Advances in Brief

Treatment of Malignant Gliomas Using Ganciclovir-hypersensitive, Ribonucleotide Reductase-deficient Herpes Simplex Viral Mutant

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Abstract

We have demonstrated that attenuated mutants of herpes simplex virus (HSV) have therapeutic potential for malignant brain tumors. In this report, we tested a ribonucleotide reductase-deficient (RR-) HSV mutant as an experimental treatment for malignant brain tumors. The HSV-RR- mutant hrR3, containing an Escherichia coli lacZ gene insertion in the ICp6 gene that encodes the large subunit of RR, was used in this study. We examined the cytotoxic effect of hrR3 (0.1 plaque-forming unit/cell) on the U-87MG human glioblastoma cell line in vitro. Only 0.2% of U-87 cells were alive 67 h postinfection. Drug sensitivity assays demonstrated that hrR3 is hypersensitive to the antiviral agent ganciclovir. In vivo studies, 10 animals harboring U-87MG tumors were randomly divided and treated intraneoplastically with either 5 x 106 plaque-forming units of hrR3 or medium alone. The viral treatment group showed significant inhibition of tumor growth (P < 0.01; one-sided Wilcoxon rank test). Expression of the lacZ gene in hrR3, visualized by 5-bromo-4-chloro-3-indoly-1-D-galactopyranoside histochemistry, could be detected in treated tumors. The therapeutic potential of this HSV-RR- mutant for malignant gliomas is discussed.

Introduction

Despite many recent advances in neurosurgical techniques, radiation therapy, and chemotherapy, the prognosis for patients with malignant brain tumors has not improved dramatically. The 5-year survival for glioblastoma multiforme, the most malignant glioma, is still 5.5% or less (1, 2). This led us to examine novel therapeutic approaches utilizing genetically engineered viruses (3—5). Recent experimental studies indicate that engineered viruses derived from HSV-13 might have therapeutic potential for the treatment of human malignant gliomas. Previously, we demonstrated that a TK- mutant dlspkt could destroy human malignant glioma cells in an animal brain tumor model (3, 4). The HSV-TK gene encodes a key enzyme in the de novo synthesis of nucleotide precursors. This deletion compromises DNA replication in nondividing cells, including those in the mammalian nervous system (6—9). We hypothesized that the HSV-TK- mutants might effectively treat brain tumors while sparing normal brain cells. However, in considering clinical trials of this therapeutic approach, an important issue is the resistance of the HSV-TK- mutant to commonly used antiviral agents such as ACV and GCV. In the central nervous system, the major side effect of using replication-competent viruses as therapeutic agents is the possible production of encephalitis.

For example, with dlspkt, the 50% lethal dose after i.c. inoculation of CD-1 mice was 106 pfu (5). Therefore, although dlspkt is sensitive to foscarnet and vidarabine (3), it would be beneficial to use a HSV mutant that is sensitive to GCV or ACV and less neurovirulent.

RR is also a key enzyme in the de novo synthesis of DNA precursors, catalyzing the reduction of ribonucleotides to deoxyribonucleotides (10). HSV-1 encodes its own RR, which is composed of two nonidentical subunits (11). The large subunit (M, 140,000), designated ICp6, is tightly associated to the small subunit (M, 38,000). HSV-RR is required for efficient viral growth in nondividing cells but not in many dividing cells (12—15), indicating that the HSV-RR- mutant might have therapeutic potential for malignant gliomas while maintaining antiviral drug sensitivity. We studied the efficacy of HSV-RR- mutant hrR3, which contains an Escherichia coli lacZ gene insertion in the ICp6 gene, against malignant human gliomas. The presence of the lacZ gene in hrR3 allows identification of virally infected tumor cells using β-galactosidase histochemistry. Furthermore, because this mutant is hypersensitive to GCV, it may be a more clinically useful therapeutic agent for malignant gliomas than HSV-TK- mutants.

Materials and Methods

Viruses and Cell Lines. HSV-1 wild type strain KOS was kindly provided by Donald M. Coen (Harvard Medical School, Boston, MA). HSV-RR- mutant hrR3, which possesses the structural gene of E. coli lacZ inserted into the RR large subunit (ICp6) gene of HSV-1 KOS (13), was kindly provided by Sandra K. Weller (University of Connecticut Health Center, Farmington, CT). Stocks of viruses were generated in African green monkey kidney cell (Vero) cultures as described (3). Virus titration was performed as described elsewhere (3). Human glioblastoma cell lines U-87MG, T98G, U-138MG, and A172 were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's minimal essential medium, supplemented with 10% inactivated fetal calf serum and antibiotics.

Cell Culture Cytotoxicity. Viruses were infected onto subconfluent monolayers of U-87MG cells in 25-cm2 tissue culture flasks at a MOI of 0.1 plaque-forming unit; i.n., intraneoplastically; HBSS, Hanks' balanced salt solution.

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Received 4/28/94; accepted 6/9/94.

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1 This study was supported in part by NIH Grants CA60176 and NS323677.

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3 The abbreviations used are: HSV-1, herpes simplex virus type 1; TK-, thymidine kinase deficient; RR-, ribonucleotide reductase deficient; ACV, 9-[2-hydroxyethoxy]-methylguanine (acyclovir); GCV, 9-[4-hydroxy-1-(hydroxymethyl) ethoxy]methylguanine (ganciclovir); ICp6, infected cell protein 6; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside; i.e., intraneoplastically; HBSS, Hanks' balanced salt solution.
s.c. Glioma Therapy and X-gal Staining. U-87MG tumors were removed aseptically from the flanks of host mice, minced into 1-mm pieces, and transplanted into additional mice for study. Mice harboring s.c. tumors (>6 mm in diameter) were randomly divided (n = 5/group) and treated i.n. with either 5 × 10^6 pfu of hrR3 virus suspended in 0.05 ml HBSS or with HBSS alone. Treatment was repeated in an identical fashion 10 days later. The tumor diameter was measured by external caliper measurements. Tumor growth ratio was determined as:

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\frac{1 \times w \times h}{(I \times w \times b)_{day 0}}
\]

in which I is length, w is width, and h is height. Statistical differences in growth ratios were assessed by use of one-sided Wilcoxon rank test.

For pathological studies, tumor-bearing mice (>10 mm in diameter) were treated with a single injection of 5 × 10^6 pfu of hrR3 and sacrificed on days 3, 7, and 14 postinjection. Animals were perfused by 2% parafomaldehyde-5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid-2 mM magnesium chloride in 0.1 M 1,4-piperazinediethanesulfonic acid buffer, pH 7.3. The s.c. tumors were removed, placed in fixative for 1 h, and submerged in cold phosphate-buffered saline. Tumors were then placed overnight in substrate solution (containing 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40), washed with phosphate-buffered saline, and incubated overnight in cold phosphate-buffered saline containing 30% sucrose and 2 mM magnesium chloride. After being frozen on dry ice, tumors were sectioned on a cryostat. The sections were mounted onto gelatin-coated glass slides and counterstained with hematoxylin and eosin solution.

Results

In Vitro Cytopathic Efficacy. To determine whether a HSV-RR^-mutant could destroy malignant glioma cells, human glioma cell lines U-87MG, T98G, U-138MG, and A172 were infected with hrR3 (MOI, 0.1). All four cell lines were efficiently destroyed by hrR3 within 5 days. With U-87 cells, a cytopathic effect appeared on day 1 postinfection and >99% cytotoxicity was evident on day 3 (Fig. 1). Cells became round, lost normal morphological features, and lifted off the plate. The cytopathic efficacy of hrR3 to U-87MG cells was almost the same as that of dsptk (3). β-Galactosidase expression in hrR3-infected cells was also examined. Infected cells exhibiting a cytopathic effect were stained with X-gal (data not shown).

GCV Hypersensitivity of HSV-RR^-Mutant. We compared the GCV sensitivity of hrR3 with that of the parental wild type HSV strain KOS. hrR3 was approximately 10 times more sensitive to GCV than to KOS, with a median effective dose of 4–5 ng/ml, while the median effective dose for KOS is 40–50 ng/ml (Fig. 2). This is similar to the results of Coen et al. with ACV (16).

In Vivo Treatment and X-gal Histochemistry. We next studied the effect of hrR3 infection on s.c. xenografts. U-87MG tumor fragments were transplanted into BALB/c-nu/nu mice. Growing tumors (>6 mm in diameter) were evident by week 3, at which time tumors were treated with i.n. injections of hrR3 or HBSS (control). There was no significant difference in tumor diameter between control and hrR3-treated tumors for the first few days after the first injection of hrR3. From day 10 onward the tumor size between the two groups diverged. When the experiment was terminated on day 38 (Fig. 3) because of tumor size in the control animals, the mean tumor growth rate was significantly inhibited in hrR3-treated tumors compared to control tumors treated with medium alone.

Fig. 1. In vitro cytopathic efficacy of hrR3 on U-87MG cells. Duplicate flasks of U-87MG cells were infected with hrR3 at a MOI of 0.1. Viable cells were determined and compared with mock infected cells on days 1–4 postinfection. Cell survivals at 18 and 67 h postinfection are 90.7 and 0.2% of controls. Points, means; bars, SEM.
positive X-gal staining cells were observed at injection sites and around the periphery of the tumor. This may be due to leakage of inoculum within the tumor capsule and the presence of actively growing tumor cells at the periphery (Fig. 4a). Some blue cells were present outside of the tumor capsule but these did not persist at later time points. On days 7 and 14, staining of peripheral cells disappeared and the area of positive X-gal staining within the tumor was expanded, which suggests spread of the virus (Fig. 4, b and c). At higher magnification, these blue tumor cells appeared necrotic, losing the typical morphological appearance of a U-87MG cell (Fig. 4d).

Discussion

Our previous studies demonstrated that HSV-TK− mutants have therapeutic potential for malignant brain tumors (3, 4). In this study, we examined the effect of HSV-RR− mutants on malignant glioma cells. Both HSV-TK− and HSV-RR− mutants can replicate in dividing cells but not in nondividing cells due to mutations in key enzymes for nucleotide metabolism (3, 6, 13). Our model depends on the ability of HSV mutants to replicate in actively growing glioma cells while sparing normal, postmitotic brain cells and effectively destroying malignant glioma tumors in vivo with minimal collateral damage. We are characterizing different viral mutations in order to optimize the efficacy of this approach. In this study, we demonstrate that the HSV-RR− mutant hrR3 destroyed human U-87MG cells in vitro and in vivo as well as the HSV-TK− mutant dlspk.

An important difference between these HSV mutants is that hrR3 is hypersensitive to ACV and GCV while dlspk is resistant. In the case of mutant virus replication outside the tumor, HSV-TK− mutants are resistant to the most commonly used antiherpetic nucleoside analogues and are therefore currently difficult to treat, whereas HSV-RR− mutants are hypersensitive to ACV and GCV. Lack of RR leads to reduced deoxynucleotide triphosphate pool synthesis, which could increase inhibition of DNA replication by deoxynucleotide triphosphate-competitive inhibitors such as GCV-TP (16, 17).

Another difference between the behavior of HSV-TK− and RR− mutants is temperature sensitivity of viral growth. HSV-RR− is severely compromised in its ability to produce infections and synthesize viral DNA at 39.5°C in vitro, while HSV-TK− can grow as well as wild type at elevated temperatures (6, 13, 14, 18). The molecular basis for this sensitivity is not clear. In considering clinical trials of HSV-RR− mutants, this behavior may influence the therapy. Direct inoculation of HSV-RR− mutants in the tumor may lead to local inflammation and then local fever. The killing activity of the mutants might be decreased in vivo due to the elevated temperature. On the other hand, one of the symptoms of encephalitis is high fever. HSV-RR− mutants in this case might be attenuated for replication in normal
brain. Therefore, this temperature sensitivity of HSV-RR could either protect the host or diminish the efficacy of the treatment. We demonstrated that hrR3 significantly inhibited the growth of s.c. U-87MG human malignant glioblastomas as well as dlspkt, suggesting that HSV-RR mutants could destroy tumor cells effectively in vivo.

There are several HSV-RR mutants available for therapy (13, 14, 18). In this study, we used hrR3, which contains E. coli lacZ gene in its genome. The presence of the lacZ gene in the genomic DNA of HSV mutants provides a sensitive means to track not only viral infection within the tumor but also ectopic spread of the virus outside the tumor. We demonstrated positively X-gal-stained U-87MG cells in vitro and in vivo, suggesting viral replication and spread. Further characterization of viral spread with HSV mutants containing lacZ will help to understand the mechanism of HSV tumor therapy.

In order for this therapy to become an effective clinical choice, it is important that different attenuated HSV strains and mutants affecting neurovirulence be examined. A fine balance must be achieved between optimizing the efficacy of tumor cell killing, minimizing spread of the virus in non-tumor tissue, and safety options.

Acknowledgments

We thank Dr. Sandra K. Weller for providing hrR3, Dr. Donald M. Coen for providing KOS, and Dr. Herbert J. Manz (Department of Pathology, Georgetown University Medical Center) for assisting in pathological analyses.

References

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