Effect of Adenoviral-mediated Thymidine Kinase Transduction and Ganciclovir Therapy on Tumor-associated Endothelial Cells

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Abstract

Transduction of the herpes simplex virus thymidine kinase (HSV-tk) into vascular endothelial cells using a replication-defective adenoviral vector (Ad.CMV-tk) to confer sensitivity to ganciclovir (GCV) was investigated. The cytotoxic sensitivity of bovine aortic endothelial cells (BAEC) to GCV following Ad.CMV-tk transduction at multiplicity of infection of 100 was ten-fold that of 9L glioma cells in vitro. Deoxyribonucleic acid fragmentation was detected in these BAEC. A co-culture experiment using BAEC transduced with Ad.CMV-tk (BAEC-tk) and 9L cells expressing β-galactosidase (9L-Lac Z) showed about 70% tumoricidal effect under the conditions of one BAEC-tk cell in 10 9L-Lac Z cells.

Tumor-bearing Fisher 344 rats, an experimental brain tumor model, received Ad.CMV-tk intratumorally at 7 days after tumor implantation, and were subsequently treated with intraperitoneal GCV (100 mg/kg). Histological examination found the vascular endothelial cells adjacent to 9L glioma tissue revealed apoptosis. These results suggest that vascular endothelial cells are an attractive target for adenoviral-mediated HSV-tk gene therapy.

Key words: endothelial cells, bystander effect, deoxyribonucleic acid fragmentation, adenoviral vectors, herpes simplex virus-thymidine kinase, ganciclovir

Introduction

Adenoviral-mediated gene therapy based on the transfer of a suicide gene into tumor cells has been effective for curing brain tumors in animal models. Such viral-mediated transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene has been used to confer cytotoxic sensitivity to nucleoside analogues, such as ganciclovir (CCV), in a variety of tumor cells in vitro and in vivo. HSV-tk gene transduction followed by GCV exposure causes the killing of glioma cells directly. However, the target for cytotoxic therapy using suicide gene transduction includes both the tumor cells and tumor-associated vascular endothelial cells, since vascular endothelial cells are also infected even if the viral vehicle is injected intratumorally.

Proliferating tumor-associated vascular endothelial cells are a favorable target for viral-mediated gene therapy for three reasons. First, vascular endothelial cells are easily accessible through direct contact with the blood stream, which is useful for the drug contact following gene delivery. Second, vascular endothelial cells are also good targets for gene modification by adenoviral vectors. Angiogenesis in solid tumors is a critical step for tumor growth. Since malignant brain tumors, in particular, are strongly angiogenic neoplasms, dependence on the blood supply is a characteristic feature of these tumors. Therefore, vascular endothelial cells containing the suicide gene, which activates the cytotoxic agent administered systemically, provides a therapeutic strategy for depriving the tumor cells of their vital blood supply. Third, tumor-associated endothelial cells expressing therapeutic genes can be expected to sensitize non-transduced tumor cells to cytotoxic agent by way of the bystander effect as well as tumor cell to tumor cell contact.

Adenovirus has attractive features as a vehicle to transfer genes into central nervous system neoplasms compared with retrovirus. In particular, higher titers of the virus can be achieved to allow...
better levels of expression in treated tissues including glioma cells and vascular endothelial cells adjacent to the tumor.\(^\text{4,22,23}\) Previously, retroviral-mediated HSV-tk gene transduction in vivo has resulted in killing of vascular endothelial cells, followed by necrosis of the tumor tissues which leads tumor tissues.\(^{24}\)

The present study investigated the adenoviral-mediated gene transduction efficiency and sensitivity to GCV in both bovine aortic endothelial cells (BAEC) and 9L rat gliosarcoma cells to examine whether vascular endothelial cells transduced with adenoviral vector expressing suicide gene are sensitized and whether vascular endothelial cell-glioma cell contact leads to an innocent bystander effect similar to that seen in glioma cell-glioma cell contact by a co-culture experiment with BAEC and 9L. An animal model of glioma, induced 9L glioma in the rat, was used to assess if adenoviral vectors transfer genes to cerebral microcapillary endothelial cells in vivo, and the killing mechanism of the vascular endothelial cells was assessed to detect apoptosis or programmed cell death as determined by deoxyribonucleic acid (DNA) fragmentation analysis in vitro and in situ apoptosis detection in vivo.

**Materials and Methods**

I. **In vitro adenoviral-mediated cytotoxic sensitization**

Cell culture and preparation of recombinant adenovirus: 9L rat gliosarcoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). BAEC were maintained in DMEM containing 10% FBS, 100 μg/ml heparin, and 30 μg/ml endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, Mass., U.S.A.).

The recombinant adenoviruses Ad.CMV-Lac Z and Ad.CMV-tk derived from adenovirus type 5 were prepared by homologous recombination in human embryonic kidney cell line (HEK) 293 as previously described.\(^{12,13}\) In brief, Ad.CMV-tk was prepared by cotransfection of pJM17 and a shuttle plasmid containing HSV-tk gene driven by the cytomegalovirus (CMV) promoter/enhancer. The virus stocks were purified by two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 7.4), and 1 mM MgCl₂, and stored at −80°C. Viral titers were determined by the plaque-forming unit (pfu) assay in semisolid cultures of HEK 293 cells.

**Evaluation of transduction efficiency:** 9L cells and BAEC were infected with Ad.CMV-Lac Z at multiplicity of infection (MOI) values of 10 and 100. Forty-eight hours after infection, cells were lysed in phosphate buffer saline (PBS) by three freeze-thaw cycles in an Eppendorf tube. β-Galactosidase activity was quantified using o-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma Chemical, St. Louis, Mo., U.S.A.) as substrate. The ONPG substrate solution (10 μl of 4 mg/ml of ONPG in a buffer consisting of 20 mM NaH₂PO₄, 0.1 mM MgCl₂, 40 mM β-mercaptoethanol, pH 7.3) was then added. The reaction was stopped with 1 M Na₂CO₃ after incubation at 37°C for 30 minutes. The absorption was measured at 420 nm and the relative activity calculated.

**Evaluation of cytotoxic sensitization:** 9L cells and BAEC (5 × 10⁴ cells/100 μl each) were infected with Ad.CMV-Lac Z and Ad.CMV-tk at MOI values of 10, 50, 100, and 500 and plated into individual wells of a 96-well microtiter plate (Microtest III; Becton Dickinson, Linden Park, N.J., U.S.A.) 12 hours after adenoviral infection. Cells were treated with varying doses of GCV for 72 hours. The cells were then fixed after 72 hours by adding 10 μl of 25% glutaraldehyde to each well for 15 minutes. After several washes with PBS, the cells were stained with 200 μl of 0.05% methylene blue. The dye was eluted by agitation with 0.33 N-HCl for 15 minutes and the absorbance at 595 nm was measured in a microplate reader (Model 350; Bio-Rad, Hercules, Cal., U.S.A.). Cell viability was determined within the linear range and standardized to a control curve.

**Evaluation of in vitro bystander effect:** The viability of wild type BAEC treated with GCV was measured in the presence of BAEC transduced with Ad.CMV-tk (BAEC-tk) at the MOI of 100. Briefly, wild type BAEC were mixed with BAEC-tk cells at different ratios and then plated in a 6-well plate at 1 × 10⁵ cells/well. Cells were treated with 10 μM of GCV for 72 hours. The cytotoxicity assay was performed as described above.

In order to test whether this effect was also observed in glioma-endothelial cell contact, a co-culture experiment was performed with BAEC-tk and 9L glioma cells with stably transduced β-galactosidase gene using CREBAG2 retroviral producer cells (9L-Lac Z). 1 × 10⁵ of 9L-Lac Z cells and different amounts of BAEC-tk cells (1 × 10⁴, 2 × 10⁴, 5 × 10⁴, 8 × 10⁴, 1 × 10⁵) were plated together and then exposed to 10 μM of GCV. Seventy-two hours after treatment, the cells were stained with X-gal, or harvested and subjected to the β-galactosidase assay as described above. The relative activity in each condition was calculated as a percentage of the activity of 9L-Lac Z without BAEC-tk.

**Evaluation of DNA fragmentation:** BAEC (5 × 10⁴ cells) following adenoviral infection at MOI of 0,
100, and 500 were exposed to GCV (10 μM) for 12 hours then harvested, washed, and incubated in 100 μl of 50 mM Tris (pH 8.0), 10 mM ethylenediaminetetra-acetic acid (EDTA), 0.5% sodium dodecyl sulfate, and 0.5 μg/ml proteinase K for 3 hours at 50°C. Fifty microliters of 0.5 mg/ml ribonuclease A was added and incubation continued for an additional hour. The digested samples were incubated with 100 μl of 10 mM EDTA (pH 8.0) containing 2% (w/v) low-melting point agarose, 0.25% bromophenol blue, and 40% sucrose at 70°C. The DNA was separated in gels containing 2% agarose/TAE buffer (40 mM Tris-acetate and 1 mM EDTA [pH 8.0]) and visualized by ultraviolet illumination after ethidium bromide staining.

II. In vivo adenoviral-mediated cytotoxic sensitization

Adenoviral-mediated cytotoxic sensitization model: Adult male Fisher 344 rats (200–250 g) were purchased from Charles River Laboratories (Wilmington, Mass., U.S.A.). All animal studies confirmed to the guidelines of the Animal Care and Use Committee of Dana-Farber Cancer Institute.

Rats were anesthetized by intraperitoneal injection of nembutal. Twenty thousand 9L gliosarcoma cells suspended in 10 μl Hank’s balanced salt solution were injected over 30 seconds into the rat caudate nucleus to a depth of 4.5 mm using a small animal stereotactic frame (Kopf) and a 25 gauge Hamilton syringe. The needle was left in place for 1 minute and then withdrawn slowly. Seven days after tumor implantation, the rats were treated with 6 μl of Ad.CMV-Lac Z (1.2 × 10<sup>9</sup> pfu) or Ad.CMV-tk (1.2 × 10<sup>9</sup> pfu) by injection into the established 9L brain tumors using the same stereotactic coordinates. GCV treatment began 2 days after viral inoculation at a dose of 100 mg/kg body weight by intraperitoneal injection. The rat brains were harvested the next day for further histological analysis.

Evaluation of β-galactosidase gene transduction: The brains were removed and cryoprotected in 20% sucrose in PBS and frozen in ornithine carbamoyltransferase. Coronal sections (6 μm) were taken from the tumor implantation site and immersed in X-gal staining solution (5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 1 mM MgCl<sub>2</sub>, and 1 mg/ml X-gal in PBS) as described. Finally the sections were counterstained with nuclear fast red.

Evaluation of proliferation potential: Paraformaldehyde-fixed and paraffin-embedded brain sections were prepared. After deparaffinization, sections were pretreated using a microwave oven for 6 minutes at 650 W and 2.45 GHz, followed by blocking of endogenous peroxidase with 0.3% hydrogen peroxidase in methanol. Non-specific binding was blocked with 10% normal horse serum and 1% bovine serum albumin (BSA) for 30 minutes. After a brief rinse, the sections were immunoreacted with a proliferating cell nuclear antigen (PCNA) monoclonal antibody diluted 1:500 in PBS with 1% BSA and 0.1% Tween 20 at room temperature for 30 minutes followed by biotinylated anti-mouse immunoglobulin G for 30 minutes. The sections were then treated with avidin-biotin-horseradish peroxidase complex for 1 hour and 3'-diaminobenzidine was applied as a chromogen. Sections were counterstained with 1% methylene green.

Evaluation of in situ cell apoptosis: Paraaffin-embedded sections were processed to identify individual apoptotic cell death using The Apo Tag Plus In Situ Apoptosis Detection Kit Peroxidase (Oncor, Gaithersburg, Md., U.S.A.). In brief, after deparaffinization the sections were treated with 20 μg/ml of proteinase K at room temperature for 15 minutes. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase in PBS. Detection with anti-digoxigenin peroxidase was performed using 3-3' diaminobenzidine for 6 minutes. Each tissue section was counterstained with 1% methylene green.

Results

I. In vitro adenoviral-mediated cytotoxic sensitization

Evaluation of transduction efficiency in BAEC and 9L cells showed that using the same MOI of Ad.CMV-Lac Z infection, β-galactosidase activity in BAEC was higher than that in 9L (Fig. 1). Evaluation of the cytotoxic sensitization by transduction of Ad.CMV-tk showed that both 9L cells and BAEC were killed by GCV treatment and the effect was dependent on GCV dose and MOI. The median lethal dose for GCV cytotoxicity to BAEC at a MOI of 100 was 0.08 μM, which showed ten-fold sensitivity compared with 9L cells (Fig. 2).

Evaluation of the bystander effect showed that wild type and Ad.CMV-tk-transduced BAEC mixed at different ratios and then exposed to 10 μM of GCV had lower cell viability than the cells treated with GCV separately and then mixed (Fig. 3 upper). Wild type BAEC cells were only 13.7 ± 3.4% viable even if as few as 10% of the mixed cell population were HSV-tk positive. Co-culture of 9L-Lac Z cells with BAEC-tk showed the same effect, as demonstrated by β-galactosidase activity (Fig. 3 lower), and positive X-gal staining (Fig. 4). This decrease in β-galactosidase activity was dependent on the proportion of BAEC-tk cells in the co-culture (Fig. 3 lower).
Evaluation of DNA fragmentation showed that BAEC transduced with HSV-tk at a MOI of 500 underwent internucleosomal DNA fragmentation following exposure to 10 μM GCV (Fig. 5).

**II. In vivo adenoviral-mediated cytotoxic sensitization**

Evaluation of gene transduction by the Ad.CMV-Lac Z using X-gal staining showed blue coloration of the vasculature, indicative of β-galactosidase activity in the vascular endothelial cells (Fig. 6A). Evaluation of proliferation potential using PCNA immunohistochemical staining demonstrated that vascular endothelial cells as well as tumor cells were strongly positive for PCNA (Fig. 6B). Evaluation in **in situ** cell apoptosis showed that vasculatures associated with the induced 9L gliosarcoma contained apoptotic cells as determined by direct immunoperoxidase detection of the digoxigenin-labeled internucleosome DNA fragment (Fig. 6C). In contrast, no cell apoptosis was detected in a control group (data not shown).

**Discussion**

The transduction efficiency of retroviral vector into endothelial cells was clearly dependent on MOI, when the concentration of the vector was varied. Transduction efficiency of retrovirus into endothelial cells may also be affected by combination with diethylaminoethyl-dextran as the polycation. In comparison, adenoviral gene transduction has higher infectious efficiency in vitro and in vivo. Adenoviral vectors can be used to transduce transgenes into vascular endothelial cells from their luminal surface and abluminal side in vivo. The present study assessed whether the HSV-tk-GCV system using adenoviral vector injected intratumorally can also infect tumor-associated vascular endothelial
cells, finding that transduction efficiency using adenovirus was better into endothelial cells than 9L gliosarcoma cells. Transgene expression was also dependent on MOI. The sensitivity to GCV following Ad.CMV-tk infection was also compared, showing that BAEC are more sensitive than 9L cells. Moreover, inter-nucleosomal DNA fragmentation in BAEC was also observed after GCV exposure.

Current gene therapy studies have suggested that HSV-tk expression in glioma cells is associated with a bystander effect such that cells adjacent to cells expressing HSV-tk also become sensitive to
These non-transduced glioma cells are also killed, by the so-called “innocent bystander effect,” which has been demonstrated by the collapse of tumor-associated vasculature as well as toxic metabolites through cell-to-cell contact. Metabolic cooperation, mediated by gap junctions, is a major contributor to this phenomenon. This study also found that non-transduced glioma cells which are mixed with HSV-tk-transduced vascular cells.

Fig. 5 Internucleosomal deoxyribonucleic acid (DNA) fragmentation in bovine aortic endothelial cells after exposure to ganciclovir (GCV). DNA was isolated from cells infected with adenoviral vector containing herpes simplex virus thymidine kinase gene (Ad.CMV-tk) at multiplicity of infection (MOI) of 0 and 500 or β-galactosidase gene (Ad.CMV-Lac Z) used as a control and exposed to 10 μM GCV. DNA fragmentation was assessed by electrophoresis in 2% agarose gels. Lane 1: Ad.CMV-Lac Z (MOI 500), GCV (-); lane 2: Ad.CMV-Lac Z (MOI 500), GCV (+); lane 3: Ad.CMV-tk (MOI 500), GCV (-); lane 4: Ad.CMV-tk (MOI 500), GCV (+).

Fig. 6 A: Photomicrograph of endothelial cells in a capillary adjacent to the induced 9L tumor infected with adenoviral vector containing β-galactosidase gene showing focal strong nuclear staining indicating transduction. X-gal stain, ×400. B: Photomicrograph showing proliferating cell nuclear antigen expression of endothelial cells in a capillary adjacent to the induced 9L tumors. Counterstained with methylene green, ×400. C: Photomicrograph showing apoptotic cells in the microvasculature adjacent to the induced 9L tumor infected with adenoviral vector containing herpes simplex virus thymidine kinase gene followed by ganciclovir treatment. Counterstained with methylene green, ×400.
endothelial cells were sensitized to GCV treatment. These in vitro results suggest that vascular endothelial cells are a favorable target for HSV-tk-GCV therapy.

Anti-tumor therapy using viral vectors expressing HSV-tk delivered by intratumoral injection is considered to be more efficient compared to administration by intracarotid injection as a specific anti-angiogenesis therapy. GCV is always administered systemically following intratumoral gene transduction, so the vascular endothelial cells are the first to be exposed to GCV. The in vivo experiment showed that vascular endothelial cells within tumor tissue are a good target for cell cycle-dependent HSV-tk gene therapy, because of their growth potential shown by the present and previous immunohistochemical analyses with bromodeoxyuridine, or Ki-67. Tumor-associated endothelial cells in the experimental glioma model also demonstrated apoptosis following Ad.CMV-tk and GCV treatment, and the X-gal staining study showed that infection by adenoviral vector had occurred. The degree of vascularity of gliomas and the proliferation activity of vascular endothelial cells are correlated with the histological grade of malignancy. These results suggest that tumor-associated vascular endothelial cells infected with Ad.CMV-tk were sensitized to GCV due to the high proportion of cells in the G1-S phase and the high gene transduction efficiency achieved by adenoviral vectors.

Extensive microvasculatures are observed within or adjacent to malignant gliomas, so growth is considered to be dependent on angiogenesis. Collapse of the vascular component cells, especially the vascular endothelial cells, may be a feasible way to interrupt the oxygen and nutrient delivery to tumor cells, and prevent angiogenesis surrounding the tumor tissue.

To assess the possibility of this viral vector-mediated anti-angiogenesis strategy, a novel targeted anti-endothelial gene therapy model is presently being developed.

References

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Commentary

This paper takes the strategy of utilizing the reaction between thymidine kinase and ganciclovir which has been used to provide direct tumor cell damage in experimental situations and also clinically. The focus of previous experiments has been the tumor cells themselves, and this paper studies the effect of this gene therapy strategy on the blood vessels associated with tumors. It has been known for some time that tumor blood vessels are different from normal brain blood vessels, and it has been hoped that these differences could provide the basis for differential therapy that might be capable of destroying the tumor as a whole. This paper suggests that ganciclovir can produce apoptosis in vascular endothelial cells associated with the 9L glioma after transduction of the cells with adenovirus mediated thymidine kinase. It is encouraging to see this work which may in fact provide a new avenue of destruction by attacking the vascular supply of tumors in addition to the direct cytotoxic effects and the "bystander" effects previously seen with this form of gene therapy.

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Dr. T. Tanaka has reported the successful transduction of the herpes simplex virus thymidine kinase (HSV-tk) gene using a replication-defective adenoviral vector into vascular endothelial cells. In this study the author clearly showed that the cytotoxic sensitivity of the tumor-associated endothelial cells to ganciclovir was markedly augmented following the HSV-tk transduction. One of the key issue regarding gene therapy using viral vectors is to restrict the gene expression to target cells. Therefore, certain promoter/enhancer system specific to endothelial cells should be devised. Angiogenesis or neovascularization are generally considered to be closely related to endothelial cell proliferation that is essential for malignant tumor growth, so the present study of HSV-tk gene transduction is expected to contribute to the growth control of malignant brain tumors in terms of anti-angiogenesis therapy in the future.

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Since glioma cells aggressively infiltrate into normal brain tissue and are resistant to conventional therapy including surgery, radiation, chemotherapy, and immunotherapy, we need to develop a new therapy for these tumors. Gene therapy is one of the most promising therapies. To succeed in gene therapy, it is important to develop an effective vector. In clinical trials, a lot of investigators have used retrovirus vectors as a gene delivery system. However, their transduction efficiency is unfortunately confirmed to be very low in humans. Next generation vectors including adenovirus vectors and adeno-associated virus vectors are possible candidates. Therefore, this paper that deals with adenovirus vectors is timely. Adenovirus vectors have attractive features as a vector, though they have disadvantages of high immunogenicity. Some clinical trials using adenovirus vectors have been reported and their usefulness has been confirmed. Almost all of these therapies are considered to make tumor cells themselves the targets. A lot of conventional therapies are similar. However, it is thought to be difficult to kill all invasive glioma cells by these strategies. So, we need a new idea. The fact that the author chose vascular endothelial cells as a target for gene therapy is new and interesting. However, I am afraid that there was no detailed comparison between the antitumor effect of gene therapy for tumor cells and that for vascular endothelial cells, although the author discussed their combined effect. I hope that further studies and success in developing a new gene therapy that make vascular endothelial cells the targets will follow.

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