ABSTRACT

Gene therapy with thyroglobulin (TG) promoter and a prodrug/suicide gene combination may prove useful as a treatment for thyroid carcinoma. However, most poorly differentiated and anaplastic thyroid carcinomas have lost the ability to express the TG gene expression accompanied by loss of transcription factors [thyroid transcription factor-1 (TTF-1), TTF-2, or Pax-8] interacting with the TG promoter. In anticipation of loss of transcription factors [thyroid transcription factor-1 (TTF-1), gene and the luciferase gene, TG promoter activity was detected in a normal rat thyroid cell line (FRTL-5), but not in a dedifferentiated line of thyroid cells (FRT) expressing Pax-8 but not TTF-1, TTF-2, or TG [TTF-1(+)/TTF-2(-)/Pax-8(+)/TG(-)], or in a human papillary thyroid carcinoma cell line [BHP15-3; TTF-1(-)/TTF-2(-)/Pax-8(-)/TG(-)], a human pulmonary cell line [H441; TTF-1(+)/TTF-2(-)/Pax-8(-)/TG(-)], or a dog kidney epithelial cell line [MDCK; TTF-1(-)/TTF-2(-)/Pax-8(+)/TG(-)]. Co-transfection of the TTF-1 expression vector stimulated TG promoter activity in FRT and BHP15-3 dedifferentiated thyroid cells, but not in H441 pulmonary cells. Only weak activation was observed in MDCK kidney cells. We then constructed recombinant adenovirus vectors, AdTTF-1 and AdTGTK. AdTTF-1 contains cytomegalovirus promoter and rat TTF-1 cDNA; AdTGTK carried the TG promoter-driven HSV-TK gene. Infection with AdTGTK and combined with GCV treatment induced a cytotoxic effect in FRT-5 cells but not in dedifferentiated thyroid or nonthyroid cells. Cotransduction of AdTTF-1 and AdTGTK permitted 90% cytotoxicity for BHP15-3 and >95% cytotoxicity for FRT, as well as for BHP7-13 and BHP18-21 thyroid cancer cell lines [bothTTF1(+)/TTF2(-)/Pax8(-)/TG(-)]. In contrast, little cytotoxicity was seen for H441 and MDCK cell lines even with 300 µg/ml of ganciclovir. These results suggest that cotransduction of a TG promoter-controlled suicide gene and the TTF-1 gene by adenoviral vectors confers transcriptionally targeted gene-mediated cytotoxicity in poorly differentiated thyroid carcinoma cells unable to express the TG gene.

INTRODUCTION

The thyroid gland is a relatively common site of malignant neoplasms, giving rise to 90% of all endocrine cancers (1). Papillary thyroid carcinoma, the most common thyroid cancer, is effectively treated with surgery and radioiodine and is likely to show long-term survival after initial treatment (2, 3). However, anaplastic thyroid carcinoma constitutes about 5–14% of all thyroid carcinomas and is highly malignant with a median survival of 2–6 months, rapidly invading adjacent structures and metastasizing throughout the body, especially to the lungs (4, 5). In addition, 15% of patients with follicular thyroid carcinoma have distant metastasis at the time of diagnosis and thereafter face a 90% occurrence of mortality within 15 years (6). Since no effective therapy is available in patients with these aggressive types of thyroid carcinoma, development of novel therapeutic approaches including gene therapy are an urgent priority.

Recently, adenoviral vectors have been used to deliver the HSV-TK2 gene prior to GCV administration as a strategy for treatment of various types of malignant tumors (7, 8). These approaches have relied on viral promoters to achieve high expression of toxic genes (9–11). One drawback of adenoviral vectors is that they can infect nonneoplastic cells as well as tumor cells, inducing toxic effects in normal tissues. This problem might be circumvented if toxic gene expression was restricted to tumor cells using cancer-specific promoters. For example, α-fetoprotein (12) and carcinoembryonic antigen (13–15) gene promoters have been used against hepatocellular and gastrointestinal carcinoma, respectively. However, no thyroid carcinoma-specific gene has been isolated.

In the case of thyroid carcinoma, use of promoters of thyroid-specific genes would be relatively safe for stimulating targeted expression of a toxic gene in thyroid cancer cells; in case of excessive thyroid destruction, the main function of the thyroid gland could be replaced by administration of thyroid hormone, as is done when operative or radioiodine therapies ablate most or all normal thyroid tissue. Recent reports have shown that the thyrotropin receptor and the Na+/I–symporter genes are occasionally expressed in anaplastic thyroid carcinoma (16–18); these genes are well characterized in terms of promoters and interacting factors (19–23). Despite these advantages, the genes are expressed in several nonthyroid tissues (24–26).

Although recent studies (27, 28) demonstrated very low levels of the TG gene expression in thymus and kidney detected by reverse transcription-PCR, the promoter of the TG gene shows thyroid-specific activities as demonstrated in transgenic mice carrying a TG promoter-driven HSV-TK gene; GCV-induced cell ablation after gene transfer was limited to the thyroid gland (29). Thyroid-restricted activity was also shown by systemic administration of the adenoviral vector containing the TG promoter (30). The promoter region of the TG gene interacts with at least three transcription factors: TTF-1, TTF-2, and Pax-8 (Fig. 1A) (31). TTF-1 is a homeodomain-containing, DNA-binding protein expressed in thyroid and lung (32, 33). Pax-8, a member of the family of paired box-containing genes, is expressed in thyroid and kidney (34, 35). TTF-2 cDNA has recently been cloned and shown to be a member of the forkhead family of transcription factors and is expressed only in the thyroid (36). The tissue specificities in gene expressions of these transcription factors were determined by Northern blot and in situ hybridization analyses (32, 34, 36, 37).

Differentiated thyroid carcinomas express the TG gene (38). However, some poorly differentiated or metastatic thyroid carcinomas show only diminished TG expression (39, 40). Furthermore, most anaplastic thyroid carcinomas do not express the TG gene; expression

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2 The abbreviations used are: HSV-TK, herpes simplex virus-thymidine kinase; GCV, ganciclovir; TG, thyroglobulin; TTF, thyroid transcription factor; CMV, cytomegalovirus; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactoside.
failure is accompanied by loss of some thyroid-specific transcription factors (18, 40). In anticipation of developing transcriptionally targeted gene therapy against TG-nonproducing thyroid carcinomas, we investigated the effect of TTF-1 gene transfer upon the TG promoter activity and the cytotoxic effect obtained by the TG promoter-driven HSV-TK gene transferred prior to GCV exposure in several thyroid carcinoma cell lines as well as nonthyroid cell lines.

MATERIALS AND METHODS

Cell Culture. FRTL-5 rat thyroid cells, a kind gift from Dr. L. D. Kohn (Metabolic Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases, NIH, Bethesda, MD), were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum and a mixture of six hormones including bovine thyroid-stimulating hormone (10 milliunits/ml), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (41). FRT-nonfunctioning rat thyroid cells, also kindly donated by Dr. L. D. Kohn, were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum.

Three human papillary thyroid carcinoma cell lines, BHP7-13, BHP15-3, and BHP18-21, were kindly provided by Dr. J. M. Hershman (Endocrine Research Laboratory, West Los Angeles Veterans Affairs Medical Center, Los Angeles, CA). BHP18-21 cells, expressing Pax-8 but neither the TG nor the TTF-1 gene, were isolated from BHP18-21 cells. The BHP cells were maintained in RPMI 1640 supplemented with 10% FCS (42).

H441 (HTB-174; American Type Culture Collection, Manassas, VA), a human pulmonary adenocarcinoma cell line, was maintained in RPMI 1640 supplemented with 10% FCS. MDCK (RIKEN RCB0995; Wako, Saitama, Japan), a canine kidney epithelial cell line, was cultured in DMEM supplemented with 10% FCS. HeLa (CCL-2; American Type Culture Collection) cells were grown in Eagle’s minimal essential medium with 10% calf serum.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from cells by the guanidine isothiocyanate extraction method (43), and Northern blot analyses were performed as previously described (44). The rat TTF-1 cDNA from +1 to +331 bp was excised from the TTF-1 expression vector, RcCMV-THA; it was the kind gift of Dr. R. Di Lauro (Stazione Zoologica A. Dohrn, Naples, Italy) (32). Rat TTF-2 (bases 1598–2317) and human TG (bases 112–519) cDNA were obtained by reverse transcription-PCR from rat and human thyroid mRNA, respectively, as described previously (45). Rat β-actin and mouse Pax-8 cDNA were kindly donated by Dr. L. D. Kohn. All probes were radiolabeled using a random primer labeling kit (Takara Shuzo, Kyoto, Japan).

Transient Expression Analysis. To generate chimeric constructs containing the 5′-flanking region of the rat TG gene from −827 to +39 bp and the luciferase gene, pTGLuc-827, the genomic sequence of the TG gene excised with BamHI and HindIII from pTGCAT (19), kindly provided by Dr. L.D. Kohn, was inserted into pGL3-Basic vector (Promega, Madison, WI) cut with BamHI and HindIII. To construct pTGLuc-433 and pTGLuc-170, pTGLuc-827 was cut with NruI and PvuII (−433 bp) or BstEII (−170 bp), and termini were self-ligated. All plasmid preparations were purified by CsCl gradient centrifugation (46).

All cell lines used were transfected by electroporation (Gene Pulser; Bio-Rad, Richmond, CA) as described previously (25). Cells were harvested, washed, and suspended at 5 × 10^6 cells/ml in 0.8 ml of PBS. Either 20 μg of pTGLuc-827, equivalent molar amounts of the deletion mutants, or 20 μg of positive control pGL3-Control were added with 10 ng of pLC/CMV (Renilla luciferase expression plasmid; Promega) and 4 μg of pRC-CMV (ThA expression vector) or its control vector, pRC/CMV. Cells were pulsed (300 V, 300 μF, 960 μF), plated on three wells in a 6-well culture plate, and cultured for 48 h. Firefly and Renilla luciferase activity were determined using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities expressed by the TG promoter were normalized with the Renilla luciferase activity.

Construction of Recombinant Adenovirus Vector. Recombinant replication-deficient adenoviral vectors were constructed by the homologous recombination method. In brief, the rat TTF-1 expression cassette containing the CMV promoter and bovine growth hormone gene polyadenylation signal was excised from Rc/CMV-THA by NruI and PvuII and ligated into the EcoR site of pQBI-AdBN (Quantum Biotechnologies, Montreal, Quebec, Canada) to yield a transfer vector. The linearized plasmid was cotransfected with the calcium phosphate method into cultured 293 cells with a type 5 adenovirus gene from which the E3 and E6 regions had been deleted. Adenovirus recombinants carrying the TTF-1 expression cassette (AdTTF-1) were identified by PCR and electrophoretic mobility shift assays using extracts from infected 293 cells and an oligonucleotide probe containing the TTF-1-binding element in rat thyrotrpin promoter (20). For generation of AdTGTK, the expression cassette was constructed by replacing the luciferase gene in pTGLuc-688 with the HSV-TK gene excised from pMC1HSVtk (Life Technologies, Inc., Rockville, MD). The adenoviral transfer plasmid, pQBI-AdBN was digested with XhoI and NotI and ligated with the expression cassette excised by SalI and NotI. After formation of recombinant adenoviruses, individual clones were screened with PCR and in vitro cytototoxic assay using FRTL-5 cells. AdLaCZ, containing a CMV promoter-controlled LacZ gene, was provided by Quantum Biotechnologies. Recombinant adenoviruses were plaque purified, harvested 48 h after infection of 293 cells, and purified by double cesium chloride gradient ultracentrifugation (47). Viral titers were determined by plaque assays using cultured 293 cells.

Evaluation of Efficiency of Recombinant Adenoviral Infection. Cells were seeded at 4 × 10^3 cells/well in 96-well culture plates. The next day, the cells were infected with AdLaCZ at various MOIs. Two days later, the cells were fixed with 0.05% glutaraldehyde in PBS and then incubated with X-gal substrate solution (20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM MgCl₂, and 1 mg/ml X-gal in PBS) at 37°C for 2 h.

In Vitro Cytotoxic Assay with Adenovirus Infection. The sensitivity of adenovirus-infected cells to GCV was measured with a nonradioactive cell proliferation assay according to the manufacturer’s protocol (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). One day after plating 4 × 10^3 cells/well in triplicate wells of 96-well culture plates, adenoviral vectors were infected at the MOIs indicated. Sixteen hours after infection, increasing concentrations 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 a fraction representing the absorbance shown by GCV-treated cells divided by that shown by cells without GCV treatment (mean ± SE). For a cytotoxic assay to assess long-term effects of adenovirus vectors, 2 × 10^5 cells/well were plated in triplicate wells of 96-well culture plates. After GCV was added, incubation was continued; cytotoxic assays and replacements of medium were performed on days 0, 2, 4, 6, and 8.

RESULTS

Expression of Thyroid-specific Transcription Factors. In the present study, rat and human thyroid cell lines losing the ability of the TG gene expression were used. BHP15-3, BHP7-13, and BHP18-21v thyroid cancer cell lines were established from human papillary thyroid carcinoma and showed various dedifferentiation phenotypes including anaplastic form (42). To verify expression of thyroid-specific transcription factors involved in the TG gene expression in cultured thyroid cancer cells and nonthyroid cell lines (Fig. 1A), we performed Northern blot analyses using TTF-1, TTF-2, and Pax-8 cDNAs (Fig. 1B). As previously reported (31), FRTL-5 differentiated rat thyroid cells expressed either TTF-1, TTF-2, or Pax-8. Losses of TTF-1 and TTF-2 expression were observed in FRTL differentiated rat thyroid cells. BHP15-3 expressed neither TTF-1, TTF-2, nor Pax-8. In contrast, Pax-8 mRNA only was detected in BHP7-13 and BHP18-21v cells. In accord with the tissue distributions of the transcription factors (Fig. 1A), the MDCK canine kidney epithelial cell line expressed Pax-8, but not TTF-1 and TTF-2, and the H441 human pulmonary adenocarcinoma cell line exhibited the TTF-1 gene expression. TG mRNA was not detected in any cell line other than FRTL-5.

Effect of TTF-1 Gene Overexpression on the TG Promoter. To examine whether overexpression of TTF-1 could restore TG promoter activity in TG-nonproducing thyroid cells, we transduced the chimeric plasmid containing the rat TG promoter between −827 and +39 bp.
and the luciferase gene (pTGLuc-827) with or without the TTF-1 expression vector into FRTL-5, FRT, BHP15-3, H441, and MDCK cells (Fig. 2). In the absence of TTF-1 cotransfection, promoter activity of the TG gene was detected only in FRTL-5 cells. Overexpression of TTF-1 gene activated the TG promoter in FRT and BHP15-3 dedifferentiated thyroid cells. In contrast, only faint activation was observed in H441 and MDCK nonthyroid cells. These results indicate that the transduced TTF-1 gene may be effective in activating TG promoter activity, but only in cells derived from the thyroid gland.

We also investigated the effect of TTF-1 overexpression on 5′-deletion mutants of the TG promoter (Fig. 3). In both FRT and BHP15-3 thyroid cell lines, deletion up to −170 bp did not alter the effect of TTF-1 on the TG promoter activity. These results showed that the minimal promoter region up to −170 bp is responsible for TTF-1-induced activation of the TG promoter in dedifferentiated thyroid cells. In H441 cells, overexpression of TTF-1 did not activate any deletion mutants of the TG promoter. In comparison with thyroid cells, MDCK cells exhibited only faint activation in any deletion mutants after cotransfection of TTF-1 expression vector.

Generation of Adenovirus Vectors and Transduction Efficiency. To develop transcriptionally targeted gene therapy of TG-nonproducing thyroid carcinomas using the transactivating effect of TTF-1 on the TG promoter, we constructed recombinant adenovirus vectors AdTTF-1 and AdTGTK (Fig. 4). In AdTTF-1, rat TTF-1 cDNA was placed under control of the CMV promoter. AdTGTK contains the TG promoter-driven HSV-TK gene with the SV40 late polyadenylation signal sequence. The DNA fragment of the TG gene between −2688 and −139 bp was inserted in AdTGTK, since pTGLuc-688 exhibited the highest TTF-1-induced luciferase activity in the BHP15-3 thyroid carcinoma cell line. An additional polyadenylation signal sequence was inserted upstream of the TG promoter to reduce background transcription.

We determined the efficiency of adenoviral vector-mediated gene transfer by transduction with AdLacZ (Fig. 4). Expression of β-galactosidase was detected by X-gal staining at 48 h after infection with AdLacZ (MOI, 1–3000; data not shown). Approximately 50% of BHP15-3 cells were stained blue at a MOI of 30. At a MOI of 100, in FRTL-5 and BHP18-21v cells, 50% of attached cells were transduced.

Fig. 1. TG-related DNA-binding proteins and transcripts. A, Schematic representation of rat TG gene promoter occupancy by the DNA-binding proteins present in thyroid follicular cells (29). ( ), TTF-1, ( ) TTF-2, ( ) Pax-8. Interactions of the ubiquitous factor and Hex are also represented. Tissue distributions of these thyroid-specific transcription factors in adults are indicated. B, expression of transcription factors interacting with the TG promoter in thyroid and nonthyroid cell lines. Equal amounts of total RNA (20 μg/lane) are subjected to sequential Northern blot analyses using rat TTF-1, rat TTF-2, mouse Pax-8, and β-actin cDNA probes.

Fig. 2. The promoter activity of the TG gene and the ability of TTF-1 transduction to activate the TG promoter in thyroid and nonthyroid cell lines. A luciferase reporter construct containing the TG gene between −827 and +39 and pLC/CMV (Renilla luciferase expression vector) with or without the TTF-1 expression vector was transfected into the cell lines indicated. Luciferase activities were normalized with respect to the Renilla luciferase activity and are presented relative to that of the positive control plasmid (pGL3-Control). Each value represents the mean ± SE for three separate experiments in triplicate. ND, not determined.

Fig. 3. The ability of TTF-1 transduction to activate luciferase constructs containing 5′-deletion mutants of TG promoter in FRT (A), BHP15-3 (B), H441 (C), and MDck (D) cells. Luciferase activities were normalized with respect to the Renilla luciferase activity and are presented relative to that of the positive control plasmid (pGL3-Control). Each value represents the mean ± SE for three separate experiments in triplicate.
BHP7-13, FRT, and H441 cells required 300 MOI to infect 50% of cells. MDCK and HeLa cells exhibited the lowest transduction efficiency: a MOI of 1000 was needed to infect 50% of cells. Since partial cytopathic effects were occasionally seen when 95–100% of the cells were infected with higher MOI of AdLacZ, we performed the following experiments with the MOIs described above.

**GCV Sensitivity of Thyroid Cancer Cells and Nonthyroid Cells Infected with Adenoviral Vectors.** Thyroid and nonthyroid cell lines were infected with AdTTF-1 and/or AdTGTK adenovirus vectors to investigate the toxic effect of TK expressed by the TG promoter at MOIs indicated in Fig. 5. 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. As a control, cytotoxicity of AdTTF-1 or AdTGTK was compared to that of AdLacZ.

In FRTL-5 cells infected with AdTGTK alone, 70% cell death occurred with 1 μg/ml of GCV, and 90% cell death was observed using 10 μg/ml GCV induced cytopathic effects. These results showed that the TG promoter in AdTGTK was activated in TG-producing thyroid cells. In contrast, infection with AdLacZ + AdTGTK resulted in only a small cytopathic effect in FRT dedifferentiated thyroid cells (Fig. 5B). The GCV sensitivity in thyroid carcinoma cell lines producing no TG also was not enhanced by cotransduction of AdLacZ + AdTGTK (Fig. 5, C–E). These findings are consistent with expression of TG mRNA and the TG promoter activities measured by luciferase assays in these cell lines (Figs. 1 and 2). Transduction of AdTTF-1 + AdLacZ resulted in no increases in GCV-induced cytopathic effects (Fig. 5, B–E).

To evaluate the effect of TTF-1 expression on the TG promoter-mediated cytotoxicity in TG-nonproducing thyroid cell lines, we simultaneously infected dedifferentiated thyroid cells with AdTTF-1 and AdTGTK. As shown in Fig. 5, B–E, GCV sensitivities in FRT and BHP thyroid carcinoma cells were increased by cotransduction of AdTTF-1 and AdTGTK in comparison with those induced by AdLacZ + AdTGTK. Cytotoxicity occurred at GCV concentrations as low as 1 μg/ml in all thyroid cell lines infected with AdTTF-1 + AdTGTK. More than 90% cell death was seen using 10 μg/ml GCV in FRT cells or 100 μg/ml in BHP7-13 and BHP18-21v cells. Although BHP15-3 thyroid carcinoma cells do not express any thyroid-specific transcription factors, cotransduction of AdTTF-1 and AdTGTK was able to increase sensitivity to GCV (Fig. 5E). However, the effect of TTF-1 on the TG promoter-dependent cytotoxicity in BHP15-3 cells appeared to be weaker than those in thyroid cells that expressed Pax-8. In comparison to the GCV-induced cytotoxic effect in cells infected with AdTGTK and AdLacZ, enhancement of GCV sensitivity by cotransduction of AdTTF-1 and AdTGTK in FRT cells (IC50, 88 ± 12-fold) was significantly (P < 0.05) higher than that in BHP15-3 cells (35 ± 9-fold).

The long-term effects of adenoviral vectors on cytotoxicities of BHP18-21v cells were assessed at 10 and 30 μg/ml GCV which ordinarily produced only partial cytopathic effects 4 days after drug...
weaker activation (10, cotransduction the TG promoter in HeLa cells. As shown in Fig. 7C, cotransduction of AdTTF-1 and AdTGTK, as well as AdTFT-1 and AdLacZ, did not increase sensitivity to GCV in either nonthyroid cell line. In addition, we infected HeLa cells with the adenovirus vectors, since a previous report (48) showed that cotransfection of rat TG promoter with the TTF-1 expression vector activated the TG promoter in HeLa cells. As shown in Fig. 7C, cotransduction of AdTTF-1 and AdTGTK in HeLa cells resulted in a significantly weaker activation (10 ± 2-fold) of sensitivity to GCV than that in BHP15-3 (Fig. 5E).

DISCUSSION

In anticipation of developing transcriptionally targeted gene therapy of thyroid cancer, we chose to use a tissue-specific promoter, since no thyroid carcinoma-specific protein has yet been identified. Expression of the *TG* gene is strictly restricted to the thyroid gland, and its translated product is most abundant in thyroid tissue. Moreover, TG is expressed in most differentiated thyroid carcinomas, and serum TG concentration is used as a marker for detecting growth and relapse of thyroid tumors. However, the expression of TG is often undetectable in anaplastic carcinoma and papillary carcinoma with extrathyroidal extension and nodal metastases, for which conventional surgical or radioiodine treatment are insufficient (40). Reflecting deficient TG expression, luciferase activities and cytopathic effects were not induced by the TG promoter in TG-nonproducing thyroid carcinoma cell lines examined in this study. Reactivation or enhancement of TG promoter activity that otherwise is diminished in thyroid carcinoma cells is necessary for efficient gene therapy against these life-threatening cancers. We, therefore, transduced such cells with TTF-1, a major regulator of the TG promoter.

In this study, we showed that TTF-1 could reactivate the TG promoter in rat FRT cells and human thyroid carcinoma cell lines but not in nonthyroid cells. This finding is in agreement with previous studies. Transfection of TTF-1 expression vector increased chloramphenicol acetyltransferase activity expressed by the TG promoter in FRT cells but not in BRL rat liver cells (20). Recently, Chun et al. (49) also reported that overexpression of TTF-1 restored the TG promoter activity in ARO and WRO thyroid carcinoma cell lines but not in COS-7 and BRL nonthyroid cells. In addition, Mascia et al. (50) demonstrated reactivation of the chromosomal TG promoter by stable transfection of the *TTF-1* gene in FRT cells. To uncover the mechanism accounting for the thyroid-cell-restricted TTF-1 effect on the TG promoter, we performed deletion analyses of the TG promoter. Transient transfection of *S*-deletion mutants indicated that the region between −170 and +39 bp included important elements for TTF-1-induced activation of the TG promoter. This region has been known as the minimal promoter of the *TG* gene conferring its thyroid-specific expression and contains binding sites for Pax-8 and TTF-2 as well as three sites for TTF-1.

Northern blot analysis in this study divided the dedifferentiated thyroid cell lines examined into two groups. The first group, including FRT, BHP7-13, and BHP18-21v cells, expressed Pax-8 but neither TTF-1 nor TTF-2. Another group, including only BHP15-3, did not express any thyroid-specific transcription factor. Transduction of the *TTF-1* gene reactivated the TG promoter activity and induced the cytopathic effect mediated by the TG promoter in both expression-defined cell types. On the other hand, the MDCK kidney cell line expressed Pax-8, but showed only weak activation of the TG promoter by TTF-1. Moreover, neither H441 cells nor pulmonary epithelium expressed TG despite expression of the *TTF-1* gene to an extent similar to that in the thyroid gland (Fig. 1 and Ref. 32). Similar differences in activities of TTF-1 between thyroid and nonthyroid cells have recently been reported (18). Thus, overexpression of TTF-1 activated the rat TPO promoter, which has a structure similar to that of the TG promoter, in the NPA thyroid carcinoma cell line which does not express TG, TPO, TTF-1, or Pax-8. However, only poor activation by TTF-1 occurred in HeLa cells, a human cervical cancer line. Differences in the TTF-1 effect between various cells may be a result of the effects of phosphorylation (20, 51) and redox state (52)

![Fig. 6. Long-term effect of GCV on the cell viability of BHP18-21v cells infected with AdTTF-1 and/or AdTGTK. After infection of BHP18-21v with adenoviral vectors at MOIs of 100 ± 100, cells were exposed to 10 or 30 μg/ml GCV. Cell viability was assayed on days 0, 2, 4, 6, and 8 after adding GCV. Each data point is the mean ± SE (n = 6) and is expressed relative to data from untreated cells as a percentage.](image-url)

![Fig. 7. In vitro cytotoxic effect of GCV in nonthyroid cell lines infected with AdTTF-1 and/or AdTGTK. After infection of H441 (A), MDCK (B), and HeLa (C), cells with adenoviral vectors at MOIs of 300 ± 300, 1000 ± 1000, and 1000 ± 1000, respectively, were treated with varying doses of GCV for 4 days. Cell viability was then assayed. Survival of cells was presented as a percentage of the absorbance with cells not exposed to GCV. Data represent the mean ± SE (n = 6 or 9).](image-url)
promoter needs to be assessed in various organs. Nonetheless, TTF-1 action on the TG cells maintains Pax-8 expression as well as the functions of renal cells expressing TTF-1 and lung-specific surfactant proteins (55), and MDCK lines preserving phenotypes of original organs. Thus, H441 cells lower than that in thyroid cell lines. In addition, our results and those of other investigators (49) indicated that transduction of the TTF-1 gene apparently did not activate the TG promoter in nonthyroid cell lines preserving phenotypes of original organs. Thus, TTF-1 action on the TG promoter needs to be assessed in various organs in vivo, since toxicity to normal organs is a critical limiting factor in strategies for gene therapy.

The cytotoxic effects of the TK gene can be activated by treatment with the synthetic nucleoside analogue GCV. TK converts GCV into triphosphate-GCV, which results in termination of DNA synthesis (57, 58). An important advantage of the TK gene is that cytotoxicity induced by triphosphate-GCV only affects cells that are dividing. Therefore, only minimal toxicity should result for normal cells with a low mitotic index. Taken together with thyroid cell specificity of TTF-1-activated TG promoter activity, we believe that this therapeutic strategy may be safe in vivo.

Another benefit of using TK as a toxic gene is its ability to produce a “bystander” effect in vivo (59). This effect reflects the fact that the triphosphate nucleoside analogue can be transferred from one cell to another through gap junctions (60). Indeed, >95% cell death was observed in thyroid carcinoma cell lines in our study despite infections of adenoviral vectors at MOIs capable of transduction into only 50% of cells. Reduction of infection dose of adenoviral vector made possible by the bystander effect helped to avoid cytotoxic effects from the adenoviral vector itself (data not shown). In contrast, TTF-1 protein expressed by a transduced vector cannot be transferred to adjacent cells; therefore, additional studies to determine the optimal ratio of the virus dose will be needed. Nonetheless, the present approach requires transduction of both vectors in the same cells. To maximize the effectiveness of this approach and to minimize the toxicity of the adenoviral vector, additional studies using a bicistronic vector harboring both the TG promoter-TK and CMV promoter-TTF-1 constructs will also be important.

Anaplastic carcinomas have a rapidly progressing course and represent a poor survival rate due to not only local tumor invasion but also distant metastases in major organs essential to survive. Strict tissue specificity in the toxic gene expression is necessary for treatment of metastatic thyroid cancer without severe organ toxicity. In this study, we demonstrated that cotransduction of TTF-1 with the TG promoter by adenoviral vectors reactivated the TG promoter in differentiated thyroid cells but exhibited only little effect in nonthyroid cells. Although additional studies in vivo using metastatic thyroid cancer models are necessary, this strategy shows promise for transcriptionally targeted gene therapy of metastatic thyroid cancers in which expression of TG has diminished or disappeared.

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15. Brand, K., Loser, P., Arnold, W., Bartels, T., and Strauss, M. Tumor cell-specific transduction of AdTTF-1 and AdTGTGK in HeLa cells. However, TTF-1-induced sensitization to GCV in HeLa cells was significantly lower than that in thyroïd cell lines. In addition, our results and those of other investigators (49) indicated that transduction of the TTF-1 gene apparently did not activate the TG promoter in nonthyroid cell lines preserving phenotypes of original organs. Thus, H441 cells express TTF-1 and lung-specific surfactant proteins (55), and MDCK cells maintain Pax-8 expression as well as the functions of renal epithelial ion channels (56). Nonetheless, TTF-1 action on the TG promoter needs to be assessed in various organs in vivo, since toxicity to normal organs is a critical limiting factor in strategies for gene therapy.

The cytotoxic effects of the TK gene can be activated by treatment with the synthetic nucleoside analogue GCV. TK converts GCV into triphosphate-GCV, which results in termination of DNA synthesis (57, 58). An important advantage of the TK gene is that cytotoxicity induced by triphosphate-GCV only affects cells that are dividing. Therefore, only minimal toxicity should result for normal cells with a low mitotic index. Taken together with thyroid cell specificity of TTF-1-activated TG promoter activity, we believe that this therapeutic strategy may be safe in vivo.

Another benefit of using TK as a toxic gene is its ability to produce a “bystander” effect in vivo (59). This effect reflects the fact that the triphosphate nucleoside analogue can be transferred from one cell to another through gap junctions (60). Indeed, >95% cell death was observed in thyroid carcinoma cell lines in our study despite infections of adenoviral vectors at MOIs capable of transduction into only 50% of cells. Reduction of infection dose of adenoviral vector made possible by the bystander effect helped to avoid cytotoxic effects from the adenoviral vector itself (data not shown). In contrast, TTF-1 protein expressed by a transduced vector cannot be transferred to adjacent cells; therefore, additional studies to determine the optimal ratio of the virus dose will be needed. Nonetheless, the present approach requires transduction of both vectors in the same cells. To maximize the effectiveness of this approach and to minimize the toxicity of the adenoviral vector, additional studies using a bicistronic vector harboring both the TG promoter-TK and CMV promoter-TTF-1 constructs will also be important.

Anaplastic carcinomas have a rapidly progressing course and represent a poor survival rate due to not only local tumor invasion but also distant metastases in major organs essential to survive. Strict tissue specificity in the toxic gene expression is necessary for treatment of metastatic thyroid cancer without severe organ toxicity. In this study, we demonstrated that cotransduction of TTF-1 with the TG promoter by adenoviral vectors reactivated the TG promoter in differentiated thyroid cells but exhibited only little effect in nonthyroid cells. Although additional studies in vivo using metastatic thyroid cancer models are necessary, this strategy shows promise for transcriptionally targeted gene therapy of metastatic thyroid cancers in which expression of TG has diminished or disappeared.
GENE THERAPY WITH TTF-1 AND THYROGLOBULIN PROMOTER


Transcriptional Activation of the Thyroglobulin Promoter Directing Suicide Gene Expression by Thyroid Transcription Factor-1 in Thyroid Cancer Cells

Hiroki Shimura, Hideyo Suzuki, Asako Miyazaki, et al.

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