The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors

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ABSTRACT Due to lack of effective therapy, primary brain tumors are the focus of intense investigation of novel experimental approaches that use vectors and recombinant viruses. Therapeutic approaches have been both indirect, whereby vectors are used, or direct to allow for direct cell killing by the introduced virus. Genetically engineered herpes simplex viruses are currently being evaluated as an experimental approach to eradicate malignant human gliomas. Initial studies with $\gamma_{34.5}$ mutants, R3616 (from which both copies of the $\gamma_{34.5}$ gene have been deleted) and R4009 (a construct with two stop codons inserted into the $\gamma_{34.5}$ gene), have been assessed. In a syngeneic scid mouse intracranial tumor model, recombinant herpes simplex virus can be experimentally used for the treatment of brain tumors. These viruses and additional engineered viruses were subsequently tested in human glioma cells both in vitro and in vivo. Using a xenogeneic scid mouse intracranial glioma model, R4009 therapy of established tumors significantly prolonged survival. Most importantly, long-term survival was achieved, with histologic evidence that R4009 eradicated intracranial tumors in this model. Furthermore, the opportunity to evaluate $\gamma_{34.5}$ mutants that have enhanced oncolytic activity, e.g., R8309 where the carboxyl terminus of the $\gamma_{34.5}$ gene has been replaced by the murine homologue, MyD116, are considered.

Malignant gliomas are the most common primary brain tumors of humans, accounting for 30% of all primary central nervous system (CNS) tumors in adults; they are divided into two types: (i) anaplastic astrocytoma and (ii) glioblastoma multiforme. Primary malignant brain tumors in the United States are estimated to occur at an incidence of 14.7 per 100,000 people, and 10,000–15,000 new cases are diagnosed annually (1, 2). Multimodal approaches, such as surgery, radiation, and chemotherapy, have only extended median survival rate of patients with malignant gliomas from 14 weeks to 1 year and the 5-year survival rate for glioblastoma multiforme, the most malignant of gliomas, is still 5.5% or less (3, 4). The disease is characterized by local tumor recurrence with relentless regrowth, causing neurologic dysfunction and ultimately death. Thus, treatment of malignant gliomas remains a difficult therapeutic challenge.

While substantial progress has been made in understanding the molecular biology of tumors, its translation to significantly improved clinical outcome has not occurred. Malignant gliomas are ideal candidates for molecular based therapies as: (i) metastases are rare, (ii) imaging studies allow precise monitoring of outcome, and (iii) delivery techniques allow for targeting of therapeutics. In the recent years, various investiga
gators have pursued two approaches for treatment of CNS tumors: vector therapy and direct virus therapy. The first approach uses vectors (both viral and nonviral) to insert immune-stimulating or drug susceptibility genes, including the herpes simplex virus thymidine kinase gene (HSV-tk) (5–33), into tumor cells as summarized in Table 1. These vectors generally do not have significant direct effects on the tumor cells, but act as a means for inserting a genetic message into the cell. The success of such an approach depends on at least two factors: (i) the selection of the correct gene for transfer, and (ii) the selection of the correct vector for in vivo use. Most vector therapy studies use either retroviruses or adenoviruses for gene delivery. Each of these vectors has significant theore
tical disadvantages, including lack of replication and inadequate long-term gene expression (34).

The second approach uses genetically engineered viruses to directly kill tumor cells. To date, modified herpes simplex viruses (HSV) have been the mainstay of experimental viral therapy for malignant gliomas. This latter approach uses the inherent cytopathic effects attendant with cell destruction, which results from the normal life cycle of the virus and possible additional contributions from the native host immune response to achieve tumor cell destruction. An ideal candidate for direct virus therapy should be avirulent, replication competent, and oncolytic, yet maintain susceptibility to existing antiviral therapies. The status of such approaches is summarized below.

Direct Virus Therapy

As noted, virus therapy of brain tumors has chiefly used genetically engineered HSV, summarized in Table 2; nevertheless, this discussion will focus on the use of genetically engineered $\gamma_{34.5}$ negative HSV as an experimental therapeu
tic agent for CNS tumors. HSV is a neurotropic DNA virus

Abbreviations: CNS, central nervous system; HSV, herpes simplex virus; pfu, plaque-forming unit.

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with a well-defined segmented genome. It is ubiquitous in the adult population as evidenced by the fact that 90% of people over 30 years of age have acquired antibodies to HSV, indicating prior infection. Wild-type HSV is capable of replicating in both neurons and glia, resulting in necrotizing encephalitis and widespread hemorrhagic necrosis usually localized to the inferiomedial portion of the temporal lobe. However, certain genetically engineered HSV lack neurovirulence and, thus, may be safe for direct intratumoral administration. The rationale for using modified HSV as experimental antiangioma agents has resided in the fact that these viruses retain the ability to replicate in dividing tumor cells but are avirulent in the surrounding terminally differentiated cells of the CNS (which lack enzymes required for virus replication). This selectivity of recombinant HSV can be exploited as a means to destroy tumors without injuring adjacent normal brain tissue.

Genetically engineered HSV studied for glioma therapy have included mutations in viral thymidine kinase, DNA polymerase, ribonucleotide reductase, and the \( \gamma_34.5 \) gene (13, 35–42). Optimization of the therapeutic index has been a fundamental goal of studies of these genetically engineered HSV. Tumoricidal effects in vitro and in vivo in multiple glioma models (mouse, rat, and human glioma cell lines and human glioma explants) are demonstrable. In vivo models include tumor size reduction in subrenal capsules and flank cutaneous implants, but, more importantly, increased survival and tumor cures in intracranial implant models. These effects are reproducible in vivo for both immune-deficient (scid and nude) (35, 36, 38, 39) and immune-competent models (mice and rats) (13, 40–42). However, limitations to some mutants exist, namely lack of efficacy (DNA polymerase mutants); resistance to the two predominant HSV therapeutics in clinical use, acyclovir and ganciclovir (thymidine kinase negative mutants); retained capacity to induce encephalitis (thymidine kinase and DNA polymerase mutants); lack of an acceptable animal model due to significant differences in human and mouse or rat susceptibility to genetically engineered HSV (ribonucleotide reductase mutants); and potential loss of susceptibility of tumor cells previously treated with alkylating agents (ribonucleotide reductase mutants).

**Perturbations in the Expression of the HSV \( \gamma_34.5 \) Gene: Background.** We have used molecular-based strategies for the treatment of experimental gliomas utilizing genetically engineered HSV with perturbed expression of the \( \gamma_34.5 \) gene in murine models. The goal of these studies is to combine decreased neurovirulence of genetically engineered viruses with direct virus cytotoxicity for malignant cells. The product of the \( \gamma_34.5 \) gene appears responsible for the neurovirulence of HSV and is essential for the replication of HSV in the CNS (43). Murine intracerebral inoculation of viruses deleted in \( \gamma_34.5 \) (R3616) or with stop codons in both the copies of this gene (R4009) resulted in plaque-forming unit (pfu)/Ld90 values in excess of \( 10^9 \) as compared with the wild-type gene (HSV-1) or the restored virus (HSV-1 F[R]), which produced pfu/Ld90 values <\( 10^{2.5} \). This observation prompted an extensive evaluation of the natural history of these viruses in both murine and guinea pig models, using the classical portals of entry for HSV infection of humans (44). These viruses have significantly decreased capacities to induce disease in ocular and genital models of mice and guinea pigs and also have a reduced potential to establish latency and be reactivated. In human cell lines, the resulting protein of this gene prevents a cellular stress response that ordinarily occurs with the onset of viral DNA synthesis (45). Thus, the lack of \( \gamma_34.5 \) causes premature shut off of host protein synthesis due to phosphorylation of translation initiation factor eIF-2\( \alpha \) by the activated protein kinase PKR (46).

Knowledge of the phenotypic properties of R3616 and R4009 provided a foundation for evaluating these mutants for **in vitro** and **in vivo** therapy of malignant gliomas. Initially, both the viruses were tested **in vitro** against a variety of rat, mouse, and human glioma cell lines, revealing several important findings: (i) R3616 and R4009 were incapable of replicating in rat glioma and other rat tumor cells and, thus, were not oncolytic; (ii) R3616 and R4009 were lytic in mouse glioma cells; (iii) human glioma cells were susceptible to these engineered HSV. The replication competence of R3616 and R4009 in the murine and human cells defined peak titers of \( 10^9 \) and \( 10^8 \) pfu/ml, respectively, 24 hr postinfection. These observations led to the development of both syngeneic and xenogeneic murine models for the evaluation of these viruses.

**The Syngeneic Intracranial Glioma Model.** Recognizing the work of others investigators, an intracranial syngeneic model of malignant glioma in **scid** mice was established to compare the effectiveness of experimental therapy with R3616 and R4009. The MTS39MG glioma cell line, established from a spontaneous glioma from a VM/Dk (H-2b) mouse, was used to establish intracranial tumors, as previously described (38). Briefly, graded concentrations of tumor cells were stereotactically inoculated into the right caudate nucleus and animal survival was determined. A dose-response curve of 16, 22, and 39 days median survival of mice was observed for \( 10^5 \), \( 10^4 \), and \( 10^3 \) number of tumor cells injected, respectively. This led in the selection of a standard dose of \( 5\times10^4 \) MTS39MG cells per mouse inoculum for subsequent studies because it resulted in a reproducible and useful median survival of 22 days and 100% lethality by 25 days.

The efficacy of R3616 and R4009 as therapy for gliomas was tested in three series of experiments as previously described (38). At first, tumor cells and R3616 or R4009 were admixed and stereotactically inoculated simultaneously at various concentrations in a Winn-type assay. Mice receiving \( 10^5 \) MTS39MG cells mixed with \( 2 \times 10^5 \) or \( 2 \times 10^6 \) pfu of R3616 survived for a median of 20 and 26 days, respectively (Peto-Wilcoxon analysis, \( P < 0.009 \) and \( P < 0.002 \), respectively). In parallel experiments, when \( 2.5 \times 10^5 \) or \( 2.5 \times 10^6 \) pfu of R4009 were mixed with \( 5 \times 10^5 \) MTS39MG cells and implanted intracranially, the median survival rate increased to 24 and 30 days, respectively, as compared with 22 days for the animals receiving saline (\( P < 0.001 \)).

In the second experiment, tumor cells were implanted and allowed to divide over 3 days before direct intratumoral inoculation of the genetically engineered HSV at various multiplicities of infection (virus particles per total number of tumor cells implanted). The median survival of the treated animals increased from 15 days (saline treated) to 21 days when R3616 therapy was delayed until 72 hr after implantation (\( P < 0.0067 \)). Similarly, intratumoral injection of R4009 resulted in a prolongation in median survival days from 21 days (saline-treated) to 30 days. Neither assay yielded any long-term survivors and tumor growth was the cause of death in all mice.

Finally, the increased survival of glioma-bearing animals was proven attributable to engineered HSV. Oncolytic effects of direct virus therapy were assessed by administering ganciclovir intraperitoneally daily from days −1 and 5 days relative to tumor-virus injection (day 0). Tumor-bearing animals treated with ganciclovir at 50 mg/kg intraperitoneally and R4009 survived for a similar duration as tumor-bearing animals.
receiving saline, having a median survival of 19 days. In contrast, mice that received MT539MG cells exposed to 2.5 × 10^6 pfu of R4009 virus per cell and given daily intraperitoneal injections of saline survived significantly longer with a median survival rate of 128 days.

Notably, from a safety perspective, intracranial injection of up to 2.5 × 10^6 pfu of either R3616 or R4009 into scid mice had no discernable effect over a 90-day observation period. In contrast, intracranial injection of 10^3 pfu of the wild-type HSV-1(F) resulted in 100% mortality within 7 days of inoculation with significant encephalitic necrosis of their brain tissue, strengthening the argument that the γ34.5 deletion viruses are safe for experimental therapy of CNS tumors. Attempts to explant HSV from brain tissue of all animals were uniformly unsuccessful. Furthermore, histopathology of sequential brain sections harvested at 15 and 30 days postvirus inoculation did not demonstrate encephalitis. Together these studies indicate that γ34.5 HSV can be used experimentally for the treatment of brain tumors without the requirement for alternative therapies (antiviral drugs) or the risk of encephalitis in the scid intracranial glioma model. Additionally, when compared with R3616, R4009 more efficiently destroyed tumor cells in vitro and extending survival of animals in vivo, an effect most likely due to the enhanced replication competence of R4009 (38).

The Xenogeneic Intracranial Glioma Model. R3616 and R4009 were next evaluated in human glioma cells both in vitro and in vivo. Additionally, other viruses with γ34.5 mutations tested in vitro included R939 (stop codon at the carboxyl terminus), and R908 (41-codon deletion in frame after codon 72). For each specific mutation, repaired viruses were constructed [R3616(R), R939(R), and R908(R)] to demonstrate the restoration of the wild-type phenotype, confirming that the observed biologic properties were attributable to the mutations. These experiments used two established human glioma cell lines, U251MG (from a patient with glioblastoma multiformer) and D54MG (from a patient with anaplastic astrocytoma). Normal CNS cells such as human astrocytes were also used for in vitro analyses. Replication competence of the genetically engineered viruses was established in the human cells. All the γ34.5 negative viruses replicated in both the glioma cell lines with the average yield ranging from 10^2 pfu/ml for R3616 to 10^5 pfu/ml for R4009 (24 hr postinfection). As demonstrated with replication in the murine glioma cells, the titers of the engineered HSV were less than wild-type HSV-1(F), 10^6 pfu/ml. Analyses of viral proteins by immunoblotting with an antibody to the immediate early protein ICP27 demonstrated significant amounts of virus specific proteins.

Analysis of viral DNA by in situ hybridization with a biotinylated HSV probe confirmed efficient viral DNA replication in human glioma cells when infected with the engineered HSV. These findings were comparable to that seen in glioma cells when infected with the wild-type or the restored viruses.

The direct cytolitic effect of these engineered viruses was measured quantitatively in vitro on malignant cells by the alamarBlue assay (Accumed, Westlake, OH), which assesses cell viability. As compared with the maximum cell viability (100%), reductions in alamarBlue dye conversion produced by the virus-induced cell lysis were generated as a function of the multiplicity of infection. All the human glioma cell lines tested were sensitive to the cytotoxic effects of the engineered HSV. Importantly, the γ34.5 altered viruses required >1000-fold more virus for cell killing of human astrocytes obtained from normal human cerebral cortical tissue.

A series of in vivo experiments were performed to establish the effects of these genetically engineered viruses. First, to confirm the absence of neurovirulence, all genetically engineered viruses were evaluated by intracerebral injections, resulting in LD_{50} > 10^6 pfu—data similar to earlier observations with R3616 and R4009. Second, tumor development in the xenogeneic scid mouse model was assessed histopathologically following the inoculation of 10^6 D54MG cells into the CNS. Three days later, the animals were killed and the distribution of the tumor was ascertained by histopathologic examination of fixed brain tissue. As shown in Fig. 1, a large tumor mass can be observed in the right hemisphere at the site of injection, with smaller masses in the dorsal portion of the third ventricle and in the lateral ventricle. For the in vivo therapeutic glioma model, R4009 was selected since it extended survival of animals in the syngeneic murine model longer than R3616. Both U251MG and D54MG cells were used as intracranial glioma xenograft with 10^6 glioma cells implanted as described previously. Untreated scid mice injected intracranially with U251MG cells had an average mean survival rate of 34 days, whereas mice receiving D54MG cells became moribund at a more rapid rate with an average median survival of 20 days. Tumors produced by D54MG are more aggressive than U251MG tumors, providing an opportunity to compare the effects of R4009 on slow-growing versus more rapid-growing tumors in the same host strain. Notably, both human tumors were less aggressive in vivo than the murine tumor previously studied.

Both Winn-type and delayed-treatment studies were performed. In the Winn-type assay, 1 or 10 × 10^6 pfu of R4009 were mixed with 10^6 U251MG tumor cells before injection. In these studies, the median survival time increased from 33 days.
in saline-treated control animals to 48 and 49 days, respectively. Importantly, long-term survivors (>75 days) accounted for 20 and 30% of the U251MG-bearing mice. Likewise, when the more aggressive D54MG cells were admixed with the virus and implanted intracranially, R4009 significantly prolonged survival at 0.5 × 10⁶ and 5.0 × 10⁶ pfu of virus injected. Compared with the median survival of 18 days for control (animals receiving 10⁶ D54MG glioma cells mixed with an equivalent volume of saline), all tumor-bearing animals treated with the lower pfu of R4009 survived and 40% of mice that received the higher pfu of R4009 were long-term survivors.

Since Winn-type assays are less stringent models for demonstrating antitumor effects, delayed treatment was undertaken to more closely mimic clinical therapy. Thus, when U251MG cells were implanted and inoculated with the virus 5 days later, median survival times increased to 36 and 56 days for 1 and 10 multiplicity of infection, respectively, as compared with saline-treated animals (33 days). Some of the virus-treated animals (25%) were long-term survivors, whereas all the saline-treated animals died. Since D54MG glioma cells killed mice at a much more rapid pace than U251MG cells, the inocula of R4009 administered 5 days after induction of the tumors was increased. Although these mice survived significantly longer (P < 0.015) than the mock-treated control mice, the 2-fold higher dose did not increase the number of long-term survivors (only 10% in either group with HSV-treated tumors). The survival of treated and control animals is displayed in Fig. 2.

Third, the kinetics of virus replication in the U251MG tumor were evaluated. Animals bearing U251MG gliomas were injected intracranially with 10⁶ or 10⁷ R4009 5 days after tumor induction. Brain tissue was harvested at 3, 7, and 11 days postinfection and an HSV replication assay was performed. Virus was detected 3 days postinoculation, with peak viral recovery occurring approximately around 7 days. HSV-1(F) was used as positive control but none survived beyond day 6.

Fourth, detailed examination of tissue specimens was performed to assess status of the tumor. Moribund animals (saline-treated) and long-term, tumor-bearing survivors (virus-treated) were killed, and brain tissues were fixed and subjected to routine hematoxylin/eosin staining. The saline-treated, tumor-bearing brains revealed large, space-occupying tumors, whereas survivors killed at 75–80 days had no evidence of discernible tumors. Hematoxylin/eosin-stained coronal sections of brains of treated mice with tumor resolution appeared relatively normal, as shown in Fig. 3. Surprisingly, when assayed for virus, some of the brains from long-term survivors harbored residual HSV, suggesting that replicating (gliotic) astrocytes may provide a reservoir for virus replication in an otherwise mitotically quiescent tissue. The architecture of the surrounding brain appeared normal. This observation may be unique to scid mice, which are unable to elicit normal immune response to control the viral infection. Similar studies using a γ34.5 deletion mutant derived from strain 17 demonstrated no recovery of replicating virus in immune competent mice bearing melanomas (42). However, persistent HSV infection in normal brain tissues is clearly of concern. Since the γ34.5 deletion viruses retain the HSV thymidine kinase gene, they remain susceptible to acyclovir or ganciclovir. Theoretically, antiviral treatment can commence after a significant oncolytic effect has been achieved but before toxicity to brain parenchyma occurs. (The engineered HSVs are sensitive to acyclovir with an EC₅₀ value of 0.5 μg/ml, which is consistent with known susceptibility of HSV.)

**Engineered Viruses with Enhanced Oncolytic Activity: Homologous Cellular Genes.** When cells of human origin, such as neuroblastoma, human foreskin fibroblasts (HFS), and HeLa, are infected with HSV having perturbed expression of γ34.5, host protein synthesis ceases with the onset of DNA replication. In contrast, infection of Vero cells results in sustained protein synthesis (45–48). Host protein shut off has been mapped to the carboxyl terminus of γ34.5 gene (48). A stretch
of 64 amino acids at the carboxyl terminus domain of the gene is homologous to a corresponding stretch of a murine protein, MyD116, and a hamster protein, GADD34 (48–51). MyD116 represents a subset of myeloid differentiation primary response genes (50) and GADD34 is the hamster homologue of MyD116 that is expressed during DNA damage and growth arrest (51). Viruses constructed with the substitution of the homologous region of the MyD116 at the carboxyl terminus of the γ34.5 gene are unable to cause premature shut off of total protein synthesis in infected human cells (52). This may be advantageous for tumor therapy, since R8309 allows continued protein synthesis while maintaining decreased neurovirulence.

Replication competence of R8309 was assessed in the aforementioned human glioma cell lines, U251MG and D54MG. Replication peaked at 48 hr postinfection at 4 × 10^6 pfu/ml in U251MG cells and 2 × 10^6 in D54MG cells (multiplicity of infection of 1). The alamarBlue cytoxicity assay revealed that R8309 effectively lysed tumor cells in vitro. An in vivo CNS toxicity assay performed in scid mice resulted in a pfu/LD50 ratio > 2.65 × 10^9 pfu. To assess the effectiveness of R8309 on established tumors, virus was injected in scid mice with D54MG gliomas. Two doses of R8309, 2.65 × 10^6 pfu and 2 × 10^6 pfu, were used and resulted in a median survival rate increase from 19 days in control animals to 26 days at 2 × 10^6 pfu of R8309. The higher inoculum of virus was not associated with a significant increase in survival. The Kaplan–Meier survival plots for these studies appear in Fig. 4.

Conclusion

Over the past 10 years, many investigative teams have attempted to use viruses as either indirect or direct modalities for the treatment of experimental brain tumors. Indeed, the use of retrovirus-transformed cells that contain the gene for HSV thymidine kinase followed by ganciclovir therapy is currently under investigation in human trials (5). Such studies are grounded in the recognition that ganciclovir selectively kills those cells that are capable of metabolizing this drug, namely because of expression of HSV thymidine kinase. These landmark studies provide a basis for novel approaches to the treatment of the most common and uniformly fatal tumor of the CNS. The clinical effectiveness of this approach remains to be established. Even if efficacious, alternative approaches to the management of brain tumors will be necessary.

The utilization of engineered HSV viruses provide an alternative approach to brain tumor therapy. The seminal recognition of the role of the γ34.5 gene in neurovirulence is essential to the development of safe viruses for intracerebral administration (43). Furthermore, the data summarized indicate for the first time to our knowledge, that engineered HSV viruses can confer long-term survival with uniform tumor reduction after the establishment of malignant human gliomas in the CNS of scid mice. These survivors have no evidence of residual tumor, although a small percentage have persistent virus, a concern that will require further investigation. Regardless, the observation of long-term survival with tumor eradication is a landmark in experimental CNS antitumor therapy.

In these studies, an enhanced survival effect was best illustrated by a virus containing stop codons in the γ34.5 gene. The use of such a virus in human studies must be carefully considered as revertants are theoretically likely to occur, resulting in a virulent virus with the potential for causing encephalitis. Thus, our future efforts will explore genetically engineered viruses that represent specific gene deletions or foreign gene inserts to avoid the potential for such reversion. One such example is the generation of a virus that contains the MyD116 sequence. This virus has enhanced in vivo antitumor activity that is currently explored to optimize its activity. A few comments are in order about requirements for the evaluation of experimental viral therapeutics. Studies such as the ones described above require further validation before progressing to human therapies. First, extensive histopathological and immunohistochemical evaluations of treated CNS tissues are necessary to confirm absence of tumor and residual HSV. Second, the selection of the proper animal models and associated tumor cells are of paramount importance. Clearly, selection of an animal system that is not permissive for HSV replication (i.e., the rat) can lead to erroneous conclusions. Similarly, the utilization of cells that do not parallel human tumors can similarly lead to irrelevant conclusions. Third, the development of models that most closely mimic human disease is most desirable. Winn-type assays provide excellent screening methods but do not provide conditions more analogous to human tumors. Finally, before evaluating any HSV construct in humans, detailed assessment of virulence in the Aotus trivirgatus will be mandatory. Aotus is exquisitely sensitive to HSV (53) and provide a highly stringent model to assess neurovirulence of candidate therapeutic HSV for malignant brain tumors.

Perturbations in the expression of the γ34.5 gene provide a foundation for future experimental viral therapy of malignant gliomas. Fundamental to the development of human therapeutics is the requirement for enhanced knowledge of the behavior of these viruses in biologic systems. Application of this knowledge to devising successful therapeutic for the treatment of brain tumors may provide an opportunity to extend substantially lifespan of patients with malignant gliomas.

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