Retroviral Transfer of Herpes Simplex Thymidine Kinase Gene into Glioma Cells Causes Targeting of Gancyclovir Cytotoxic Effect

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Abstract

The thymidine kinase (tk) gene of herpes simplex virus type 1 (HSV-1) was transduced into three glioma cell lines (T98, U251MG, T9) using a retrovirus vector. The supernatants of viral producer cell line PA317/LTRNL was used for infection and three transduced cell lines (T98/LTRNL, U251MG/LTRNL, T9/LTRNL) were established. The toxicities of the anti-herpetic drugs, acyclovir and gancyclovir, were evaluated in vitro. The cytotoxicities of acyclovir and gancyclovir to the HSV-1 tk gene-transduced cells increased 100-1000 fold compared to the non-transduced parental cell lines. The cytotoxic effect of gancyclovir was higher than that of acyclovir, requiring a concentration of less than 0.31 μM to obtain 50% inhibition. Deoxyribonucleic acid analysis of the gancyclovir-treated cells demonstrated fragmentation, suggesting that apoptosis is involved in the mechanism of cell death. The HSV-1 tk gene-transduced cells were co-cultured with parental cells and treated with gancyclovir. More than 95% of cells were killed in a mixture ratio of 1:1, suggesting that the “bystander effect” operated in this system. Selective transduction of HSV-1 tk gene into glioma cells using a retroviral vector and treatment with gancyclovir is a promising therapy for patients with malignant glioma.

Key words: malignant glioma, herpes simplex virus, thymidine kinase, retroviral vector, apoptosis, bystander effect

Introduction

Glioma is an invasive, rapidly growing tumor. The prognosis for patients with malignant glioma is always poor because the results of conventional treatment using surgery, radiotherapy, and chemotherapy are often disappointing. Recently, targeting therapies have been introduced, such as radioimmunotherapy using radiolabeled monoclonal antibody, adoptive immunotherapy with cytotoxic T cells, or gene therapy with toxic gene in an attempt to overcome this formidable neoplasms. Malignant glioma contains many dividing cells, in contrast to normal brain cells such as neurons and glial cells which are in general non-dividing cells. The resultant metabolic differences are useful for targeting therapy with retrovirus-mediated gene transfer because retroviruses can only integrate into the genome of dividing cells.

Thymidine kinase (tk) produced by the herpes simplex virus type 1 (HSV-1) tk gene offers a selective killing mechanism for dividing cells. This enzyme is not harmful to mammalian cells, but catalyzes phosphorylation of specific nucleoside analogs, such as acyclovir or gancyclovir, to the nucleoside monophosphate. The nucleoside monophosphate is then phosphorylated by cellular kinase to the nucleoside triphosphate and incorporated into deoxyribonucleic acid (DNA), leading to inhibition of DNA synthesis and cell death. The high specificity of viral tk for these analogs allows selective killing of cells expressing HSV-1 tk gene. Therefore,
retrovirus-mediated HSV-1 tk gene transfer following treatment with the nucleoside analogs may be an effective targeting therapy for malignant glioma.

We previously reported the construction of a novel retroviral vector and transduction of the HSV-1 tk gene into human leukemia cell lines. Here we describe the transduction of the HSV-1 tk gene into glioma cells with our novel vector, which in conjunction with treatment using nucleoside analogs, demonstrates selective killing of the glioma cells in vitro.

Materials and Methods

I. Establishment of viral producer cell

The ecotropic packaging cell line 220 and the amphotropic packaging cell line PA31722) were maintained in minimum essential medium (MEM) with 10% fetal bovine serum (FBS). Retrovirus vector pLTRNL was described previously. Stable viral producer was established by transfection of pLTRNL DNA (20 µg) into 5 x 105 y-2 cells using the calcium phosphate precipitation method in 10-cm tissue dishes. One day after transfection, the culture medium was collected and applied to amphotropic PA317 cells in the presence of Polybrene (Sigma, St. Louis, Mo., U.S.A.) (5 mg/ml). Infected cells were selected in medium containing G418 (neomycin analog) and established viral producer cell PA317/LTRNL.

II. Establishment of HSV-1 tk-transduced cell lines

T9 (rat gliosarcoma cell line), U-251MG (human glioma cell line), and T98 (human glioma cell line) were maintained in MEM with 10% FBS. PA317/LTRNL cells were maintained in medium with G418 until they reached 80% confluency, then in medium without G418 for 24 hours. The virus-containing medium was removed, filtered through a 0.22-µm pore filter, and stored at -70°C until use. The HSV-1 tk gene-transduced cells (T98/LTRNL, U251MG/LTRNL, T9/LTRNL) were obtained by infection with the virus-containing medium in the presence of Polybrene. Briefly, parental cell lines (T9, U251MG, T9) were mixed at various ratios (0:10, 1:1, 9:1, 10:0), plated in 24-well plates at a density of 1 x 10^4/ml, and cultured with gancyclovir (20 µM). The number of cells was counted after 3 and 6 days.

In some experiments, porous membrane filters (Falcon Cell Culture Inserts; Beckton Dickinson, Lincoln Park, N.J., U.S.A.) were placed in the 6-well plates to inhibit cell to cell contact. 1.5 x 10^4 T9 cells were seeded on the membrane filter and the same number of T9/LTRNL cells were seeded in the bottom of the same well. In other wells, 1.5 x 10^4 cells of T9 and T9/LTRNL were mixed and then seeded in the well without the membrane filters. The cultures were maintained and the numbers of cells were counted after 6 days.

V. DNA extraction and agarose gel electrophoresis

The procedure was basically the same as that of Hogquist et al. Briefly, cells cultured with gancyclovir (20 µM) were pelleted and resuspended in cold lysis buffer (500 µl) containing 20 mM Tris-HCl (pH 7.4), 10 mM ethylenediaminetetra-acetic acid (EDTA), and 0.2% Triton X-100 for 20 minutes. The lysate was centrifuged for 20 minutes at 4000 rpm. 100 µg/ml proteinase K was added and the mixture incubated at 50°C for 6 hours, then digested with ribonuclease at 50 µg/ml for 2 hours at 37°C. The DNA in the viscous solution was extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1), then precipitated with ethanol at -20°C. Electrophoresis was carried out on 1% agarose gel in 90 mM Tris/borate buffer (pH 8.0) containing 2 mM EDTA. The gel was stained with ethidium bromide at 1 µg/ml and visualized under ultraviolet light.

Results

I. Cytotoxicity of acyclovir and gancyclovir

The growth curves in Fig. 1 show the cytotoxic effects of gancyclovir or acyclovir on T98 and T98/LTRNL. The concentration of gancyclovir to obtain the cytotoxicity of acyclovir and gancyclovir were assayed using parental wild-type cells and HSV-1 tk gene-transduced cells in 24-well plates at a density of 1 x 10^4/ml. After 24 hours, acyclovir and gancyclovir were added at various concentrations (0-320 µM) and the cells were incubated for a further 3 days. The cytotoxicity was determined by the trypan blue dye exclusion method, by comparing the number of surviving cells in the presence and absence of the nucleoside analog.

IV. Bystander effect under gancyclovir treatment

Parental cells (T9, U251MG) and HSV-1 tk-transduced cells (T9/LTRNL, U251MG/LTRNL) were mixed at various ratios (0:10, 1:1, 9:1, 10:0), plated in 24-well plates at a density of 1 x 10^4/ml, and cultured with gancyclovir (20 µM). The number of cells was counted after 3 and 6 days.
50% inhibition of T98/LTRNL cell growth was less than 0.31 μM and more than 80% inhibition was observed at 1.25 μM. In the concentration range of 0–20 μM, gancyclovir showed no marked toxicity to wild-type cells but achieved remarkable toxicity to HSV-1 tk-transduced cells. The cytotoxicity of acyclovir increased about 100 fold against transduced cells compared to wild-type cells. Acyclovir seems to be less effective because a higher concentration is necessary to inhibit growth compared to gancyclovir. U251 MG/LTRNL and T9/LTRNL showed similar susceptibility to gancyclovir (Fig. 2). The cytotoxicity of gancyclovir increased more than 1000 fold against transduced cells compared to wild-type cells. The concentrations of gancyclovir required to obtain 50% inhibition of both transduced cell lines were less than 0.31 μM.

II. DNA analysis
DNA extracted from cells (U251MG, U251MG/LTRNL) cultured with gancyclovir was analyzed by agarose gel electrophoresis. Figure 3 shows the degradation of DNA to oligoclonal bands as a ladder pattern detected 6 days after gancyclovir treatment.

III. Bystander effect
The bystander effect was clearly observed in a short-term culture with gancyclovir. When T9 and T9/LTRNL cell cultures were mixed equally, more than 95% of both cell lines were killed 6 days after treatment with gancyclovir. Similar results were obtained when U251MG and U251MG/LTRNL were mixed. Even when the mixtures contained only 10% of U251MG/LTRNL, nearly 99% of the cells were killed (Fig. 4). However, the bystander effect was not observed in cultures using the membrane filter, in which no contact between T9 and T9/LTRNL cells occurred. The number of T9 cells grown on the membrane filter was not inhibited after 6-day culture while almost all T9/LTRNL cells grown on the bottom of the same well did not survive (Table 1).
Fig. 3  Agarose gel electrophoresis of DNA extracted from cultured U251MG cells. lanes 1: molecular standards, 2: DNA extracted from U251MG cells cultured with 20 µM of gancyclovir for 6 days, 3: DNA extracted from U251MG/LTRNL cells cultured with 20 µM of gancyclovir for 6 days.

Table 1  Effect of gancyclovir on growth of mixed cultures with or without porous membrane separator

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Discussion

Retroviral vectors are useful vehicles to achieve gene transfer, and have been used successfully to deliver foreign genes selectively to tumor cells because they only integrate in the genome of dividing cells. To increase the efficiency of transduction of the foreign gene, the infection titer of viral producing cells must be increased. We previously constructed a novel retroviral vector pLTRNL containing the HSV-1 tk gene, but without the poly A signal of HSV-1 tk, which increases the titer of the viral producing cells. In this study, we used this vector to make viral producer cell line PA317/LTRNL and established three HSV-1 tk gene-transduced glioma cell lines.

The expression of the HSV-1 tk gene alone is not harmful. The toxic effect depends on enzymatic conversion of acyclovir and gancyclovir to toxic nucleoside intermediates that inhibit DNA synthesis. In this study, the toxicity of gancyclovir to HSV-1 tk-expressing cells was greater than that of acyclovir. The concentration of gancyclovir required to achieve 50% inhibition of cell growth was less...
than 0.31 µM. As the plasma concentration of gancyclovir is about 40 µM 1 hour after intravenous injection of 5 mg/kg of gancyclovir into humans, this concentration is practical for clinical use.

Apoptosis is a type of cell death which was initially described as a physiological process of cell selection during development. In contrast to necrosis, there is no marked inflammatory reaction and the process is relatively slower. An apoptotic cell induces the suicidal cascade in response to stimuli and destroys itself. Apoptosis is caused by both physiological stimuli and cytotoxic treatment with many anticancer agents such as cisplatin or etoposide. The main morphological characteristics of apoptosis are nuclear fragmentation and cellular breakdown in apoptotic vesicles. Internucleosomal DNA fragmentation is an important biochemical feature. 180-200 base pair fragments are readily shown as a typical ladder pattern by electrophoresis, while in necrosis the DNA breakdown is random and appears as a smear. In our experiments, DNA degradation to oligoclonal bands was clearly observed in gancyclovir-treated U251MG/LTRNL cells, indicating that the inhibition of DNA synthesis in HSV-1 tk-transduced cells by the nucleoside intermediates leads to the process of apoptosis.

Culver et al. originated the term "bystander effect" to describe the death of wild-type tumor cells neighboring HSV-1 tk-transduced tumor cells in vivo. Wild-type tumor cells mixed with tumor cells transduced with the HSV-1 tk gene were injected into normal mice treated with gancyclovir. Complete regression was observed in nearly all animals containing 50:50 cell mixtures and in some animals with cell mixtures containing as few as 10% HSV-1 tk-expressing cells. The mechanism mediating this bystander effect is not fully understood.

This study demonstrated the bystander effect in vitro without the host immune response. The bystander effect was not induced when T9 cells and T9/LTRNL cells were cultured in the same well but separated by a membrane filter. This suggests that direct cell contact is necessary, and humoral factors are not related to this bystander effect. Further studies on the mechanism of bystander effect will be based on this observation. The bystander effect is an attractive characteristic because even though the efficiency of the gene transduction to glioma cells is low, a much greater killing effect of glioma cells can be expected.

Our results show that once HSV-1 tk genes are transduced to glioma cells, these cells are easily killed by treatment with gancyclovir. Clinical use of retrovirus-mediated gene transfer is possible because the gene is inserted only in dividing tumor cells, not neurons and glial cells. The efficiency of gene transduction can be increased by intratumoral injection of viral producer cells. This depends on the fact that the brain is a relatively immunologically privileged site, permitting the viral producer cell, which is a murine fibroblast and immunologically incompatible with humans, to continue the process of gene transduction to tumor cells for a long time.

Retrovirus-mediated HSV-1 tk gene transfer followed by treatment with gancyclovir is a promising therapy for patients with malignant glioma. Clinical trials have already started in the United States. Further investigation using animal models will be done in the future.

Acknowledgments

We wish to thank Dr. T. Friedmann (Dept. of Pediatrics, Univ. of California, San Diego, Cal., U.S.A.) for kindly providing retroviral plasmids.

References


Neurol Med Chir (Tokyo) 34, June, 1994


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