Abstract. A proof-of-concept study is presented using dual gene therapy that employed a small hairpin RNA (shRNA) specific for mammalian target of rapamycin (mTOR) and a herpes simplex virus-thymidine kinase (HSV-TK) gene to inhibit the growth of tumors. Recombinant adeno-associated virus (rAAV) vectors containing a mutant TK gene (sc39TK) were transduced into HeLa cells, and the prodrug ganciclovir (GCV) was administered to establish a suicide gene-therapy strategy. Additionally, rAAV vectors expressing an mTOR-targeted shRNA were employed to suppress mTOR-dependent tumor growth. GCV selectively induced death in tumor cells expressing TK, and the mTOR-targeted shRNA altered the cell cycle to impair tumor growth. Combining the TK-GCV system with mTOR inhibition suppressed tumor growth to a greater extent than that achieved with either treatment alone. Furthermore, HSV-TK expression and mTOR inhibition did not mutually interfere with each other. In conclusion, gene therapy that combines the TK-GCV system and mTOR inhibition shows promise as a novel strategy for cancer therapy.

Introduction

Gene therapy holds great promise for treating intractable tumors, and suicide gene therapy is one of the most promising strategies (1). The herpes simplex virus (HSV) thymidine kinase (TK) gene is the most widely applied suicide gene in clinical trials (2-5). TK catalyzes the phosphorylation of the prodrug ganciclovir (GCV) to generate the monophosphate form, which is converted by cellular kinases into di- and triphosphate forms that inhibit DNA synthesis (6) and induce apoptosis (7). Furthermore, by exerting the so-called ‘bystander effect’, toxic metabolites of GCV can kill neighboring non-transfected tumor cells, enhancing cytotoxicity (8). However, the effects of the TK-GCV system are suboptimal, and combination with another strategy is usually recommended (4,5).

Mammalian target of rapamycin (mTOR) is an evolutionally conserved serine/threonine protein kinase and is a target of antitumor therapy because it regulates cell growth and metabolism and also because its aberrant activation occurs in many types of tumors (9-11). In addition to rapamycin, the prototypical inhibitor of mTOR, several rapamycin analogs (rapalogs) show improved pharmacokinetic bioavailability and reduced toxicity (12-14). Moreover, rapalogs such as temsirolimus and everolimus have been approved by the United States Food and Drug Administration (FDA) for treatment of renal cell cancer and lymphoma (14,15). However, the mechanism of mTOR inhibition deserves further scrutiny because many tumors are extremely heterogeneous regarding their sensitivity to rapalogs. This can be explained in part by the complexity of mTOR signaling. mTOR comprises the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which interact in a negative feedback loop (11). Thus, inactivation of mTORC1 after the administration of traditional rapalogs leads to paradoxical activation of Akt, the upstream stimulator of mTORC1, by reactivating either the insulin receptor substrate-1 pathway or the mTORC2 pathway, diminishing the antitumor effects
of a single rapalog (14-16). Further, simultaneous inhibition of mTORC1 and mTORC2 pathways or suppression of all mTORCs and PI3K-Akt pathways using chemical inhibitors is often unacceptably toxic to normal cells despite the improved antitumor effects (11,14). Therefore, a highly sophisticated approach is required if mTOR is to be considered as a therapeutic target for gene therapy.

Here, we developed a novel dual gene therapy strategy and confirmed its potential as a combination gene therapy in a uterine cervical carcinoma model. We employed a suicide gene (HSV-TK) and its prodrug (GCV) to inhibit DNA synthesis and a small hairpin RNA (shRNA) that targeted mTOR expression. Recombinant adeno-associated virus (rAAV) vectors were used for each strategy (17,18). Herein, we show that the combination of the TK-GCV system and mTOR inhibition was efficacious in our model system.

**Materials and methods**

**Cell culture and reagents.** Human cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-Invitrogen), GlutaMAX-1 (2 mM) and 1% penicillin (100 IU/ml)/streptomycin (50 µg/ml) in a humidified atmosphere containing 5% carbon dioxide at 37°C.

**Construction of a recombinant adeno-associated virus.** The rAAV vectors were constructed as previously described (19,20). The rAAV2 plasmid expressing splice-corrected 39TK (sc39TK) under the control of the CMV promoter was constructed by replacing the BamHI-SalI site. The PCR product of the sc39TK gene from pcDNA3.1(+) was inserted into the BamHI-SalI site of the pSP72-self complementary AAV-GFP vector (20). The rAAV2 vectors that expressed mTOR-shRNA or control shRNA driven by an H1 promoter were constructed as described previously (19). The sequences of the mTOR-shRNA were as follows: 5'-GAT CCG AATGT TAC AATGC AGA ATA GCA TTT GTG AACATT CCT TTT TGG AAA AGC T-3' (sense) with a BamHI linker and 5'-AGC TTT TCC AAA AAAGAATGT TGA CCA ATG CTA TTC TCT TGT AAT AGGCATT GGT CAA CAT TCG-3' (antisense) with a HindIII linker. Nucleotides specific for mTOR are underlined.

**Western blot analysis and immunocytochemical analysis.** For western blot analysis, proteins were separated using gel electrophoresis through SDS-polyacrylamide gels and then transferred to a PVDF membrane. The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% (w/v) skim milk. After washing with TBST, the membranes were incubated overnight at 4°C with antibodies against mTOR (Cell Signaling Technology #2983, Boston, MA, USA), GFP (Millipore, #AB16901, Temecula, CA, USA) or TK (from William Summers, Yale University, USA), diluted with TBST containing 1% skim milk. After washing with TBST, the membranes were incubated for 1 h at room temperature with the secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Bands were detected using an ECL system (Thermo Scientific, Rockford, IL, USA). For immunocytochemistry, cells were plated in 6-well plates and infected with rAAV2-sc39TK at various MOIs. Cells were blocked with TBS containing 5% bovine serum albumin (BSA). After washing, the anti-TK antibody (1:200) and Cy3-labeled secondary antibody (1:500) were sequentially added to the fixed cells. Fluorescence signals were analyzed using a fluorescence microscope.

**Cell cycle analysis.** HeLa cells were infected with either rAAV2-shCont or rAAV2-shmTOR. After 48 h, the cells were trypsinized and fixed with ice-cold ethanol. The cells were then incubated with 0.05% propidium iodide and analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA) or TK (from William Summers, Yale University, USA), GFP (Millipore, #AB16901, Temecula, CA, USA) or TK (from William Summers, Yale University, USA), diluted with TBST containing 1% skim milk. After washing with TBST, the membranes were incubated for 1 h at room temperature with the secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Bands were detected using an ECL system (Thermo Scientific, Rockford, IL, USA). For immunocytochemistry, cells were plated in 6-well plates and infected with rAAV2-sc39TK at various MOIs. Cells were blocked with TBS containing 5% bovine serum albumin (BSA). After washing, the anti-TK antibody (1:200) and Cy3-labeled secondary antibody (1:500) were sequentially added to the fixed cells. Fluorescence signals were analyzed using a fluorescence microscope.

**Construction of an adenovirus vector.** The adenovirus vector was generated using the adenovirus vector generation system (Clontech Laboratories, Inc., Palo Alto, CA, USA). The adenovirus vector was constructed by inserting the TK-shRNA cassette into the AAV genome. The adenovirus vector was then amplified and purified for use in the study.

**Cytotoxicity assay.** Exponentially growing HeLa cells were seeded in 6-well plates. After overnight incubation, cells were infected with the rAAV2-sc39TK virus at a multiplicity of infection (MOI) of 1,000. At day 1 post-infection, the cells were re-seeded in 96-well plates (10,000 cells per well), and different concentrations of GCV (Cymerene, Roche Diagnostics, Indianapolis, IN, USA) were added to the media, followed by incubation for 4 days. Each treatment condition was tested in triplicate. The CCK-8 assay was performed according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cell viability was calculated relative to that of control cells.

**Data presentation and statistical analysis.** Data were reported as the mean ± standard deviation (SD). Differences between experimental groups and controls were determined using the Mann-Whitney test and were considered significant at two-tailed p<0.05.

**Results**

**Recombinant adeno-associated virus vectors.** The expression of the GFP or mutant TK (sc39TK) genes was controlled by the constitutive promoter (pCMV) in the rAAV2-GFP or
rAAV2-sc39TK vectors, respectively (Fig. 1A). The sc39TK gene was generated by introducing a five-codon substitution and a silent mutation in the GCV-resistant splice acceptor and donor sequences (22). These mutations enhance the drug sensitivity of tumor cells (20,22). In contrast, the expression of the control shRNA (shCont) and the mTOR-shRNA (shmTOR) was regulated by the H1 promoter in the rAAV2-shCont and the rAAV2-shmTOR vectors, respectively (Fig. 1B). The GFP gene under control of the CMV promoter was used as a reporter gene for expression of the corresponding shRNAs.

**Figure 1. Characteristics of rAAV vectors.** (A) rAAV2-GFP and rAAV2-sc39TK. The expression of genes encoding the GFP and mutant TK (sc39TK) was under control of the CMV promoter. (B) rAAV2-shCont and rAAV2-shmTOR. The control shRNA and the mTOR-specific shRNA were inserted into the rAAV2 cassettes, and their expression was driven by the H1 promoter. The GFP gene under control of the CMV promoter was used as a reporter gene for expression of the corresponding shRNAs.

Suicide gene therapy using the sc39TK-GCV system and mTOR inhibition. To investigate the effects of combined gene therapy, the mice were subjected to the treatments as follows: i) rAAV2-shCont, ii) rAAV2-shCont with GCV, iii) rAAV2-sc39TK and rAAV2-shmTOR without GCV, and iv) rAAV2-sc39TK and rAAV2-shmTOR with GCV. On day 17, tumors generated by cells infected with rAAV2-shmTOR and rAAV2-sc39TK were smaller than those generated by cells that were infected with rAAV2-shCont (Fig. 4A). Administration of GCV further reduced the growth of tumors generated by engrafted cells infected with rAAV2-sc39TK and rAAV2-shmTOR, and the tumor volumes were smallest in this group (Fig. 4A). On day 29 after implantation, compared with the group of mice engrafted with cells infected with rAAV2-shCont, there was a decrease in tumor volume of 72.9±6.3% in the group not treated with GCV but implanted with cells co-infected with rAAV2-sc39TK and rAAV2-shmTOR and rAAV2-sc39TK were smaller than those generated by cells that were infected with rAAV2-shCont (Fig. 4A). Administration of GCV further reduced the growth of tumors generated by cells co-infected with rAAV2-sc39TK and rAAV2-shmTOR, and the tumor volumes were smallest in this group (Fig. 4A).

Combination gene therapy using the sc39TK-GCV system and mTOR inhibition. To investigate the effects of combined gene therapy, the mice were subjected to the treatments as follows: i) rAAV2-shCont, ii) rAAV2-shCont with GCV, iii) rAAV2-sc39TK and rAAV2-shmTOR without GCV, and iv) rAAV2-sc39TK and rAAV2-shmTOR with GCV. On day 17, tumors generated by cells infected with rAAV2-shmTOR was significantly reduced compared with those induced by cells infected with rAAV2-shCont (Fig. 3D). In xenografted mice, the volume of tumors induced by engrafted cells infected with rAAV2-shmTOR was significantly reduced compared with those infected with rAAV2-sc39TK (Fig. 3D).
Figure 2. Suicide gene therapy using the sc39TK-GCV system. (A) Transduction efficiency of rAAV2-GFP or rAAV2-sc39TK in HeLa cells at MOI 1,000. Scale bars, 100 µm. (B) Western blot analysis of TK expression 48 h after infection of HeLa cells with rAAV2-sc39TK. β-actin served as a loading control. (C) Cytocidal effects on HeLa cells by sc39TK expression 96 h after GCV treatment. (D) Effects of GCV on tumors expressing sc39TK in xenografted mice. GCV was injected intraperitoneally every day from day 14 to 29. *p<0.05. **p<0.005. n≥3.

Figure 3. mTOR inhibition using rAAV-mediated RNAi. (A) Equivalent transduction efficiency 48 h post-infection (MOI 1,000) in HeLa cells transduced with rAAV2-shCont or rAAV2-shmTOR. Scale bars, 100 µm. (B) Western blot analysis for mTOR inhibition by rAAV2-shmTOR. β-actin served as a loading control. (C) Cell cycle analysis of HeLa cells infected with rAAV2-shmTOR or rAAV2-shCont at 48 h post infection. (D) Analysis of tumor growth in mice xenografted with HeLa cells expressing shmTOR. *p<0.05. **p<0.005. n≥3.
Effects of combination gene therapy on transgene expression. We further investigated whether combined transduction of cells with rAAV2-sc39TK and rAAV2-shmTOR mutually inhibited the expression of the transgenes. It is of note that there is no GFP signal from rAAV2-sc39TK (Fig. 1A). In the HeLa cells infected with both rAAV2-sc39TK and rAAV2-shmTOR, the...
GFP signals were comparable to those from rAAV2-shmTOR alone (Fig. 5). Furthermore, TK expression by cells infected with rAAV2-sc39TK was not inhibited by co-infection with rAAV2-shmTOR. Moreover, when the cells were infected at MOI ≤ 5,000, neither the GFP signal nor TK expression level was reduced (data not shown).

Discussion

In the present study, we demonstrate for the first time to our knowledge that the combination of suicide gene therapy (sc39TK-GCV system) and mTOR inhibition using an mTOR-specific shRNA enhanced antitumor effects without mutual interference of transgene expression. Taking into consideration that the tumors increase in size because of proliferation (increase of cell number) and growth (increase of cell size) of the individual tumor cells, the cytociidal effects of the sc39TK-GCV system (Fig. 2) combined with the cytostatic effect of mTOR inhibition (Fig. 3) serve as an ideal antitumor mechanism (24). The sc39TK-GCV system effectively inhibits the proliferation of tumor cells by inhibiting DNA synthesis (1,6,20), whereas the mTOR-specific shRNA inhibits the growth of tumor cells by downregulating tumorigenic pathways (19).

The TK-GCV system has been evolved in many aspects. The original version of HSV-TK was modified to sc39TK to enhance its antitumor effects upon GCV treatment (20,22,25). A theranostic (simultaneous therapy and diagnosis) approach for individualized therapy was successfully achieved using TK activity that incorporates therapeutic benefits such as GCV and diagnostic imaging agents such as 18F-FHBG (a radiolabeled penciclovir analog for positron emission tomography) (4,20). However, because the therapeutic efficacy of the TK-GCV system alone is usually insufficient (4,5), the TK-GCV system has been tested in combination with other antitumor strategies such as another suicide gene, conventional chemotherapy, radiotherapy, and immunotherapy (1). In fact, the combination here of TK-GCV with RNAi is a relatively new approach to cancer therapy (26).

mTOR is an emerging target of antitumor gene therapy because it functions in the PI3K/Akt/mTOR signaling pathway that mediates tumorigenesis (13,27). mTOR regulates the growth, senescence, survival, and metabolic homeostasis of cells (11). Elevated mTOR activity is present in many tumors, and suppression of mTOR induces tumor regression (9,10). Moreover, gene therapy targeting mTOR is a promising antitumor approach, because, as an evolutionarily conserved target, relatively few mutant forms are known (13,28). However, mTOR inhibitors have been found to be successful for treating only a few types of tumors such as renal cell carcinoma or lymphoma, and the responses of many other tumors to such drugs are highly heterogeneous (13). In this regard, the RNAi approach used in the present and previous studies shows promise because mTOR-shRNA inhibits both mTORC1 and mTORC2 without serious off-target effects (19,23).

Using rAAV as a vector for gene therapy may have contributed substantially to the success of the combination approach. Wide applicability to a variety of tumors and long-term expression of transgenes without serious toxicity are advantages of using rAAV vectors (29,30). Furthermore, rAAV has been FDA-approved for gene therapy (29,31). We have used rAAV vectors for transgene packaging, delivery, and expression and for in vitro and in vivo monitoring in our previous studies (20,32-34). In the present study, the transduction efficiencies were well balanced between the HSV-TK gene and the mTOR-shRNA (Figs. 2 and 3). Transgene expression was well maintained until the end of the experiments (Fig. 4) without mutual interference (Fig. 5). The demonstration of the combined effects of the dual gene therapy strategy might have been difficult without this expertise of rAAV vectors.

However, some questions remain unanswered regarding the utility of the dual gene therapy presented here. In the TK-GCV system, actively dividing tumor cells are killed directly, and the non-dividing quiescent tumor cells may be indirectly affected by toxic GCV metabolites through the bystander effect. Whether or not the bystander effect contributes to tumor inhibition in conjunction with mTOR inhibition is unknown (8). Additionally, the present study does not address techniques for delivery to patients or the effects of treatment for >1 month (3). These questions require further study.

In the present proof-of-concept study, we demonstrate that the TK-GCV system combined with an mTOR-specific shRNA enhances the suppression of tumor growth compared to the use of either technique alone. This promising result may lead to the development of a novel gene therapy strategy against intractable tumors.

Acknowledgements

This study was supported by grants from Medical Research Center Program (2008-0062286 to H. Lee), Basic Science Research Program (NRF-2011-004821 to H.N. Woo), and Nuclear Research and Development Program (NRF-2014M2B2A9030104 and NRF-2012M2A2A7035589 to W.W. Lee), Republic of Korea.

References


