An Oncolytic HSV-1 Mutant Expressing ICP34.5 under Control of a Nestin Promoter Increases Survival of Animals even when Symptomatic from a Brain Tumor

Hirokazu Kambara,[1] Hideyuki Okano,[2,3] E. Antonio Chiocca,[1] and Yoshinaga Saeki[1]

[1]Dardinger Center for Neuro-oncology, Department of Neurological Surgery, The Ohio State University Comprehensive Cancer Center, Columbus, Ohio; [2]Department of Physiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan; and [3]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Abstract

Oncolytic herpes simplex virus-1 (HSV-1) mutants possessing mutations in the *ICP34.5* and *ICP6* genes have proven safe through clinical trials. However, *ICP34.5*-null viruses may grow poorly in cells due to their inability to prevent host-cell shut-off of protein synthesis caused by hyperphosphorylation of eukaryotic initiation factor 2α. To increase tumor selectivity, glioma-selective expression of ICP34.5 in the context of oncolysis may be useful. Malignant gliomas remain an incurable disease. One molecular marker of malignant gliomas is expression of the intermediate filament nestin. Expression of nestin mRNA was confirmed in 6 of 6 human glioma lines and in 3 of 4 primary glioma cells. Normal human astrocytes were negative. A novel glioma-selective HSV-1 mutant (rQNestin34.5) was thus engineered by expressing ICP34.5 under control of a synthetic nestin promoter. Replication, cellular propagation, and cytotoxicity of rQNestin34.5 were significantly enhanced in cultured and primary human glioma cell lines compared with control virus. However, replication, cellular propagation, and cytotoxicity of rQNestin34.5 in normal human astrocytes remained quantitatively similar to that of control virus. In glioma cell lines infected with rQNestin34.5, the level of phospho-eukaryotic initiation factor 2α was lower than that of cells infected by control rHsvQ1, confirming selective ICP34.5 expression in glioma cells. *In vivo*, rQNestin34.5 showed significantly more potent inhibition of tumor growth compared with control virus. Treatment in the brain tumor model was instituted on animal's display of neurologic symptoms, which usually led to rapid demise. rQNestin34.5 treatment doubled the life span of these animals. These results show that rQNestin34.5 could be a potent agent for the treatment of malignant glioma. (Cancer Res 2005; 65(7): 2832-9)

Introduction

Oncolytic viruses are being tested in human clinical trials for a variety of cancers (1–8). Initial data related to their safety has been encouraging in that it is evident that viral doses exist which are not toxic in humans either by systemic administration or by direct intratumoral injection in a variety of tumors. However, evidence for clinical efficacy remains elusive.

Mutants based on herpes simplex virus-1 (HSV-1) have been among the most widely studied oncolytic viruses. Two basic types of mutations have been placed in the viral genome to render it gtumor-selective.h One has entailed deletion of the viral *UL39* locus, encoding the viral large subunit of ribonucleotide reductase (9–11). The second has entailed deletion of both copies of the viral γ_1 *34.5* gene. Whereas these deletions render the mutant HSV-1 more tumor-selective, they can also attenuate the titer of progeny virions (particularly the γ_1 *34.5* deletion) in infected cells, thus possibly limiting overall efficacy (12).

To overcome this possible limitation, we have generated another type of general mutant in which one copy of the γ_1 *34.5* gene is reinserted into the *UL39*-deleted, γ_1 *34.5*-deleted viral genome under control of a tumor-specific promoter (12). We have been seeking tumor tissue-specific promoters for use against human malignant gliomas. One gene that is up-regulated in malignant glioma is the one encoding for the intermediate filament, nestin (13). This protein is expressed during neuronal embryogenesis but its expression is "shut-off" in the adult brain. We have thus hypothesized that a mutant HSV-1 in which a nestin promoter element drives expression of γ_1 *34.5* might provide increased tumor specificity by replicating only in glioma cells and not in astrocytes even if they are proliferating. Herein, we show that this novel mutant (rQNestin34.5) does possess favorable replicative and oncolytic characteristics in glioma cells versus astrocytes.

Materials and Methods

Engineering of the rQNestin34.5 oncolytic virus. We employed the HSVQuick method to engineer rQNestin34.5. Portions of this methodology were previously published (14, 15). First, a transcriptional cassette consisting of the nestin enhancer element, contained in the 3′-terminal 714 nucleotides of the second intron in the nestin gene linked to the minimal promoter of the heat shock protein 68 (hsp68), was generated as described in ref. 16. This cassette was then subcloned into a shuttle vector in which the nestin-hsp78 promoter/enhancer element drives expression of the HSV-1 γ_1 *34.5* gene. This shuttle vector was used to transform *E. coli* carrying the bacterial artificial chromosome called fHsvQuik1, which has two *flp* recombination sites within this *UL39* locus. FLP-mediated, site-specific recombination between the shuttle vector and fHsvQuik-1 results in formation of a bacterial artificial chromosome containing the nestin-hsp68 promoter-enhancer element driving expression of the γ_1 *34.5* gene within the deleted *UL39* locus. Vero cells are transfected with this bacterial artificial chromosome and a Cre recombinase-expression vector. The prokaryotic vector sequences from the shuttle vector possess flanking *loxP* sites which are removed by Cre recombinase. The recombinant rQNestin34.5 virus is thus generated and packaged in these cells and harvested as transfected Vero cells are lysed per routine.

Note: Portions of this work were done when authors were at the Molecular Neuro-oncology Laboratories, Neurosurgery Service, Massachusetts General Hospital, Harvard Medical School (Boston, MA).

Requests for reprints: E. Antonio Chiocca or Yoshinaga Saeki, Dardinger Center for Neuro-oncology, Department of Neurological Surgery, The Ohio State University Medical Center Comprehensive Cancer Center, James Cancer Hospital and Solove Research Institute, N-1017 Doan Hall, 410 West 10th Avenue, Columbus, OH 43210. Phone: 614-293-9312; Fax: 614-293-4281; E-mail: Chiocca-1@medctr.osu.edu or saeki-1@medctr.osu.edu.

Southern blot analysis. Viral DNA was isolated after lysis of infected Vero cells with SDS-proteinase K, repeated phenol-chloroform extraction, and ethanol precipitation. DNA was digested with *Hin*dIII (New England Biolabs, Beverly, MA), separated by agarose gel electrophoresis, and transferred to a nylon membrane (Amersham Corp., Arlington Heights, IL). Probes included the *Xho*I-*Nco*I nestin-hsp68 hybrid promoter fragment from the shuttle vector, the *Nde*I-*Xho*I hsp68 promoter fragment from the shuttle vector, and the *Hin*dIII-*Bam*HI γ34.5 fragment from pBSKγ34.5. Probe labeling and hybridization were done with the enhanced chemiluminescence (ECL) system (Amersham) according to the protocol of the manufacturer.

Cell culture. Human glioma cell lines (U251, U87dEGFR, T98G, Gli36d5, U138, and MGH238), Vero cells, and primary gliomas cells were cultured with DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin/mL, and 10 mg of streptomycin/mL. Human astrocytes were purchased from ScienCell Research Laboratories (San Diego, CA) and cultured with astrocyte medium supplemented with 2% fetal bovine serum, 100 units of penicillin/mL, and 10 mg of streptomycin/mL. All cells were cultured at 37°C in an atmosphere containing 5% carbon dioxide.

Reverse transcription-PCR. Total mRNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and first-strand cDNA was synthesized from mRNA fraction using SuperScript First-Strand cDNA Synthesis System (Invitrogen). PCR amplification was done for human nestin using the primers 5′-ggcagcgttggaacagaggttgga-3′ and 5′-ctctaaactggagtggtcagggct-3′ and for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primers 5′-ggtcggagtcaacggatttggtcg-3′ and 5′-cctccgacgcctgcttcaccac-3′ as internal control. The cDNA products of the reverse transcription reaction were denatured at 95°C for 15 minutes followed by a 40-cycle PCR reaction (94°C for 60 seconds, 60°C for 60 seconds, and 72°C for 60 seconds) for nestin and by a 25-cycle PCR reaction for GAPDH. PCR products were separated by agarose gel electrophoresis.

Immunoblot assays for eukaryotic initiation factor 2α phosphorylation. U251, U87dEGFR, or astrocytes were seeded at 2.5×10^5 cells/well in six-well plates. Cells were infected with rHsvQ1 or rQNestin34.5 at various multiplicities of infection (MOI, 0.004-0.4) or mock infected. To harvest protein, medium was aspirated and 200 μL of protein loading dye [50 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 100 mmol/L DTT, 0.1% bromophenol blue] were added. Cells were scraped and collected into sterile 1.5-mL tube. Lysates were then boiled for 5 minutes to denature the solubilized proteins. Proteins were electrophoretically separated by SDS-PAGE, transferred electrically to a nitrocellulose membrane, and reacted with either a monoclonal anti–eukaryotic initiation factor 2α (eIF-2α) antibody or a peptide antibody specific for the Ser51-phosphorylated form of eIF-2α (Cell Signaling Technology, Beverly, MA). Proteins were detected using an ECL-Plus kit (Amersham Pharmacia Biotech, Piscataway, NJ) as recommended by the manufacturer.

***In vitro* virus replication assay.** One day before infection, cells were seeded on six-well plates to a density that allowed them to become subconfluent by the day of infection. Viruses, 1×10^4 or 1×10^3, were used to infect cells at the day-0 time point. Three hours after infection, cells were washed with glycine saline solution (pH 3) for removal of unattached viruses. Cells and supernatant were then harvested on day 3, at which time titration was done. Viral spread was measured by fluorescent microscopy at the day-3 time point.

***In vitro* virus cytotoxicity assay.** The day before infection, 1.0×10^5 cells were seeded on 12-well plates. At the day-0 time point, 1×10^4 plaque-forming units (pfu) of viruses were added. Surviving cells were counted at the day-4 time point by enumeration through a Coulter counter.

Animal studies. Nude (*nu/nu*) mice were obtained from the Cox 7 breeding facility, Massachusetts General Hospital. BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). S.c. tumors were obtained by injection of 5×10^5 cells into the flanks of athymic mice (six animals per group for human U87dEGFR glioma cells). Seven days after tumor implantation, animals with similar tumor volumes were randomly divided and viruses were injected intratumorally at 2×10^5 pfu/dose in 10-μL volumes on days 1, 3, 5, and 7. Animals were euthanized at the day-28 time point. Tumor volumes were measured with external calipers as previously described (12). Brain tumors were obtained by stereotactic injection of 2×10^5 cells into the right frontal lobe (2 mm lateral and 1 mm anterior to the bregma at a depth of 3 mm). Seven or fourteen days after tumor implantation, animals were randomly divided and viruses were injected intratumorally at a dose of 3×10^5 pfu in 5-μL volumes (10 animals per group for the 7-day virus injection; 6 animals per group for the 14-day virus injection). The survival time for each group was monitored. The time course of neurologic symptom progression is further detailed in Results. For neurotoxicity experiments, BALB/c mice were stereotactically injected in the right frontal lobe (depth of 3 mm) with 5-μL volumes of virus at different dilutions up to the highest titers obtainable. Animals were checked daily for 30 days. All animal studies were done in accordance with guidelines issued by Massachusetts General Hospital Subcommittee on Animal Care. Viral inoculation and care of animals harboring viruses were done in approved BL2 viral vector rooms.

Results

Nestin expression in glioma cells. The intermediate filament nestin is expressed in neural stem cell (17) and malignant glioma cells (13). In fact, nestin mRNA was relatively abundant in human

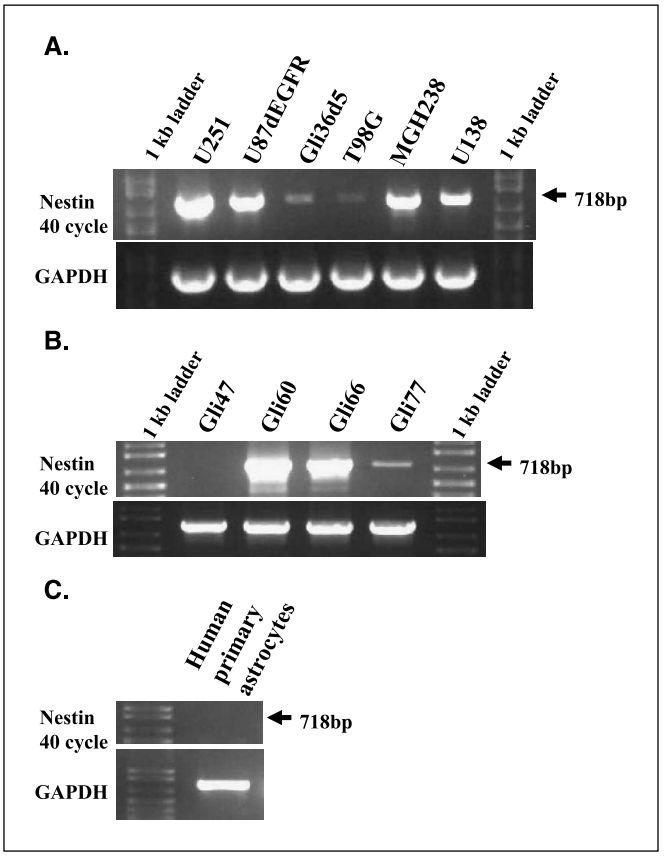

Figure 1. Nestin reverse transcription-PCR (RT-PCR) in gliomas. *A,* RT-PCR analysis of glioma cell lines. The upper and lower primers were designed to hybridize to exon 1 and exon 4 of the human nestin gene. Total mRNA was isolated from cells using TRIzol reagent and reverse transcribed with SuperScript First-Strand cDNA Synthesis System (Invitrogen). PCR was done using the cDNA as templates. The amplified product corresponding to nestin measured 718 bp and was readily detected as a band in U251, U87dEGFR, MGH238, and U138, whereas faint bands were detected with Gli36d5 and T98G. GAPDH was used as internal control and its amplification was done for 25 cycles. *B,* RT-PCR analysis of primary glioma cells. The 718-bp nestin-specific band was readily detected in Gli60 and Gli66, whereas only a faint band was detected with Gli77 and no nestin product amplified in Gli47 cells. *C,* RT-PCR analysis of human primary astrocytes. No nestin product was amplified, even after 40 cycles, with RNA isolated from human astrocytes. The intensity of the GAPDH amplification reaction is very similar in *A*, *B*, and *C*, thus providing an internal control for exposure and loading.

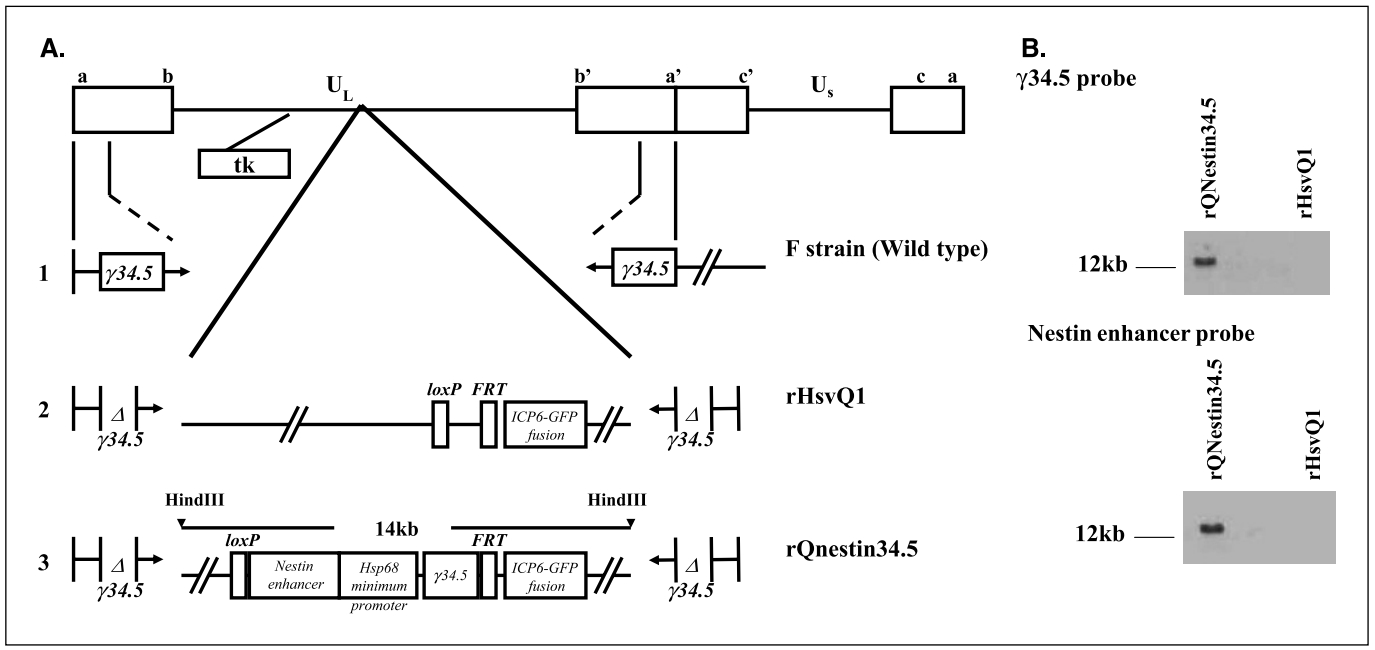

Figure 2. Construction of oncolytic HSVs and Southern blotting analysis. *A,* schematic maps of HSV strain F (wild-type), rHsvQ1 (double *UL39*-γ_1 *34.5* mutant), and rQNestin34.5. All strains contain the typical HSV-1 genome with its two unique segments (U_L and U_S, respectively), each flanked by inverted repeat elements (*ab* and *ca*, respectively). The locations of diploid γ_1 *34.5* genes and of the thymidine kinase gene (*tk*) are shown in the top construct, representing wild-type F strain HSV. In the middle construct, the insertion of a green fluorescent protein (*GFP*) cDNA into *UL39* and the deletions within γ_1 *34.5* are shown for rHsvQ1. These consist of a deletion of about 1,000 bp in the coding region. The bottom construct shows the site of recombination of the hybrid promoter (nestin enhancer and hsp68 minimum promoter)-γ_1 *34.5* expression cassette into *ICP6*, giving rise to the novel mutant oncolytic virus rQNestin34.5. The approximate sizes of the *Hin*dIII fragment from rQNestin34.5 are provided. *B,* hybridization of *Hin*dIII-digested viral DNA to a γ_1 *34.5* probe and to a hybrid nestin promoter probe. After digestion of viral DNA with *Hin*dIII and separation of fragments by electrophoresis, a γ_1 *34.5* probe was employed to detect the expected 14-kb *Hin*dIII fragment in rQNestin34.5. A hybrid hsp68-nestin promoter probe was used to detect hybridization to the expected 14-kb fragment of rQNestin34.5. *Hin*dIII-digested rHsvQ1 DNA did not hybridize to either probe, as expected.

U251, U87dEGFR, MGH238, and U138 glioma cell lines and less so in human Gli36d5 and T98G glioma cell lines (Fig. 1*A*). In primary gliomas, relatively strong expression was detected in human Gli60 and Gli66 cells, with human Gli77 cells exhibiting relatively weak and human Gli47 cells showing no expression (Fig. 1*B*). Nestin mRNA was not detected in normal human astrocytes (Fig. 1*C*). In summary, 6 of 6 human glioma cell lines and 3 of 4 primary gliomas expressed nestin, whereas human astrocytes did not.

Engineering of a nestin-driven oncolytic herpes simplex virus 1. The above studies suggested that a nestin promoter-driven oncolytic virus might provide a logical construct to

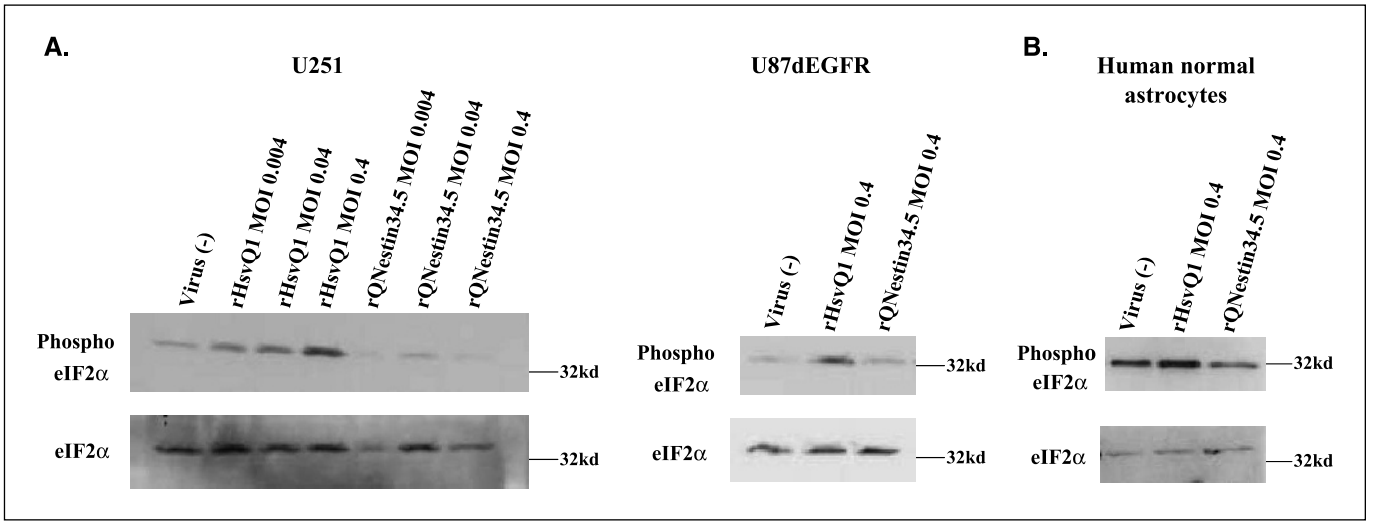

Figure 3. Western blot analysis of eIF-2a and phospho-eIF-2a in virally infected cells. *A,* rHsvQ1 infection of U251 cells increases phosphorylation of eIF-2α with increased MOI of virus 24 hours later. rQNestin34.5 infection, however, did not increase the level of phospho-eIF-2a in either U251 or U87dEGFR glioma cells. *B,* infection of astrocytes with either rHsvQ1 or rQNestin34.5 did not lead to gross alterations in the phosphorylation state of eIF-2α. The levels of eIF-2α (*bottom lanes*) are approximately equal in all lanes (except for the U251, rQNestin34.5 MOI of 0.004 lane), providing a control for protein transfer and loading.

achieve antiglioma effects. We thus engineered a new mutant HSV-1 in which an artificial nestin promoter linked to the minimal promoter from hsp68 (16) was placed upstream of the HSV-1 *ICP34.5* gene in the context of a viral mutant with deletions in both the endogenous *ICP34.5* gene and the *ICP6* gene (Fig. 2*A*). The gross genetic structure of the new mutant virus was confirmed by Southern blot analysis (Fig. 2*B*).

Immunoblotting analyses for assay of eukaryotic initiation factor 2α phosphorylation. In virally infected cells, *ICP34.5* gene expression leads to dephosphorylation of the translation initiation factor, eIF-2α. Without ICP34.5, this factor is hyperphosphorylated from activity of the double-stranded RNA-dependent protein kinase (PKR; ref. 18). To confirm that rQNestin34.5 infection of glioma cells leads to eIF-2α dephosphorylation, human U251 glioma cells were infected with parental virus, rHsvQ1, or rQNestin34.5 at different MOI. There was an MOI-dependent increase in the phosphorylation state of eIF-2α in rHsvQ1-infected cells, whereas there was an MOI-dependent decrease in the phosphorylation state of eIF-2α in rQNestin34.5-infected cells (Fig. 3*A*). Similar results were observed with human U87dEGFR glioma cells. In normal human astrocytes, the phosphorylation state of eIF-2α was relatively elevated even with vehicle. Infection with either rHsvQ1 or rQNestin34.5 did not seem to alter these levels significantly (Fig. 3*B*). These results indicated that rHsvQ1 induced phosphorylation of eIF-2α in an MOI-dependent fashion in glioma cells. Conversely, rQNestin34.5 infection resulted in a decrease in the level of phosphorylated eIF-2α in glioma cells. In astrocytes, viral infection did not seem to significantly affect the baseline elevated levels of phosphorylated eIF-2α.

***In vitro* assays of rQNestin34.5 selectivity.** We next sought to determine if rQNestin34.5 could replicate and kill glioma cells in a selective fashion. We employed three assays: (1) a virus yield assay to measure number of progeny virions generated from cell infection, (2) a plaque-size assay to measure the ability of the virus to propagate in infected cultures, and (3) a dose-response assay to measure cytotoxicity. In the first assay, established human glioma cells and primary human glioma cells were infected with rQNestin34.5 or control rHsvQ1. Yields of rQNestin34.5 were always higher than those of rHsvQ1 in all tested cells, with a range from 3 orders to 1 order of magnitude (Fig. 4*A*). Conversely, there was no significant difference in viral yields between rHsvQ1- and rQNestin34.5-infected astrocytes (Fig. 4*B*).

For the second assay, human glioma cells were infected with each virus and then their propagation was measured by changes in plaque size. In both cell lines, the size of plaques and overall propagation was significantly elevated after rQNestin34.5 infection when compared with rHsvQ1 infection (Fig. 4*C* and *D*).

For the third assay, six established glioma cell lines and two primary glioma cells were infected with rQNestin34.5 or control rHsvQ1. There was significantly increased cytotoxicity in all glioma cells infected with rQNestin34.5 (Fig. 5*A*). Conversely, more than 90% of human astrocytes were alive 4 days after infection with either virus (Fig. 5*B*).

These experiments revealed that rQNestin34.5 replicated to higher levels, propagated more efficiently, and killed glioma cells to a higher degree than parental rHsvQ1. However, in normal astrocytes, there were no significant differences detected between these two viruses.

***In vivo* assays of efficacy.** We then sought to determine the *in vivo* antitumor effects of rQNestin34.5. After establishing human U87dEGFR glioma tumors in the flanks of athymic mice, intratumoral inoculation with each virus was done. rQNestin34.5 completely inhibited tumor growth and volumetric measurements revealed complete absence of tumor 21 days after injection, whereas rHsvQ1 was not as effective in inhibiting tumor growth (data not shown). An intracerebral tumor model was then used. After establishing human U87dEGFR glioma tumors in the right frontal lobes of athymic mice, intratumoral inoculation with each virus was done. We initially tested injections of virus 7 days after tumor cell implantation: 7 of 9 mice injected with rQNestin34.5 survived for more than 90 days, at which time they were euthanized for histologic analyses, whereas only 2 of 10 mice injected with rHsvQ1 survived for more than 90 days (Fig. 6*A*). Animals injected with vehicle were all dead by day 21 after tumor cell implantation. We next tested injections of virus 14 days after tumor cell implantation: 4 of 8 mice injected with rQNestin34.5 survived for more than 90 days, at which time they were euthanized. Conversely, 0 of 10 mice injected with rHsvQ1 survived for more than 35 days (Fig. 6*B*). These experiments indicated that rQNestin34.5 possessed potent anti-glioma effects when compared with rHsvQ1.

rQNestin34.5 doubles the survival of animals once they manifest neurologic symptoms from their brain tumors. The experimental treatment of animals with brain tumors is always idealized. Treatments are always instituted at a determined time point after brain tumor implantation before neurologic symptoms can be observed. When such symptoms occur, demise is relatively rapid usually within 24 to 48 hours. For the human U87dEGFR model in athymic mice, such symptoms usually occur at days 17 to 19 after tumor implantation with animal demise by day 21. In humans afflicted with malignant glioma, treatments are always instituted after the onset of symptoms (seizure, apathy, speech anomaly, and hemiparesis). We thus sought to determine if rQNestin34.5 possessed a significant anticancer effect in mice even after they displayed signs of neurologic impairments. Nineteen days after tumor implantation, all animals displayed such symptoms (mild hemiparesis, lack of grooming, or lethargy). Animals were thus treated with vehicle, rHsvQ1, or rQNestin34.5 injections. Figure 6*C* shows that survival of animals in the latter group was significantly increased compared with the former. These results indicated that rQNestin34.5 could prolong the life of animals that displayed neurologic symptoms from their tumors.

Histopathologic analyses of brains from long-term surviving animals. Pathologically, the brains of animals with brain tumors, surviving more than 90 days after treatment, showed that the tumor-containing hemisphere was now devoid of growing tumor, although there was evidence for gliosis (data not shown). At lower magnification, the ipsilateral ventricle seemed enlarged likely due to an *ex vacuo* phenomenon rather than true hydrocephalus because the size of the contralateral ventricle was normal (data not shown).

***In vivo* toxicity.** To show that rQNestin34.5 was not more clinically toxic than control rHsvQ1, immunocompetent BALB/c mice were injected intracerebrally with each virus in a dose-escalating fashion. Wild-type HSV-1 (F strain) was employed as a positive control. Injected animals were observed for 30 days. All mice injected with 10^7 pfu (the maximum dose tested) of rHsvQ1 and rQNestin34.5 survived for 30 days, whereas the LD_{50}

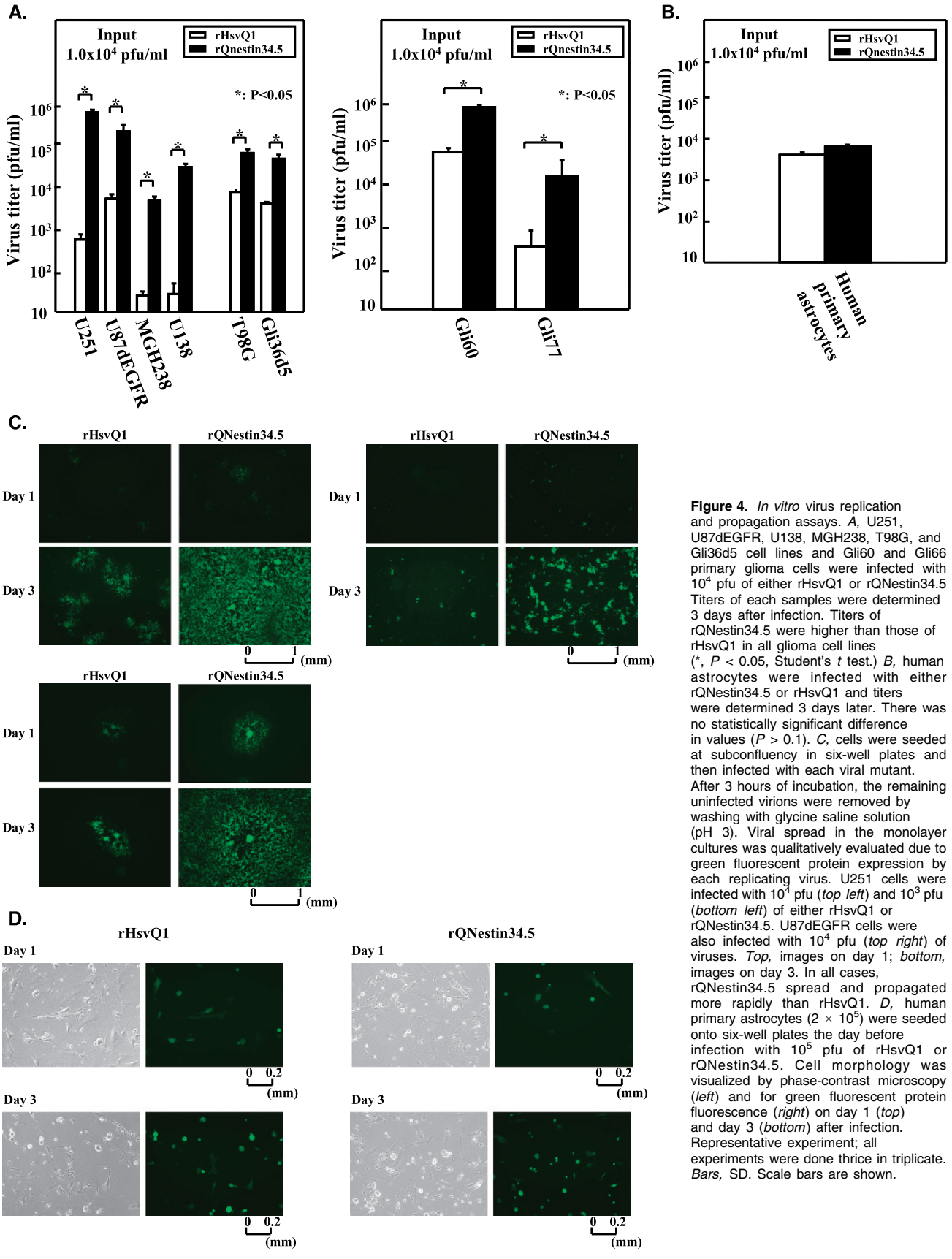

Figure 4. *In vitro* virus replication and propagation assays. *A,* U251, U87dEGFR, U138, MGH238, T98G, and Gli36d5 cell lines and Gli60 and Gli66 primary glioma cells were infected with 10^4 pfu of either rHsvQ1 or rQNestin34.5 Titers of each samples were determined 3 days after infection. Titers of rQNestin34.5 were higher than those of rHsvQ1 in all glioma cell lines (*, $P < 0.05$, Student's t test.) *B,* human astrocytes were infected with either rQNestin34.5 or rHsvQ1 and titers were determined 3 days later. There was no statistically significant difference in values ($P > 0.1$). *C,* cells were seeded at subconfluency in six-well plates and then infected with each viral mutant. After 3 hours of incubation, the remaining uninfected virions were removed by washing with glycine saline solution (pH 3). Viral spread in the monolayer cultures was qualitatively evaluated due to green fluorescent protein expression by each replicating virus. U251 cells were infected with 10^4 pfu (*top left*) and 10^3 pfu (*bottom left*) of either rHsvQ1 or rQNestin34.5. U87dEGFR cells were also infected with 10^4 pfu (*top right*) of viruses. *Top,* images on day 1; *bottom,* images on day 3. In all cases, rQNestin34.5 spread and propagated more rapidly than rHsvQ1. *D,* human primary astrocytes (2×10^5) were seeded onto six-well plates the day before infection with 10^5 pfu of rHsvQ1 or rQNestin34.5. Cell morphology was visualized by phase-contrast microscopy (*left*) and for green fluorescent protein fluorescence (*right*) on day 1 (*top*) and day 3 (*bottom*) after infection. Representative experiment; all experiments were done thrice in triplicate. *Bars,* SD. Scale bars are shown.

for HSV-1-inoculated animals was $\sim 10^4$ pfu, as previously reported. These findings confirmed that reintroduction of ICP34.5 under control of the nestin promoter was clinically tolerated by animals.

Discussion

A variety of mutant (19, 20) and engineered (21, 22) strains of viruses are being tested in glioma therapies. Effectiveness of the therapy will be improved by increasing viral replication in glioma cells and by restricting viral replication in astrocytes and other brain cells. To accomplish this goal, we have pursued reengineering HSV-1 so that genes needed for efficient replication are under control of glioma-specific promoters. In this report, we were interested in determining if the promoter elements for nestin could be adapted to engineer an HSV-1 mutant with improved selectivity for glioma versus astrocytic cells. We showed the following: (1) nestin expression is up-regulated in glioma cell lines and primary glioma samples; (2) a new oncolytic virus (rQNestin34.5) replicates to higher levels in glioma cells, is more cytotoxic to glioma cells, and spreads more in monolayer cultures than a parental construct (3); (3) the replication and cytotoxicity of this new oncolytic virus against human astrocytes are similar to that of the parental construct of which safety in the clinical setting has been confirmed in phase I clinical trials (3); (4) this new oncolytic virus leads to greater than 50% long-term survival of animals with established gliomas when therapy is instituted at early time points of disease; and (5) even when animals become symptomatic of their brain tumor, treatment with this oncolytic virus leads to a significant increase in survival.

Routinely, models of experimental gliomas have been idealized in terms of instituting experimental treatments at a predetermined time point after tumor cell inoculation, usually when tumors are small and animals appear normal in behavior and asymptomatic. The appearance of symptoms in animals with brain tumors produces severe neurologic morbidity and need for euthanasia (or death) in a relatively rapid time frame. These symptoms include lack of grooming, weight loss, and inertia. Usually within 24 to 48 hours more severe symptoms such as hemiparesis appear, which necessitate euthanasia or lead to death within 24 to 48 hours. With the U87dEGFR glioma model, lack of grooming, weight loss, and inertia begin on days 17 to 18 after tumor cell implantation. By day 20, hemiparesis is evident, with animal demise occurring by days 21 to 22. Experiments in the past with oncolytic viruses, as well as with other agents, usually institute treatment by days 3 to 10 well in advance of any detectable symptoms. With rQNestin34.5 we were able to show that even when treatment was instituted on day 19 (i.e., when animals were obviously symptomatic) we could double animal life span from diagnosis. We argue that this represents a significant advance. In humans afflicted with malignant glioma, the average time from diagnosis to death is ~ 1 year (23). Any treatment that doubles this would be considered significant.

The intermediate filament, nestin, is expressed in normal brain development and then becomes down-regulated and expression is not observed in normal brain. Nestin becomes reexpressed in glial malignancies (13) and we were able to confirm this finding in glioma cell lines as well as in 3 of 4 primary human glioma cells. This confirms that nestin may be a marker for glial malignancy.

The genetic structure of the *UL39-γ_1 34.5* parental mutant (rHsvQ1) should be very similar to that of MGH1 (24) and of G207 (25). G207 was an HSV-1 mutant tested in clinical trials for humans with malignant glioma and shown to be safe up to the highest dose that could be tested (3). G207 and other mutants in the *γ_1 34.5* gene are restricted in their replicative ability because PKR activity is stimulated by infection, leading to phosphorylation of the translation factor, eIF-2α (18). This in turn shuts off translation and efficient viral replication. The wild-type HSV-1 *γ_1 34.5* gene product activates a cellular phosphatase that dephosphorylates eIF-2α, restoring translation and leading to increased

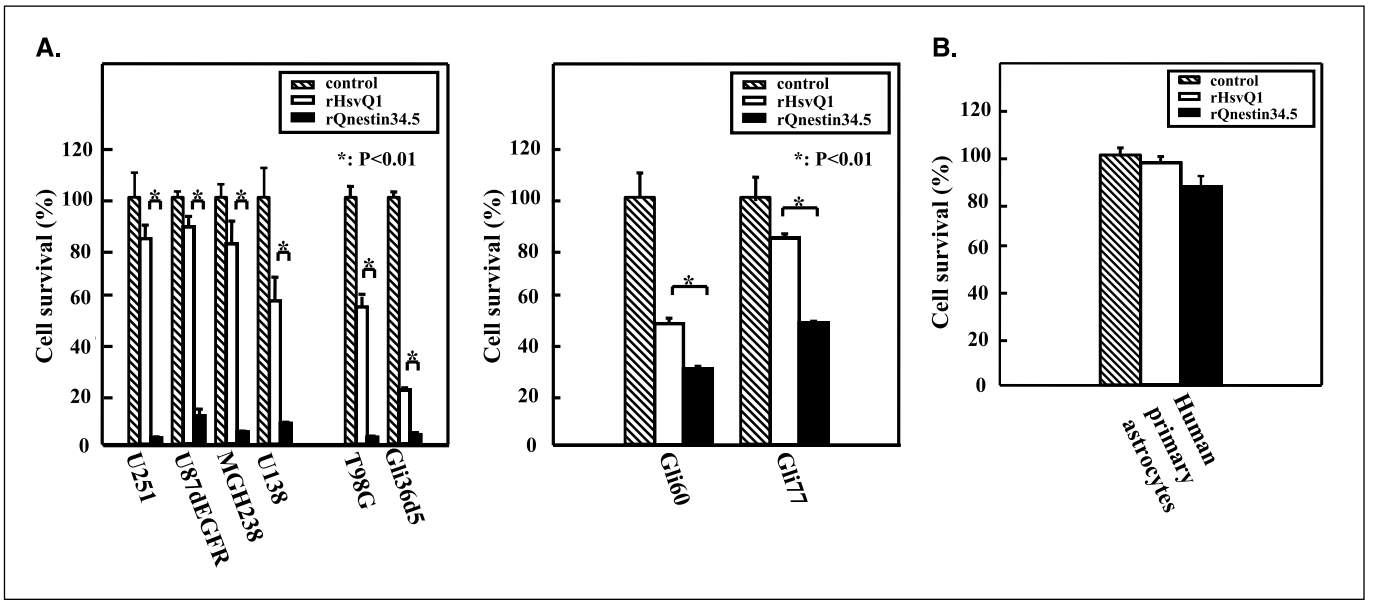

Figure 5. Cytotoxicity assays. *A,* cells (10^5) were seeded into 12-well plates. The following day, cells were infected with 10^4 pfu of either rHsvQ1 or rQNestin34.5 and incubated further for 4 days. The number of surviving cells was then assayed using a Coulter counter. rQNestin34.5-infected cells survived less than rHsvQ1-infected cells (*, $P < 0.01$, Student's *t* test). *B,* no significant difference in number of surviving astrocytes was detected after infection with either virus. Representative experiment; all experiments were done thrice in triplicate. *Bars,* SD.

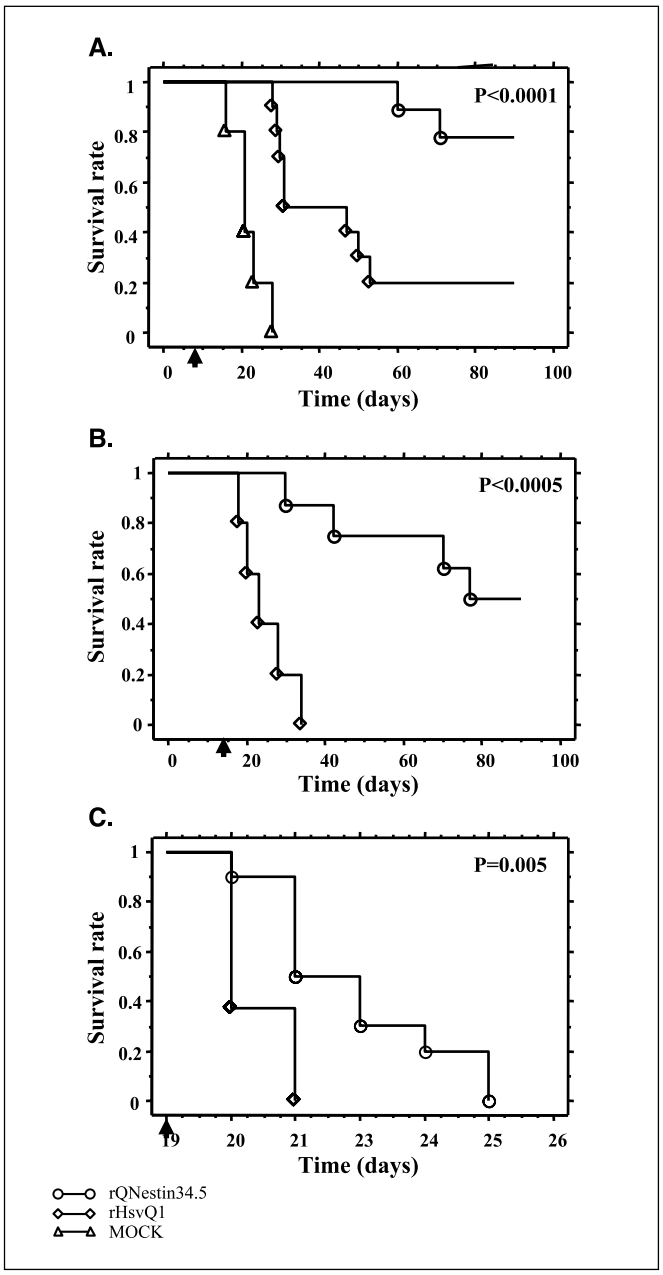

Figure 6. *In vivo* assays of efficacy. *A,* Kaplan-Meier survival curves are shown for animals treated when intracerebral tumors are 7 days old. Human U87dEGFR glioma cells (2×10^5 cells) were inoculated into the brains of athymic mice on day 0. Viruses (3×10^5 pfu) were injected in tumors on day 7 (*arrow*). There was a statistically significant difference in survival among the groups ($P < 0.0001$, Wilcoxon log-rank). *B,* Kaplan-Meier survival curves are shown for animals treated when intracerebral tumors are 14 days old. Human U87dEGFR glioma cells (2×10^5 cells) were inoculated into the brains of athymic mice on day 0. Viruses (3×10^5 pfu) were injected in tumors on day 14 (*arrow*). There was a statistically significant difference in survival among the groups ($P < 0.0005$, Wilcoxon log-rank). *C,* clinical symptoms of mild hemiparesis, lethargy, and absence of grooming were clinically detected on day 19 (*arrow*) at which time 2.5×10^6 pfu viruses were stereotactically injected into tumors. There was a statistically significant difference in survival among the groups ($P = 0.005$, Wilcoxon log-rank).

viral progeny production (18). Our results clearly show that the levels of phospho-eIF-2α in glioma cells infected with rQNestin34.5 are decreased compared with the levels in glioma cells infected with rHsvQ1. Conversely, in human astrocytes, the levels of phosphorylated eIF-2α are relatively similar in both rQNestin34.5- and rHsvQ1-infected cells. These results strongly argue that rQNestin34.5 oncolytic selectivity results from increased viral progeny production in glioma cells due to the ability of rQNestin34.5 to prevent the shut-off of protein synthesis mediated by PKR antiviral defense mechanism.

The *in vitro* and the *in vivo* results further show that the ability of rQNestin34.5 to replicate to higher levels in glioma cells translates to an increased therapeutic effect when compared with the parental rHsvQ1 virus. As discussed before, the finding that such a therapeutic effect can be translated even at very late time points in the course of the disease in animals argues that rQNestin34.5 possesses potent oncolytic activities. In mice, intracerebral toxicity studies show that its clinical safety parallels that of rHsvQ1. This suggests that the therapeutic window of rQNestin34.5 must be larger than that of clinically used HSV-1 mutants. However, additional histologic, immunopathologic studies in mice without tumors and in susceptible primates are needed before testing in human clinical trials. In the past, additional concerns involved the possible activation of virulent, latent wild-type HSV-1 on injection of an oncolytic HSV-1. Whereas theoretically this remains possible, primate studies and clinical studies have not shown this to occur. Another concern may relate to recombination of the nestin promoter-enhancer sequences into a wild-type HSV-1. Such recombination events are likely to lead to an attenuated HSV-1 because they would substitute the action of strong viral promoters with a relatively weak cell-specific cellular promoter.

Our results with rQNestin34.5 and those of others who have reported on the use of other tumor-specific promoters to drive expression of the γ_1 *34.5* gene (26–28) confirm and validate our original concept of using a tumor-specific promoter to drive expression of this viral gene and thus improve the selectivity of oncolysis. The original article described use of a B-Myb promoter and generating a virus designated as Myb34.5 (12). We compared rQNestin34.5 with Myb34.5 and found that the former was more effective than the latter in lysing glioma cells that expressed high levels of nestin, whereas in glioma cells that expressed low levels of nestin the efficacies of the two viruses were similar (Kambara et al., data not shown). rQNestin34.5 thus may possess advantages compared with Myb34.5 for applications to glioma therapy. One other advantage is that the B-Myb promoter will be expressed in cycling cells whereas the nestin promoter will only be expressed in glioma cells in the brain. The only other cell type in the brain that could be potentially targeted by rQNestin34.5 may be represented by neural stem cells within he subventricular zone and hippocampus. We argue, however, that current treatments of glioma (Radiation and chemotherapy) are also likely active against the human subventricular zone cell population due to its ability to divide, and thus possible rQNestin34.5 effects would have to be evaluated in this context.

In conclusion, rQNestin34.5 shows encouraging evidence of potent preclinical effects against malignant glioma models and should be further developed as a possible therapeutic modality.

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