Enhanced Efficacy of Transcriptionally Targeted Suicide Gene/Prodrug Therapy for Thyroid Carcinoma with the Cre-loxP System

Yuji Nagayama,1 Eijun Nishihara, Makoto Iitaka, Hiroyuki Namba, Shunichi Yamashita, and Masami Niwa

Departments of Pharmacology 1 [Y. N., M. N.] and Nature Medicine [E. N., H. N., S. Y.], Nagasaki University School of Medicine, Nagasaki 852-8523, and Department of Internal Medicine 4, Saitama Medical College, Saitama, 350-0495 [M. I.], Japan

Abstract

Our recent study demonstrates the feasibility of the thyroglobulin (TG) promoter in transcriptionally targeted gene therapy for thyroid carcinomas expressing TG, albeit less effectively than the constitutive viral promoter. The present study was, therefore, designed to enhance the activity of the TG promoter with the Cre-loxP system. Our data demonstrate that the in vitro cytotoxic effect of herpes simplex virus thymidine kinase/ganciclovir obtained with the TG promoter and the Cre-loxP system is 5–10-fold higher than that with the TG promoter alone. Enhanced tumor growth inhibition was also observed in in vivo tumor models. These data indicate the usefulness of the Cre-loxP system to enhance the activity of a tissue (or tumor)-specific promoter in transcriptionally targeted cancer gene therapy.

Introduction

Thyroid cancers are the most common endocrine malignancy, accounting for ~1% of all cancers (1). We have recently focused our interest toward the development of gene therapy for thyroid cancers (2–5).

Selectivity and efficacy are the most critical factors for successful gene therapy, especially for cancer gene therapy using a suicide gene approach. Tumor (or tissue)-specific promoters have been used widely to selectively target tumor cells (6). One drawback of this transcriptional targeting approach is, however, the relatively low expression levels of such promoters, often resulting in insufficient therapeutic efficacy in cancer gene therapy. This is also the case in gene therapy for thyroid carcinomas using a suicide gene of HSV-TK2 and the TG promoter, one of the thyroid-specific promoters (4). Thus, our recent studies with retrovirus-mediated gene transfer approach have demonstrated that, although a sufficient cell killing effect was readily observed when the HSV-TK gene was placed under the control of the strong, constitutive CMV promoter in all thyroid cell lines tested, irrespective of their TG expression levels (2, 3), the cytotoxic effect of HSV-TK was less in thyroid carcinoma cells expressing low levels of TG (FRTC cells) when its expression was controlled by the TG promoter (4). It is crucial to overcome this issue from a clinical point of view, because, although most well-differentiated thyroid carcinomas express relatively high levels of TG, there is a general tendency toward lower TG expression in less differentiated thyroid cancers (1).

In this study, therefore, we attempted to enhance TG promoter activity while maintaining tissue specificity by using a novel method employing the Cre-loxP system with the recombinant adeno virus approach.

Materials and Methods

Cell Lines. Three different thyroid cell lines were used in this study. FRTL5 cells are a normal, differentiated rat thyroid cell line, expressing the high levels of TG mRNA in the presence of thyrotropin as demonstrated previously (4). FRTC cells are a transformed FRTL5 cell line (4) with low levels of TG mRNA expression. FRO cells are an anaplastic thyroid carcinoma cell line that does not express TG mRNA (4). The culture conditions of each cell line have been described previously (4); FRTL5 and FRTC cells were maintained in the presence of 2 milliunits/ml thyrotropin throughout this study.

In Vitro Cytotoxic Assay with Retrovirus Infection. Retrovirus vectors LNC/TK and LNTGTK (Fig. 1A) have been constructed previously in our laboratory (2, 4). Retrovirus vector transfection, viral infection, and viral titration were performed as described previously (2). Polyclonal populations of transfected cells were selected with 800 µg/ml G418 (Geneticin; Wako, Osaka, Japan) for 2 weeks. In the in vitro cytotoxic assay, the cells were seeded at 1–5 × 10³ cells/well in 96-well microtiter plates. On the next day, the cells were treated with various concentrations of GCV (Hoffman-La Roche Ltd., Basel, Switzerland) in 100 µM of medium. The cell survival was quantitated with a Cell Counting kit (Wako) 4 days later. Survival ratios were expressed as percentages relative to untreated controls.

Construction of Recombinant Adenovirus Vectors. Recombinant adeno virus vector AxCaLacZ (Fig. 1B) was the generous gift of Dr. I. Saito (7). AXTGTK, AXTGNCre, and AxCaLNLNTK (Fig. 1B) were constructed on the basis of the COS-TPC method established previously (8). Briefly, the expression cassette containing the 0.9-kb rat TG promoter and the HSV-TK gene was excised from pLNTGTK (4) by HindIII and ClaI digestion, blunt-ended, and ligated into the SwaI site of the cosmid vector pAXclw (7) to yield pAXNTGTK. For AXTGNCre, the TG promoter was released from LNTGTK (4) by HindIII and BamHI digestion, blunt-ended, and ligated into the SwaI site of pAXAwNcre (7). pAxCaLNLNTK was constructed by subcloning the HSV-TK gene, which had been excised from LNTGTK (4) by BamHI and ClaI digestion, and blunt-ended into the SwaI site of pAXCALNLw (7).

293 cells were transfected with pAXNTGTK, pAXTGNcre, or pAxCaLNLNTK together with EcoT221 digested Ad5-dlx DNA-terminal protein complex (8) by the calcium phosphate method. Recombinant adenoviruses were generated through homologous recombination and propagated in 293 cells. Preparation of the virus stocks and concentration and purification of the viruses were performed as described previously (9).

In Vitro LacZ Gene Expression with Adenovirus Infection. Cells were seeded at 1 × 10³ cells/well in 24-well culture plates. The next day, the cells were infected with AxCaLacZ at the MOI indicated. Two days later, the cells were stained with 5-bromo-4-chloro-indolyl-β-D-galactopyranoside as described previously (7).

In Vitro Cytotoxic Assay with Adenovirus Infection. Subconfluent cells in 6-well culture plates were infected with the recombinant adenoviruses at the MOI indicated. The next day, the cells were seeded at 1–5 × 10³ cells/well in 96-well microtiter plates. One day later, the cells were treated with various concentrations of GCV in 100 µL of medium. Cell survival was quantitated 4 days later as described above. Survival ratios were expressed as percentages relative to untreated controls.

In Vivo Cytotoxic Effect with Adenovirus Infection in a s.c. Tumor Model in Nude Mice. Male nude mice (Charles-River Japan, Tokyo, Japan), 6–7 weeks of age, received s.c. injections on both flanks with 1 × 10³ FRTC cells. Two weeks later, recombinant adenoviruses were directly injected into...
the tumors from four directions on 4 consequent days. The total amounts of injected viruses were $1 \times 10^9$ TCID$_{50}$/tumor. AxNTGNCre and AxCALNLNTK were mixed at a ratio of 2:1. i.p. injections of 100 mg/kg GCV were then performed daily for 2 weeks (days 1–14). Tumor volumes were calculated as described previously (2). The results were expressed as percentages relative to tumor size on day 1. All mice were maintained in Nagasaki University Animal Facility, and all animal studies were conducted in accordance with the principles and the procedures outlined in the Guide for the Care and Use of Laboratory Animals at Nagasaki University School of Medicine.

Statistical analysis was performed using an unpaired Student’s $t$ test.

The tissue specificity and relative potency of the retrovirus vectors LNCTK and LNTGTK (Fig. 1A), in which the suicide gene HSV-TK was placed under the control of the CMV promoter and the TG promoter, respectively, were examined in three different thyroid cell lines, FRTL5, FRTC, and FRO cells. As we have shown previously (4), FRTL5 cells express the highest levels of TG mRNA, with its transcripts being readily detectable by Northern blot analysis. FRTC cells express very low levels of TG transcripts, which could be detected only by reverse transcription-PCR but not by Northern blot. In contrast, FRO cells did not express TG mRNA.

The surviving curves of control and retrovirus-infected cells treated with GCV for 4 days are shown in Fig. 2. LNCTK infection comparably sensitized all of the cells to GCV. Thus, sensitivity to GCV was increased ~1700 to ~2700-fold (the mean of at least two independent experiments) in all three cell lines infected with LNCTK. In contrast, the efficacy of LNTGTK infection was dependent on TG expression status; LNTGTK infection sensitized FRTL5 and FRTC cells ~1700- and ~130-fold, respectively, to GCV but did not sensitize FRO cells. These data suggest that the TG promoter is as efficacious as the CMV promoter in FRTL5 cells expressing high levels of TG but is less efficient in FRTC cells with very low TG expression.

To obtain a higher therapeutic efficacy with the TG promoter in thyroid carcinoma cells expressing low levels of TG, like FRTC, we attempted to enhance the TG promoter activity. To this end, we used the Cre-loxP system (7) and constructed new recombinant adenovirus vectors AxNTGNCre and AxCALNLNTK (Fig. 1B). In AxNTGNCre, the Cre recombinase gene was placed under the control of the TG promoter. In AxCALNLNTK, a 1.3-kb stuffer sequence, including the neo-resistant gene and polyadenylation signal, was placed between the CAG promoter (10) and HSV-TK gene. This stuffer sequence was flanked by the loxP sequences. In theory, the Cre recombinase would only be expressed when the TG promoter is active (in TG-expressing thyroid cells), which would remove the stuffer sequence and consequently induce HSV-TK expression under the transcriptional control of the CAG promoter (Fig. 1B). The CAG promoter is indeed the strongest, versatile promoter ever reported (10). The efficacy of double infection of these adenoviruses was compared with that of AxNTGNCre, another newly constructed adenovirus in which the HSV-TK gene is under the direct control of the TG promoter.

The efficiency of adenovirus infection of thyroid cells was first examined with AxCALAcZ infection, which contains the Escherichia coli LacZ gene under the control of the CAG promoter (Fig. 1B). LacZ expression was detected by 5-bromo-4-chloro-indolyl-$eta$-D-galactopyranoside staining. Almost 100% of the cells were stained blue at a MOI of 100 in FRTL5 and FRTC and at a MOI of 30 in FRO cells. With the higher MOI, the staining intensity in each of the cells increased further (data not shown).

In the in vitro cytotoxic assay with the recombinant adenovirus
vectors (Fig. 3), the cells were infected with AxNTGTK or AxNTGNCre/AxCALNLNTK (2:1 ratio) at a MOI required for infecting all cells (100 for FRTL5 and FRTC and 30 for FRO cells). In a preliminary study, a 2:1 ratio of AxNTGNCre and AxCALNLNTK gave the best result (data not shown). As shown in Fig. 3, FRTL5 and FRTC cells were sensitized to GCV ~125- and ~40-fold, respectively, by AxNTGTK infection and ~710- and ~440-fold by AxNTGNCre/AxCALNLNTK double infection, thus demonstrating a ~10-fold higher efficacy of AxNTGNCre/AxCALNLNTK than AxNTGTK in TG-expressing thyroid cells. By contrast, neither AxNTGTK nor AxNTGNCre/AxCALNLNTK were effective in FRO cells.

The relative therapeutic efficacy of AxNTGTK and AxNTGNCre/AxCALNLNTK was also compared in an in vivo s.c. tumor model in nude mice using FRTC cells. As shown in Fig. 4, daily administration of 100 mg/kg GCV resulted in significant growth inhibition in tumors infected with AxNTGTK or AxNTGNCre/AxCALNLNTK (2:1); the effect of the latter was much more profound than the former. Thus, tumor sizes after 2 weeks of treatment with GCV were 3412.5 ± 1108.7 mm3 (mean ± SE; 3830.4% compared with day 1) in tumors infected with AxCALacZ, 980.1 ± 131.3 mm3 in those infected with AxNTGTK (1100.7% compared with day 1; 28.7% compared with the AxCALacZ group, P < 0.01) and 383.1 ± 20.4 mm3 in those infected with AxNTGNCre/AxCALNLNTK (430.1% compared with day 1; 11.2% compared with the AxCALacZ group, P < 0.01; 39.1% compared with the AxNTGTK group, P < 0.01). Therefore, the higher efficacy of AxNTGNCre/AxCALNLNTK double infection was also observed in in vivo experiments, although even AxNTGNCre/AxCALNLNTK double infection could only lead to tumor growth inhibition, not tumor regression.

Tissue (or tumor)-specific promoters have been widely used for transcriptionally targeted cancer gene therapy, although their expression levels are generally lower than the constitutive viral promoter (6). The Cre-loxP system has been successfully introduced recently as a means to enhance the transcriptional activity of such tissue-specific promoters by Saito et al. (11). They demonstrate that LacZ expression by the AFP promoter can be enhanced ~50-fold by using this system in AFP-expressing hepatocarcinoma cells (11). This system has also been used for the on/off switching strategy of a gene of interest (7, 12) and for the production of a recombinant adenovirus, which carries a gene the product of which is toxic for virus-producing 293 cells (13).

In this article, we demonstrate that this system is also capable of enhancing the transcriptional activity of TG promoter in TG-expressing thyroid cells in in vitro and in vivo while maintaining tissue specificity. Thus, tissue-specific thyroid cancer gene therapy using a combination of the TG promoter and the Cre-loxP system appears more efficacious than that using the TG promoter alone in thyroid carcinoma cells, particularly those expressing low levels of TG. However, in our opinion, the magnitude of enhancement of the therapeutic efficacy in HSV-TK/GCV by this approach seems somewhat less striking than we expected from the original report mentioned above (11). In other words, the antitumor effect obtained with this new approach in this study is as yet unsatisfactory to us. The cytotoxic effect of a therapeutic gene may not necessarily be correlated with LacZ expression levels. Further study will be necessary to improve the efficacy.

It should be noted that, with a higher MOI (≥300 for FRTL5 and FRTC cells and ≥100 for FRO cells), although the more profound efficacy was apparently observed in FRTC cells infected with AxNTGTK or AxNTGNCre/AxCALNLNTK, the sensitivity to GCV was also slightly increased by the adenovirus infection (2.5–10-fold; data not shown) in FRO cells, implying that the TG promoter may be somewhat “leaky” at a very high MOI. Similar results have also been reported in AFP promoter (11).

In conclusion, we demonstrate the enhancement by the Cre-loxP system of the activity of the TG promoter in tissue-specific gene therapy for thyroid carcinomas expressing TG. Our data suggest that this system may also be applicable with tissue (or tumor)-specific promoters for other cancer gene therapies with a transcriptional target.

Acknowledgments

We thank Dr. I. Saito at Tokyo University and Dr. J. Miyazaki at Osaka University for the cosmid vectors and the CAG promoter, respectively, and Dr. Basil Rapoport at Cedars-Sinai Medical Center, Los Angeles, California, for critical review of the manuscript. We are also grateful to Hoffman La-Roche Ltd., Basel, Switzerland for GCV.

References


Enhanced Efficacy of Transcriptionally Targeted Suicide Gene/Prodrug Therapy for Thyroid Carcinoma with the Cre-\textit{lox}\textit{P} System

Yuji Nagayama, Eijun Nishihara, Makoto Iitaka, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/13/3049

Cited articles
This article cites 12 articles, 2 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/13/3049.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/13/3049.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.