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Targeted Expression of Toxic Genes Directed by Pituitary Hormone Promoters: A Potential Strategy for Adenovirus-Mediated Gene Therapy of Pituitary Tumors*

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ABSTRACT

Pituitary adenomas cause clinical manifestations because of mass effects and excess hormone production. This group of tumors represents a tractable target for gene therapy because they are rarely metastatic and because reductions in tumor size and function, in addition to those achieved after surgery, may be of clinical benefit. In this report we describe a strategy for targeting the expression of toxic genes to pituitary cells using adenoviral vectors. Pituitary hormone promoters (human GH or glycoprotein hormone α-subunit) were used to express either a marker gene [β-galactosidase (β-gal)] or a toxic gene [herpes simplex virus thymidine kinase (TK)]. In GH-producing GH_3 cells and in α-subunit-producing pituitary tumor cell lines, recombinant adenoviruses containing either the α-subunit promoter (AdαGal; AdαTK) or the GH promoter (AdGHGal; AdGHTK) were expressed at high levels. Using histological studies and assays for β-gal activity, expression was shown to persist for at least 21 days, and it was relatively selective for pituitary cell lines. Cytotoxicity studies were performed using the TK-containing vectors and treatment with ganciclovir. Both AdGHTK and AdαTK caused greater than 95% cytotoxicity of GH_3 and αT3 cells, respectively, at a viral dose (multiplicity of infection, 5 plaque-forming units/cell) that induced minimal toxicity using control viruses. Little cellular toxicity was seen using a nonpituitary cell line (T47D breast tumor cells). The AdGHTK virus also caused marked reduction in the size of GH_3 cell tumors that were propagated in nude mice. These studies suggest that adenoviral vectors carrying human pituitary gland specific promoters may be useful for developing gene therapy strategies for the treatment of pituitary adenomas. (*J Clin Endocrinol Metab* **84:** 786–794, 1999)

PITUITARY adenomas are typically benign tumors that arise in the sella turcica of the brain. They comprise up to 15% of primary intracranial neoplasms (1). Pituitary adenomas create clinical manifestations because of the overproduction of hormones or because of mass effects of the enlarging tumor within the confined space of the sella turcica. There are five major types of pituitary tumors, reflecting the different cell types in the pituitary gland. These tumor types include ACTH-producing tumors that cause Cushing's disease, prolactinomas that hypersecrete PRL resulting in reproductive abnormalities, GH-secreting tumors that cause the syndromes of acromegaly and gigantism, gonadotropin-secreting tumors that hypersecrete LH and/or FSH, and TSH-producing tumors that result in hyperthyroidism. Because of its anatomical location in the sella turcica, mass effects of pituitary adenomas frequently include visual field defects resulting from compression of the optic nerves, other cranial nerve deficits, hypopituitarism, and, occasionally, invasion into surrounding structures.

Current therapies for pituitary tumors include surgery, radiotherapy, and pharmacological approaches for a subset of tumor types (2). For microadenomas (<1 cm), transsphenoidal surgery is successful in the majority (70–90%) of cases in the hands of an experienced surgeon. However, larger macroadenomas are rarely cured by surgery alone (3, 4). Medical therapies are available for selected types of pituitary tumors. In the case of prolactinomas, dopamine agonists markedly reduce hormone secretion and reduce tumor size in most patients (5). However, dopamine agonists do not cure the tumors, and they recur if the medications are discontinued. Somatostatin analogs have also been used for the treatment of GH-secreting adenomas and for TSH-secreting adenomas. Finally, radiotherapy is used as an adjunctive treatment after surgery and in rare instances as primary treatment for pituitary tumors. Although radiotherapy is often effective, several years are usually required before it has full impact, and potential complications include hypopituitarism, secondary tumors, and occasional neural deficits (4).

Recently, gene therapy using adenoviral vectors to deliver the herpes simplex virus-thymidine kinase (HSV-TK) followed by ganciclovir (GCV) administration has been developed as a strategy for the treatment of various types of malignant tumors (6, 7). Partial efficacy has been shown in

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tumors carried in animal models (8–13) as well as in trials of human malignancies. In general, these approaches have relied on viral promoters to achieve high level expression of the suicide gene (9, 10). One of the limitations of this type of adenoviral vector is that they can infect nontumorous cells as well as tumor cells, leading to potential toxic effects in normal tissues. However, this problem might be circumvented if toxic gene expression could be restricted to tumor cells using cell-specific promoters.

Pituitary tumors, because of characteristic expression of hormone genes, represent an attractive model for developing targeted expression of toxic genes. A variety of pituitary cell-type specific promoters have been identified. These include the promoters of the major pituitary hormones as well as receptors for hypothalamic releasing hormones and a number of transcription factors involved in the development of the pituitary gland. For example, transient gene expression studies (14) and transgenic experiments (15, 16) have documented restricted expression of the GH and glycoprotein hormone α-subunit promoters in pituitary cells (17, 18). In addition, the transcriptional activity of these promoters is relatively high, suggesting that they might be capable of delivering high levels of toxic genes to pituitary tumor cells. In this report, we describe experiments using adenoviruses containing the human GH and α-subunit promoters to target genes to pituitary cell lines and transplanted pituitary tumors.

Materials and Methods

Cells and culture conditions

GH_3 GH- and PRL-producing pituitary tumor cells and HEK293 embryonic kidney cell lines were obtained from American Type Culture Collection (Manassas, VA). The α-subunit-secreting pituitary tumor cell line, αT3–1 (hereafter referred to αT3 cells), was provided by Dr. P. Mellon (University of California-San Diego). T47D breast cancer cells were provided by V. Craig Jordan (Northwestern University Medical School, Chicago, IL).

GH_3 cells were grown in Optimem containing 5% FBS. HEK293 cells were maintained in DMEM supplemented with 10% FBS. αT3 cells were grown in DMEM-Ham's F-12 supplemented with 10% FBS at 37 C with 5% CO_2. T47D cells were cultured in RPMI with 10% FBS.

Generation of recombinant adenoviral vectors

The recombinant type 5 (Ad5 309/356) adenoviral vector has a backbone in which the E3 region has been deleted. This virus also carries a 2-bp deletion in the E4 6/7 open reading frame (19). Ad5 309/356 was constructed by ligating viral DNA fragments from 0–75.0 map units of dl 309 and 75.0–100 map units or dL 356. A cassette containing a pituitary hormone-specific promoter and β-galactosidase (β-gal) or the herpes simplex virus type 1 thymidine kinase (HSV-TK) gene was inserted in place of the E1 deletion. Two pituitary hormone-specific promoters were used: the human GH promoter and the human α-subunit promoter. The human GH promoter consists of nucleotides -336 to $+58$ from the human GH 5′-flanking region, and the human α-subunit promoter consists of nucleotides -846 to $+45$ of the human α-subunit 5′-flanking region. These sequences were derived by PCR from human genomic DNA, cloned into pGem3Zf(+) (Promega Corp., Madison, WI), and the sequences were verified by DNA sequencing. The HSV-TK sequences were derived from plasmid pPNT by PCR amplification using specific primers. The HSV-TK sequences were cloned into pGem7Zf(+) (Promega Corp.) and verified by DNA sequencing. The HSV-TK fragment was ligated to each promoter such that HSV-TK expression is under the control of pituitary hormone-specific promoters, and transcription is terminated by the simian virus 40 polyadenylation signal. Subsequently, the promoter-HSV-TK gene fragment was cloned into a pcDNA3-based vector downstream of adenoviral sequences between map units 0 and 1.2, which comprise the lefthand inverted terminal repeat and packaging signal of the adenovirus (Fig. 1). The *Escherichia coli lacZ* gene encoding β-gal and the preceding nuclear localization signal were ligated to each of the promoter-containing plasmids by standard cloning techniques. Subsequently, the promoter-β-gal fragments were cloned into a pcDNA3-based vector downstream of adenoviral sequences between map units 0 and 1.2 (Fig. 1).

The adenoviral transfer plasmids were digested with *Hin*dIII and *Xba*I. The fragment containing the left end adenoviral sequences, the promoters, and the HSV-TK or β-galactosidase genes were then ligated with *Xba*I-digested Ad5 309/356 DNA representing 3.8–100 map units. The Ad5 309/356 is a recombined virus deleted of the early region 3 (E3). The ligation products were then transfected into 293 cells to allow production of recombinant adenoviral DNA and packaging into virions. The recombinant adenoviruses contain the HSV-TK or *E. coli* β-galactosidase genes fused to the human GH promoter (AdGHTK, AdGHGal) or the human α-subunit promoter (AdαTK, AdαGal). Individual clones of AdGHTK, AdGHGal, AdαTK, and AdαGal were purified and titrated using plaque assays. The expression cassette in the adenoviral vectors was confirmed by DNA sequencing of viral DNA using specific primers.

As a positive control for expression and detection of β-galactosidase activity, parallel studies were carried out using AdCMVGal, an adenovirus vector similar in design, but with the cytomegalovirus (CMV) promoter, nuclear localization signal, and β-galactosidase as the reporter gene.

Evaluation of efficiency of recombinant adenoviral infection and recombinant gene expression

The transduction efficiency of adenoviral vectors in pituitary tumor cell lines was tested using AdGHGal, AdαGal, and AdCMVGal. Cells were plated in 12-well culture plates at a density of 2×10^5 cells/well. The next day, infections of cell lines were carried out by the addition of viral solutions to cell monolayers and incubation at 37 C for 1 h with brief agitation every 15 min. After the addition of culture medium, infected cells were returned to the 37 C incubator, and medium was changed 24 h later. For studies using a range of multiplicity of infection (MOI) doses, triplicate wells were infected with each virus at plaque-forming units (PFU) of 1, 5, 10, or 25 for 48 h. For long term expression studies, cells were infected using a MOI of 5 for 1, 2, 3, 5, 7, 10, 14, or 21 days. Cells were fixed with 1.0% glutaraldehyde for 10 min, washed with phosphate-buffered saline (PBS; pH 7.4), and then incubated with X-gal substrate solution (10.0 mmol/L potassium ferrocyanide, 10.0 mmol/L potassium ferricyanide, 1 mmol/L $MgCl_2$, 20% Nonidet P-40, and 0.1% X-Gal in PBS) at 37 C for 2 h to evaluate β-gal expression at the histological level.

Triplicate wells of infected cells were also used to measure β-gal activity using *O*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate (Sigma Chemical Co., St. Louis, MO). Culture medium was aspirated, cell lysis solution was added, and lysates were mixed with the ONPG substrate solution and incubated in 37 C for 2 h. The reaction was stopped with 100 μL 1 mol/L Na_2CO_3. Absorption was measured at 405 nm, and β-gal activity was calculated using a standard curve.

Western blot analysis

Cells were plated in six-well culture dishes at a density of 1.5×10^6 cells/well. The next day, cells were infected with AdGHTK or AdαTK at a MOI of 10 PFU/cell. Forty-eight hours after infection, cell cultures were washed twice with PBS, then scraped into 0.3 mL sample buffer containing 20 mmol/L Tris-HCl (pH 7.5), 2 mmol/L dithiothreitol, 0.5 mol/L KCl, 1 mmol/L phenylmethylsulfonylfluoride, and 20% glycerol. After three cycles of freezing in a dry ice-ethanol bath and thawing in a 37 C water bath, samples were centrifuged at $12{,}000 \times g$ for 20 min at 4 C, and the supernatants were stored at -70 C until assayed. Solubilized samples were separated by SDS-PAGE using 4.5% stacking and 10% separating gels cast in a minigel apparatus (Hoeffer, San Francisco, CA). After electrophoresis, the gels were rinsed with transfer buffer containing 25 mmol/L Tris base, 192 mmol/L glycine, and 20% methanol. Electroblotting of the proteins to nitrocellulose paper was performed at 30 mA overnight at 4 C. Residual gels were stained with Coomassie blue (0.012% Coomassie blue, 40% methanol, and 7% acetic acid) to evaluate the transfer of proteins.

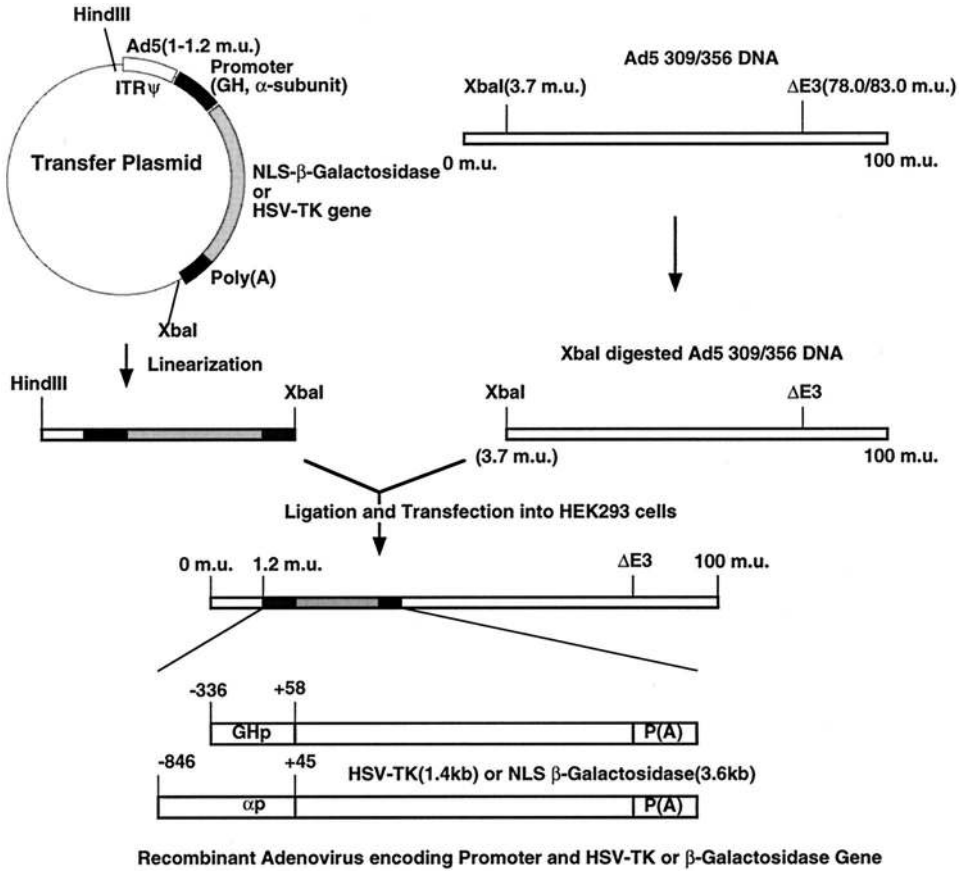

FIG. 1. Generation of recombinant adenovirus vectors. The transfer plasmid containing the extreme lefthand adenoviral DNA sequences, promoters, and β-gal or HSV-TK gene were digested with *Hin*dIII and *Xba*I to linearize DNA. The linearized fragments were ligated onto *Xba*I-digested Ad5 309/356 DNA representing 3.8–100 map units. The ligation products were then transfected into 293 cells, whereby the resulting recombinant adenoviral DNA was replicated and packaged into virions. The recombinant adenoviruses carried the HSV-TK or *E. coli* β-gal gene fused to the human GH promoter (AdGHTK, AdGHGal) and the human α-subunit promoter (AdαTK, AdαGal).

After electroblotting, nonspecific protein binding was blocked using 3% milk in PBS for 2 h. Antibody against HSV-TK (rabbit polyclonal specific for HSV-TK; provided by Dr. S. M. Albelda, University of Pennsylvania Medical Center, Philadelphia, PA) was diluted 1:800 in 3% milk in PBS and then incubated overnight at 4 C followed by three 10-min washes in 0.1% Tween-20 in PBS. Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Promega Corp.; 1:5000) was added for 1 h at room temperature. After three washes in 0.1% Tween-20 in PBS, chemiluminescent substrate solution (LumiGlo, Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and incubated for 1 min. Samples were blot-dried and exposed to film for 3 min.

GCV sensitivity of AdGHTK- and AdαTK-infected cells

The sensitivity of adenovirus-infected cells to GCV was measured with a nonradioactive cell proliferation assay according to the manufacturer's protocol (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega Corp.). The day after plating 4×10^3 cells in triplicate wells of 96-well plates, adenoviral vectors were infected at different MOIs (0, 1, 5, 10, 100, and 500 PFU/cell). Sixteen hours after infection, increasing concentrations (0, 1, 5, 10, and 50 μg/mL) of GCV were added, and fresh medium containing GCV was added every 2 days. Cell viability was assayed on day 4 after adding GCV. The percent survival of cells is presented as the fraction of the absorbance found in the GCV-treated cells divided by that in the cells without GCV treatment (mean ± SD). For long term studies of infected tumor cells, MOIs of 0, 1, 5, and 10 were used, and 5 μg GCV were added to each well. Cell viability was assayed on days 0, 2, 4, 6, and 8 after adding GCV.

Treatment of tumor-bearing mice with adenoviral vectors in vivo

All studies involving the use of nude mice were approved by the Northwestern University Medical School animal care and use committee. GH_3 cells (1×10^7) were injected into the flank area of adult (7-week-old) athymic male nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN). Three weeks after injection of GH_3 cells, tumors of about 1.0 cm in diameter developed, and the mice were divided into four groups: 1) AdGHTK injection and GCV treatment (n = 8), 2) AdGHTK injection without GCV treatment (n = 8), 3) AdGHGal injection and GCV treatment (n = 8), and 4) injection of dialysis buffer used for adenovirus preparation with GCV treatment (n = 8). Adenoviral vectors (0.5×10^9 PFUs) in a total volume of 100 μL dialysis buffer were injected into a growing tumor from three directions on 2 successive days. The following day, GCV was administered ip at 100 mg/kg once daily for 10 days. The size of the tumor was measured every 2 days with calipers in three dimensions. Tumor size is presented as cubic millimeters. αT3 cells (1×10^7) were also injected into male and female nude mice, but no tumors developed.

Results

Gene transfer efficiency of AdGHGal, AdαGal, and AdCMVGal

The efficiency of adenoviral vector-mediated gene transfer to pituitary hormone producing GH_3 and αT3 cells was assessed after infection with AdGHGal, AdαGal, or AdCMVGal adenoviruses. Expression of β-galactosidase was detected by X-gal staining, and the enzymatic activity of β-galactosidase was quantitated in cell lysates. Consistent with the strong activity of the CMV promoter, 95–100% of GH_3 cells and αT3 cells were stained blue at 48 h after infection with AdCMVGal (MOI of 5–10 PFU/cell; data not shown). The intensity of X-gal staining was even stronger using a greater viral titer. Using the GH promoter-driven adenovirus, AdGHGal (MOI of 10 PFU/cell), 50–60% of GH_3

cells were stained at 48 h after infection (Fig. 2B). The percentage of GH_3 cells expressing β-galactosidase increased with time and reached 95–100% by 4–5 days after infection (Fig. 2C). Similar results were seen in αT3 cells using the α-subunit promoter-driven adenovirus, AdαGal. About 65–70% of αT3 cells were stained blue at 48 h (Fig. 2E), and the fraction of infected cells increased to 95–100% by 4–5 days after infection (Fig. 2F).

The intensity of X-gal staining was variable even though 100% of cells were stained blue. β-Gal activity in cell lysates was therefore measured to obtain a more quantitative index of the level of expression. Results with AdGHGal and the AdαGal vectors are expressed relative to the ubiquitously expressed AdCMVGal (MOI of 10 PFU/mL) to allow comparisons between different cell types. GH_3 cells infected with AdGHGal at an MOI of 10 PFU/cell had 16.2% of the activity of AdCMVGal. The activity of αT3 cells infected with AdαGal was 28.7% of the level of AdCMVGal (Fig. 3).

In an effort to assess the cell specificity of the AdGHGal and AdαGal vectors, they were each infected into the cell line producing the other hormone. The AdGHGal vector exhibited 24.1% of the activity in αT3 cells compared to GH_3 cells. The AdαGal vector was expressed in GH_3 cells at about 18.1% its activity in αT3 cells. In T47D breast cancer cells, AdGHGal expression was undetectable, and AdαGal showed 6.9% of the β-galactosidase activity seen in αT3 cells (Fig. 3).

Analysis of long term expression of the reporter gene demonstrated a gradual increase in β-gal activity in GH_3 cells and αT3 cells infected with AdGHGal or AdαGal, respectively, with peak expression occurring 5–7 days after infection (Fig. 4). Thereafter, the activity decreased, but it was still maintained until 21 days after infection. For cells infected with AdCMVGal, β-gal activity increased sharply and peaked earlier, but also decreased faster than in cells infected with AdGHGal or AdαGal.

Expression of TK

Western blot analyses were performed to examine the expression of the HSV-TK protein 48 h after infection with either AdGHTK or AdαTK. As shown in Fig. 5, expression

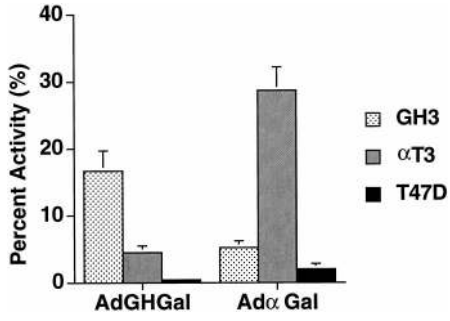

FIG. 3. Cell-specific expression of adenoviral vectors. GH_3 cells, αT3 cells, and T47D cells were infected with AdCMVGal, AdGHGal, and AdαGal at a MOI of 10. The β-gal activity was assayed at 48 h after infection. The activity of cells infected with AdGHGal and AdαGal was calculated as a percentage of the activity of AdCMVGal-infected cells. Results are expressed as the mean ± SD.

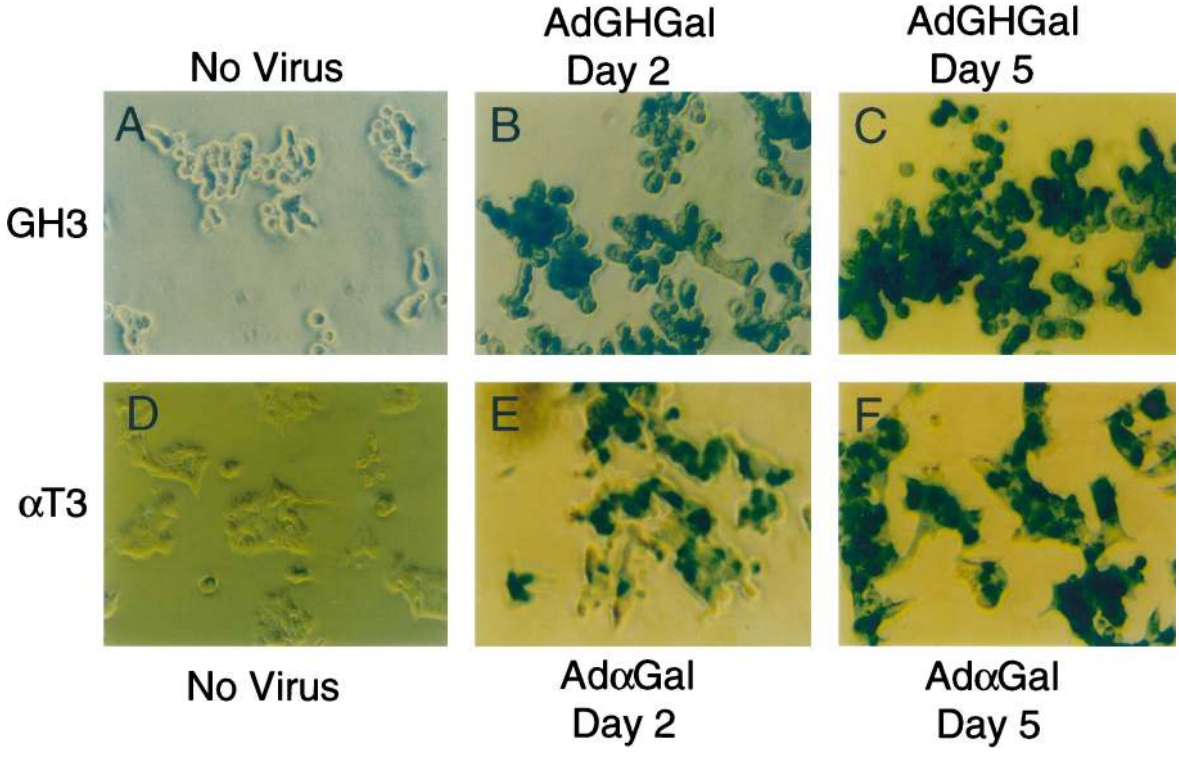

FIG. 2. The expression of β-gal in GH_3 cells and αT3 cells infected with AdGHGal or AdαGal (MOI of 10). Cells were fixed with 1.0% glutaraldehyde for 10 min, washed with PBS (pH 7.4), and then incubated with X-gal substrate solution at 37 C for 2 h. A, GH cells without virus; B, GH_3 cells at 2 days after infection of AdGHGal; C, GH_3 cells at 5 days after infection of AdGHGal; D, αT3 cells without virus; E, αT3 cells at 2 days after infection of AdαGal; F, αT3 cells at 5 days after infection of AdαGal.

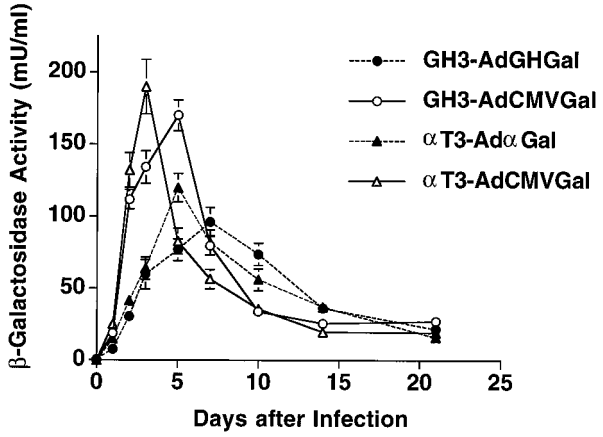

FIG. 4. Long term course of *lacZ* gene expression of GH_3 and αT3 infected with adenoviral vectors (5 PFU/cell of AdCMVGal and 10 PFU/cell of AdGHGal and AdαGal). Triplicate wells were used to measure *lacZ* gene expression by assessing β-galactosidase activity using ONPG as substrate. Infected cell lysates were added to the ONPG substrate solution and incubated at 37 C for 2 h. The reaction was stopped with 100 μL 1 mol/L Na_2CO_3. Absorption was measured at 405 nm, and the activities of individual wells were calculated by a standard β-gal activity curve. Results are expressed as the mean ± SD.

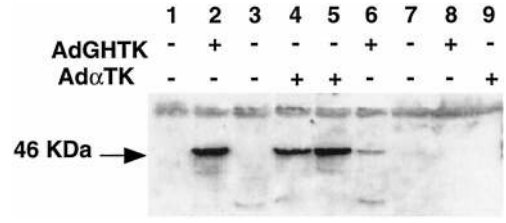

FIG. 5. Western blot analysis of HSV-TK protein. Cell extracts were analyzed for expression of the HSV-TK protein with a rabbit polyclonal antibody 48 h after infection with 10 PFU/cell of AdGHTK or AdαTK. The anti-HSV-TK antibody recognized a band at 46 kDa. Each sample was prepared using equal numbers of cells, and equal loading was confirmed by Coomassie blue staining (not shown). Lanes 1, 2, and 5, GH_3 cells; lanes 3, 4, and 6, αT3 cells; lanes 7–9, T47D.

of TK was detected in AdGHTK-infected GH_3 (lane 2) and αT3 cells (lane 6) and in AdαTK-infected αT3 (lane 4) and GH_3 cells (lane 5). The level of TK expression in AdGHTK-infected αT3 cells (lane 6) was weaker than that in GH_3 cells (lane 2), whereas AdαTK expression was similar in the two cell types (lanes 4 and 5). T47D cells infected with AdGHTK (lane 8) or AdαTK (lane 9) did not express the TK protein. These results demonstrate that the viral vectors express TK protein and confirm that expression occurs in a pituitary cell-preferential manner.

Ganciclovir sensitivity of GH_3 cells and αT3 cells infected with adenoviral vectors

Adenoviral vectors expressing the TK gene were used to investigate the sensitivity of tumor cell lines to expression of the toxic gene. To activate TK-mediated cytotoxicity, cells were exposed for 4 days to a range of GCV doses, and cell viability was determined using a cell proliferation assay. Without viral infection, high doses of GCV (50 μg/mL) induced partial cytopathic effects (~25% cell death) using either GH_3 or αT3 cells (data not shown). In addition, cytopathic effects were seen when adenoviral vectors were infected at high MOIs (>100 PFU/cell). For these reasons, experiments were performed using varying amounts of adenoviral vectors and a range of GCV doses. As controls, the cytotoxicity of the TK-expressing vectors was compared to that of the β-Gal-expressing vectors.

Using the AdGHTK virus, cytotoxicity increased in proportion to the amount of virus and in response to increasing amounts of GCV (Fig. 6A). For example, at a MOI of 10 PFU/mL, cytotoxicity of GH_3 cells infected with AdGHTK occurred with GCV concentrations as low as 1 μg/mL, and complete cell death occurred using 10 μg/mL GCV (Fig. 6A). Similar results were seen in αT3 cells infected with the AdαTK virus. There was 90% cell death at a dose of 5 μg/mL of GCV and a MOI of 10 PFU/mL (Fig. 6C). At doses of 100–500 PFU/mL, the AdαTK virus was toxic to αT3 cells, even at 1 μg/mL GCV. Little or no cytopathic effect was seen with the control AdGHGal and AdαGal viruses, except at very high amounts of the vectors (500 PFU/mL; Fig. 6, B and D).

We also evaluated the effects of the AdGHTK virus in αT3 cells and the AdαTK virus in GH_3 cells to assess whether TK expression from these pituitary hormone promoters is cell type specific. As shown in Fig. 6, E and F, the GCV sensitivity induced by these viruses was similar to that seen in cell lines that actively express the hormone promoters. These findings are consistent with the expression of β-Gal by these promoters in both cell lines (Fig. 3). In contrast, T47D breast cancer cells infected with AdGHTK or AdαTK did not show GCV sensitivity at low doses of adenoviral vectors (1–10 MOI; Fig. 6, G and H). These experiments indicate that AdGHTK or AdαTK viruses confer dose-dependent GCV sensitivity to pituitary, but not breast cancer, cell lines. However, because the promoters are active in both pituitary cell lines, their effects are not specific for the types of hormones produced by these cell lines.

The long term effects of the adenoviral vectors on cell growth were assessed at a dose of GCV (5 μg/mL) that is not toxic in the absence of viral infection. Cells were infected with varying doses of virus (MOIs of 0, 1, 5, and 10), and cell viability was determined during an 8-day period of treatment with GCV (Fig. 7). In GH_3 cells infected with AdGHTK (MOI of 1), there was 70% growth inhibition after 8 days (Fig. 7A). At higher doses of AdGHTK (5 and 10 PFU/mL), there was greater than 95% tumor cell growth inhibition. The growth rate of GH_3 cells was not affected by the AdGHGal virus, indicating that growth inhibition is caused by the expression of the TK gene. Similar results were seen in αT3 cells when they were infected with the AdαTK virus (Fig. 7B). At 1 PFU/mL, AdαTK caused 60% growth inhibition, and there was greater than 95% inhibition of αT3 cell growth at 5 and 10 PFU/mL. In contrast, there was no growth inhibition of αT3 cells infected with the AdαGal virus.

Effect of the AdGHTK virus on GH_3 cell tumors in vivo

GH_3 cells and αT3 cells were injected sc into nude mice in an effort to develop an *in vivo* model for assessing the effects of the recombinant adenoviruses. Tumors (601.7 ± 217.1 mm^3) developed with the GH_3 cells, but not with αT3 cells (data not shown). Therefore, subsequent experiments were performed using tumors developed from GH_3 cells.

Injection of the GH_3 cell tumors with AdGHTK virus, and

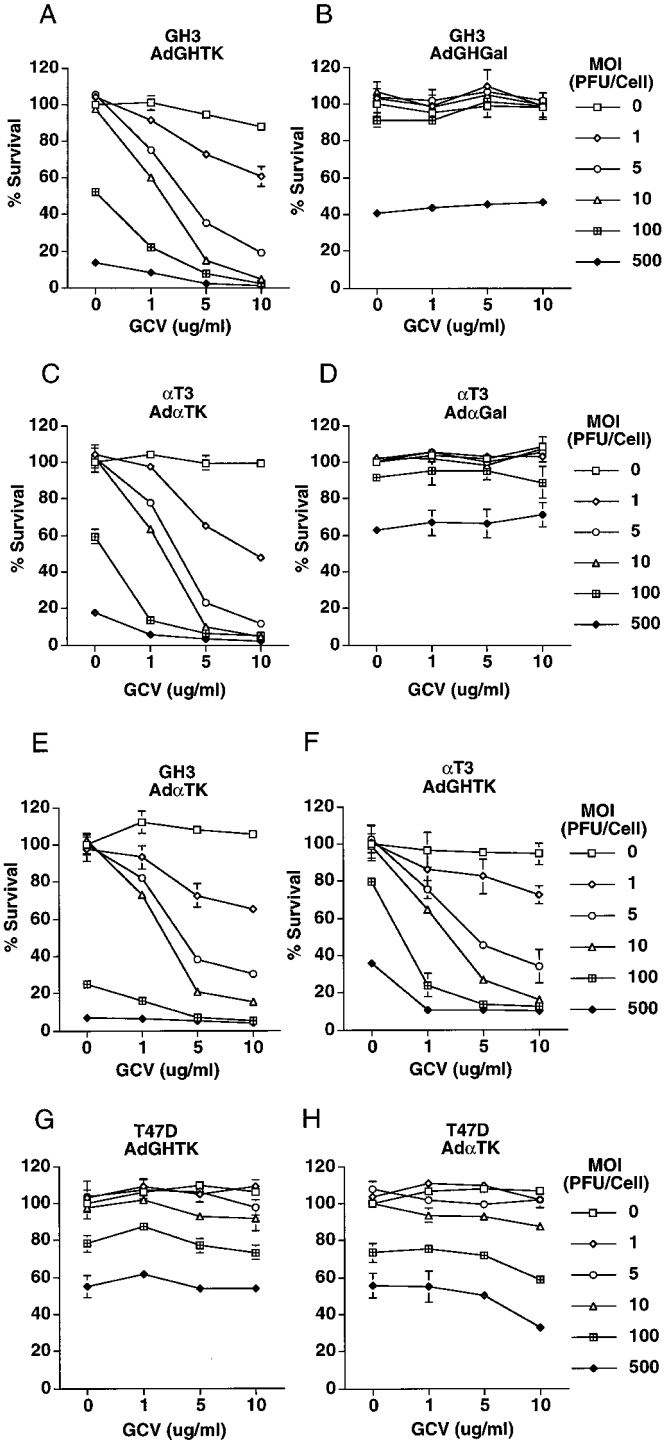

FIG. 6. GCV sensitivity of cells infected with adenoviral vectors. After infection of cell lines at different MOI of adenoviral vectors, cells were treated with varying doses of GCV for 4 days, and the cell viability was determined by a cell proliferation assay. The percent survival of cells was presented as a percentage of the absorbance found in the GCV-treated cells divided by that in the cells without GCV treatment (mean ± SD). A, GH_3 cells infected with AdGHTK; B, GH_3 cells infected with AdGHGal; C, αT3 cells infected with AdαTK; D, αT3 cells infected with AdαGal; E, GH_3 cells infected with AdαTK; F, αT3 cells infected with AdGHTK; G, T47D cells infected with AdGHTK; H, T47D cells infected with AdαTK.

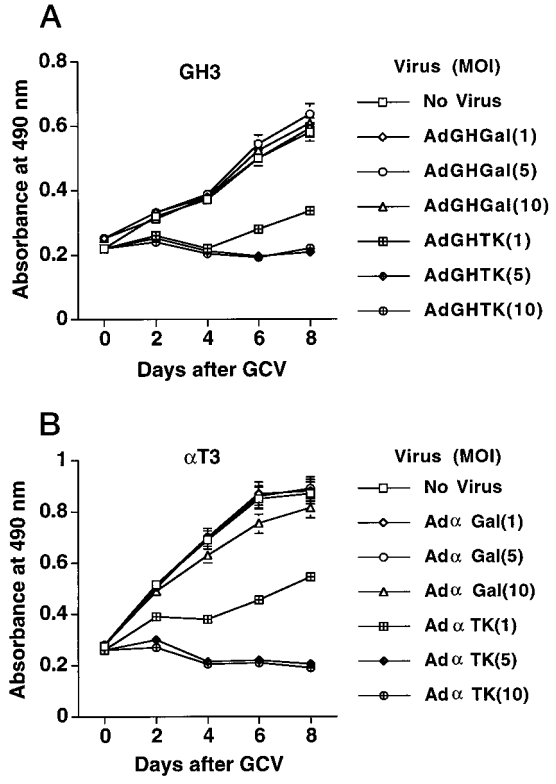

FIG. 7. Long term effect of GCV on growth of GH_3 cells and αT3 cells infected with adenoviral vectors. MOIs of 0, 1, 5, and 10 of viruses were achieved, and 5 μg GCV were added to each well. The cell viability was assayed on days 0, 2, 4, 6, and 8 after adding GCV. Results are expressed as the mean ± SD. A, GH_3 cells infected with AdGHTK and AdGHGal; B, αT3 cells infected with AdαTK and AdαGal.

treatment with GCV caused a dramatic regression in the tumors (Fig. 8). To quantitate the effect of the virus, several different tumors were injected with AdGHTK or with the nontoxic control, AdGHGal. All eight GH_3 cell tumors that received AdGHTK demonstrated regression after GCV treatment (Fig. 9A), whereas all eight tumors that were injected with AdGHTK, but were not treated with GCV, increased in size (1299.9 ± 570.4 mm^3; Fig. 9B). Tumors that were injected with the control virus, AdGHGal, in the presence of GCV (1304.5 ± 432.2 mm^3; Fig. 9C) or with vehicle in the presence of GCV treatment (1627 ± 642.5 mm^3; Fig. 9D) also increased in size.

Discussion

Gene therapy is an appealing strategy for the treatment of pituitary tumors for several reasons. Pituitary adenomas are biologically benign, relatively localized, and rarely metastasize. Therefore, direct administration of adenoviruses has a greater chance of reaching most of the tumor cells. In contrast to more aggressive forms of cancer, incomplete destruction of pituitary tumor cells is unlikely to be associated with rapid recurrence or metastases. Although an ultimate goal is to completely eliminate residual pituitary tumor cells, a partial reduction of tumor size would be of clinical benefit by virtue of reducing tumor mass effects on surrounding structures and should also lower hormone levels.

In this report, we describe initial attempts to target a toxic

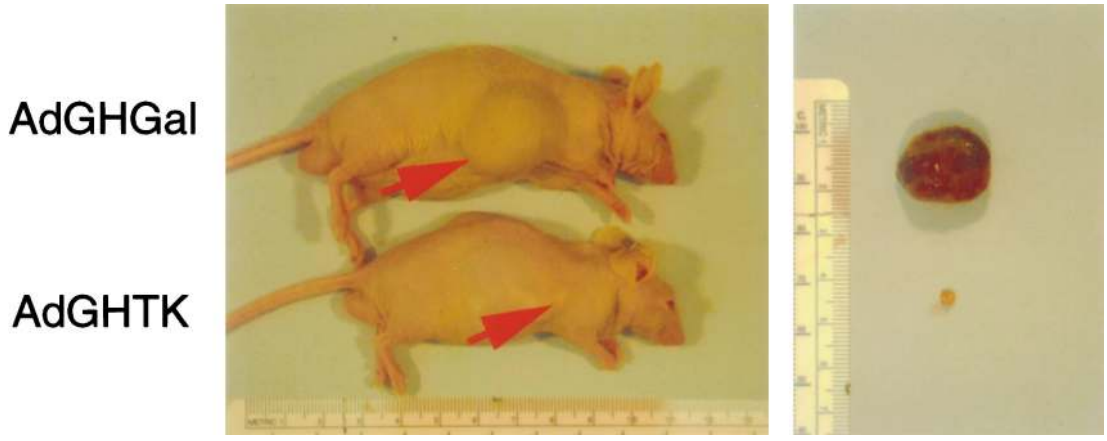

FIG. 8. Injection of adenoviral vectors into GH_3 cell tumors in nude mice. Tumors were injected 1.0×10^9 PFUs of AdGHTK or AdGHGal adenoviral vectors followed by treatment with GCV (100 mg/kg, ip, once daily for 10 days). Excised tumors are shown in the panels at the *right*.

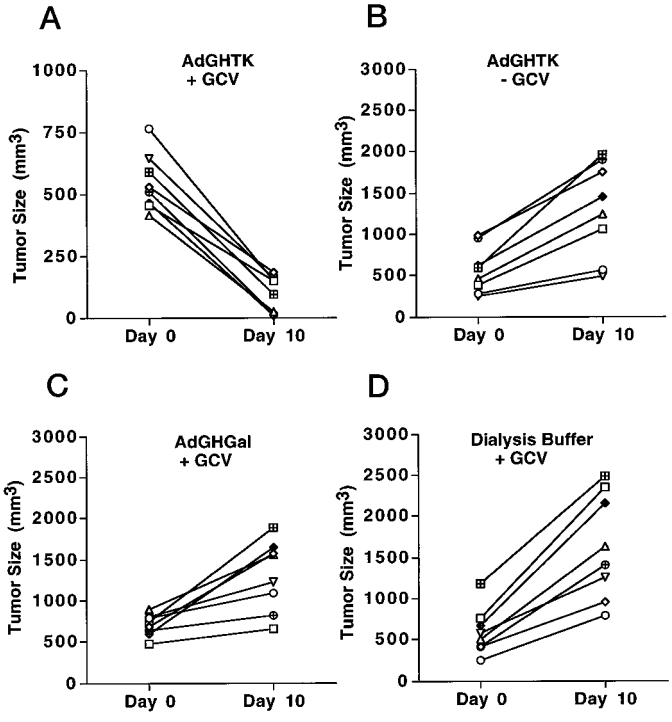

FIG. 9. Effect of injection of adenoviral vectors into preestablished GH_3 cell tumors of nude mice. The growing tumors received an intratumoral injection of 1.0×10^9 PFUs of the indicated adenoviral vectors, and GCV was administered ip at 100 mg/kg once daily for 10 days. Tumor size (presented as cubic millimeters) was measured before and 10 days after GCV treatment. A, AdGHTK and GCV treatment; B, AdGHTK without GCV treatment; C, AdGHGal and GCV treatment; D, dialysis buffer used for adenovirus preparation and GCV treatment.

gene to rodent pituitary tumor cell types. We have shown that 0.4 kb of the human GH promoter and 0.9 kb of the human α-subunit promoter are sufficient to express the β-gal and TK genes. Parallel studies of these two genes offer several advantages. The β-gal gene allows determination of infection efficiency using histological analyses and allows quantitation of promoter activity based on the level of expressed enzyme activity. On the other hand, the TK gene confers cytotoxicity in the presence of GCV, more closely mimicking the ultimate goal of cell killing in pituitary tumors.

The choice of promoter fragments for these studies was based in part on known functional sequences involved in expression and cell specificity, but also on practical limitations involving the amount of DNA that can be inserted into the modified adenoviral genome. In the case of the GH promoter, the cell type-specific expression of the 5′-flanking region has been confirmed in several lines of transgenic mice (16). The rat GH promoter, between −181 and +2 bp, was sufficient to be expressed specifically in the pituitary of transgenic mice (15). Expression was seen in some lactotrophs as well as somatotropes. In other transgenic models, GH promoter activity was greater when sequences were extended to −310 bp (20). Transient expression studies using 500 bp of the human GH promoter demonstrated efficient expression in GH_3 cells (14). Deletion of the human GH gene to −285 bp preserved cell-specific expression, but further deletions caused loss of specific expression (21). For this reason, the human GH promoter used in this study (−336 to +58) contains this region. However, it is unclear at present whether this fragment confers maximal expression and cell specificity.

The expression and regulation of the α-subunit promoter have been studied extensively. In transgenic mice, the mouse α-promoter is strongly expressed in gonadotrope and thyrotrope cells using promoter sequences between 0.48–4.6 kb (22). Experiments using the diphtheria toxin gene linked to 313 bp of the bovine α-promoter caused ablation of gonadotope cells, but not thyrotrope cells, suggesting that distinct regulatory elements may be involved in thyrotrope- and gonadotrope-specific expression (18). In thyrotrope tumor cells, additional upstream sequences (−507 bp) are required for thyrotrope-specific expression (23). Because it is of interest to target each of the various types of glycoprotein hormone-producing tumors (α-subunit, TSH, LH, and FSH) (3), we used −846 to +45 bp of the human α-promoter sequence, which should be sufficient for expression in gonadotrope and thyrotrope cells (24). This promoter sequence has been shown previously to be expressed strongly in αT3 cells (25, 26), a finding consistent with our studies using adenoviral vectors that carry the human α-promoter.

An important question is whether the human GH and

α-subunit promoters target gene expression in a cell type-specific manner. Our studies provide mixed evidence in this regard. Using β-gal as an indicator, it is clear that each of these promoters is expressed in both GH_3 cells and αT3 cells. Thus, promoter activity is not completely restricted to cell lines that produce the respective hormones. This finding is not unexpected for several reasons. First, these cell lines are not fully differentiated, a feature of most cells that proliferate rapidly in culture. Second, the α-promoter is known to be expressed in GH_3 cells (27–29). To our knowledge, there are no previous studies of GH promoter activity in αT3 cells. However, these cells are thought to represent an early pluripotential progenitor of the pituitary cell lineage (30). Recently, several transcription factors, such as LH-2 (31) and Ptx1 (32), have been shown to be expressed in multiple pituitary cell types and to regulate the expression of several different pituitary hormone promoters. These and other pituitary transcription factors might account for some degree of leaky expression of the hormone promoters.

It is noteworthy that a substantial fraction (~37%) of human GH-secreting pituitary tumors cosecrete the α-subunit (33, 34), and up to 59% stain positive for both GH and α-subunit (35–37). Similarly, about 30% of TSH-secreting tumors (producing the α-subunit in combination with the TSH β-subunit) also produce GH (38). Thus, there appears to be some overlap of α-subunit and GH expression in neoplastic pituitary cells.

Although the α-subunit and GH promoters overlap in their expression in different pituitary cell lines, there is evidence that the GH and α-subunit promoters are expressed preferentially in GH_3 and αT3 cells, respectively. For example, the α-subunit promoter was expressed in GH_3 cells at 18% of the level in αT3 cells. Similarly, the activity of the GH promoter in αT3 cells was about 30% of that seen in GH_3 cells. In addition, AdGHGal and Adα Gal expression was much lower in the nonpituitary T47D breast cancer cells. Consistent with the β-gal results, there was no GCV-induced cytotoxicity when T47D cells were infected with AdGHTK or AdαTK. We also did not detect AdGHGal or Adα Gal expression in the livers of rats injected in the tail vein with 1×10^{10} PFU of virus (data not shown). By comparison, a similar amount of AdCMVGal was strongly expressed in liver, lung, and other organs. Further studies of the cell specificity of these promoters are required, because leaky expression of targeted genes could lead to undesired toxicity to normal tissues.

The cytotoxic effects of the TK gene can be activated by treatment with the synthetic nucleoside analog GCV. TK converts GCV into triphosphate-GCV, which results in the termination of DNA synthesis (39, 40). In principle, treatment with GCV provides an additional dimension of therapeutic opportunity by allowing the activity of the toxic gene to be modulated by the dose of the nucleoside analog. An important advantage of the TK gene is that the cytotoxicity induced by triphosphate-GCV only affects cells that are dividing. Therefore, one might expect minimal toxicity for normal pituitary cells, which have a low mitotic index. Consistent with this idea, we did not observe GCV-induced cytotoxicity in primary rat pituitary cell cultures infected with AdGHTK and AdαTK (data not shown).

Consistent with the β-gal expression data, GH_3 cells and αT3 cells infected with AdGHTK or AdαTK at a MOI of 5–10 PFU/cell showed cytotoxicity at 5–10 μg/mL GCV. The therapeutic dosage of GCV for human CMV isolates tested *in vitro* in several cell lines ranges from 0.2–3 μg/mL (39). Higher concentrations of GCV (>10 μg/mL) inhibit the growth of some mammalian cells *in vitro*. In our study, 50 μg/mL GCV induced some cell death in the absence of viral infection. These findings suggest that the amounts of TK expression produced by the AdGHTK or AdαTK are sufficient to sensitize pituitary cells to therapeutic doses of GCV.

One of the benefits of using TK as a toxic gene is its ability to generate an *in vivo* "bystander" effect. In a dose-effect study, it has been shown that complete regression of tumors was observed when only 10% of the tumor cells expressed TK (41). This effect reflects the fact that the triphosphate nucleoside analogs can be transferred from one cell to another through gap junctions. In our *in vivo* studies, AdGHTK caused significant regression of GH_3 cell tumors 10 days after GCV treatment. All other groups, including AdGHTK injection in the absence of GCV, demonstrated continued growth of the tumors. In other studies, 2×10^9 PFUs of adenoviral vectors were cytopathic to hepatocellular carcinomas when injected into tumors (83 mm^3) *in vivo* (8). In our study, effective tumor regression was achieved without an apparent cytotoxic effect of the adenoviral vectors alone, even though the initial tumor volume (601.7 ± 217.1 mm^3) was much greater, and the dose of the viral vectors was lower. These findings may reflect the impact of the bystander effect to induce regression of the AdGHTK-injected GH_3 cell tumors or the efficacy of the GH promoter. Further studies to optimize the doses of virus and GCV will be of interest.

In cell culture experiments, high doses of the adenoviral vectors were cytopathic, even without expression of the TK gene. At a high multiplicity of infection, the E1 region becomes unnecessary for viral replication (42). This may reflect the high level of expression of viral gene products, some of which are cytotoxic or trigger host cellular immune responses (43, 44). Although this effect is not necessarily deleterious in the context of tumor cell destruction, it could lead to inadvertent toxicity in normal cells. Recently, the E4 region of adenovirus has been shown to be necessary for viral replication (45). The E4 region contains several open reading frames that encode a variety of regulatory proteins that shut off host protein synthesis and induce normal viral DNA replication (46, 47). In the future, deletion of the E1 and E4 regions should be advantageous compared to the current E1-deleted vector.

Although many hurdles remain before gene therapy might be applied to human pituitary tumors, it represents a potentially valuable adjunct to existing therapeutic options. Because of the advantages of local administration of adenoviral vectors, gene therapy might be used initially in conjunction with neurosurgery. Recently, there have been a number of important advances in specific medical therapy of pituitary tumors (2). Dopamine agonists represent an effective modality for the treatment of prolactinomas, and somatostatin analogs provide a useful means for suppressing GH and TSH production by pituitary tumors. Efforts to develop specific antagonists for the GH receptor also represent a potentially promising treatment for GH hypersecretion (48). Thus, there are several emerging strategies for designing specific therapies for different types of pituitary tumors, and

ultimately, it may be possible to use independent approaches for more effective management of pituitary tumors.

In conclusion, we have demonstrated that adenoviral vectors containing pituitary hormone promoters efficiently transfer marker genes as well as a toxic gene to pituitary tumor cells *in vitro* and *in vivo*. These or similar adenoviral vectors have potential for gene therapy of human pituitary adenomas.

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References

1. **Daniels GH, Martin JB.** 1995 Neuroendocrine regulation and diseases of the anterior pituitary and hypothalamus. In: Isselbacher KJ, Braunwald E, Wilson JD, Martin JB, Fauci AS, Kasper DL, ed. Harrison's principles of internal medicine, 13th ed. New York: McGraw-Hill; 1891–1918.
2. **Shimon I, Melmed S.** 1998 Management of pituitary tumors. Ann Intern Med. 129:472–483.
3. **Lee EJ, Jameson JL.** 1997 Gonadotropin (FSH and LH) pituitary tumors. In: Wierman ME, ed. Disease of the pituitary: diagnosis and treatment. Totowa: Humana Press; 247–272.
4. **Melmed S.** 1997 General aspects of the management of pituitary tumors by surgery or radiation therapy. In: DeGroot LJ, ed. Endocrinology, 3rd ed. Philadelphia: Saunders; 497–503.
5. **Schlechte JA.** 1997 Differential diagnosis and management of hyperprolactinemia. In: Wierman ME, ed. Disease of the pituitary: diagnosis and treatment. Totowa: Humana Press; 71–77.
6. **Ali M, Lemonine N, Ring CJA.** 1994 The use of DNA viruses as vectors for gene therapy. Gene Ther. 1:367–384.
7. **Anderson WF.** 1998 Human gene therapy. Nature. 392(Suppl):25–30.
8. **Kaneko S, Hallenbeck P, Kotani T, et al.** 1995 Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. Cancer Res. 55:5283–5287.
9. **Vincent AJPE, Vogels R, Someren GV, et al.** 1996 Herpes simples virus thymidine kinase gene therapy for rat malignant brain tumors. Hum Gene Ther. 7:197–205.
10. **Elshami AA, Kucharczuk JC, Zhang HB, et al.** 1996 Treatment of pleural mesothelioma in an immunocompetent rat model utilizing adenoviral transfer of the herpes simples virus thymidine kinase gene. Hum Gene Ther. 7:141–148.
11. **Vincent AJ, Esandi MC, Avezaat CJ, et al.** 1997 Preclinical testing of recombinant adenoviral herpes simplex virus-thymidine kinase gene therapy for central nervous system malignancies. Neurosurgery. 41:442–451.
12. **Block A, Chen SH, Kosai K, Finegold M, Woo SL.** 1997 Adenoviral-mediated herpes simplex virus thymidine kinase gene transfer: regression of hepatic metastasis of pancreatic tumors. Pancreas. 15:25–34.
13. **Ross HM, Hirschowitz EA, Russi TJ, et al.** 1997 Adenoviral thymidine kinase prodrug gene therapy inhibits sarcoma growth *in vivo*. J Surg Res. 70:7–11.
14. **Lefevre C, Imagawa M, Dana S, Grindlay J, Bodner M, Karin M.** 1987 Tissue-specific expression of the human growth hormone gene is conferred in part by the binding of a specific trans-acting factor. EMBO J. 6:971–981.
15. **Lira SA, Crenshaw EB, Glass CK, Swanson LW, Rosenfeld MG.** 1988 Identification of rat growth hormone genomic sequences targeting pituitary expression in transgenic mice. Proc Natl Acad Sci USA. 85:4755–4759.
16. **Schaufele F.** 1994 Regulation of expression of the growth hormone and prolactin genes. In: Imura H, ed. The pituitary gland, 2nd ed. New York: Raven Press; 91–116.
17. **Alarid ET, Windle JJ, Whyte DB, Mellon PL.** 1996 Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development. 122:3319–3329.
18. **Kendall SK, Saunders TL, Jin L, et al.** 1991 Targeted ablation of pituitary gonadotropes in transgenic mice. Mol Endocrinol. 5:2025–2036.
19. **Swaminathan S, Thimmapaya B.** 1996 Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. J Mol Biol. 258:736–746.
20. **Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM.** 1989 Transgenic mice with inducible dwarfism. Nature. 339:538–541.
21. **Cattini PA, Peritz LN, Anderson TR, Baxter JD, Eberhardt NL.** 1986 The 5′-flanking sequences of the human growth hormone gene contain a cell specific control element. DNA. 5:503–509.
22. **Kendall SK, Gordon DF, Birkmeier TS, et al.** 1994 Enhancer-mediated high level expression of mouse pituitary glycoprotein hormone α-subunit transgene in thyrotropes, gonadotropes, and developing pituitary gland. Mol Endocrinol. 8:1420–1433.
23. **Sarapura VD, Wood WM, Gordon DF, Ocran KW, Kao MY, Ridgway EC.** 1990 Thyrotrope expression and thyroid hormone inhibition map to different regions of the mouse glycoprotein hormone α-subunit gene promoter. Endocrinology. 127:1352–1361.
24. **Pennathur S, Madison LD, Kay TW, Jameson JL.** 1993 Localization of promoter sequences required for thyrotropin-releasing hormone and thyroid hormone responsiveness of the glycoprotein hormone α-gene in primary cultures of rat pituitary cells. Mol Endocrinol. 7:797–805.
25. **Horn F, Windle JJ, Barnhart KM, Mellon PL.** 1992 Tissue-specific gene expression in the pituitary: the glycoprotein hormone alpha-subunit gene is regulated by a gonadotrope-specific protein. Mol Endocrinol. 6:2143–2153.
26. **Chedrese PJ, Kay TW, Jameson JL.** 1994 Gonadotropin-releasing hormone stimulates glycoprotein hormone alpha-subunit messenger ribonucleic acid (mRNA) levels in α T3 cells by increasing transcription and mRNA stability. Endocrinology. 134:2475–2481.
27. **Kim DS, Ahn SK, Yoon JH, et al.** 1994 Involvement of a cAMP-responsive DNA element in mediating TRH responsiveness of the human thyrotropin alpha-subunit gene. Mol Endocrinol. 8:528–536.
28. **Gurr JA, Kourides IA.** 1989 Regulation of the transfected human glycoprotein hormone alpha-subunit gene by dexamethasone and thyroid hormone. DNA. 8:473–480.
29. **Yarwood NJ, Gurr JA, Sheppard MC, Franklyn JA.** 1993 Estradiol modulates thyroid hormone regulation of the human glycoprotein hormone α subunit gene. J Biol Chem. 268:21984–21989.
30. **Windle JJ, Weiner RI, Mellon PL.** 1990 Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endocrinol. 4:597–603.
31. **Roberson MS, Schoderbek WE, Tremml G, Maurer RA.** 1994 Activation of the glycoprotein hormone α-subunit promoter by a LIM-homeodomain transcription factor. Mol Cell Biol. 14:2985–2993.
32. **Tremblay JJ, Lanctot C, Drouin J.** 1998 The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim-homeodomain gene Lim3/Lhx3. Mol Endocrinol. 12:428–441.
33. **Oppenheim DS, Kana AR, Sangha JS, Klibanski A.** 1990 Prevalence of α-subunit hypersecretion in patients with pituitary tumors: clinically nonfunctioning and somatotroph adenomas. J Clin Endocrinol Metab. 70:859–864.
34. **Ishibashi M, Yamaji T, Takaku F, Teramoto A, Fukushima T.** 1987 Secretion of glycoprotein hormone α-subunit by pituitary tumors. J Clin Endocrinol Metab. 64:1187–1193.
35. **Furuhata S, Kameya T, Tsuruta T, Naritaka H, Toya S.** 1994 Colocalization of growth hormone (GH) and glycoprotein subunit α in GH-producing pituitary adenomas in acromegalic patients. Acta Neuropathol. 87:568–571.
36. **Hofland LJ, van Koetsveld PM, Verleun TM, Lamberts SW.** 1989 Glycoprotein hormone α-subunit and prolactin release by cultured pituitary adenoma cells from acromegalic patients: correlation with GH release. Clin Endocrinol (Oxf). 30:601–611.
37. **Osamura RY, Watanabe K.** 1987 Immunohistochemical colocalization of growth hormone (GH) and α subunit in human GH secreting pituitary adenomas. Virchows Arch A Pathol Anat Histopathol. 411:323–330.
38. **Smallridge RC, Smith CE.** 1983 Hyperthyroidism due to thyrotropin-secreting pituitary tumors. Diagnostic and therapeutic considerations. Arch Intern Med. 143:503–507.
39. **Smee DF, Boehme R, Chernow M.** 1985 Intracellular metabolism and enzymatic phosphorylation of 9-(1,3-dihydroxy-2-propoxymethyl) guanine and acyclovir in herpes simplex virus-infected and uninfected cells. Biochem Pharmacol. 34:1049–1056.
40. **Mattews T, Boehme R.** 1988 Antiviral activity and mechanism of action of ganciclovir. Rev Infect Dis. 10:S490–S494.
41. **Mooten FL.** 1986 Tumor chemosensitivity conferred by inserted herpes thymidine kinase gene: paradigm for a prospective cancer control strategy. Cancer Res. 46:5276–5281.
42. **Jones N, Shenk T.** 1979 Isolation of adenovirus type 5 host range deletion mutants defective for transformation in rat embryo cells. Cell. 17:683–689.
43. **Zhang Y, Schneider RJ.** 1994 Adenovirus inhibition of cell translation facilitates release of virus particles and enhances degradation of the cytokeratin network. J Virol. 68:2544–2555.
44. **Valentine RC, Pereira HG.** 1965 Antigens and structure of the adenovirus. J Mol Biol. 13:13–20.
45. **Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovesdi I.** 1996 A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. J Virol. 70:6497–6501.
46. **Ketner G, Bridge E, Virtanen A, Hemstrom C, Pettersson U.** 1989 Complementation of adenovirus E4 mutants by transient expression of E4 cDNA and deletion plasmids. Nucleic Acids Res. 17:3037–3048.
47. **Bridge E, Ketner G.** 1990 interaction of adenoviral E4 and E1b products in late gene expression. Virology. 174:345–353.
48. **Chen WY, Chen N, Yun J, Wagner TE, Kopchick JJ.** 1994 *In vitro* and *in vivo* studies of the antagonistic effects of human growth hormone analogs. J Biol Chem. 269:15892–15897.