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A tandemly repeated thyroglobulin core promoter has potential to enhance efficacy for tissue-specific gene therapy for thyroid carcinomas

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Recombinant adenoviruses, carrying herpes simplex virus thymidine kinase (*HSVtk*) genes, were developed to evaluate the possibility of tissue-specific gene therapy for thyroid carcinomas. The *HSVtk* gene was driven by a minimal thyroglobulin (TG) promoter (AdTGtk) and a tandemly repeated minimal TG promoter (Ad2×TGtk) to obtain thyroid-specific cell killing ability. The transduction of *HSVtk* genes by infection with Ad2×TGtk followed by ganciclovir (GCV) treatment showed more powerful cytotoxicity for TG-producing FRTL5 cells, a rat normal thyroid cell line, and FTC-133 cells, a human follicular thyroid carcinoma cell line, than when infected with AdTGtk *in vitro*. The cell killing ability of Ad2×TGtk was 10- to 30-fold higher than that of AdTGtk and similar to that of AdCMVtk, which carries *HSVtk* under the control of CMV promoter. Whereas after treatment with adenovirus/GCV to non–TG-producing cell lines (undifferentiated thyroid carcinoma cell lines and carcinoma cell lines from other tissues), Ad2×TGtk and AdTGtk needed more than 100-fold concentrated GCV to reach IC_{50} compared to AdCMVtk. We confirmed the enhanced efficacy of Ad2×TGtk for tissue-specific cytotoxicity *in vivo*. After adenovirus/GCV treatment for FTC-133 tumor-bearing nude mice, Ad2×TGtk enhanced tumor growth inhibition and survival rates compared to AdTGtk. Tumor growth inhibition and survival rates by Ad2×TGtk were similar to that by AdCMVtk. Moreover, any toxic effect for rat normal tissues was not revealed after intravenous injections with Ad2×TGtk and intraperitoneal administrations with GCV *in vivo*, whereas severe liver damages were observed after treatment with AdCMVtk/GCV. These data indicate a beneficial effect of Ad2×TGtk for tissue-specific gene therapy for TG-producing thyroid carcinomas without toxicity for normal tissues.
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The suicide gene/prodrug system is one of the common strategies for cancer gene therapy. Several methods using this system have been developed for delivering human herpes simplex virus thymidine kinase (*HSVtk*) genes to mammalian cells.[1–13] Recombinant adenoviruses, which are replication-incompetent, have advantages as a gene delivery system. They can be produced in high titers, and infection transduces genes with high efficacy into most tissues without integration of the transduced genes into genomic DNA of host cells. Another advantage is that a favorable promoter can be chosen for the expression of transduced genes with this system. Several groups have reported adenovirus-mediated gene therapy models using HSVtk expression driven by strong nonspecific promoters[1–8] or promoters with restricted expression.[9–13]

Thyroid carcinomas are the most common endocrine neoplasia. They are pathologically classified into papillary, follicular, anaplastic, and medullary cancers. Papillary and follicular thyroid cancers are the two most frequent entities, usually referred to as differentiated thyroid carcinoma, with frequencies of 50–70% and 10–15%, respectively, in all thyroid carcinomas.[14] Immunostaining for thyroglobulin (TG) is almost always positive in both papillary and follicular thyroid tumors.[14,15] Anaplastic thyroid carcinoma is one of the most aggressive human cancers and referred to undifferentiated thyroid carcinoma, with a frequency of 5–15% in the cases. It usually shows no or tiny amount of TG secretion. Medullary tumors derive from the calcitonin-secreting parafollicular C cells of the thyroid. Thus, they do not express TG.[14,15]

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Differentiated thyroid carcinomas, generally accepted to have a good prognosis, often transform to the cells that can invade into surrounding tissues or can metastasize to lymph nodes and lungs.[14] Approximately 30% of patients with these carcinomas experiences recurrence after initial treatment.[16] These cases are usually treated with radioactive iodine therapy or anticarcinogenic agents although effects of these treatments are limited. Differentiated thyroid carcinomas often show anaplastic changes and the patients with these carcinomas are killed by dissemination of these transformed cells. Thus, a novel clinical approach for treating these carcinomas is required.

Differentiated thyroid carcinomas, in many cases, are able to produce and release TG.[14] The TG is a glycoprotein precursor for thyroid hormone biosynthesis,[17] and can be detected only in the thyroid follicular cells.[18] The presence of tissue specificity is thought to be based on the cell-specific function of the promoter of TG.[19,20] The promoter region of TG has binding sites for thyroid transcriptional factor-1 (TTF-1), TTF-2, and Pax-8. These *trans*-acting factors positively regulate the expression of TG at the transcriptional level.[21–32] The rat TG promoter, especially 0.2 kbp upstream from the transcriptional start site (TSS), is established to play an important role in the thyroid hormone–specific TG expression.[21] Based on these findings, it is possible to speculate that gene therapy using TG promoter is useful for the treatment of TG-producing carcinomas.

Zeiger et al[1] demonstrated that FRTL5 cells, which are derived from normal rat thyroid cells, were killed by stable transfection with TG*tk* genes followed by addition of ganciclovir (GCV) to culture. They also demonstrated that recombinant adenovirus, injected through the intracardiac route, was useful for delivering *lacZ* genes under the control of CMV promoters into thyroid cells of neonatal rats. Braiden et al[33] reported that TG*tk* genes induced cell-specific cytotoxicity by infection with recombinant retroviruses, in which the cell lines could permanently express TG*tk* gene as retroviruses integrated their own genes into genomic DNA of host cells. We previously reported that cell-specific gene expression for TG*tk* was successfully mediated by recombinant adenoviruses *in vitro*.[34] The Cre–*lox*P system has been introduced into adenovirus-mediated gene therapy in order to enhance cell killing ability without losing tissue specificity.[35] Nagayama et al[36] tried this system for experimental gene therapy for thyroid carcinomas using *HSVtk* genes as a suicide gene driven by a TG promoter. By using stable transfectants but not infection with viruses, Kitazono et al[37] reported that the enhancer sequence of the TG promoter increased transcriptional activity in combination treatment with histone deacetylase inhibitor or sodium butyrate *in vitro*. These studies confirmed that infection with recombinant adenoviruses expressing TG*tk* genes is available for gene therapy in TG-producing thyroid carcinomas. However, proven clinical methods have not yet been established because of their unknown efficiency, unestablished cell specificity, and undetermined side effects *in vivo*.

In the present study, we established a tandemly repeated TG promoter fused to the *HSVtk* gene for cell-specific gene therapy for thyroid carcinomas. And we examined the effect of this recombinant gene by infection with recombinant adenoviruses both *in vitro* and *in vivo*.

Materials and methods

Cell lines and cell cultures

Continuous lines of cultured cells, FRTL5 cells, a normal rat thyroid cell line,[38] COS1 cells, SV40-transformed CV-1 cells which were derived from a monkey kidney carcinoma, HepG2 cells, a human hepatoma cell line, and 293 cells, a human embryonal kidney cell line were purchased from the American Type Culture Collection (Rockville, VA). FTC-133 cells, a human follicular thyroid carcinoma cell line, were purchased from the European Collection of Animal Cell Cultures (OJG, UK). Continuous cell lines, 8505C and TCO-1 cells, human undifferentiated thyroid carcinoma cell lines, and Panc1, a human pancreatic carcinoma cell line, were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cultured rat hepatoma dRLH-84 cells[39] were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

FRTL5 cells were grown in HamF-12K medium supplemented with 5% calf serum (CS), 100 U/mL penicillin, and 100 μg/mL streptomycin (PCSM), and six hormones or growth factors (6H) (4 U/L bovine TSH, 5 U/L insulin, 5 U/L transferrin, 2.5 nM hydrocortisone, 10 μg/L somatostatin, and 10 μg/L glycyl-L-histidyl-L-lysine acetate) as the cell bank recommended. HepG2, COS1, dRLH-84, TCO-1, and Panc1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), PCSM, and 6H. FTC-133 cells were grown in medium of DMEM/F-12 supplemented with 10% FBS, PCSM, and 6H. Cultured 293 cells were grown in minimal essential medium (MEM), supplemented with 10% FBS and PCSM. Cultured 8505C cells were also grown in MEM, supplemented with 10% FBS, PCSM, and 6H. Rat fibroblasts were derived from skin of Fisher rats (Nippon SLC, Hamamatsu, Japan) and maintained in DMEM with 10% FBS, PCSM, and 6H.

Plasmid construction and preparation of recombinant adenovirus

The plasmid of pTGCAT5′-41, containing the rat TG promoter region from −284 to +39 bp fused to the *CAT* gene,[21] was kindly provided by Dr R Di Lauro (Napoli, Italy). The plasmid pBS-tk, which includes the *HSVtk* gene fused to a bovine growth hormone gene polyadenylation signal (polyA),[6] was a kind gift from Dr SLC Woo (Baylor College of Medicine). The plasmid pJM17 was kindly provided by Dr S Refetoff (University of Chicago). The recombinant adenovirus AdTKluc,[40] carrying the luciferase gene driven by the TK promoter, was also kindly provided by Dr S Refetoff (University of Chicago). The plasmids pΔE1sp1B, pCA14, and pJM13 were purchased from Microbix Biosystems (Toronto, Canada).

To create the plasmid including the *HSVtk* gene driven by the TG promoter, the *Bam*HI–*Xho*I fragment of the *HSVtk* gene with polyA from pBS-tk was inserted into the multiple cloning site of pGEM7Z (Promega, Madison, WI)

(pGEM7Z-tk). Then the fragment of *HSVtk* with polyA was cut out at *Bam*HI and *Xba*I sites from pGEM7Z-tk and inserted into pBluescript (Stratagene, La Jolla, CA) (pBlueS-tk). The *Eco*RI–*Hin*dIII fragment of the TG promoter gene from pTGCAT5′-41 was subcloned into the multiple cloning site of pGEM7Z (pGEM7Z-TG). Then the TG promoter gene was cut out from pGEM7Z-TG at *Bam*HI and *Bam*HI sites and transferred to a *Bam*HI site of pBlueS-tk upstream of the *HSVtk* gene (pBlueS-TGtk). In this step, the pBlueS-2×TGtk plasmid, containing tandemly repeated two TG promoters upstream of the *HSVtk* gene in pBluescript, was also constructed. Proper directions of the TG promoters in pBlueS-TGtk and pBlueS-2×TGtk were checked by the digestion with *Hin*dIII. The *Eco*RI –*Xba*I fragments of TG*tk* and 2×TG*tk* from pBlueS-TGtk and pBlueS-2×TGtk were inserted into pΔE1sp1B at the corresponding sites (pΔE1-TGtk and pΔE1-2×TGtk, respectively).

For making a plasmid including the *HSVtk* gene under the control of the CMV promoter, the *Hin*dIII–*Bam*HI fragment of pBlueS-tk was inserted into the multiple cloning site of pCA14 to make pCA14-tk. pCA14-tk and pJM13 were used to make recombinant adenoviruses. We also constructed an adenovirus vector expressing β-galactosidase (β-gal; *lacZ*) under the control of the CMV promoter as previously described.[41]

To construct a plasmid expressing *lacZ* driven by the TG promoter, the coding region of the *lacZ* gene with polyA from pSV-β-gal (Promega, Madison, WI) was subcloned into the multiple cloning site of pΔE1sp1B at the *Hin*dIII and *Bam*HI sites (pΔE1-lacZ). The *Hin*dIII–*Hin*dIII segment of the TG promoter gene from pBlueS-TGtk was ligated into the *Hin*dIII site of pΔE1-lacZ (pΔE1-TGlacZ). Proper direction of the TG promoter in pΔE1-TGlacZ was checked by the digestion with *Eco*RI.

To generate replication-defective recombinant adenoviruses, pΔE1-TGtk, pΔE1-2×TGtk, or pΔE1-TGlacZ was cotransfected with pJM17 into 293 cells by the calcium phosphate precipitation method to produce AdTGtk, Ad2×TGtk, and AdTGlacZ, respectively. In making AdCMVtk, pCA14tk and pJM13 were cotransfected. Each recombinant adenovirus was isolated from a single plaque. Integrity of the construct was checked by DNA digestion with proper restriction enzymes. Recombinant adenoviruses were expanded in 293 cells, and purified by double cesium chloride gradient ultracentrifugations. After dialysis, the adenoviruses were stored in 10% glycerol at −80°C. Plaque-forming assays were repeated more than three times to determine the titer of each recombinant adenovirus.

For creating a reporter plasmid, including the *luciferase* gene driven by the TG promoter, the *Bam*HI–*Hin*dIII fragment from pTGCAT5′-41 containing the TG promoter was subcloned at *Bgl*II and *Hin*dIII sites of the multiple cloning site of pGL3-Basic vector (Promega, Madison, WI) (pGL3-TGluc). To make a reporter plasmid expressing the luciferase under the control of a tandemly repeated TG promoter, the TG promoter region was cut out at *Hin*dIII and *Hin*dIII sites from pBlueS-2×TGtk and inserted into the *Hin*dIII site of pGL3-TGluc (pGL3-2×TGluc). Proper direction of the TG promoter in pGL3-2×TGluc was checked by the digestion with *Hin*dIII.

Transient transfection and reporter assay procedures

FRTL5 cells were plated in 24-well cell culture plates at a density of 4×10^4 cells per well 24 hours prior to transfection. pGL3-TGluc or pGL3-2×TGluc genes (500 ng/well) were transfected as reporters using calcium phosphate precipitation method. The same amounts of pGL3-Basic or pGL3 promoter plasmids (Promega, Madison, WI) were also transfected as negative and positive controls, respectively. The plasmid of β-gal (Promega, Madison, WI) (250 ng/well) was cotransfected for evaluating transfection efficiency. Cells were incubated with 0.5 mL of medium containing transfection mixture for 12 hours and the medium was freshly changed. Incubation was continued for an additional 24 hours. Then medium was discarded and cells were washed twice with PBS. Cells in each well were harvested with 10 mM Tris Cl (pH 7.4), 1 mM EDTA, and 150 mM NaCl and lysed with 50 μL of reporter assay reagent (Promega, Madison, WI). Cell lysate (10 μL each) was used for luciferase and β-gal assay. Luciferase activity was determined by luciferase assay system (Promega, Madison, WI) using Lumat LB9501 (Berthold Japan, Tokyo, Japan). Assay for β-gal activity was performed by the method previously described.[42]

Cultured cells of FTC-133, 8505c, TCO-1, dRLH-84, HepG2, COS1, Panc1, and rat fibroblasts were plated in 24-well culture plates at a density of 2×10^4 cells/well 24 hours prior to transfection. The methods of cotransfection and reporter assays were the same as FRTL5 cells. All data of luciferase activity were corrected for β-gal activity to account for variation in transfection efficiency and expressed as percentages for the results using pGL3 promoter. Each transfection was conducted in triplicate, and data represent the mean±SD from more than three individual experiments.

In vitro *β-gal expression by infection with recombinant adenoviruses*

FRTL5 cells were plated in 24-well plates at a density of 8×10^4 cells/well 24 hours prior to infection with recombinant adenoviruses. Cells were then infected with AdCMVlacZ or AdTGlacZ at the doses of 0–200 multiplicity of infection (MOI) for 2–24 hours in minimal volume of HamF-12K medium supplemented with 2% CS, PCSM, and 6H. As pilot tests showed that infection with 100 MOI of AdCMVlacZ to FRTL5 cells for 12 hours led to nearly maximal β-gal induction without toxic effect of adenovirus, we chose this condition. The medium was freshly changed and incubation was continued for an additional 24 hours. Then β-gal activity was determined by the methods previously described.[42] HepG2, COS-1, and dRLH-84 cells were plated in 24-well plates at a density of 4×10^4 cells/well. Procedures for infection with recombinant adenoviruses and β-gal assay were the same as FRTL5 cells. All data of β-gal activity by AdTGlacZ were expressed as percentages for activity by AdCMVlacZ. Each box and bar shows the mean±SD from more than three independent experiments.

In vitro *cytotoxic effect by adenovirus/GCV*

FRTL5 cells were plated in 96-well plates at a density of 5×10^3 cells/well 24 hours before infection with adenovi-

ruses. Cells were infected with 100 MOI of recombinant adenoviruses for 12 hours in minimal volume of HamF-12K medium supplemented with 2% CS, PCSM, and 6H. Medium was changed to fresh HamF-12K supplemented with 5% CS, PCSM, and 6H in the presence of various concentrations (0–100 μM) of GCV (F Hoffmann-La Roche, Basel, Switzerland) and incubation was continued for 2–6 days. Then MTT assay was performed by CellTiter 96™ Nonradioactive Cell Proliferation Assay (Promega, Madison, WI) according to the protocol from the manufacturer, using Microplate reader Model 550 (BIO-RAD, Hercules, CA).

Ten MOI of adenoviruses were used for the experiments for FTC-133 because infection with more than 30 MOI of AdCMVtk showed toxic effects for FTC-133 cells even in the absence of GCV, and 5–20 MOI of AdCMVtk had enough effect for cytotoxicity for FTC-133 cells in the presence of GCV. AdCMVlacZ showed strong cytotoxic effect for FTC-133 cells in the absence of GCV, whereas AdTKluc did not show any toxic effect for each cell line in the presence of 100 μM GCV. Thus, we used AdTKluc as a negative control in this study. FTC-133 cells were plated in 96-well plates at a density of 2×10^3 cells/well 12 hours prior to infection with adenoviruses. Cells were infected with adenoviruses for 2 hours in minimal volume of medium supplemented with 2% FBS, PCSM, and 6H. Procedures after infection with adenoviruses were the same as FRTL5 cells. Other cell lines tested in this study were used according to the same protocol as FTC-133 cells.

To evaluate the differences of the virus titer-dependent cytotoxic effects between Ad2×TGtk and AdTGtk, FRTL5 and FTC-133 cells were infected with various doses of adenoviruses. Incubation was continued with 30 μM GCV for 5 days. Then cell viability was determined by MTT assays as described above. All data of cell viability were expressed as the percentages relative to the untreated controls (mock) in the absence of GCV. Each cytotoxicity assay *in vitro* was conducted in triplicate, and data represent the mean±SD from more than three individual experiments.

Comparison of bystander effect between Ad2×TGtk and AdTGtk

FTC-133 cells, plated in six-well plates, were infected with 30 MOI of each adenovirus in medium containing 2% FBS (total 800 μL) for 2 hours at 37°C. Infected or noninfected cells were washed with FBS-free medium for three times and incubation was continued for an additional 6 hours with fresh medium containing 10% FBS in the absence of adenovirus. Cells were then harvested and mixed in tubes at the various ratios for infected and noninfected cells in medium including 10% FBS and 30 μM GCV. Mixed cells were plated into 24-well plates at a density of 2.0×10^4 cells/well. Incubation was continued for 5 days and MTT assays were performed. Each cell lysate, stained with MTT and lysed with the lysis buffer, was transferred into a 96-well plate (100 μL each) and OD_{570} was measured to determine cell viability.

Tumor growth inhibition by adenovirus/GCV in vivo

To establish a tumor-bearing animal model, cultured FTC-133 cells were harvested and injected subcutaneously into a balb-c nu/nu mouse (Nippon SLC, Hamamatsu, Japan) at a density of 5×10^6 cells in 100 μL of FBS-free medium. Tumor volumes were measured with vernier calipers and calculated from the following formula: (length×width×height/2), which is derived from the formula for an ellipsoid ($\pi d^3/6$). When the tumors reached an average size of 250 mm^3, they were injected with 1×10^9 plaque-forming units (pfu) of AdTKluc, AdCMVtk, AdTGtk, or Ad2×TGtk at day 0 with a 1-mL insulin syringe. Intraperitoneal injections of GCV (100 mg/kg body weight) were started 24 hours after the adenovirus injection at day 1. Administration of GCV was continued once a day until day 14. Tumor volumes were measured as described above every 2 or 3 days. The data of tumor volumes were expressed as the percentage relative to the values of day 0 just before the injection of adenoviruses in each group. Each point and bar shows the mean±SEM of more than five mice in each group. Survival rates were also determined as the percentages of the number of alive mice for total numbers of mice for each group.

In vivo *toxic effect by infection with adenoviruses*

Fisher male rats were purchased from Nippon SLC and maintained under standard conditions. Each adenovirus was injected into a jugular vein of a rat at the dose of 5×10^9 pfu in 200 μL of PBS. To rats infected or noninfected with AdCMVtk, AdTGtk, or Ad2×TGtk, GCV was intraperitoneally given 24 hours after viral injection at the dose of 100 mg/kg body weight/day. Administration of GCV was continued up to day 10. Three hundred microliters blood was saved from a jugular vein of each rat 3 days after starting GCV treatment to measure serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels using a commercial kit (Sigma, St. Louis, MO). Rats were sacrificed 24 hours after the last GCV injection and tissues of liver, kidney, and spleen were taken and fixed in 30% chloroform. Rats injected with AdCMVtk were sacrificed on day 5 because all the rats in this group were dead within 10 days after starting treatment with GCV. The tissue specimens were embedded in paraffin, sectioned, and stained with hematoxylin–eosin for pathological examination.

Results

Induction of cell-specific β-gal expression by AdTGlacZ

In order to evaluate whether the TG promoter can work in cell-specific manner, β-gal activity was measured in TG-producing or non–TG-producing cells after infection with AdCMVlacZ or AdTGlacZ (Fig 1). The β-gal was expressed in all cell lines when the cells were infected with AdCMVlacZ. In contrast, β-gal activity induced by AdTGlacZ was only detected in TG-producing FRTL5 cells. In HepG2, COS1, and dRLH-84 cells, which did not produce TG, β-gal activity was not induced by infection with AdTGlacZ (Fig 2). These results indicated that the TG promoter works only in TG-producing cells and that the system of infection with adenovirus carrying the TG

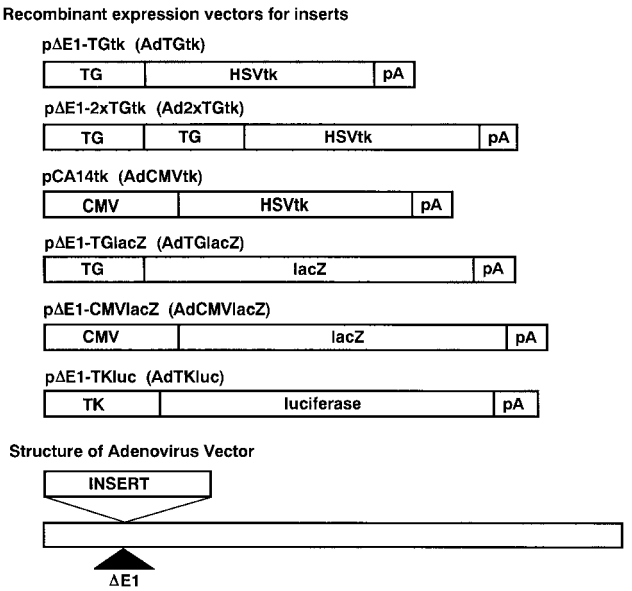

Figure 1 Schematic representation (not drawn to scale) of the structure of the plasmids and the adenovirus vectors. The pΔE1-TGtk carries the TG promoter fragments (from −284 to +37), which were fused to the *HSVtk* genes containing bovine growth hormone gene polyadenylation signals (pA). The pΔE1-CMVtk carries the same *HSVtk* genes fused to the CMV promoters. The pΔE1-TGlacZ and the pΔE1-CMVlacZ have the *lacZ* genes instead of the *HSVtk* genes in the pΔE1-TGtk and pΔE1-CMVtk, respectively. The pΔE1-2×TGtk carries tandemly repeated TG promoters, fused to the *HSVtk* genes. These plasmids were cotransfected into 293 cells with pJM17 or pJM13 (E1 deleted) to generate recombinant adenoviruses. The terms in parentheses are the nomenclatures of recombinant adenoviruses.

promoter is useful for thyroid cell–specific expression of proteins.

Transcriptional activation induced by the TG promoter

Transcriptional activity induced by the TG promoter was estimated by using the transient transfection system. When the luciferase activity, which was induced by the reporter plasmid containing the luciferase gene driven by the SV40 promoter (pGL3 promoter; Promega), was defined as 100%, the activity induced by the TG promoter increased to 330% in FRTL5 cells (Fig 3). When a tandemly repeated TG promoter was used (2×TGluc), the activity further increased. The increase induced in a system using 2×TGluc was observed not only in FRTL5 cells but also in FTC-133 cells, which were derived from a human follicular thyroid carcinoma. However, the increase induced in these systems using the TGluc or 2×TGluc reporter plasmids was not observed in cells that cannot produce TG. These results indicate that TG-producing cell-specific work is present in the action of TG and 2×TG promoters and that a 2×TG promoter is more powerful than a single TG promoter in the induction of gene expression.

Cytotoxic effect of GCV on cells infected with recombinant adenoviruses

The effect of GCV on cell viability was estimated in the FRTL5 cells infected with recombinant adenoviruses. Re-

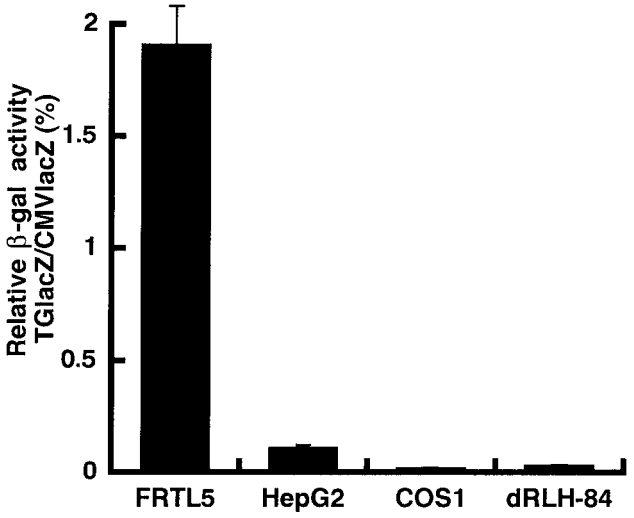

Figure 2 Comparison of β-gal activity expressed in FRTL5, HepG2, COS1, and dRLH-84 cells, infected with AdTGlacZ. Cells were infected with AdTGlacZ or AdCMVlacZ and β-gal activity was measured as related in *Materials and methods*. The vertical axis indicates the percentages of β-gal activity of the TGlacZ group for the activity of the CMVlacZ group. Each box and bar shows the mean±SD from more than three independent experiments.

combinant expression vectors were constructed as shown in Figure 1, and replication-defective recombinant adenoviruses were generated in 293 cells as related in methods. As

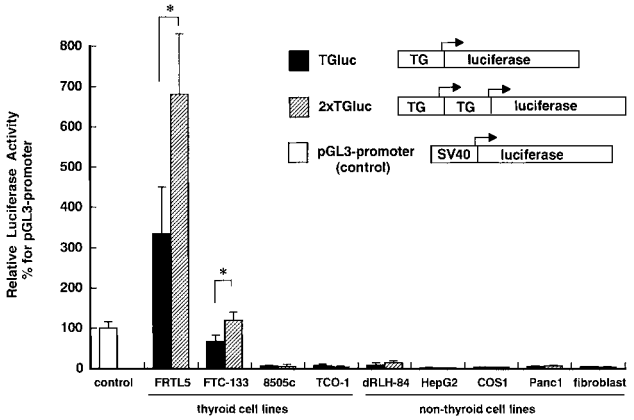

Figure 3 Cell-specific enhanced transcriptional activity by a tandemly repeated TG promoter. Indicated pGL3-TGluc, pGL3-2×TGluc, or pGL3 promoter plasmids were cotransfected with β-gal plasmids into each cell line, and luciferase and β-gal activities were determined, as described in *Materials and methods*. The following cell lines were used: FRTL5 (rat normal thyroid), FTC-133 (human follicular thyroid carcinoma), 8505C and TCO-1 (human undifferentiated thyroid carcinoma), dRLH-84 (rat hepatoma), COS1 (monkey kidney carcinoma), Panc1 (human pancreatic carcinoma), and rat fibroblasts. After 24 hours of incubation, cells were harvested and luciferase and β-gal activities were determined. All data of luciferase activity were corrected for β-gal activity to account for variation in transfection efficiency and expressed as percentages for the results using pGL3 promoter (control; the results using pGL3 promoter). Each transfection was conducted in triplicate, and data represent the mean±SD from more than three individual experiments. Statistical significance was evaluated by Student's *t*-test. $^*P<.05$.

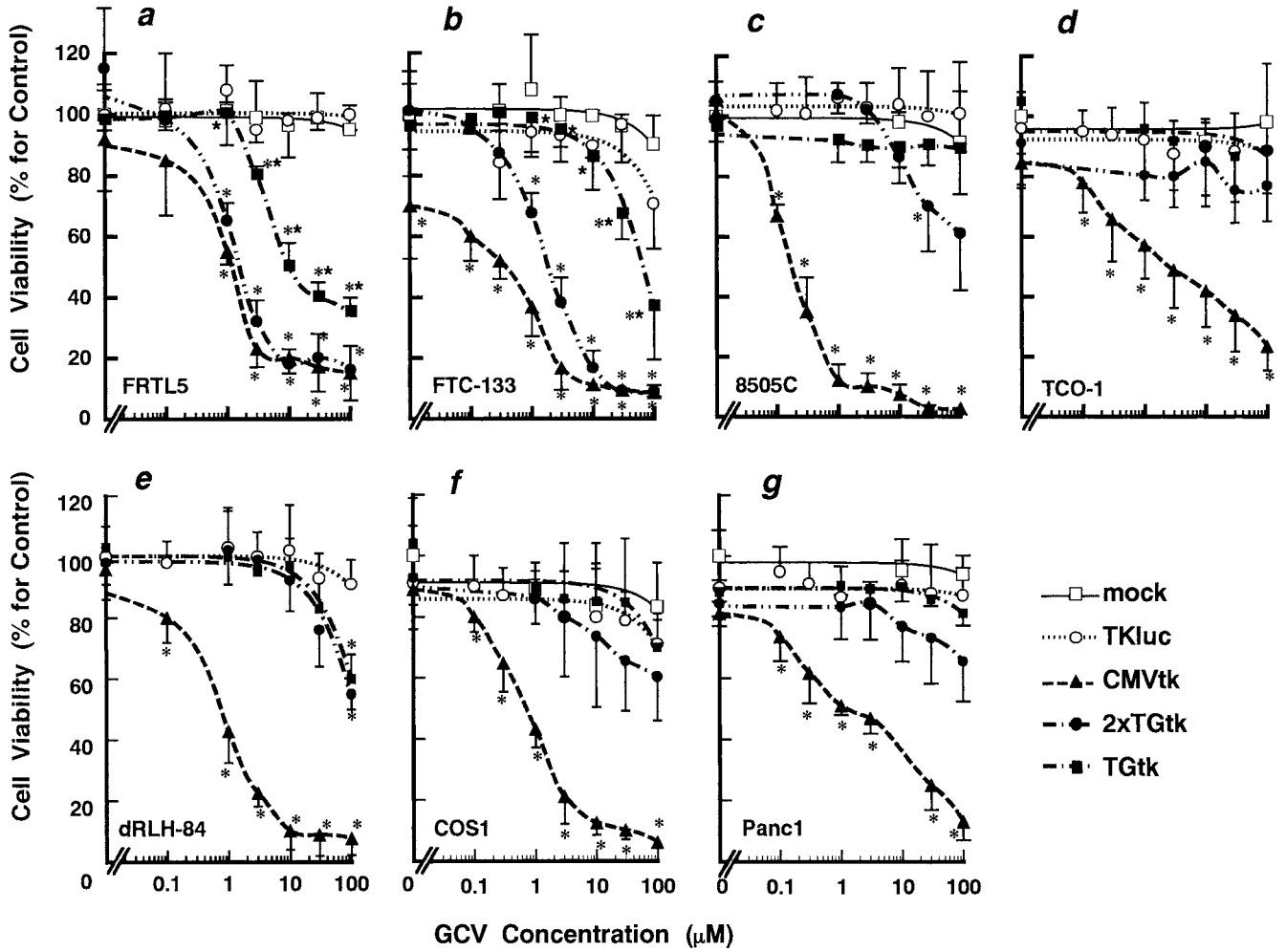

Figure 4 The GCV dose-dependent cytotoxicity by recombinant adenoviruses and GCV. The cell lines used were described in Figure 3. FRTL5 **(a)** or other indicated cells **(b–g)** were infected with 100 or 10 MOI of adenoviruses, respectively, and treated with GCV at indicated concentrations in 96-well plates. Five days after infection, cell viability was measured as described in *Materials and methods*. All data of cell viability were expressed as the percentage relative to the untreated controls in the absence of GCV. Each point and bar shows the mean ± SD from more than three independent experiments. Mock means the cell group treated with GCV without infection with adenovirus. Statistical significance was evaluated by analysis of variance. **P*<.05 compared to TKluc group. **P*<.05, TGtk vs. 2×TGtk group.

controls, we used two groups: one (mock) was without infection, and the other was infected with AdTKluc. In a study of time course, GCV-induced cytotoxicity was significantly greater in CMVtk, TGtk, and 2×TGtk groups than that in mock and TKluc groups during 2–6 days of incubation of the cells with GCV. The significant decrease in cell viability was observed from the second day of incubation in the former three groups (data not shown).

Cell-specific viability was examined by using FRTL5 (Fig 4a), FTC-133 (Fig 4b), 8505C (Fig 4c), TCO-1 (Fig 4d), dRLH-84 (Fig 4e), COS1 (Fig 4f), and Panc-1 (Fig 4g). Cells were infected with 10 MOI (in case of FRTL5, 100 MOI were infected) of each adenovirus and incubated with 0–100 μM GCV for 5 days. Cell viability in control groups (mock or TKluc) was not affected by GCV in any cell lines. In contrast, the viability in the AdCMVtk-infected group markedly decreased in all cell lines. Infection with AdTGtk or Ad2×TGtk showed a GCV concentration–dependent decrease in viability in FRTL5 and FTC-133 cells, and the decrease was significant at 1.0 μM GCV. The decrease, however, was not observed in other cells. The IC_{50} values obtained by GCV treatment were 4, 11, and 60 μM in FRTL5 cells, and 1, 2, and 40 μM in FTC-133 cells after infection with AdCMVtk, Ad2×TGtk, and AdTGtk, respectively. These results suggested that the HSVtk/GCV system is useful for induction of TG-producing cell-specific cytotoxicity when AdTGtk or Ad2×TGtk was used as a vector, and that the cytotoxic potency by Ad2×TGtk is stronger than that induced by AdTGtk.

As has been shown in Figure 3, approximately 5-fold higher activity of luciferase was observed in FRTL5 cells compared to that in FTC-133 cells both in TGluc and 2×TGluc groups. This result suggested that FRTL5 cells are able to produce more TG compared to FTC-133 cells. However, both AdTGtk and Ad2×TGtk showed nearly the same cytotoxicity comparing between FRTL5 and FTC-133

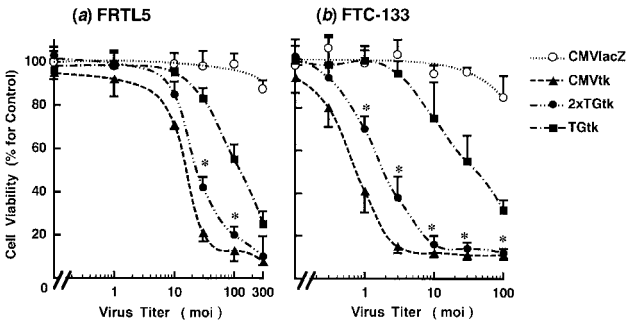

Figure 5 The virus titer–dependent sensitivity to GCV. Cytotoxicity assays were performed as described in Figure 4. Indicated doses of adenoviruses were infected to FRTL5 **(a)** and FTC-133 cells **(b)** and cells were incubated with 30 μM GCV for 5 days. Cell viability was determined as described in *Materials and methods*. All data of cell viability were expressed as the percentage relative to TKluc group in the absence of GCV. Each point and bar shows the mean ± SD from more than three independent experiments. Statistical significance was evaluated by analysis of variance. **P*<.05 compared to TGtk group.

cells, even using 100 MOI of adenoviruses for FRTL5 and 10 MOI for FTC-133 cells. These results indicated that the difference of infectivity should be present between two cell lines. We determined the infectivity of adenovirus for these cell lines using infection with AdTKluc. Approximately 25- and 20-fold higher activity of luciferase was obtained in FTC-133 cells at 5 and 30 MOI, respectively, when compared to those in FRTL5 cells (data not shown). This result indicates that infectivity with adenovirus for FTC-133 cells is higher than that for FRTL5 cells. This is a beneficial phenomenon for the adenovirus-mediated gene therapy for thyroid carcinomas.

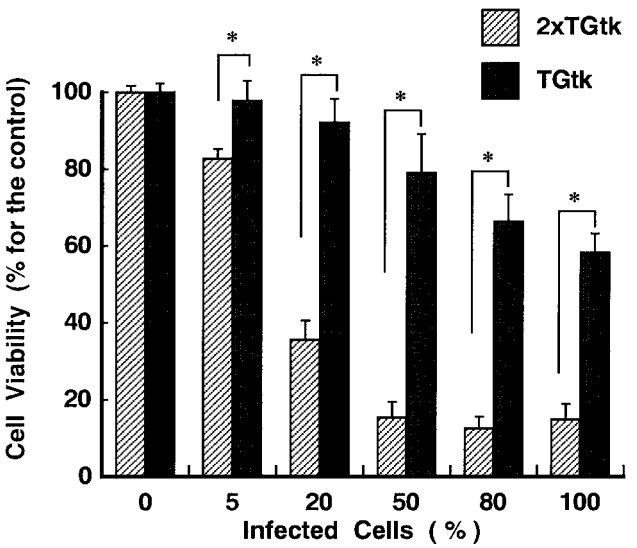

Figure 6 Comparison of bystander effect between Ad2×TGtk and AdTGtk in FTC-133 cells. Indicated percentages of adenovirus-infected cells were mixed with noninfected cells and cell viability was determined after 5 days of incubation in the presence of 30 μM GCV as described in *Materials and methods*. Each datum represents the mean ± SD from more than three independent experiments. Statistical significance was evaluated by Student's *t*-test. **P*<.05.

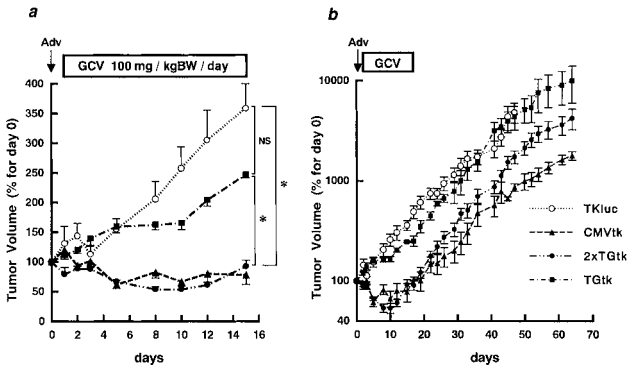

Figure 7 Tumor growth inhibition by adenovirus/GCV *in vivo*. A human follicular thyroid carcinoma cell line, FTC-133 cells, was transplanted to balb-c nu/nu mice. Adenoviruses (1×10^9 pfu) were injected into tumors on day 0, and 100 mg/kg body weight GCV was intraperitoneally given once a day for 14 days. The data of tumor volumes were expressed as the percentages relative to the values of day 0 in each group. The volume changes during a GCV administration period **(a)** and a long period **(b)** are shown. Each point and bar shows the mean ± SEM from more than five mice in each group. The number of mice was decreased during a long period of observation because of death of mice in each group. Statistical significance was evaluated by analysis of variance. **P*<.05.

Adenovirus titer–dependent cytotoxicity was also determined as shown in Figure 5. When cells were infected with AdTGtk, approximately 6- and 18-fold higher titers were needed to reach IC_{50} values, compared to Ad2×TGtk in FRTL5 and FTC-133 cells, respectively. The bystander effect was observed in cells infected with Ad2×TGtk at the percentage of 5% (Fig 6). A mixture of 50% infected and 50% uninfected cells led cell viability to the same levels as that from 100% infected cells with Ad2×TGtk. However, infection with AdTGtk showed no or minimal bystander

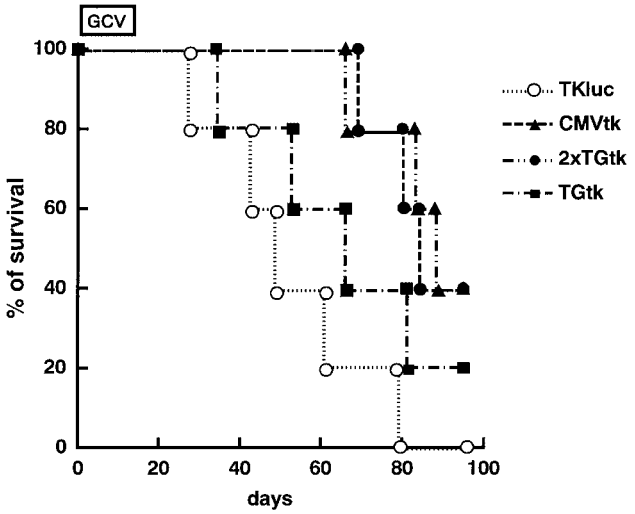

Figure 8 Survival rates of tumor-bearing nude mice. Treatment for tumor-bearing nude mice was described in Figure 7. Survival rates were determined as the percentages of the number of alive mice for total numbers of mice for each group.

effect in this cell line. This result suggested that efficient tk expression by the 2×TG promoter further increased efficiency of bystander effect. The results from Figures 4–6 indicated that Ad2×TGtk more efficiently worked for cytotoxicity in TG-producing cells than AdTGtk.

Effect of viral infections on tumor growth in vivo

FTC-133 cells were transplanted to nude mice and the changes in the tumor volume were estimated *in vivo*. As shown in Figure 7a, tumor volume increased in a time-dependent manner even during injection of GCV in the control (TKluc) group. In contrast, decrease in the volume was observed in a group treated with Ad2×TGtk after the beginning of GCV. The inhibition of tumor growth in this group continued during the period of GCV and the growth curve of tumors was similar to that of CMVtk group. However, the significant difference between control and the TGtk group was not observed. Tumor volumes, however, began to increase after the period of GCV administration even in Ad2×TGtk or AdCMVtk group. However, the time required to reach 1000% of tumor volume was longer in these groups (42 and 50 days, respectively) than that (26 days) in the control (AdTKluc) group (Fig 7b).

Survival rates of tumor-bearing nude mice were shown in Figure 8. The mice infected with AdTKluc began to die on day 27. In this group, all mice were dead on day 80. In contrast to this group, the beginning of death was observed on day 66 in the group infected with Ad2×TGtk. The calculated time lag of decrease in survival rate was approximately 40 days between control and 2×TGtk group (Fig 8).

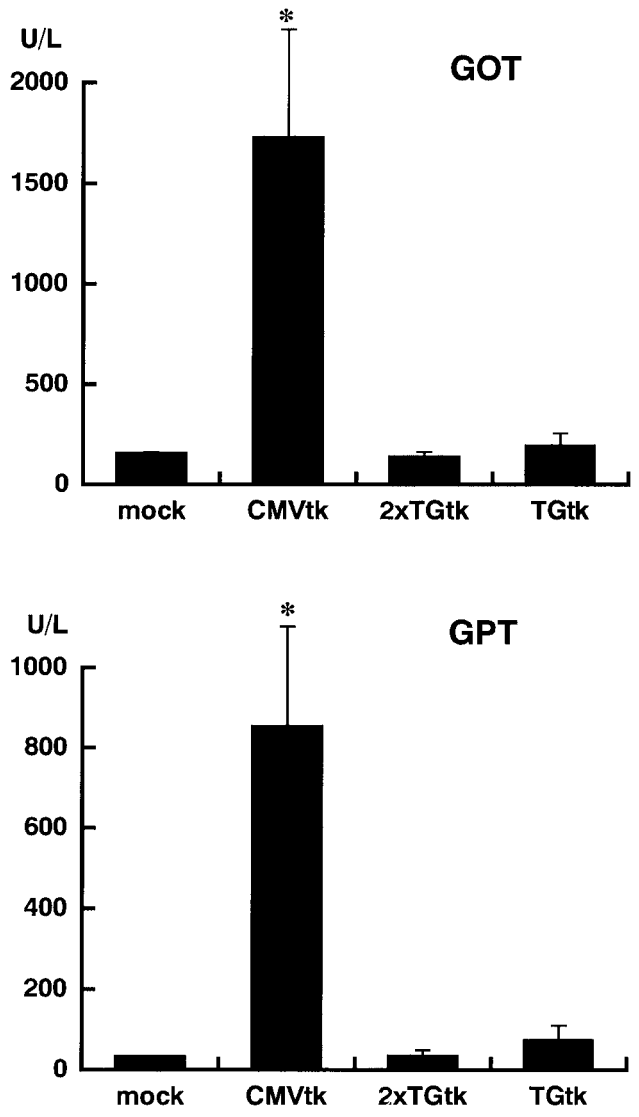

Figure 9 Rat serum transaminase levels after an intravenous injection with adenoviruses and intraperitoneal administrations with GCV. Each adenovirus was injected into a jugular vein of a Fisher rat and GCV was intraperitoneally given. Serum levels of GOT **(upper panel)** and GPT **(lower panel)** were measured 3 days after starting GCV treatment. Statistical significance was evaluated by Student's *t*-test. **P*<.01 compared to mock group.

Toxic effect of HSVtk/GCV system

The toxic effect of AdTGtk/GCV or Ad2×TGtk/GCV was analyzed in rats. As markers for liver damage, serum GOT and GPT levels were measured during HSVtk/GCV treatment. Enzyme levels drastically increased in rats treated with AdCMVtk/GCV. However, no significant elevation of transaminase levels was observed in animals given Ad2×TGtk or AdTGtk followed by GCV (Fig 9). These results indicate that infections with Ad2×TGtk and AdTGtk do not induce damage of hepatocytes, although AdCMVtk does. Pathological examination revealed hepatic necrosis associated with lymphocyte infiltration in rats treated with AdCMVtk/GCV (data not shown). In these animals, damages in kidneys and spleens were also observed. In contrast, no abnormal pathological findings were observed in livers, kidneys, and spleens of rats treated with Ad2×TGtk or AdTGtk.

Discussion

As is well documented, tissue-specific promoters are suitable for virus infection–induced gene therapy for cancers. However, it has been indicated that tissue-specific promoters are too weak to be used for killing target cells. In order to increase the cytotoxic sensitivity, many trials for improvement of methods have been studied. Nagayama et al[36] introduced the Cre–*lox*P system in order to increase the tissue-specific cytotoxicity in gene therapy for thyroid carcinomas, in which they used *HSVtk* genes as a suicide gene under the control of the TG promoter. They demonstrated that a double infection of adenoviruses, carrying TG-Cre and CAG-*lox*P-tk genes, had more efficacy for killing TG-producing cells than transducing *TGtk* genes alone. Kitazono et al[37] constructed the TG enhancer/TG promoter *HSVtk* gene and stably transfected it into cells, and they obtained a result that sensitivity to GCV in this system is higher than that in a system using TK promoter in FTC-133 cells. They also introduced histone deacetylase inhibitors and sodium butyrate in combination with 8-bromo-cAMP to enhance transcriptional activation

of the TG promoter. However, the *in vivo* effects, especially side effects, of these compounds have not yet been evaluated.

In this study, we introduced a tandemly repeated TG promoter, which is fused to the *HSVtk* gene and developed an Ad2×TGtk as one of the infecting adenoviruses. For the preparation of the Ad2×TGtk, the following two points were considered: the first is how to maintain cell specificity, and the second is how to enhance the cytotoxic effect of HSVtk/GCV system. Musti et al[21] demonstrated that tissue-specific transcriptional activity requires 167-bp nucleotides upstream of TSS. Further, they showed that the minimal TG promoter extending up to −284 bp upstream from the TSS has 2.4-fold higher transcriptional activity than an 827-bp-long TG promoter. Thus, we used the minimal TG promoter including 284-bp nucleotides upstream from TSS. As demonstrated in *Results*, the Ad2×TGtk, which was prepared as described, had a strong potential for GCV-induced cytotoxicity with a TG-producing cell-specific manner.

GCV dose-dependent or virus titer-dependent cytotoxicity assays demonstrated that Ad2×TGtk had more efficient effect for killing cells with tissue specificity than AdTGtk. The reason why the Ad2×TGtk was stronger than AdTGtk in cell killing ability is not clear. We observed this phenomenon both *in vitro* and *in vivo*. Transcriptional activity of a 2×TG promoter was higher than a single TG promoter in transient transfection studies, indicating that the number of binding sites for factors regulating TG gene expression is increased by a tandemly repeated TG promoter. As reported, binding sites for thyroid-specific transcription factors, such as TTF1, TTF2, and Pax8, are present on a TG promoter including 284-bp nucleotides upstream from TSS. Although it is possible that the greater effect of Ad2×TGtk is owing to the increase in the number of binding sites for transcriptional factors on TG promoters, the precise mechanism of induction of the strong effect in Ad2×TGtk is not certain. The increase in transcriptional activity of the 2×TG promoter, compared to the single TG promoter, was only twice in FRTL5 and FTC-133 cells as shown in transfection experiments. However, the increase in sensitivity to GCV in TG-producing cells was more dramatic after infection with Ad2×TGtk than AdTGtk. Kitazono et al[37] showed a similar phenomenon between luciferase activity and cytotoxicity. They compared the activity of the TG promoter and enhancer TG promoter, which included enhancer sequence upstream of the TG promoter. They observed that luciferase activity by transfection of the enhancer TG luciferase reporter plasmid was only 1.7-fold higher than that by the TG luciferase reporter. However, they observed that 10- to 100-fold more concentrated GCV was needed for cytotoxicity by AdTGtk than by Ad enhancer TGtk *in vitro*. These results suggest that potency of cytotoxicity by the *HSVtk* gene is not always parallel to its promoter strength. The way of expression of luciferase protein and induction of luciferase activity is rather simple, whereas the pathway of cytotoxicity by *tk* genes is more complicated. The cytotoxicity results from transduction of *tk* genes, expression of tk protein, phosphorylation of GCV by tk, triphosphorylation of GCV by endogenous kinase, cytopathic effect by triphosphorylated GCV, and bystander effect. We compared the bystander effect between Ad2×TGtk and AdTGtk in FTC-133 cells to clarify the difference of cytotoxicity by infection with these adenoviruses. Interestingly, strong bystander effect was observed after treatment with Ad2×TGtk/GCV, but not with AdTGtk/GCV. Efficient tk expression may enhance efficiency of bystander effect. Thus, it is suggested that enhanced efficacy of bystander effect should be the one of the factors for enhanced cytotoxicity by Ad2×TGtk.

The higher activity of the 2×TG promoter was observed in FRTL5 cells, a normal thyroid cell line, than in FTC-133 cells, a follicular thyroid carcinoma cell line. No activity was observed in two undifferentiated thyroid cancer cell lines. However, cytotoxicity in FRTL5 cells by Ad2×TGtk was similar to that in FTC-133 cells. We think that these phenomena were due to the difference of infectivity of adenovirus in the cell lines. Approximately 25-fold higher luciferase activity in FTC-133 cells was observed after infection with AdTKluc than in FRTL5 cells. Approximately 10- to 20-fold more AdCMVtk or Ad2×TGtk was needed to reach the IC_{50} values in FRTL5 cells, compared to FTC-133 cells. Thus, we believe that Ad2×TGtk should have similar or more beneficial effect for TG-producing carcinoma than normal thyroid cells in clinical trials. We cannot expect therapeutic effects for undifferentiated thyroid carcinomas by the Ad2×TGtk/GCV system.

We observed powerful cytotoxic effects of Ad2×TGtk not only in *in vitro* but also in *in vivo* studies. Tumor growth–inhibitory potential of Ad2×TGtk is almost similar to that of AdCMVtk. However, tumors began to enlarge after discontinuation of GCV even in mice treated with Ad2×TGtk, although the time of progression was delayed in this group compared to that in the control group.

In this study, we demonstrated one method of gene therapy in which a tandemly repeated TG promoter is fused to the *HSVtk* gene, and observed that the Ad2×TGtk has a TG-producing carcinoma-specific cytotoxic potential in the presence of GCV. Our system using Ad2×TGtk/GCV may have utility in the treatment of TG-producing thyroid carcinomas and warrants further development.

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