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Original Articles

Liposomal insulin promoter–thymidine kinase gene therapy followed by ganciclovir effectively ablates human pancreatic cancer in mice

James X. Wu [a], Shi-He Liu [a], John J. Nemunaitis [b], F. Charles Brunicardi [a,*]

[a] David Geffen School of Medicine, UCLA, Los Angeles, CA, USA
[b] Mary Crowley Cancer Research Centers, Dallas, TX, USA

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ABSTRACT

PDX1 is overexpressed in pancreatic cancer, and activates the insulin promoter (IP). Adenoviral IP–thymidine kinase and ganciclovir (TK/GCV) suppresses human pancreatic ductal carcinoma (PDAC) in mice, but repeated doses carry significant toxicity. We hypothesized that multiple cycles of liposomal IP-TK/GCV ablate human PDAC in SCID mice with minimal toxicity compared to adenoviral IP-TK/GCV. SCID mice with intraperitoneal human pancreatic cancer PANC-1 tumor implants were given a single cycle of 35 μg iv L-IP-TK, or four cycles of 1, 10, 20, 30, or 35 μg iv L-IP-TK (n = 20 per group), followed by intraperitoneal GCV. Insulin and glucose levels were monitored in mice treated with four cycles of 35 μg iv L-IP-TK. We found that four cycles of 10–35 μg L-IP-TK/GCV ablated more PANC-1 tumor volume compared to a single cycle with 35 μg. Mice that received four cycles of 10 μg L-IP-TK demonstrated the longest survival ($P < 0.05$), with a median survival of 126 days. In comparison, mice that received a single cycle of 35 μg L-IP-TK/GCV or GCV alone survived a median of 92 days and 68.7 days, respectively. There were no significant changes in glucose or insulin levels following treatment. In conclusion, multiple cycles of liposomal IP-TK/GCV ablate human PDAC in SCID mice with minimal toxicity, suggesting non-viral vectors are superior to adenoviral vectors for IP-gene therapy.

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Introduction

Pancreatic cancer remains a terrible and challenging disease, representing only 2.8% of new cancers but ultimately responsible for 6.8% of all cancer deaths [1]. Pancreatic cancer has demonstrated remarkable resistance to conventional therapies. Only a quarter of patients with localized disease survive 5 years beyond diagnosis [1].

Pancreatic duodenal homeobox 1 (PDX1) is a gene specific to pancreatic tissue, is aberrantly expressed in pancreatic cancer, and facilitates oncogenesis [2,3]. Taking advantage of PDX1-mediated stimulation of the insulin promoter, we previously developed a novel recombinant gene therapy that targets PDX1 expressing cells: a recombinant rat insulin promoter–viral thymidine kinase (IP-TK) gene [4]. Aberrant PDX1 expressed by pancreatic cancer cells would bind and activate the insulin promoter, driving the expression of viral thymidine kinase, sensitizing them to ganciclovir (GCV) [4,5]. Previously, we demonstrated adenoviral IP-TK/GCV therapy ablates human pancreatic cancer in vitro and in vivo in SCID mice [4,6–9].

Adenoviral IP-TK/GCV therapy is limited by several factors. These include toxicity to healthy tissue, resulting in hyperglycemia with repeat dosing, as well as reduced efficacy of repeat doses due to induction of viral antigen targeting antibodies [8]. Even a single virus infusion will result in antibody development, which has led to myriad strategies to circumvent this therapeutic obstacle [10–13]. Given that a number of studies have demonstrated successful gene delivery to cancer cells using non-viral liposomal vectors [14–16], we hypothesized that multiple cycles of liposomal naked IP-TK DNA followed by GCV can efficiently ablate human PDAC in mice with less toxicity and retain efficacy with repeat dosing.

Materials and methods

Cell lines, plasmid vectors and antibodies

The human pancreatic cancer cell line PANC-1 was purchased from the American Type Culture Collection (ATCC, Bethesda, MD), and was maintained in DMEM medium (Invitrogen, MD) supplemented with 100,000 units/l of penicillin, 100,000 μg/l of streptomycin and 10% fetal bovine serum. Plasmid DNA IP-lacZ and IP-TK were constructed as described previously [7,17]. Rabbit and goat anti-HSV-TK antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cy3 conjugated anti-rabbit IgG antibodies were purchased from Sigma (St. Louis, MO).

Preparation of liposomal IP-TK complex (L-IP-TK)

L-IP-TK was prepared fresh at room temperature. Formulated liposomes (Gradalis Inc, at Dallas, TX) (DOTAP or DOTAP:chol) were diluted to a final concentration of 4 mM in 300 μl final volume with 5% dextrose in water. Plasmid DNA was diluted to a concentration of 1 μg/μl, and then mixed with an equal volume of 4 mM DOTAP to give a final concentration of 50 μg/100 μl.

Abbreviations: IP, insulin promoter; TK, thymidine kinase; GCV, ganciclovir; PDAC, pancreatic ductal adenocarcinoma.

* Corresponding author. Tel.: +1 310 562 1635; fax: +1 (424) 259-7390.
E-mail address: CBrunicardi@mednet.ucla.edu (F.C. Brunicardi).

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Animals and gene delivery

SCID mice were housed in a BL-4 facility and cared for under the guidelines in *The Care and Use of Laboratory Animals* manual prepared by the Institute of Laboratory Animal Resources, the Commission on Life Science, the National Research Council, and the Animal Research Committee of Baylor College of Medicine.

To investigate the maximally tolerated dose of liposomal empty vector DNA, male SCID mice 8–10 weeks of age (n = 5) were given one cycle of 20, 30, 35, 40, 50, 60, 70, or 80 μg of liposomal empty vector DNA. Mice were observed for 26 days.

To simulate intraperitoneal metastatic pancreatic cancer, male SCID mice 8–10 weeks of age were inoculated with 0.5×10^5 PANC-1 cells by intraperitoneal injection. This model of metastatic pancreatic cancer has been used in our previous studies [7]. These mice were randomized to seven groups (n = 20): (1) 1 cycle of 35 μg iv L-IP-TK/GCV, (2) 4 cycles of 1 μg iv L-IP-TK/GCV, (3) 4 cycles of 10 μg iv L-IP-TK/GCV, (4) 4 cycles of 20 μg iv L-IP-TK/GCV, (5) 4 cycles of 30 μg iv L-IP-TK/GCV, (6) 4 cycles of 35 μg iv L-IP-TK/GCV, and (7) 40 mg/kg iv GCV without IP-TK. Each cycle consisted of liposomal IP-TK iv injection on the first day followed by 2 weeks of GCV (40 mg/kg body weight twice daily) and 1 week of rest.

Tumor evaluation and survival analysis

Necropsy and tumor evaluation were performed at 77 and 120 days after treatment. At least five mice were sacrificed at each time point. Tissues were saved for further immunohistochemical analyses. Tumor volume was evaluated at each time point. Peritoneal tumors were evaluated macroscopically and microscopically and the larger (A) and smaller (B) diameters measured and recorded. Tumor volume (V; a rotational ellipsoid) was calculated according to the formula: $V\,(mm^3) = A\,(mm) \times B^2\,(mm^2) \times 0.5$. Mice were classified according to presence or absence of tumor. Mouse survival was measured from the date of initial treatment to date of death or sacrifice.

Immunohistochemistry

Pancreata and tumors were removed and fixed in 4% paraformaldehyde at 4 °C for 4 hours at the time of necropsy. Tissues were embedded in paraffin and tissue sections were prepared. For immunostaining, sections were deparaffinized in xylene and hydrated gradually through graded alcohol. Slides were then placed in a humidified chamber, overlaid with 1:100 diluted antibody against HSV-TK, and incubated overnight at 4 °C. After washing with PBS, slides were incubated with Cy3-conjugated rabbit antibody for 1 hour at room temperature. Slides were then washed with PBS and mounted with cover slides.

Detection of apoptosis in tumor xenografts and the islet cells of mice

Apoptosis in tumor and pancreatic specimens was determined with TUNEL assay (FragEL DNA Fragmentation Detection Kit, Colorimetric-TdT Enzyme; Calbiochem, La Jolla, CA) according to the manufacturer's protocol and expressed as the ratio of apoptotic cancer cells to the total number of endothelial cells in 10 fields at 100× magnification. To evaluate the effect of L-IP-TK/GCV on the endocrine pancreas, at least 10 islets per specimen were evaluated.

Insulin and glucose measurements

For the four cycles of 35 μg iv L-IP-TK treatment group, 50 μl whole blood samples were collected from each mouse and spun to separate the serum at days 14, 35, 56, and 77. This group was selected for serum insulin and glucose measurements because these mice received the greatest amount of L-IP-TK therapy. Serum samples were stored at –20 °C until completion of experiments. Glucose levels and insulin levels were measured as reported previously [18].

Statistical analysis

The unpaired Student's *t*-test was used for statistical analyses of tumor volume, glucose, and insulin levels, with $P < 0.05$ indicating significance. The χ^2 test was used for rate comparison. Log rank test was used to compare the mice survival data. Kaplan–Meier in SPSS 15.0 for Windows was used to plot survival curves.

Results

Cytotoxicity of empty vector liposomal DNA

SCID mice that received 35 μg or less of liposomal empty vector plasmid DNA had a 100% survival rate at 26 days. Mice that received 40 μg of liposomal empty vector DNA had a 50% survival rate at 26 days, and doses 50 μg and higher resulted in 0% survival at 26 days.

Table 1
Mean tumor volume and median survival after L-IP-TK/GCV therapy.

Treatment group	Mean final tumor volume (mm^3)	Median survival (days)
GCV 40 mg/kg, without L-IP-TK	730.14 ± 107.01	68.7 ± 5.1
L-IP-TK 35 μg, single cycle	23.78 ± 10.19	92 ± 4.4
L-IP-TK 1 μg, four cycles	109.40 ± 148.51	75 ± 7.7
L-IP-TK 10 μg, four cycles	5.70 ± 6.25	126 ± 3.7
L-IP-TK 20 μg, four cycles	3.00 ± 2.78	99 ± 7.0
L-IP-TK 30 μg, four cycles	3.12 ± 2.97	101 ± 11.8
L-IP-TK 35 μg, four cycles	0.56 ± 1.62	70 ± 4.3

Multiple doses of L-IP-TK/GCV reduce or ablate xenograft pancreatic tumor volume in mice

Four cycles of L-IP-TK/GCV, 10–35 μg, significantly reduced more PANC-1 tumor volume compared to a single cycle of 35 μg. Four cycles of 35 μg iv L-IP-TK/GCV resulted in the smallest remaining tumor volume among all treatment groups (versus four cycles of 30 μg L-IP-TK, next smallest mean tumor volume, $P = 0.017$). See Table 1, Fig. 1. There was no significant difference in residual tumor volume among 10, 20, or 30 μg L-IP-TK treatment groups after 4 cycles. Complete tumor ablation was noted in 50% of the 10 and 20 μg L-IP-TK treatment groups, and 57.1% of the 30 μg and 35 μg L-IP-TK treatment groups.

Multiple doses of L-IP-TK/GCV improve survival in mice

Four cycles of 10 μg L-IP-TK/GCV resulted in the longest survival in study mice, for a median survival of 126 days (versus all treatment groups $P < 0.05$; the *P* values of four cycles of 10 μg iv L-IP-TK versus a single cycle of 35 μg, as well as four cycles of 1 μg, 10 μg, 20 μg, and 30 μg of L-IP-TK were 0.035, 0.017, 0.028, 0.029, and 0.018, respectively) (Fig. 2). For median survival of each treatment group, see Table 1.

Multiple doses of L-IP-TK did not significantly affect pancreatic islets

Following multiple treatments with 35 μg iv L-IP-TK, there was no significant alteration in serum glucose or insulin levels (Fig. 3). TUNEL assay revealed extensive apoptosis in tumor tissue following L-IP-TK/GCV therapy, but no evidence of apoptosis in pancreatic islets. When control liposomal empty vector DNA/GCV therapy was given, no apoptosis was noted in tumor tissue (Fig. 4).

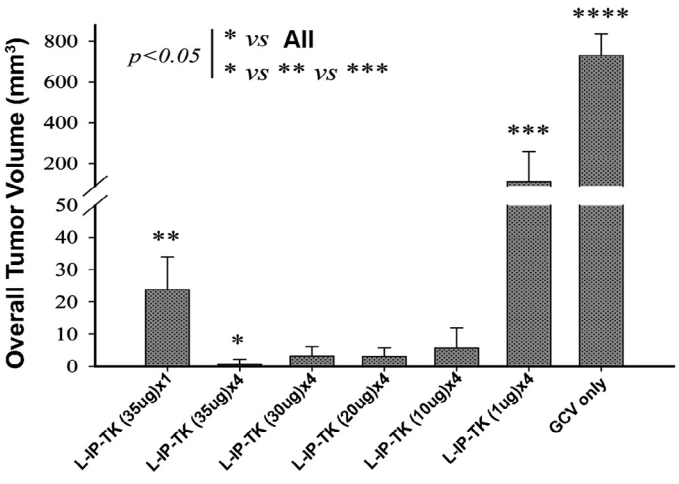

Fig. 1. Effect of L-IP-TK dose on tumor volume.