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Basic Investigations

RETROVIRAL-MEDIATED SUICIDE GENE THERAPY OF EXPERIMENTAL GLIOMA

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Objective: To establish a retroviral-mediated suicide gene therapy system for experimental glioma and test its efficacy. Methods: C6 rat glioma cells were infected with recombinant retrovirus containing HSV-tk gene. The C6/tk cell line which stably expressed tk was selected and cloned. The sensitivities of C6/tk cells to several nucleoside analogues, such as GCV, BVdU, ACV were compared by the growth inhibition studies. Antitumor effects were also observed after GCV treatment in nude mice bearing tumors derived from C6/tk cells. Results: The growth inhibition studies showed that GCV was the most efficient prodrug in this system. C6/tk cells were highly sensitive to GCV, with an IC_{50}<0.2 μmol/L, being 500-fold less than that in tk-negative C6 cells. *In vivo* studies showed significant tumor inhibition in the treatment group. Conclusion: Glioma cells can be eradicated by using retroviral-mediated suicide gene system *in vitro* as well as *in vivo*.

Key words: Suicide gene, Retrovirus, Glioma, HSV-tk gene, Gene therapy.

Malignant glioma, the most common intracranial tumor, is one of the most lethal cancers in human. The median survival time after diagnosis is less than one year for glioblastomas.[1] Because of the poor success of traditional treatment, the potential use of gene therapy is being investigated in many laboratories. One approach is the use of drug susceptibility genes ("suicide gene") for selective destruction of the tumor. The viral gene which codes for the enzyme, such as HSV-tk, was incorporated into the replicating cancer cells. Using this approach, Moolten and Wells et al. demonstrated that tumor cells become sensitive to GCV *in vitro* and *in vivo* as a result of retroviral-mediated transduction of the HSV-tk gene. Ezzeddine and Culver et al. extended these findings to demonstrate that retrovirus-producer cell lines could be used to deliver the HSV-tk gene directly to a tumor mass, using brain tumor as a model. In this study, C6 rat glioma cells were infected with recombinant retrovirus containing HSV-tk gene. The C6/tk cells which stably express tk were highly sensitive to the nontoxic prodrug GCV. The sensitivities to another two prodrugs, BVdU and ACV, were also compared in this experiment. *In vivo* studies showed significant tumor inhibition in the treatment group.

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MATERIALS AND METHODS

Plasmid Construction

PCR primers were designed according to the published sequence of the tk gene.[2] tk 5' primer: 5' GCGCGTATGGCTTCGTACCC 3', inserted an *Eco*R I restriction site before the start coden ATG; tk 3' primer: 5' TCCTTACGTGTTTCAGTTAGCCTC 3',

inserted a *Bam*H I restriction site after the stop coden. PCR amplification was carried out using the plasmid HSV106 as the template (annealing temperature 55℃, 30 cycles). The resulting 1.2 kb fragment was cloned to the plasmid pBluescript and sequenced using T7 sequencing kit (Pharmacia). Then tk gene was subcloned into the *Eco*R I/*Bam*H I sites of the retrovirus vector pLXSN (gift from Dr. A.D.Miller) to form the plasmid pLtkSN.

Cell Culture, Recombinant Retrovirus Packaging and Retroviral Infection

Both rat glioma cell line C6 and amphotropic retrovirus packaging cell line PA317 (kindly provided by Dr. A.D.Miller) were cultured in DMEM with 10% newborn calf serum (GIBCO-BRL) in a 5% CO_2 atmosphere at 37 ℃. The plasmids pLtkSN and pLXSN were transfected into PA317 cells using Lipofect Amine Transfection Reagent (GIBCO-BRL), respectively. After selected with 800 μg/ml G418 (GIBCO-BRL) for 2 weeks, the retrovirus-producing cell lines PA317/tk and PA317/0 were established from G418 resistant clones. Amphotropic retrovirus LtkSN and LXSN were harvested from the supernatant of these cells. No helper virus could be detected.

C6 cells were plated at 1×10^5 cells per 35 mm dish and infected by exposure to the retrovirus LtkSN or LXSN in the presence of 8 μg/ml polybrene (Sigma) at 30℃ for 24 h.[3] The G418 (800 μg/ml) resistant clones were selected randomly. Representative clones C6/tk and C6/0 were maintained in medium containing 200 μg/ml G418.

Identification of Gene Integration and Expression

Total cellular genomic DNA and RNA were isolated from C6/tk, C6/0 and C6 cells with isolation reagents DNA_{ZOL}^{TM} and RNA_{ZOL}^{R} (GIBCO-BRL), respectively. Then PCR and RT-PCR reaction were done with the designed 5' primer and 3' primer of tk gene using these genomic DNA and c-DNA as the templates. The plasmid pBluescript-tk which digested with EcoR I/BamH I was used as the control.

Cytotoxic Assay *In Vitro*

The cytotoxicity of prodrugs was measured by MTT assay. Target cells were plated into 96-well microplates at 2000 cells per well. Next day, cells were cultured in 200 μl/well fresh medium containing various concentrations of prodrug. Six days later, medium was replaced with 180 μl fresh medium containing 0.25 mg/ml MTT (Sigma). After 4 h of incubation at 37℃, 100 μl solubilizing reagent (20% SDS and 50% DMF in water) was added. Then after another 4 h, the A_{595} of each well was measured with a microplate reader with reference filter of 655 nm. All tests were performed in six samples, and the percentage of survival was estimated as: % Survival= A/B×100% (A: mean value of A_{595} from cells incubated with prodrug; B: mean value of A_{595} from cells incubated with medium only).

Transmission Electron Microscopy

After treated with 20 μmol/L GCV for 3 days, C6/tk and C6 cells were fixed in 2.5% glutaraldehyde and postfixed in 2% osmium tetroxide, then dehydrated in a gradient ethanol series, and embedded in epoxy resin (Epon 812). Thin sections were photographed by transmission electron microscope (JEM 100B).

In Vivo Experiment

Sixteen male BALB/c-nu/nu mice (about 20 g weight) were inoculated s.c. with 2×10^6 C6/0 and C6/tk cells in the left and right flanks, respectively, and then randomly divided into two groups: experimental group and control group. Each group consisted of 8 mice. From day 8, when the average diameter of s.c. tumor was 3–4 mm, each mouse was given i.p. injection twice a day of 100 mg/kg/d GCV or N.S. for 10 days. During the whole course of the experiment, the tumor weight was estimated based upon tumor size as described.[4]

RESULTS

Identification of the Integration and Expression of Suicide Gene

The electrophoretic analysis has shown that PCR and RT-PCR amplification products had the same bands as the control (Data not shown). Since mammalian cells do not produce HSV-tk, transcription of tk gene could be detected only in tk-transduced C6/tk cells.

Prodrug-mediated Growth Inhibition *In Vitro*

The wild type C6 cells were used as control in the cytotoxicity assay. As shown in Figure 1a. Transduction of tk gene made rat glioma cells become highly sensitive to GCV. The IC_{50} was <0.2 μmol/L, while the parental C6 cells and tk-negative C6/0 cells were relatively resistant to GCV (IC_{50}>100 μmol/L). But the efficiency of another two prodrugs BVdU and ACV was lower than that of GCV, as shown in Figure 1b, Figure 1c. Both tk-positive and negative C6 cells were relatively insensitive to BVdU and ACV.

Morphological Analysis

Transmission electron microscopy analysis revealed that the characteristic features of apoptosis exhibited in C6/tk cells after treated with 20 μmol/L GCV for 2 days. In contrast, the normal C6 cells which identically treated with GCV remained viable with intact nuclear structure (Figure 2a). The apoptosis features of C6/tk cells included the chromatin aggregation, the nuclear material condensation and migration to the periphery (Figure 2b), and subsequent breakdown of nuclear. These results confirmed that enzyme/prodrug systems mainly disrupted DNA synthesis in dividing cells and selectively killed them.

GCV Mediated Growth Inhibition *In Vivo*

All mice had similar individual tumor weight of approximate 30 mg by day 8 as determined by tumor size, as shown in Figure 3. In the control group, tumors derived from C6/tk and C6/0 cells grew fast through out the course of the experiment, though transduction of tk gene might decrease the tumorgenesis and the tumors derived from C6/tk cells were a little smaller than that from C6/0 cells. In the treatment group, profound anti-tumor effects were observed in tumors derived from C6/tk cells but not in tumors derived from C6/0 cells after GCV treatment. Some of the C6/tk derived tumors completely regressed and could not be detected on day 35.

DISCUSSION

Gene therapy for cancer represents one of the most important novel therapeutic approaches emerging in the past decade. Four major strategies of cancer gene therapy are involved in most experimental or clinical investigations: protection of normal tissues; improvement of the host antitumor response; reversion of the malignant phenotype; and direct killing of tumor cells by means of cytotoxic or drug susceptibility gene (suicide gene).[5]

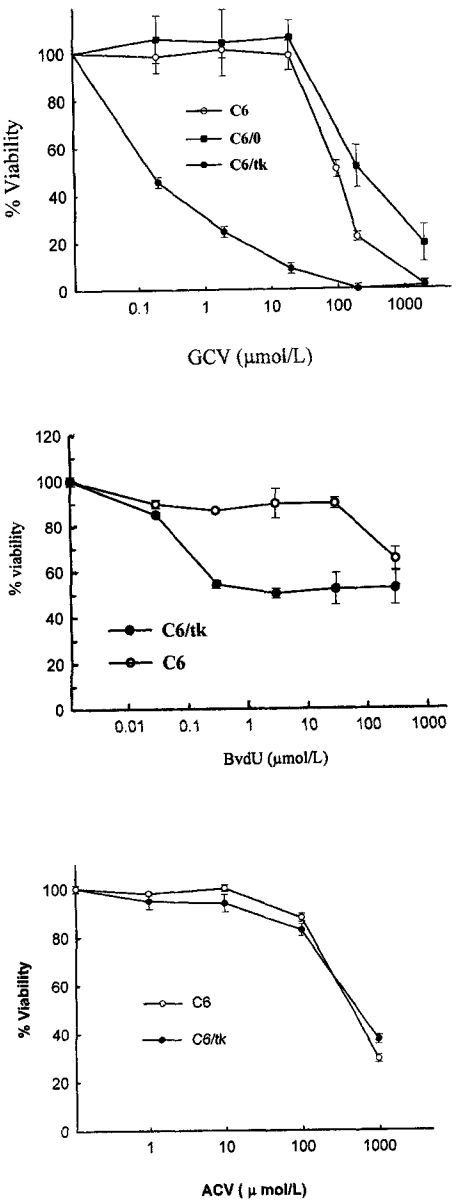

Fig. 1. Prodrug-mediated growth inhibition *in vitro* (n=5).

a. GCV mediated growth inhibition

b. BVdU mediated growth inhibition

c. ACV mediated growth inhibition

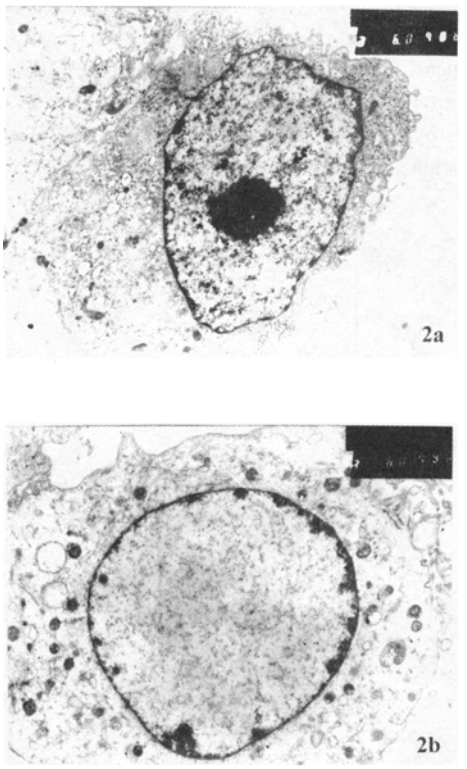

Fig. 2. Transmission electron microscopy analysis of apoptosis in suicide gene tk modified C6 cells after GCV treatment (×6000).

a. Wild type C6 cells exposed to GCV

b. C6/tk cells exposed to GCV

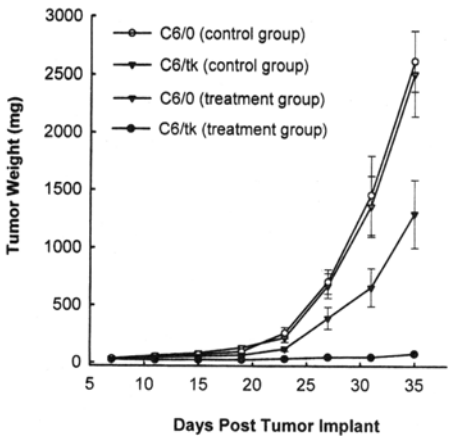

Fig. 3. GCV-mediated growth inhibition *in vivo* (n=8).

Gliomas represent nearly 40–50% of all primary brain tumors. The 5 years survival rate for patients with glioblastoma is less than 5% given current treatment modalities of surgery, radiation therapy and chemotherapy. The majority of patients die of local recurrence. Given the lack of adequate therapy for glioblastomas, new approaches to treatment of these brain tumors are needed. Retroviral-mediated suicide gene therapy for the treatment of brain tumors might be a feasible means.[6]

In 1970s', Elion et al. found that the thymidine kinase enzyme was able to convert purine and pyrimidine derivatives. tk can efficiently phosphorylate nucleoside analogues, such as the widely used antiherpes drugs GCV, BVdU and ACV, into nucleoside intermediates. They are then converted into triphosphated substrates, which are incorporated into elongating DNA during cellular division, causing chain termination and cell death.[7]

Considering that retroviruses only stably integrated their genes in target cells that are actively synthesizing DNA,[8] retroviral-mediated gene transfer might be particularly suitable for the treatment of tumors that are made up rapidly dividing cells invading a nonproliferating tissue.[9] It has been reported that in the normal adult CNS neurons and most other cells are stable in G_0, a resting phase, and do not regularly synthesize DNA. Thus, within brain, only tumor cells and endothelial cells which are necessary for tumor growth are actively proliferating. So brain tumors should be an ideal candidates for targeted gene therapy by retroviruses. Another advantage of the brain for this approach is that it is a relatively immunological privileged site and could permit the histoincompatible retrovirus-producer cells to persist and expose the tumor cells to potential transduction for a long time without immunologic rejection.

Based on the experimental data, suicide gene therapy for the treatment of malignant gliomas has been approved by the FDA and the NIH Recombinant DNA Advisory Committee in America.[10] A phase II clinical trial has begun since 1994. The studies were designed to evaluate the efficacy of *in situ* gene transfer by directly injecting retrovirus-producer cells after surgery. The results suggest that the approach is beneficial to some patients.

Our study has shown that transduction of HSV-tk gene made rat glioma cells highly sensitive to GCV with an IC_{50} less than 0.2 μmol/L. Morphological analysis showed the apoptosis features mainly existed in the nuclear, demonstrated that the cell death was caused by the disruption of DNA synthesis.

Meanwhile, BVdU and ACV were compared with GCV, but their specificity and efficiency were relatively poor. *In vivo* studies also demonstrated the efficacy of this retroviral-mediated negative selection system. Significant tumor inhibition was found in the treatment group. Also, the rat *in situ* glioma model has been established in our laboratory, the further study on the feasibility of this system is under way.

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