Toxicity studies in thymidine kinase–deficient herpes simplex virus therapy for malignant astrocytoma

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Previous studies have shown that genetically engineered thymidine kinase (tk)–defective herpes simplex virus type 1 (HSV-1) can effectively and selectively destroy gliomas in animal models. The consequences of viral infection and tumor regression must be characterized before this therapy can be applied in human trials. To study the potential for long-term toxicity, immunocompetent rats harboring 9L gliosarcomas were injected intratumorally with a tk–defective HSV-1, KOS-SB, at titers that previously have been demonstrated to cause tumor regression. In animals surviving 3 months or longer following viral treatment, there was no evidence of persistent infection or inflammation in peritumoral brain tissue or in remote systemic organs studied with routine histological and immunocytochemical analyses. Polymerase chain reaction using primers specific for HSV-1 detected HSV-1 DNA in peritumoral tissue only in animals sacrificed within 3 months of viral injection. There was no evidence of HSV-1 DNA in systemic tissues at any time after treatment. We conclude that stereotactic intratumoral injection of tk–deficient HSV can be attempted for the treatment of brain tumors without risk of systemic infection or significant toxicity to normal brain or remote proliferating tissues.

Key Words • herpes simplex virus • thymidine kinase • viral therapy • glioma

GLIOBLASTOMA multiforme is the most malignant primary cerebral neoplasm and accounts for nearly 40% of all primary brain tumors. Despite aggressive multimodality treatment including surgery, radiation, and chemotherapy, the average survival for a patient with this disease is less than 1 year. The failure of conventional methods to result in substantial improvement in survival time has prompted the development of novel therapeutic strategies that use sophisticated molecular techniques to target specifically the rapidly dividing tumor cell population.

Recent experimental studies indicate that genetically engineered viruses derived from a herpes simplex virus (HSV) or retrovirus may serve as vehicles to kill tumor cells selectively while not causing harm to adjacent normal brain tissue. In one approach, HSV type 1 (HSV-1) with a mutation in the thymidine kinase (tk) gene replicates in and destroys proliferating tumor cells. Because the tk-negative mutants can only replicate in and lyse dividing cells, the surrounding postmitotic neural cells are spared. Alternatively, retrovirus vectors bearing the HSV-tk gene can deliver this gene selectively to tumor cells in the brain and thus render them sensitive to the toxic effects of HSV-specific nucleoside analogs such as ganciclovir, whereas nondividing cells in the brain do not integrate this gene and are resistant to the drug.

Attention has also been directed toward adenoviral vectors using similar gene delivery strategies. Previously we reported the successful treatment of malignant primary brain tumors in immunocompetent rats using an HSV-1 tk-deficient mutant. Important hurdles remain in the development of such engineered viruses as oncolytic agents, including the demonstration of long-term safety and absence of systemic spread. The present studies were undertaken to determine the presence or absence of systemic and cerebral viral toxicity following intratumoral injection of engineered tk-deficient HSV.

Materials and Methods

Animal Preparation

Adult male Long–Evans rats weighing 250 to 300 g were used in the present study. All experiments were performed in compliance with guidelines provided by the University of British Columbia Committee on Animal Care.

The surgical procedures for tumor implantation and viral injection have been described previously. Briefly, animals were anesthetized by intramuscular injections of ketamine and xylazine and placed in a stereotactic headframe (David Knopf Instruments, Tujunga, CA). Five microliters of 9L rat gliosarcoma cells was injected into the rats’ frontal cortex through a 0.7-mm burr hole using a Hamilton syringe connected to the manipulating arm of the stereotactic frame. Two weeks after tumor implantation, 1 μl of tk-negative HSV-1 virus containing 10^6 plaque-forming units (PFUs) was injected into the tumor site. After various intervals, the animals were killed with an overdose of anesthetic agents and their brains and other organs were harvested for subsequent analysis.
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removed and immediately stored at −20˚C. To avoid storage effects, tissues from animals with various survival periods were all stored for 2 weeks before further processing.

Viral Vector

The tk-negative HSV-1 strain, KOS-SB, was used in the present study. This mutant has a 200-bp deletion in the coding region of the tk gene. The virus was produced in African green monkey kidney cells (Vero cell line) and harvested by centrifugation at 5000 g at 4˚C for 10 minutes. The supernatant was further centrifuged at 10,000 g at 4˚C for 2 hours to pellet the virions. Virion pellets were resuspended in phosphate-buffered saline and resuspended through a 20% sucrose gradient for 2 hours at 10,000 g at 4˚C. Pellets were then resuspended with phosphate-buffered saline at a concentration of 10^6 PFU/ml, determined using Vero cells. The viral stocks were stored in aliquots at −70˚C.

Histological and Immunocytochemical Investigations

Brain tissue was cut with a cryostat into 12-μm sections and post-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 minutes at room temperature. To determine the presence of HSV-1 antigens, sections were incubated overnight at 4˚C with rabbit anti-HSV-1 polyclonal antibody (1:10,000). Following incubation with biotinylated horse anti-rabbit serum (1:400) for 2 hours at room temperature, the sections were processed with a horseradish peroxidase avidin-biotin complex system (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature, and the immunoreaction was detected using 0.01% 3',3'-diaminobenzidine and 0.003% hydrogen peroxide. Primary antisera was omitted from the control sections and no immunoreactivity was detected, indicating the specificity of immunostaining. Additional primary antibodies used in the present study included OX42, OX18, OX6, W3/25, W3/13 HLK, and OX1, which are all monoclonal antibodies (all purchased from Sera-lab, distributed by Dimension Laboratories, Inc., Mississauga, Ontario, Canada). Adjacent sections were stained with cresyl violet for routine histological examination.

Extraction of DNA

Tissues of brain, heart, spleen, muscle, and liver were collected and weighed and then homogenized for DNA extraction. In the case of the brain, 20 to 60 mg of tissue surrounding the tumor cavity was collected by carefully scraping out adjacent peritumoral tissue on serial 12-μm frozen sections. The homogenized tissues were then treated with 10% sodium dodecyl sulfate and proteinase K (20 μg/ml) in Tris–ethylenediamine tetraacetic acid (EDTA) buffer at 55˚C for 2 hours or at 37˚C overnight followed by incubation with RNase (10 mg/ml) for 2 hours at 37˚C. After phenol–chloroform extraction and ethanol precipitation, the DNA was resuspended in Tris–EDTA buffer for polymerase chain reaction (PCR) studies.

Polymerase Chain Reaction

Pairs of oligonucleotide primers were chosen from the coding region of the HSV-tk gene. The primers were selected to flank the region containing nucleotides 252 to 1141 from the messenger RNA initiation site. Because the virus used in the present study has a 200-bp deletion from the coding region, the expected size of PCR product was approximately 0.69 kb instead of 0.89 kb. Considerations in the selection of appropriate primers also included primer length, guanine-plus-cytosine base content, and transtranscript primer complementarity. The sequence used upstream was: ATGACTTACGGCAGGTTGCT; the sequence downstream was: CTTCGCGTTATCAGTTGCC.

Aliquots of DNA from peritumoral brain tissue, as well as systemic organs harvested at various intervals, were exposed to PCR using standard methods. To determine the presence or absence of HSV-1 DNA in the tissue samples, PCR reaction products were electrophoresed on 1% agarose gels with appropriate DNA size markers and viewed on an ultraviolet transilluminator. Results were confirmed by Southern blot analysis with a tk probe.

Results

Histological Findings

Twenty-one animals were injected intratumorally with tk-negative HSV-1 2 weeks after tumor implantation. Two rats died due to overgrowth of tumors in the brainstem on Day 9 and Day 11 postinfection, respectively. The rest of the animals were killed after intervals of 3 days (three animals), 14 days (four animals), 20 days (three animals), 3 months (three animals), and 12 to 19 months (six animals) postinfection. Two normal animals were used as controls. There was no evidence of acute or delayed sickness after viral injection. However, histological examination revealed enlargement of the ventricular system in two of five animals killed within 14 days postinjection. In these animals there was a two- to threefold increase in ventricular volume (Fig. 1). In one of three animals killed after 3 months, minor ventricular enlargement (approximately a 30% increase) was observed. Ventricular size was normal in all animals surviving more than 3 months. Apart from ventricular size, there was no detectable change in either the volume of the brain or the density of cells compared to control animals at all time intervals studied.

Immunocytochemical Findings

Immunoreactivity for HSV-1 was found in ependymal cells and in the immediately subjacent white matter of animals killed within 2 weeks of viral injection. Scattered neurons in peritumoral brain tissue also showed a positive reaction for HSV-1. In contrast, neurons and astrocytes in the other regions did not show immunoreactivity for the HSV-1 (Fig. 2). In those animals surviving at least 20 days after viral injection, no HSV-1 immunoreactivity was found in any part of the brain.

To investigate the possibility of a chronic inflammatory or immune response in the brain after viral injection, sections from animals that had survived for longer than 3 months were also reacted with monoclonal antibodies for
indications of rat major histocompatibility complexes I and II, and leukocyte, macrophage, lymphocyte, and T-helper cells. No immunoreactivity was observed.

Results of Polymerase Chain Reaction

Polymerase chain reaction was applied to DNA from brain tissue and other organs of treated rats killed at various intervals to confirm the existence of the viral genome. A large quantity of amplified HSV-1 DNA was obtained from brain tissue surrounding the tumor cavity in animals killed within 1 month of virus injection. However, viral DNA was barely detectable by PCR in animals surviving 3 months and none was detected in animals surviving longer than 3 months (Fig. 3). No HSV-1 DNA was detected by PCR in tissue obtained from muscle, heart, liver, or spleen.

Discussion

The localized and nonmetastatic nature of malignant primary brain tumors renders them optimal candidates for selective local therapy such as infection with modified HSV-1 or in situ transfer of a drug susceptibility gene using retroviruses. Herpes simplex virus infection has several advantages over the retrovirus strategy, including a higher rate of tumor cell transduction and absence of oncogenic potential. Both of these systems have been used in animal models with promising initial results. Experiments published to date have focused on the feasibility of these approaches and such variables as the choice of specific viral agents, dosage, titer of virus injected, and dosing schemes for the administration of ganciclovir following transduction. The only specific toxicity study thus far failed to detect activity of a reporter gene (β-galactosidase) in major body organs of mice and rats treated with retroviral vector-producer cells. Endothelial cells were occasionally transduced in brain capillaries adjacent to the injection site. No significant inflammatory activity was seen in the meninges or brain parenchyma, apart from mild edema and reactive gliosis at the actual site of injection.

In the present study, we investigated the long-term effects of intratumoral tk-negative HSV-1 injection in immunocompetent rats. As already reported, excellent survival has been achieved using this methodology. Results from the present study show that there was an acute/subacute toxicity evidenced by viral infection of ependymal cells, as well as ventricular enlargement. However, this “communicating hydrocephalus” was not accompanied by cortical atrophy or any obvious clinical effects. There was no evidence of residual HSV-1 infection found in animals killed more than 3 months posttreatment using either immunocytochemistry or PCR amplification of DNA. We also failed to detect any persisting inflammatory or immune response in the brains of these “long-term” animals. Negative results of immunocytochemical analyses for various molecular markers of the immune response in animals 12 months after viral injection indicate that there was no chronic inflammatory reaction in the brain.

The cause of ventricular enlargement is not clear. It may be related to the acute inflammatory response detected by immunocytochemical analysis using the HSV-1 antibody early after viral injection. The HSV-1 immunoreactivity was observed in ependymal and subependymal cells during the first 2 weeks following viral injection.

![Fig. 2. Results of immunocytochemical study with a polyclonal antibody for herpes simplex virus type 1 (HSV-1) demonstrating strong reactivity in the ependymal cells of animals killed within the first 2 weeks after viral injection. After longer intervals, no HSV-1 reactivity could be demonstrated.](image)

![Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) reaction products using primers specific for the herpes simplex virus (HSV)-thymidine kinase (tk) gene. Lane 1 shows PCR product from purified tk-negative HSV-1 DNA, which is 200 bp smaller than the wild-type virus. Lanes 2 through 5 show the results from brain tissue samples. Lane 2 = nontreated animals; Lanes 3, 4, and 5 = animals killed 20 days, 3 months, and 19 months after viral injection, respectively. The HSV-1 DNA was barely detectable 3 months postinjection and undetectable after that period. Results were confirmed by Southern blotting with a tk probe.](image)
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though the route of ependymal infection remains unclear, it is most likely due to migration along extracellular fluid pathways radiating from the site of inoculation. Leakage of viral particles into the subarachnoid space and, subsequently, circulated through the cerebrospinal fluid pathways is a less likely explanation. Interestingly, immunoactivity for HSV-1 was not observed in cells of the choroid plexus.

It is not surprising that immunoactivity for HSV-1 antigens was absent in brains of animals surviving for long intervals. Previous experiments have demonstrated that HSV will establish latency in the nervous system after acute infection. During latency, the virus does not express any viral proteins but only a messenger RNA, called latency-associated transcript. Although latent wild-type virus in sensory ganglia can be reactivated under certain circumstances, it is uncertain whether tk-negative HSV-1 mutants possess this ability. We failed to detect HSV-1 using either immunocytochemical analysis or PCR in experimental animals surviving more than 3 months after viral injection. In addition, it seemed that the longer the survival period, the more difficult it was to detect viral DNA by PCR. This is interesting because viral DNA is detectable long after latency, when the virus directly infects neural tissue in the brain or sensory ganglia. Because the difference between the present study and other latency studies lies in the fact that the virus was directly injected into the tumor mass, it is likely that viral particles were eliminated with tumor regression before they reached surrounding neural tissue. Although some virus may have infected surrounding neurons and established latency, the number of infected cells may be too low to detect using current methods. This hypothesis is supported by our initial studies, which demonstrated very few immunopositive neurons within immediately adjacent surrounding brain tissue.

Failure to detect viral antigens by immunocytochemical analysis and PCR confirms that the KOS-SB virus is severely defective in replication in normal rat brain tissue, although long-term observation for potential encephalitis may still be necessary in other species. Other HSV-1 viruses with tk mutations have shown evidence of potential neural toxicity in similar experiments, with the toxicity subsequently reduced by engineering multiple genomic mutations. The pathogenicity of the virus in the brain may also be restricted by factors such as the presence of microglia and other systemic immune responses to viral infection or rapid necrosis of infected tumor cells following inoculation, thereby limiting viral survival and spread.

The development of genetically engineered viruses to treat malignant brain tumors is a rational approach to therapy for these fatal lesions; it has the advantage that it may be combined with traditional treatment strategies. However, several basic and important questions must be addressed before proceeding to widespread application in clinical trials. It is uncertain how far from the site of stereotactic inoculation an HSV will travel to enter a tumor cell and whether distant tumor cells infiltrating into normal brain can be killed. The possibility of delayed neurological or systemic consequences of viral infection and spread has not yet been adequately examined. The present study addresses the questions pertaining to viral spread by examining brain and peripheral organ tissue at the molecular level, using PCR to determine the presence or absence of HSV-1 DNA. This is a far more stringent analysis than routine histological immunocytochemical studies, but given the potential applications of such a therapy, it is imperative that its safety be conclusively demonstrated at the molecular level. Potential human application of this technique will be justified only after the system is sufficiently optimized and the pathological consequences ascertained in a high-order primate model system.

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