoter of $pCMV_0$, yielding pCMV*tk*. pRc/CMV*tk* was obtained by subcloning the HSV*tk* gene under CMV promoter into pRc/CMV (Invitrogen, Carlsbad, CA) that also contains the *neo*^{r} gene under SV40 early promoter. Plasmids were amplified in *E. coli* DH5α (Gibco-BRL), grown in LB medium containing 100 μg/mL ampicillin and purified using ion-exchange chromatograpy (Qiagen, Valencia, CA).

Liposome preparation and in vitro lipofection

DMRIE (1,2-dimyristyl oxypropyl-3-dimethyl-hydroxyethilammonium bromide) was synthesized and provided by BioSidus (Buenos Aires, Argentina). DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphatidyl ethanolamine) was purchased from Sigma. Liposomes were prepared at DMRIE:DOPE molar ratios of 1:1 by sonication. Cells were transfected with 1:6 μg DNA:nmol lipid in OptiMEM (Gibco-BRL).

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Lipoplexes (0.5 μg DNA/cm^2) were applied to cultured cells at a density of 15000 cells/cm^2 (about 30% confluence). After 6–12 hours, lipofected cells were returned to regular culture conditions. Cells were tested after 48 hours for sensitivity to GCV. For stable expression, cells were lipofected with pRc/CMV*tk* and selected with 500–700 μg/mL geneticin (Gibco-BRL) after 48 hours. Single clones were isolated and tested for their sensitivity to GCV.

β-Galactosidase assays

Assay of β-galactosidase activity with orthonitrophenyl 1-β-D-galactopyranoside (ONPG, Sigma) and staining of β-gal positive cells with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL, Sigma) were done by standard methods. The enzymatic assay was standardized with purified *E. coli* β-galactosidase (ICN, Costa Mesa, CA 574 U/mg).

Sensitivity to GCV assays

In vitro *studies.* HSV*tk*-expressing cells (5×10^3), both transiently or stably transfected, as well as their respective transiently β-gal–expressing or parental cells, were incubated for 5 days in 96-well plates with 100 μl of 10% FBS supplemented medium containing from 0.01 to 1000 μg/mL GCV (synthesized and provided by Bio-Sidus). Cell viability was quantified by the MTS test (Promega, Madison, WI). The percentage of cell survival was calculated from the ratio between the absorbances of cells incubated in the presence and in the absence of GCV. The cell sensitivity to GCV, expressed as the concentration of GCV that inhibited cell survival by 50% (IC_{50}), was estimated from dose–response curves.

In vivo *studies.* Because of GCV toxicity for smaller animals, randomized inbreed male Balb/c mice, weighing about 30 g, were employed for the *in vivo* assays. All tumor-bearing animals were euthanized after the last tumor volume determination. After 6 hours lipofection in 100-mm-diameter Petri dishes, cells were returned to regular culture conditions, and 24 hours later cells were washed with PBS, trypsinized, washed three times with serum-free culture medium, counted, resuspended in the same medium, and kept for 1 hour at 37°C before injection. Animals were injected subcutaneously in the right flank at day 0 with 3×10^5 (i) 24-hour pCMV*tk* or $pCMV_0$ transiently transfected P07 cells, or (ii) HSV*tk* stably expressing or parental P07 cells in 200 μl IMDM/F12 medium. Then, they were treated twice a day for 7 consecutive days by intraperitoneal injections of 75 mg/kg GCV diluted in saline or saline alone. Tumors were measured twice a week and volumes were calculated as $A\times B^2/2$, where A was the minor and B the major diameter of the tumor.

Results and discussion

We were able to establish early cell cultures (<15 passages) derived from four Balb/c murine adenocarcinomas: three mammary (M3, M05, and M38) and one lung (P07) adenocarcinomas that are currently maintained as subcutaneous transplantable tumors into the syngeneic host.[2]

In vitro *GCV cytotoxicity for HSV*tk-*lipofected tumor cells*

Early passages of murine adenocarcinoma-derived cell cultures were transfected *in vitro* for transient expression with lipoplex-containing pCMV*tk*. The cytotoxicity of increasing concentrations (0.01 to 1000 μg/mL) of the prodrug GCV was tested on HSV*tk*-expressing cells and control transiently expressing β-gal cells. As calculated from the estimated IC_{50}s, P07, M3, M05, and M38 cells were about 130-, 30-, 120-, and 170-fold more sensitive to GCV than the respective pCMVβ-transfected controls (Fig 1A, Table 1).

After P07 lipofection with pRc/CMV*tk* followed by geneticin selection, we cloned HSV*tk*-expressing P07 cell lines. One of the clones, P07*tk*-1, was about 400-fold more sensitive to GCV than the parental P07 control cells (Fig 1B, Table 1).

The *in vitro* sensitivity to GCV of transiently HSV*tk*-expressing cells did not correlate with their transfection efficiency. Highly lipofected P07 cells displayed similar sensitivity to GCV as weakly lipofected M05 and M38 cells (Table 1). It is worth noting that M3 cells, with relatively high lipofection efficiency, were poorly sensitive to GCV. This could partially explain the weak treatment efficacy of our *in vivo* preliminary results for M3 (data not shown). In addition, *in vitro* preliminary results derived from GCV dose–response curves of different mixtures of pCMV*tk*- and pCMVβ-lipofected cells suggest that P07, M05, and M38 transiently lipofected cells displayed a higher bystander effect than M3 (data not shown). Thus, the main factor that would allow the proposed nonviral suicide approach for M05 and M38 would be the bystander effect. For P07, this effect would be improved by the high lipofection efficiency. Taken together, all these results indicate that GCV sensitivity is intrinsic for each tumor type, probably proportional to the bystander effect and, that *in vitro* transfection efficiency is not enough to predict the GCV sensitivity of transiently lipofected cells with HSV*tk* suicide gene. Other factors that could vary in every individual tumor would play a role on the bystander effect including (i) phagocytosis of the apoptotic vesicles from dying HSV*tk* tumor cells by adjacent unmodified tumor cells, (ii) gap junction intercellular communications, and (iii) releasing of soluble factors induced by lipoplexes that leads to direct antitumor effects.[3]

In vivo *GCV cytotoxicity for* ex vivo *HSV*tk-*lipofected tumor cells*

Because only P07 cells combined both high lipofection rate and efficient destruction under clinically relevant GCV concentration (1–10 μg/mL), they became our best target for lipoplex-mediated HSV*tk*/GCV therapy. To assess the feasibility of this suicide gene approach, bypassing the problem of the low *in vivo* lipofection efficiency, we subcutaneously injected into Balb/c mice two kinds of HSV*tk*-expressing P07 cells: (i) pCMV*tk* transiently transfected P07 cells and (ii) stably transfected P07*tk*-1 cells.

Although all control animals started to display tumors about 13 days after inoculation, 80% of the mice inoculated with pCMV*tk* transiently transfected P07 cells and treated

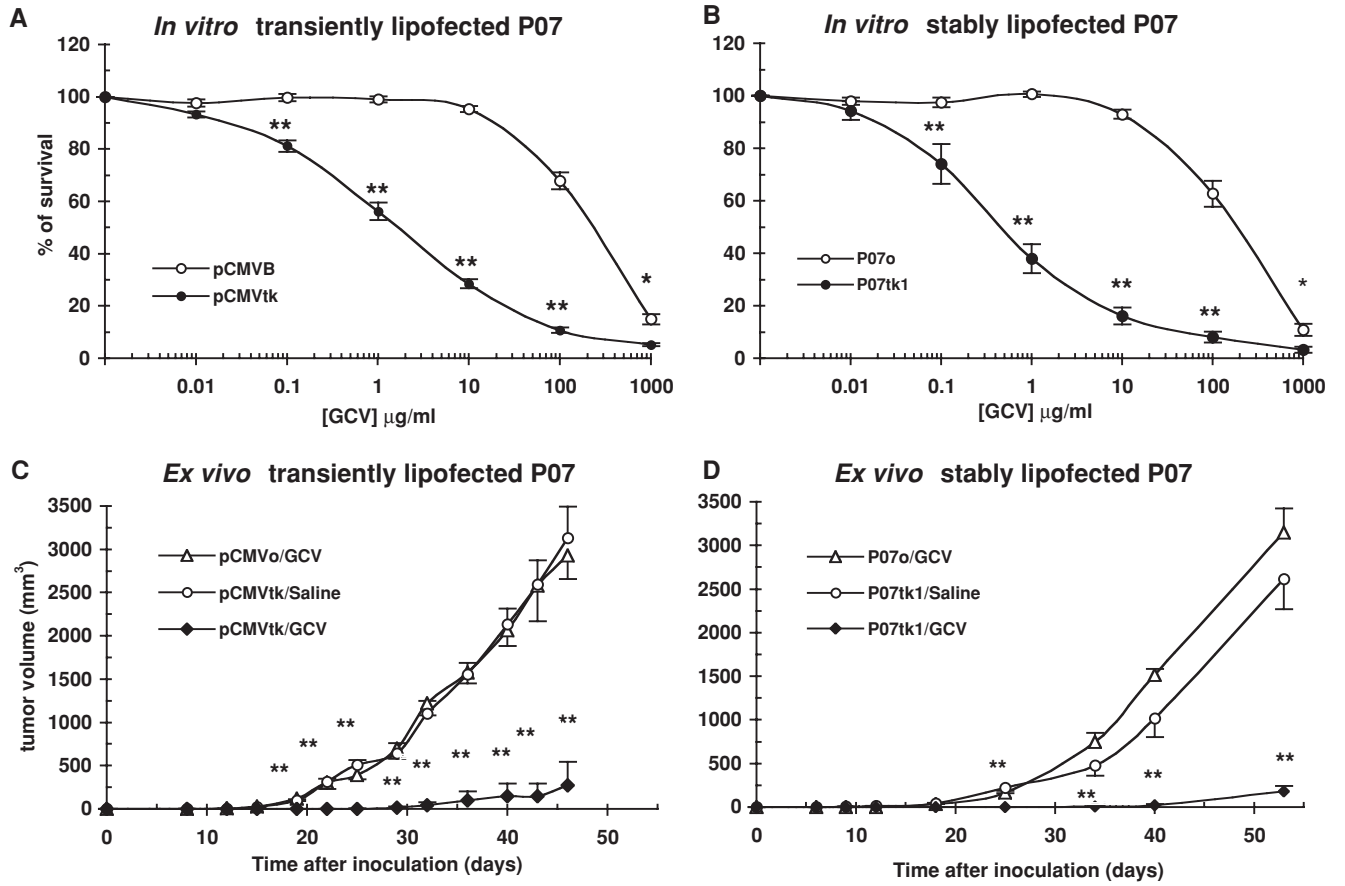

Figure 1 Sensitivity of HSV*tk*-expressing P07 cells to GCV both *in vitro* **(A, B)** and *in vivo* **(C, D)**. Transiently pCMV*tk* **(A, C)** and stably pRc/CMV*tk* **(B, D)**-lipofected cells, or pCMVβ **(A)** and $pCMV_0$ **(C)** transiently lipofected cells, and parental $P07_0$ cells **(B, D)** as respective controls, were used. *In vitro* **(A, B)** dose–response curves for GCV. The percentage of cell survival was reported as means ± standard error of the mean (SEM) of *n* independent experiments measured in duplicate (the corresponding *n* are indicated in Table 1, sixth column). *In vivo* **(C, D)** effect of GCV on tumor growth in Balb/c mice injected with transiently **(C)** and stably (**D**) HSV*tk*-expressing P07 cells. The results were expressed as means ± SEM (n=5 animals per group). Tumor volumes were derived by averaging the volumes of detectable tumors with the tumors that failed to appear (volume=0). Differences between groups were determined by analysis of variance (ANOVA). *P<.05 and **P<.01, compared to their respective control values.

with GCV for 7 days were free of tumor for over 70 days. Tumor development was substantially delayed in the remaining 20% of the mice with 21 days of latency (Fig 1C).

It is noteworthy that some tumor cells, which were not completely killed by GCV *in vitro* (Fig 1A), failed to grow in mice (Fig 1C), probably due to the generation of an antitumor

Table 1 *In vitro* β-gal expression efficiencies and sensitivity to GCV of HSV*tk*$^+$- and HSV*tk*$^-$expressing cells

	Lipofection efficiency (%)	β-gal activity mU/10^6 cells	IC_{50} for GCV (μg/mL)		IC_{50}s ratio
Cell	pCMVβ		*tk*$^-$	*tk*$^+$	*tk*$^-$/*tk*$^+$
P07	41±4 [30]	393±73 [10]	235±22	1.8±0.2	130 [14]
M3	14±2† [40]	130±32† [10]	230±64	7.9±2.4*	29 [22]
M05	7±2† [18]	24±7† [10]	280±28	2.4±1.9	116 [6]
M38	6±2† [15]	23±6† [10]	170±14	1.0±0.5	170 [6]
P07*tk*-1	n.d.	n.d.	199±32	0.48±0.11	400 [6]

The results represent means ± SEM of percentage of X-GAL–stained cells (second column), mU β-gal/million cells (third column), and GCV concentrations leading to 50% reduction in cell viability (IC_{50}) (fourth and fifth columns).

tk$^+$: transiently pCMV*tk*-lipofected cells or stably HSV*tk*-expressing P07*tk*-1 cells. *tk*$^-$: transiently pCMVβ-lipofected cells or parental $P07_0$ cells. n.d.: not determined. [*n*]: number of experiments corresponding to independent assays. Differences between groups were determined by ANOVA.

†P<.01: the expression level compared to the respective P07 value.

*P<.01: the IC_{50} for GCV of each type cell compared to the respective P07 value.

immune response. It was reported that HSV*tk* dying tumor cells are more antigenic than the unmodified tumor cells. In addition, P07 tumor secretes soluble cytokines such as granulocyte–macrophage colony-stimulating factor (GM-CSF)[2], a powerful host leucocytosis inducer that stimulates potent, specific, and long-lasting antitumor immunity.

However, all the mice inoculated with P07*tk*-1 cells and treated with GCV for 7 days presented a substantial tumor growth delay with a latency of 34 days. At that time, 80% started to display detectable tumors, with the remaining 20% free of tumor for over 70 days. All the control animals started to display tumors about 11 days after inoculation (Fig 1D). Even in a population of almost 100% *HSVtk* gene–modified tumor cells, resistant cells are present, as it was reported for HSV*tk*-expressing melanoma B16F10 cells.[4] This high rate of late recurrence (80%) could result from a small amount of surviving cells due to (i) partial or total loss of HSV*tk* expression, (ii) cell mutation to GCV resistance, (iii) inefficient treatment scheme, or (iv) permanence of few cells in G_0 during GCV treatment.[4]

All these *ex vivo* results contrast with data obtained *in vitro*: Even though stably HSV*tk*-expressing P07*tk*-1 cells synthesized about 10-fold less HSV*tk* mRNA (data not shown), *in vitro* they displayed similar bystander effect (data not shown) and 3-fold more sensitivity to GCV than transiently *HSVtk*-expressing P07 cells (Fig 1), Table 1: 400 vs. 130). Additional mechanisms, beyond the number of HSV*tk* expressing cells or the amount of recombinant enzyme synthesized may enhance the *in vivo*. GCV sensitivity of the transiently HSV*tk* expressing P07 cells. An adjuvant effect of transient transfections with lipoplexes on the *in vivo* bystander effect, was reported[3]. It was reported that lipoplex administration produces changes in the microenvironment that favor rejection of tumor cells by the host, probably related to an inflammatory reaction and activation of NK cells and macrophages.[5] This appears to be a generalized, nonspecific response of the tumor cell to transient lipofections, not found in long-term stable cell lines generated by transfection with lipoplexes.[3,5]

In conclusion, this work suggests that (i) the *in vitro* GCV sensitivity of transiently lipofected cells with *HSVtk* gene is intrinsic of each individual tumor, probably proportional to the bystander effect; (ii) the *in vitro* transfection efficiency, the number of HSV*tk*-expressing cells, or the amount of recombinant enzyme synthesized are not enough to predict the success of this treatment; and (iii) the process of transient transfection with lipoplexes may extend the effect of the HSV*tk*/GCV system.

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