Gonadal tumors of mice double transgenic for inhibin- α promoter-driven simian virus 40 T-antigen and herpes simplex virus thymidine kinase are sensitive to ganciclovir treatment

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Abstract

We have previously produced transgenic (TG) mice expressing the mouse inhibin α -subunit promoter/Simian virus 40 T-antigen (Inh α /Tag) fusion gene. The mice develop gonadal somatic cell tumors at the age of 5–7 months; the ovarian tumors originate from granulosa cells, and those of the testes from Leydig cells. In the present study another TG mouse line was produced, expressing under the same inh- α promoter the herpes simplex virus thymidine kinase gene (Inh α /TK). Crossbreeding of the two TG mouse lines resulted in double TG mice (Inh α /TK–Inh α /Tag), which also developed gonadal tumors. The single (Inh α /Tag) and double TG (Inh α /TK–Inh α /Tag) mice, both bearing gonadal tumors, were treated at the age of 5.5–6.5 months with ganciclovir (GCV, 150 mg/kg body weight twice daily i.p.) for 14 days, or with aciclovir (ACV, 300–400 mg/kg body weight per day perorally) for 2 months. During GCV treatment, the

total gonadal volume including the tumor, decreased in double TG mice by an average of 40% ($P<0.05$), while in single TG mice, there was a concomitant increase of 60% in gonadal size ($P<0.05$). GCV was also found to increase apoptosis in gonads of the double TG mice. Peroral treatment with ACV was less effective, it did not reduce significantly the gonadal volume. We also analyzed the *in vitro* efficacy of ACV and GCV treatments in transiently HSV-TK–transfected KK-1 murine granulosa tumor cells, originating from a single-positive Inh α /Tag mouse. GCV proved to be more effective and more specific than ACV in action. These results prove the principle that targeted expression of the HSV-TK gene in gonadal somatic cell tumors is potentially useful for tumor ablation by anti-herpes treatment. The findings provide a lead for further development of somatic gene therapy for gonadal tumors.

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Introduction

A promising strategy for gene therapy of tumors is to transduce them with a gene encoding a protein that is detrimental to cell growth and/or proliferation. Such 'suicide' or 'toxic' genes tested for this purpose include diphtheria toxin A subunit (DT-A), attenuated diphtheria toxin A (tox176), ricin A, and herpes simplex virus thymidine kinase (HSV-TK) (for a review see Camper *et al.* 1995). DT-A inhibits the protein synthesis mainly by ADP ribosylation of elongation factor 2. Tox176, through a mutation (128-glycine→aspartic acid), is 15- to 30-fold less active than wild-type DT-A, and thus less likely to have non-specific deleterious effects outside the targeted cell type. Ricin A inhibits protein synthesis by RNase activity specific for the 28S ribosomal RNA.

Another type of suicide gene, and the most widely used, is HSV-TK, which alone is non-toxic, but can induce

ablation of dividing target cells upon metabolic prodrug treatment. This viral enzyme monophosphorylates nucleoside analogues such as ganciclovir (GCV) and aciclovir (ACV), and the cytotoxic triphosphorylated product is incorporated into elongating DNA of proliferating cells, stopping DNA replication, and thereby causing cell death (Nishiyama & Rapp 1979, Furman *et al.* 1980, Miller & Miller 1980, Keller *et al.* 1981, Cheng *et al.* 1983, Elion 1983, Mar *et al.* 1985, Moolten 1986). This property makes it possible to treat rapidly dividing tumor cells in targeted fashion, at the same time saving the surrounding healthy tissues. The existence of the so-called 'bystander effect' overcomes one of the major limitations of gene therapy of cancer, i.e. inefficient gene transduction into the tumor mass (Freeman *et al.* 1992, 1993, Caruso *et al.* 1993). HSV-TK is currently being tested in experimental and clinical gene therapy trials as a conditional suicide gene to ablate tumor cells (Moolten & Wells 1990,

Culver *et al.* 1992, Oldfield *et al.* 1993, Ram *et al.* 1993, Chen *et al.* 1994, Freeman *et al.* 1995, Chen *et al.* 1995, Ram *et al.* 1997).

We have earlier described a transgenic (TG) mouse model, expressing the Simian virus (SV) 40 T-antigen under control of the murine inhibin- α subunit promoter (Inh α /Tag) (Kananen *et al.* 1995, 1996). The Inh α /Tag mice develop gonadal tumors at the age of 5–7 months, the ovarian tumors originating from granulosa cells, and testicular tumors from Leydig cells. In the present study we produced another TG mouse line expressing the HSV-TK gene under the inh α promoter (Inh α /TK). Due to the same promoter, we hypothesized that if the two TG lines are crossbred, the double transgenic offspring (Inh α /TK–Inh α /Tag) express both Tag and HSV-TK in the same gonadal tumors.

Hence, the aim of this study was to produce a double TG (Inh α /TK–Inh α /Tag) mouse line, in order to generate a spontaneously arising tumor model for testing the principle that targeted expression of the HSV-TK gene is suitable for the treatment of gonadal somatic cell tumors by antiherpes drugs. We hypothesized that this model mimicks the clinical situation more closely than the commonly used transplanted animal tumor models and human tumor xenografts (Khleif & Gregory 1997).

Materials and Methods

Generation and identification of TG mice

For Inh α /TK mice, an 8.3 kilobase (kb) fusion gene containing the inhibin- α promoter (6 kb), fused with the *Bgl*II/*Eco*RI (2.3 kb) fragment of the HSV-TK coding sequence (McKnight 1980) was microinjected (Hogan *et al.* 1994) into the pronuclei of FVB/N embryos. Three Inh α /TK mouse lines were produced, and females from line 1–6 were crossbred with Inh α /Tag males from an earlier produced line (IT6-M) to produce double-mutant Inh α /TK–Inh α /Tag mice. The transgenic constructs used for microinjections containing the inhibin- α promoter and Tag or HSV-TK fragments are shown in Fig. 1.

For routine genotyping, PCR analyses were carried out using DNA extracted from tail biopsies. The sequences of the PCR primers were identical to those previously described (Markkula *et al.* 1993, Kananen *et al.* 1995). Genotyping of the TG mice was performed by amplifying tail DNA in PCR as described previously except that the annealing temperature in TK-PCR was increased to 60 °C.

Mice were housed four to six per cage, after weaning at the age of 21 days, in a room with controlled light (12 h light, 12 h darkness) and temperature (21 $\pm$ 1 °C). The mice were specific pathogen free and they were routinely screened for common mouse pathogens. Avertin anesthesia was used in surgical operations and after the operations, analgesia was administered (buprenorphine, 3 μ g/mouse

Figure 1 The gene constructs used for generation of transgenic mice. (A) Inh α /Tag containing 6 kb of the mouse inhibin- α promoter (about –6000 to +71 bp) fused with the *Stu*I/*Bam*HI 2.7 kb fragment of SV40 Tag coding region. (B) Inh α /TK containing the same inh α promoter fused with the *Bgl*II/*Eco*RI 2.3 kb fragment of the HSV-TK coding region.

i.p.). The University of Turku Ethical Committee on Use and Care of Animals approved all the procedures using mice.

Histology and immunocytochemistry of gonadal sections

Gonads were fixed in freshly prepared 4% paraformaldehyde. Paraffin sections (5 μ m thick) were stained with hematoxylin/eosin for histological analysis.

For immunocytochemical detection of T-antigen, a mouse monoclonal anti-SV 40 large T-antigen antibody (1:100 to 1:5000 in PBS) (kindly donated by Dr D Hanahan, University of California, San Francisco, CA, USA) was used as the primary antibody. HSV-TK was detected with polyclonal rabbit antiserum to HSV-TK (1:100 to 1:500 in PBS) (kindly donated by Dr W C Summers, Yale University, New Haven, CT, USA). The antigen–antibody complexes were visualized using the immunoperoxidase technique (Vectastain Elite ABC Kit, Vector, Burlingame, CA, USA).

Analysis of TG-RNA expression

Snap-frozen tissues were kept at –70 °C until RNA isolation. Total RNA from a piece of tumor or from the whole gonad was extracted using TRIzol reagent (Life Technologies, Gibco-BRL, Glasgow, Scotland) according to instructions of the manufacturer. The expression of both transgenes in the same tumor tissue was shown by RT-PCR. One microgram DNase-treated (Gibco, Paisley, Scotland) RNA was transcribed by avian myeloblastosis virus–reverse transcriptase (AMV–RT) (Promega, Madison, WI, USA) and amplified using thermostable DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland)

Figure 2 Immunohistochemical staining of Inha/Tag and Inha/Tk testes. (A) Testis section of an adult Inha/Tk male showing immunoreaction of anti-HSV-TK in Leydig (LC) and Sertoli (SC) cells (magnification $\times 650$). (B) Testicular tumor section of a 7-month-old IT6-M male showing immunoreaction with anti-SV40 Tag in Leydig and Sertoli cells ($\times 650$). Scale bar, 10 μm .

in a thermal cycler. The same primer pairs and PCR programs were used as described earlier (Markkula *et al.* 1993, Kananen *et al.* 1995). The mouse β -actin (Genbank accession number X03672) forward (5' primer: 5'-CGTGGGCCCGCCCTAGGCACCA-3') and reverse (3' primer: 5'-TTGGCCTTAGGGTTCAGGGGG-3') primers were used to generate a control for the amount of RNA used in the other RT-PCR reactions. A 30% aliquot of the PCR product was resolved on 1% agarose gel and transferred onto nylon membrane (Hybond-XL, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The specificity of the RT-PCR products was determined by hybridizing the membranes with nested oligonucleotides end-labeled with [^{32}P] γ -ATP (Amersham), as described earlier (Markkula *et al.* 1993, Kananen *et al.* 1995). The blots were exposed to X-ray film (Fuji RX, Tokyo, Japan).

Aciclovir (ACV) and ganciclovir (GCV) treatments

Six single TG (Inha/Tag) and five double TG (Inha/TK–Inha/Tag) male mice, and five single and five double TG female mice, were treated at the age of 6.5 months with GCV (150 mg/kg body weight $\times 2$ i.p., Cymevene, Hoffmann La Roche AG, Basel, Switzerland) for 14 days. ACV (a generous gift from Glaxo Wellcome, Herefordshire, UK) treatment was administered to 6 single and 7 double TG male mice, and to 6 single and 5 double TG female mice, starting at the age of 5–6 months by adding ACV to drinking water (measured daily consumption 300–400 mg/kg body weight) for 2 months. The drinking water with ACV was changed every second

day. At the beginning of the experiment, the gonadal sizes were assessed during laparotomy under general anesthesia, by measuring their length, depth and width. The product of these three measures (expressed in mm^3) was taken as approximation of the total gonadal volume. At the end of the treatment period, the mice were anesthetized, blood was collected by cardiac puncture, the size of the tumors was measured as above, and the tissues collected were either frozen or fixed in Bouin's solution or 4% paraformaldehyde, dehydrated and embedded in paraffin.

In situ 3'-end labeling of DNA in apoptotic cells

Paraffin-embedded tissue samples were cut into 5 μm thick sections. Sections were rehydrated through alcohol series. Permeability of the cell membranes was increased by incubating the sections in 400 μg proteinase K (Boehringer) in 200 ml PBS for 15 min. Indigenous peroxidase activity was quenched by incubating the samples for 5 min in 5% H_2O_2 . Apoptosis was identified in the gonadal tissue sections using an *in situ* DNA 3'-end labeling kit (Oncor, Gaithersburg, MD, USA) as previously described (Vaskivuo *et al.* 2000). After apoptosis detection, the sections were lightly counterstained with hematoxylin. Apoptosis was analysed using light microscopy from an area of the section that mainly comprised tumor cells. Positive cells were counted per visual field by two independent observers using $\times 400$ magnification.

Hormone measurements

Testosterone was measured by RIA from diethyl ether extracts of the sera and testis homogenates as described

Figure 3 Histological analysis of testicular and ovarian tumors showing the Leydig cell origin of the tumors in 7-month-old Inha/Tag (A) and Inha/Tk-Inha/Tag (B) male, and granulosa cell origin of the tumors in 7-month-old Inha/Tk-Inha/Tag (C) and Inha/Tag (D) female. DF, developing follicle; GCT, granulosa cell tumor; LCT, Leydig cell tumor; ST, seminiferous tubule; (magnification $\times 200$). Scale bar, 40 μ m.

earlier (Huhtaniemi *et al.* 1985). Luteinizing hormone (LH) content of the sera and pituitary homogenates was measured by a supersensitive immunofluorometric assay for rat LH (Delfia; Wallac Oy, Turku, Finland) as described earlier (Haavisto *et al.* 1993). Progesterone was measured from diethyl ether extracts of the sera and tumor homogenates by RIA, as previously described (Vuorento *et al.* 1989). Estradiol was measured by RIA from diethyl ether extracts of the sera (Jaakkola *et al.* 1990).

Cytotoxicity assay in granulosa cells in vitro

Passages 18–21 of KK-1 murine granulosa cells (Kananen *et al.* 1995) were incubated on culture dishes (10 cm diameter) (Greiner Labortechnik, Frickenhausen, Germany) at 37 °C in a humidified atmosphere in an incubator containing 5% CO₂. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)–Hams's

F12 (1:1, with 0.365 g/l L-glutamine) (Sigma Chemical Co., St Louis, MO, USA), 10% heat-inactivated fetal calf serum (FCS; Bioclear, Berks, UK), and 0.1 g/liter gentamicin (Biological Industries, Bet-HaEmek, Israel). Near-confluent plates were used for transfections.

DNA transfections were made by using lipofectin (Gibco BRL) according to the manufacturer's protocol with pCMV-TK construct including the cytomegalovirus (CMV) promoter and the HSV-TK (pUHC13–1, Gossen & Bujard 1992). The pCMV-luc construct including the firefly luciferase coding sequence was used in control transfections to assess the specificity of cytotoxicity. Eight hours after the transfection the medium was changed, the cells were trypsinized 24 h after transfections, suspended in DMEM which contained 5% FCS but no Phenol Red (cDMEM-5) (Gibco BRL), counted under a microscope, diluted accordingly, and plated on 24-well cell culture plates (Greiner Labortechnik), 2×10^4 cells/0.5 ml, at

Figure 4 RT-PCR and Southern detection analysis of expression of Tag (A) and TK (B) mRNA in double TG (Inha/TK-Inha/Tag) tumors and single TG (Inha/Tag) tumors and Inha/TK gonads as controls. The locations of the expected hybridization signals (795 bp for Tag and 480 bp for TK) are indicated. (C) Ethidium bromide staining of the agarose gel of the RT-PCR analysis of mouse β -actin mRNA for control, to demonstrate equal quality and amount of mRNA used in each sample.

5–10% confluency at the beginning of the experiments. After 12 h, the cells were incubated in cDMEM-5 (0.5 ml/well in 24-well plates) containing 0–100 mg/l GCV or ACV, for 4 days.

The toxicity and cell-killing efficacy were assessed daily in quadruplicate, using a colorimetric Thiazol Blue (MTT) assay (Mosmann 1983). Fifty microliters of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl Blue; Sigma Chemical Co.) in serum-free DMEM without Phenol Red were added into the wells. After incubation for 4 h at 37 °C, 0.5 ml 2-propanol was added, and the plates were shaken for 15 min. The absorbance at 570 nm was measured using a Victor 1420 Multilabel Counter (EG&G Wallac, Turku, Finland), using 630 nm as the reference wavelength.

Statistical analysis

A Macintosh version of the SuperANOVA program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for one factor analysis of variance, followed by Fisher's Protected LSD *post hoc* test. The apoptosis data were analyzed using the Mann–Whitney *U* test.

Results

Expression of TK and Tag in the TG mouse gonads and gonadal tumors

In the present study, three transgenic mouse lines expressing HSV-TK under a 6.0 kb long mouse inhibin- α subunit promoter were generated (Inha/TK). Immunohistochemical analysis of the adult Inha/TK mouse testes

showed that TK was expressed both in Leydig and Sertoli cells (Fig. 2A). Expression of TK was also found in the adrenal cortex and pituitary gland (data not shown). An identical pattern of staining for Tag was found in the Inha/Tag mice previously generated (Fig. 2B). Interestingly, in the Inha/Tag mouse (Fig. 3A) as well as in the double TG Inha/TK-Inha/Tag mouse testes (Fig. 3B), only Leydig cells give rise to tumors, while the Tag-expressing Sertoli cells appear resistant to tumorigenesis. In ovaries of the Inha/TK-Inha/Tag double TG mice, tumors of granulosa cell origin are formed (Fig. 3C), which are akin to those previously found in the Inha/Tag (Fig. 3D) mice.

To further confirm the simultaneous expression of both TK and Tag in the tumors formed, RT-PCR analysis was carried out (Fig. 4). Tag expression was found in testicular and ovarian tumors of both Inha/Tag and Inha/Tag-Inha/TK mice, while the Inha/TK mice, used as a control, did not show the Tag-specific signal of about 800 base pairs (bp) in length. Respectively, RT-PCR confirmed the presence of a TK mRNA signal of expected size (480 bp) in the Inha/TK mice as well as in double TG mice expressing both TK and Tag. In addition, a shorter 250 bp truncated product of TK was detected in the testis. The results obtained agree well with earlier findings on similar truncations of the TK message in the testis (Al-Shawi *et al.* 1991, Markkula *et al.* 1993). Although the RT-PCR data must be considered qualitative, similar levels of β -actin mRNA amplification in all samples indicate that roughly similar amounts of RNA were used in each RT-PCR reaction.

Tumor size

GCV treatment decreased the total gonadal volume, including the tumor and adjoining normal gonadal tissue, in male and female double TG mice by an average of 40% ($P < 0.05$) (Fig. 5A and B), compared with single TG (Inha/Tag) male and female mice with concomitant increases of 57 and 53% ($P < 0.05$) (Fig. 5A and B) in the total gonadal volumes, respectively. Due to their worsened general condition, three of the five control females were killed before the end of the 2 week treatment period. Therefore, the gonadal weight-gain of these animals would have been even greater had they survived until the end of the experiment, and, respectively, the difference in gonadal weights between treated and control animals would have been more impressive. Figure 6 depicts a representative testicular tumor before and after GCV treatment.

ACV treatment did not show significant therapeutic effects in the form of total gonadal volume reduction (data not shown).

Apoptosis

Apoptosis was analyzed in the tumors of double and single TG mice after 2 weeks GCV treatment. Double TG mice

Figure 5 (A) The changes in total gonadal volumes of single (dashed lines) and double (continuous lines) TG female mice after a 2 week GCV treatment. The values are means of both gonads. Due to worsened general condition, three of the control females were killed before the end of the planned 14 day treatment (days 4, 9 and 11). (B) The changes in total gonadal volumes of single (dashed lines) and double (continuous lines) TG male mice after a 2 week GCV treatment. The values are means of both gonads.

had significantly more apoptotic tumor cells per visual field than single TG (Inh α /Tag) mice (Table 1). Apoptotic tumor cells were scattered around the tumor tissue in single and double TG animals. While apoptotic cells were located sparsely in the single TG mice, there was no marked difference in the location

or the number of apoptotic cells between male and female mice (Fig. 7). Apoptosis was present abundantly in tumor cells but it was also found in the non-malignant tissue. It was also found in granulosa cells of antral follicles in the ovaries and in testicular tubules (not shown).

Figure 6 The same testicular tumor, photographed upon laparotomy before (a, the outline of the tumor is highlighted), and upon autopsy (b) after a 2 week GCV treatment (bar and divisions of the scale, 1 mm).

Table 1 *In situ* 3'-end labeling analysis of apoptotic DNA in tumor tissue of single TG (Inha/Tag) and double TG (Inha/TK-Inha/Tag) mice after 2 weeks GCV treatment. The values are presented as positive cells per visual field

	Females		Males	
	Single TG (n=4)	Double TG (n=6)	Single TG (n=4)	Double TG (n=3)
Range	0–12	13–98	2–39	55–136
Median	7	23.5*	12.5	128*

* $P < 0.05$ vs single TG (Mann–Whitney *U* test).

Table 2 Concentrations of selected reproductive hormones in control single TG (Inha/Tag) and double TG (Inha/TK-Inha/Tag) mice after the 2 week GCV treatment. The values are geometric means (range of S.E.)

	Males		Females	
	Single TG (n=6)	Double TG (n=5)	Single TG (n=5)	Double TG (n=5)
Hormones				
Serum testosterone (nmol/l)	1.72 (1.22–2.42)	8.59** (5.93–12.5)	0.48 (0.44–0.52)	1.06 (0.68–1.65)
Tumor testosterone (pmol/mg)	0.10 (0.06–0.18)	0.64* (0.50–0.82)	ND	ND
Serum LH (μ g/l)	0.10 (0.05–0.20)	0.08 (0.05–0.11)	0.39 (0.27–0.57)	0.18 (0.13–0.27)
Pituitary LH (μ g/pituitary)	0.41 (0.32–0.53)	0.66 (0.48–0.90)	0.27 (0.16–0.44)	0.79* (0.59–1.04)
Serum estradiol (pmol/l)	ND	ND	7.55 (4.47–12.7)	9.02 (7.18–11.3)
Tumor progesterone (fmol/mg)	9.62 (6.05–15.3)	15.9 (8.45–29.7)	ND	ND

ND, not determined. * $P < 0.05$; ** $P < 0.01$; compared with respective single TG, tested after logarithmic transformation of the data.

Hormone levels

After GCV treatment of the male mice, their testicular and serum testosterone concentrations were significantly higher ($P < 0.05$) in double TG mice, compared with the control single TG mice. No significant differences were observed in serum LH levels (Table 2). In females, the GCV-treated double TG females had significantly higher pituitary LH levels ($P < 0.05$) compared with the single TG controls (Table 2). Serum estradiol levels in females, and tumor progesterone levels in males were not significantly different between the single and double TG animals.

ACV treatment had no effect on any of the hormone parameters measured (Table 3).

In vitro cytotoxicity

The cytotoxicity of GCV and ACV was also compared *in vitro*. After 48 h incubation in the presence of GCV, the growth of CMV-TK transfected cells was reduced to 55 and 31% of maximal growth at concentrations of 1 and 10 mg/l respectively, while the growth of the CMV-luc transfected cells was reduced to 74 and 68%, with the respective GCV doses ($P < 0.001$, TK vs. luc). After 96 h incubation, the specific cytotoxicity of GCV was more obvious, the TK-cell growth being 51, 20 and 7% at GCV concentrations 0.1, 1 and 10 mg/l compared with luc-cell growth of 84, 78 and 56% ($P < 0.001$, TK vs. luc; Fig. 8A).

When compared with GCV, ACV at similar concentrations was more cytotoxic to the cells in general, and it showed no specific cytotoxicity related with TK-expression in the cells either after 48 or 96 h of incubation (Fig. 8B).

Table 3 Concentrations of selected reproductive hormones in control single TG (Inha/Tag) and double TG (Inha/TK–Inha/Tag) mice after the 2 month ACV treatment. The values are geometric means (range of s.e.)

	Males		Females	
	Single TG (n=6)	Double TG (n=7)	Single TG (n=6)	Double TG (n=5)
Hormones				
Serum testosterone (nmol/l)	2.32 (1.81–2.98)	2.26 (1.57–3.25)	1.13 (0.81–1.56)	1.45 (0.77–2.73)
Tumor testosterone (pmol/mg)	0.10 (0.05–0.22)	0.24 (0.17–0.33)	0.02 (0.01–0.04)	0.01 (0.01–0.01)
Serum LH (µg/l)	0.09 (0.05–0.14)	0.05 (0.03–0.08)	0.08 (0.05–0.14)	0.02 (0.01–0.04)
Pituitary LH (µg/pituitary)	0.73 (0.57–0.93)	0.71 (0.50–1.01)	0.23 (0.13–0.43)	0.15 (0.08–0.30)
Serum progesterone (nmol/l)	1.68 (1.26–2.23)	2.85 (2.52–3.23)	6.00 (4.08–8.81)	6.27 (4.56–8.61)
Tumor progesterone (fmol/mg)	14.9 (8.51–25.9)	19.9 (11.0–36.1)	286 (244–336)	129 (69.5–241)

Discussion

We have designed a TG mouse model, which enables us to study the potential role of gene therapy by antiherpes treatment of HSV-TK-expressing cells. A major limitation of gene therapy of cancer is the difficulty of transducing a large enough proportion of the tumor cells. With retroviral vectors, typically less than 10% of the cells are successfully transduced with *in vivo* delivery systems (Culver *et al.* 1992). Although significantly better transduction efficiency has been shown for adenoviral vectors, 100% transduction is never achieved (Trapnell 1993). In our TG mouse model all the tumor mass expresses the HSV-TK, which enabled us to effectively block tumor growth.

In somatic gene therapy, all tumor cells need not express the HSV-TK gene because of the so-called bystander effect, which means response to the treatment of surrounding non-transduced cells (Freeman *et al.* 1992, 1993, Kolberg 1992, Takamiya *et al.* 1992). Because of this effect the regression of a tumor mass is possible even when only 10% of the tumor cells express the HSV-TK gene (Freeman *et al.* 1993). The mechanism of the bystander effect is not fully understood. The level of HSV-TK activity in transduced tumor cells (C Y Chen *et al.* 1995), uptake of the phosphorylated GCV through apoptotic vesicles into the non-transduced cells (Freeman *et al.* 1993), transfer of phosphorylated GCV through inter-cellular gap junction-mediated communication (Bi *et al.* 1993, Pitts 1994, Fick *et al.* 1995, Yang *et al.* 1998, Carystinos *et al.* 1999) as well as the independence of cell–cell contacts (Bai *et al.* 1999, Princen *et al.* 1999) have been shown to be related to the bystander effect. The bystander cells have also been shown to be able to protect the HSV-TK transduced cells from GCV-mediated cyto-

toxicity, which might either decrease the effect of therapy, or increase it by permitting prolonged synthesis of toxic metabolites in longer surviving transduced cells (Wygoda *et al.* 1997).

Although our study did not compare equal doses or modes of administration of the two antiherpes drugs, it clearly demonstrated the superior antitumoral effect of the intraperitoneally administrated GCV over peroral ACV. Recently, the possible mechanisms for superior cytotoxicity of GCV over ACV in HSV-TK-expressing cells was reported (Rubsam *et al.* 1998). ACV was shown to be less cytotoxic because of its lower degree of phosphorylation and less effective incorporation into DNA. GCV incorporated into DNA without inhibiting DNA synthesis, thus permitting cells to complete one cell division, after which they were arrested in the early S phase.

Also increased efficacy of cell killing, without concomitantly increased toxicity, has been reported by replacing GCV with ACV (Tong *et al.* 1998). However, in our *in vitro* experiments, GCV proved to be more effective and less cytotoxic than ACV. This finding raises the question about possible differences in mechanisms of action of these cytotoxic agents in different cell types. It has been shown that ACV acts as a chain terminator in DNA synthesis because of the lack of a 3'-hydroxyl group (Furman *et al.* 1980, Reardon & Spector 1989), while GCV incorporates into DNA throughout the genome and allows further DNA synthesis by having a hydroxyl group on its sugar ring analogous to a 3'-hydroxyl on dGMP (Cheng *et al.* 1983, St Clair *et al.* 1987, Rubsam *et al.* 1998). The different modes of incorporation into DNA might have critical effects on the apoptotic machinery, which has been shown to participate in many cases in cell killing of the HSV-TK/antiherpes drug systems (Freeman *et al.* 1993,

Figure 7 *In situ* 3'-end labeling of apoptotic cells (arrows) in the gonadal tumors of female double TG (A), female single TG (B), male double TG (C) and male single TG mice. Scale bar, 5 μ m.

Colombo *et al.* 1995, Samejima & Meruelo 1995, Wei *et al.* 1998, Kuriyama *et al.* 1999).

In our study, GCV was found to increase apoptosis in the double TG mice. It has been previously suggested that GCV treatment of TK transfected cells involves accumulation of p53 protein and sensitizes cells for FAS ligand, tumor necrosis factor (TNF) and TNF-related, apoptosis-inducing, ligand (TRAIL)-induced cell death (Beltinger *et al.* 1999). It has also been proposed that GCV increases the expression apoptosis agonists genes *bax* and *bak*, which participate in regulation of apoptosis at the mitochondrial level (Beltinger *et al.* 2000, McMasters *et al.* 2000).

To obtain clearer results we treated mice with prominent tumors, and therefore not all animals survived until

the end of treatment. It is noteworthy, that the survival rate was 100% in the double TG mice, whereas no female control mice survived until the end of the experiment because of aggressive tumor growth. The trend in the tumor growth curves was clear, showing an unambiguous effect of GCV on tumor growth, although a minor toxic effect of GCV cannot be ruled out. Since the gonadal sizes before and after the treatment represent the tumor and adjoining normal gonadal tissue combined, the size reduction of the tumoral part must have been even greater than reflected by change of the combined size.

The increase in testosterone levels in double TG male mice after GCV treatment apparently demonstrated functional recovery of the remaining healthy testis tissue.

Figure 8 Comparison of ganciclovir (A) and aciclovir (B) cytotoxicity in CMV-TK and CMV-luc transfected KK1 cells. The growth rates are presented as percentages of original number of plated cells. Values are means $\pm$ S.E. of quadruplicates in one of three similar experiments (** $P < 0.01$, *** $P < 0.001$ vs luc 48 h and luc 96 h).

We have earlier reported that the Tag male mice with testicular tumors produce low amounts of testosterone (Kananen *et al.* 1996). An opposite trend was observed in serum LH. These two hormonal responses after GCV

treatment are signs of recovery of normal pituitary-testicular function upon shrinkage of the testicular tumors after therapy. In females, high pituitary LH levels in double TG mice can also be taken as signs of increased

gonadal feedback and recovery of gonadal endocrine function. In conjunction with the persistence of gonadal volumes, the unaltered hormonal values after ACV treatment demonstrated the lack of tumor reduction by this treatment.

In conclusion, our results indicate that the strategy of targeted expression of the HSV-TK suicide gene can be highly effective and suitable for treatment of gonadal tumors by GCV. We are currently developing efficient, targetable vectors for gene transfer, to allow the application of the HSV-TK/GCV technique for somatic gene therapy.

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