Efficacy of Systemically Administered Oncolytic Vaccinia Virotherapy for Malignant Gliomas Is Enhanced by Combination Therapy with Rapamycin or Cyclophosphamide

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Abstract

Purpose: The oncolytic effects of a systemically delivered, replicating, double-deleted vaccinia virus has been previously shown for the treatment of many cancers, including colon, ovarian, and others. The purpose of this study was to investigate the oncolytic potential of double-deleted vaccinia virus alone or in combination with rapamycin or cyclophosphamide to treat malignant gliomas in vitro and in vivo.

Experimental Design: Rat (RG2, F98, C6) and human (A172, U87MG, U118) glioma cell lines were cultured in vitro and treated with live or UV-inactivated vaccinia virus. Viral gene [enhanced green fluorescent protein (EGFP)] expression by fluorescence-activated cell sorting, relative cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and assays for cytopathic effects were examined. S.c. murine tumor xenografts (U87MG, U118, C6) and i.c. (RG2, F98) tumor models in immunocompetent rats were treated with systemic administration of EGFP-expressing vaccinia virus (vvDD-EGFP), alone or in combination with rapamycin or cyclophosphamide, or controls. Tumor size, viral biodistribution, and animal survival were assessed. Lastly, the oncolytic effects of vvDD-EGFP on human malignant glioma explants were evaluated.

Results: vvDD-EGFP was able to infect and kill glioma cells in vitro. A single systemic dose of vvDD-EGFP significantly inhibited the growth of xenografts in athymic mice. Systemic delivery of vvDD-EGFP alone was able to target solitary and multifocal i.c. tumors and prolong survival of immunocompetent rats, whereas combination therapy with rapamycin or cyclophosphamide enhanced viral replication and further prolonged survival. Finally, vvDD-EGFP was able to infect and kill ex vivo primary human malignant gliomas.

Conclusions: These results suggest that vvDD-EGFP is a promising novel agent for human malignant glioma therapy, and in combination with immunosuppressive agents, may lead to prolonged survival from this disease.

There has been minimal improvement in the median survival of patients with malignant gliomas, and these tumors remain largely incurable despite advances in surgery, radiation, and chemotherapy (1). The need for new treatment strategies for malignant gliomas has led to the emergence of oncolytic virotherapy, oncolytic viruses able to selectively destroy tumor cells while sparing normal tissue. Much effort to date has been achieved in preclinical models of malignant gliomas using several oncolytic viruses, including herpes virus (2), adenovirus (3), reovirus (4), poliovirus (5), myxoma virus (6), and vesicular stomatitis virus (7). Some of these have already been tested in early clinical trials (8–10), and a small number of responses were found. Efficacy may be limited by factors such as the host immune response against the virus (11, 12) and limitations of systemic delivery in brain tumor patients, which may...
include the blood-brain barrier (13) and immune responses to virus in the vascular compartment.

Vaccinia virus is a double-stranded, enveloped, lytic DNA virus (14) with several advantages over other oncolytic viruses. It is easy to manipulate genetically; its replication and spread are rapid; and it is motile (actin tail dependent) and has no potential capacity to integrate into foreign DNA. It is a clinically safe and well-known virus because of its widespread use as a vaccine in the small pox eradication program. Several poxvirus-based vaccination trials for cancer are currently under investigation (15, 16), highlighting the relevance of this virus for anticancer immunotherapy.

Many strains of attenuated replicating vaccinia viruses have shown promising efficacy in the treatment of murine models of human gliomas (17) and other cancers (14, 18, 19), as well as primary cultures of human tumors (20). Because there may be a potential concern about the safety of a replicating vaccinia virus, a mutant "double-deleted" version of the Western Reserve (WR) strain [double-deleted vaccinia virus (vvDD)] with deletions of the thymidine kinase and vaccinia growth factor genes was created to enhance its safety (14). vvDD was nontoxic following i.v. delivery in nonhuman primates (21), which suggests that it may be a good candidate for the systemic oncolytic virotherapy of human tumors.

As the efficacy of oncolytic virotherapy as a single agent has thus far been unsatisfactory (8–10), this suggests that new or multiple oncolytic viruses or other combination therapies might prove to be more effective against malignant gliomas. Others have investigated oncolytic viruses in combination with chemotherapeutics, radiation therapy, or with suicide gene/prodrug systems, and have found enhanced efficacy of the oncolytic viruses toward brain tumors (22–24). Improved results have also been seen in immunocompetent animals using an oncolytic herpes virus in combination with the cyclophosphamide to suppress the immune response and provide for prolonged replication of the virus (25, 26). Recently, we and others have shown that the combination of myxoma virus plus rapamycin led to enhanced viral replication in vitro and in vivo, and improved efficacy against medulloblastomas (27) and melanoma (28). This led us to investigate the use of oncolytic vaccinia virus in combination with rapamycin or cyclophosphamide therapy for the treatment of experimental models of malignant gliomas.

To date, vvDD has not been evaluated for its efficacy or toxicity in brain tumor models. The objectives of this study were to determine the following: (a) the efficacy of an EGFP-expressing vaccinia virus (vvDD-EGFP) alone or in combination with rapamycin or cyclophosphamide as an experimental therapeutic agent against malignant gliomas in vitro and in vivo, and (b) the distribution and clearance of the virus after systemic (i.v.) delivery in immunocompetent hosts. We found that vvDD-EGFP targeted malignant glioma tumors specifically and prolonged survival in immunocompetent animal models of malignant glioma. This was further improved by the addition of rapamycin and cyclophosphamide. Although no cures were seen, there was a dramatic improvement in median survival. We also found that systemic (i.v.) administration of vvDD-EGFP in rats was feasible, safe, and well tolerated. The results of this study suggest that further investigation of the utility of oncolytic viruses in the treatment of malignant gliomas is warranted.

Translational Relevance

Malignant gliomas, an aggressive form of brain tumor, are currently incurable. In this study, we examine the activity of a novel therapeutic double-deleted vaccinia virus (vvDD). We show in animal models that vvDD is effective in prolonging survival from malignant gliomas and it is enhanced by the chemotherapeutic agents cyclophosphamide and rapamycin, which are now in clinical use. vvDD is safe in these models and is an excellent candidate to take forward in clinical trials for malignant glioma. This is an important step toward the future development of new treatments for malignant gliomas using this type of combination therapy.

Materials and Methods

Cell lines. Human and rat glioma cell lines (U87MG, U118MG, A172, F98, RG2, and C6), monkey kidney fibroblasts (CV1), and murine NIH3T3 cells were obtained from the American Type Culture Collection. All cell lines were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L glutamine, and 1% antibiotic-antimycotic (Gibco). Cell lines were maintained in an incubator at 37°C with 5% CO2 and serially passed every 3 to 4 d. Each cell line was tested routinely for mycoplasma contamination.

Vaccinia viruses. The recombinant (WR strain) vaccinia viruses vvDD-EGFP (14) and vvDD-R2RLuc were used in these studies. vvDD-R2RLuc was constructed from the plasmid pTREX-R2RLuc, which contains the red fluorescent protein (mCherry; gene synthesized commercially) fused through a foot-and-mouth disease virus 2A peptide motif to the Renilla luciferase gene (Promega Corp.). The foot-and-mouth disease virus 2A motif allows for bicistronic expression of the two genes. The R2RLuc segment was subcloned into our vaccinia shuttle plasmid pVx–EGFP (14) upstream of the vaccinia synthetic early/late promoter, replacing EGFP. After homologous recombination with the parental virus VSC20 (ref. 29; a gift from Dr. B. Moss), vvDD-R2RLuc underwent five rounds of selection in mycophenolic acid. Both viruses are based on the double-deleted platform, which lacks thymidine kinase and vaccinia growth factor described previously (14). All viruses were expanded in HeLa cells, purified on a sucrose cushion, and titered on CV1 cells (30).

Flow cytometry analysis. Cells were infected with vvDD-EGFP or mock control at a multiplicity of infection (MOI) of 1 and harvested after 0, 24, 48, and 72 h. Cells were washed twice with 1× PBS, resuspended in fluorescence-activated cell sortings (FACS) buffer (Optimized Sheath Fluid, BD Biosciences), and evaluated by FACS caliper (Becton Dickinson Canada, Inc.). Green fluorescent protein (FL1; 488 nm) and phycoerythrin (PE) (FL2; 488 nm; compensation, FL1·1.1% FL2) expression was analyzed using CellQuest software (BD Biosciences).

Cell proliferation assays. For the MITT assay, cells were infected with vvDD-EGFP at an MOI of 1, seeded in complete medium (10% FCS) into 96-well plates (5 × 104 cells/well; triplicate), and incubated at 37°C in a 5% CO2 humidified atmosphere. After 0, 24, 48, and 72 h, 10 μL of the MITT labeling reagent (5 mg/mL in PBS, Cell Proliferation Kit 1, Roche Applied Science) was added to each well. After 4 h (37°C, 5% CO2), 100 μL of solubilization solution (10% SDS in 0.01 mol/L HCL) was added and cells were incubated overnight. Microtitre plates were evaluated on a Vmax Kinetic microplate reader (Molecular Devices Corp.), and data analysis was done (SOFTWARE software version 2.32, Molecular Devices Corp.). For the combined therapy group, rapamycin...
(1–10 nmol/L/mL) was added to the cells 1 h before treatment with virus.

**In vitro** cytopathic effects (cytopathic effect assay) were visualized in six-well plates. Malignant glioma cell lines were plated until confluent and then infected with vvDD-EGFP (or mock control or UV-inactivated control) at an MOI of 1 for 2 h. At 24, 48, and 72 h after infection, cells were photographed under white light (original magnification, ×10; Nikon Eclipse TE200 microscope with a Hamamatsu ORCA100 digital camera).

**Animals.** CD-1 nude mice (female; 6–8 wks old) and Fisher 344 rats were purchased from Charles River Canada. The animals were housed in groups of three to five in a vivarium maintained on a 12-h light/dark schedule with a temperature of 22°C ± 1°C and a relative humidity of 50% ± 5%. Food and water were available ad libitum. All procedures were reviewed and approved by the University of Calgary and University Health Network (Toronto, Canada) Animal Resource Centres.

**In vivo oncolysis in a s.c. xenograft model.** To determine the antitumor effects of vaccinia virus in s.c. models, we injected C6, U87, and U118 (1 × 10⁷ cells/mouse) malignant glioma cells into the right flank of female nude mice to establish s.c. tumors. Seven days later, when tumor volumes reached 0.10 cm³ for C6, 0.13 cm³ for U87, and 0.06 cm³ for U118, the treatment group was injected i.p. with 10⁹ particle-forming unit of vvDD-EGFP in 2 mL of HBSS and the control group with 2 mL of HBSS i.p. Tumors were then measured twice a week by a blinded investigator, and tumor volume was calculated as (width)² × length × 0.52. Animals were sacrificed according to Animal Resource Centres guidelines, and tumor tissues were removed for histologic examination.

**Evaluation of toxicity of vvDD-EGFP in immunocompetent rats.** To investigate the toxicity of vvDD-EGFP in non-tumor-bearing immunocompetent rats, Fisher 344 rats (n = 12) under anesthesia (isofluorane 2%) received i.c. injections of increasing doses of vvDD-EGFP (1 × 10², 1 × 10⁴, 1 × 10⁶, 1 × 10⁸ particle-forming unit/rat; two rats per dose) using the guide-screw system (31) or 1 × 10⁹ particle-forming unit/rat or vehicle control via tail vein. Animals were followed for 42 d and weighed twice per week. Animals losing 20% body weight or having other unacceptable symptoms were sacrificed as per our Institutional Animal Care Guidelines. After sacrifice, brains and major organs were taken out and processed for histologic examination.

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**Fig. 1.** vvDD-EGFP infects human and rat brain tumor cells in vitro. A, fluorescence-activated cell sorting analysis for EGFP of malignant glioma cell lines (C6, A172, RG2, U87MG, and F98) 24 to 72 h after infection with the vvDD-EGFP at an MOI of 1. B, MTT assay of malignant glioma cells (human: U87MG, U118MG, A172; rat: C6, RG2, F98) compared with uninfected controls over 72 h. C, cytopathic effect on malignant glioma cells. Cells plated at confluence were infected the next day with vvDD-EGFP or UV/psoralen-inactivated virus (UV vvDD) at an MOI of 1. Microscopy was done 48 h after viral infection. Original magnification, ×400.
Survival studies of orthotopic glioma models in immunocompetent hosts. To investigate the efficacy of vvDD-EGFP in orthotopic glioma animal models, female Fischer 344 rats under anesthesia were injected with $1 \times 10^5$ F98 cells in 3 mL of sterile PBS using the guide-screw system (31) or were fixed to a stereotactic apparatus, and $5 \times 10^4$ RG2 cells in 3 mL of PBS were inoculated as described previously (6). Seven (F98) or five (RG2) days after tumor implantation, rats were i.v. administrated vvDD-EGFP ($1 \times 10^7$ particle-forming unit/rat in 100 mL HBSS) as a single dose or multiple doses ($1 \times 10^7$ particle-forming unit/rat every 2 d for a total of three injections). Control animals were treated with HBSS. Rats were monitored daily until they lost >20% of body weight or had trouble ambulating, feeding, or grooming; then, they were sacrificed, and their brains and major organs were collected for histologic analysis.

In vivo viral distribution studies in an orthotopic glioma model. To determine if vvDD-EGFP targets multifocal gliomas in the brain, we established a bilateral tumor model using RG2 tumor cells to mimic multifocal malignant glioma of patients. RG2 cells were implanted by stereotactic techniques as described above in immunocompetent Fischer rats. Ten days later, rats were administered i.v. vvDD-EGFP ($1 \times 10^7$ particle-forming unit/rat in 100 mL HBSS). Animals ($n = 3$) were sacrificed at 1, 3, 5, 7, and 14 d after virus administration. When sacrificed, animals were anesthetized and perfused with 50 mL of saline, followed by 30 mL of phosphate-buffered 10% formalin via cardiac catheter. Following fixation, a whole brain picture was taken with a Leica MZ-FLIII fluorescence stereomicroscope using a standard green fluorescent protein filter set and the brain, tumor, and other major organs were removed and frozen in liquid nitrogen for virus recovery assays.

Combination therapy in vitro and in vivo. To determine whether pretreatment with rapamycin promotes vvDD oncolysis of rat cell lines in vitro, viral green fluorescent protein expression and cell viability were assessed 48 or 72 h postinfection in the presence or absence of rapamycin. F98 and RG2 Cells were pretreated with rapamycin (100 nM) 1 h before virus infection, then infected with vvDD-EGFP (MOI, 0.1 or 1). After a 48-h incubation, green fluorescent protein expression was analyzed using a Zeiss inverted microscope (Axiovert 200 M) with green fluorescent protein filter and a Carl Zeiss camera (AxioCam MRc). To assess the effects of combined therapy on cell viability, an MTT assay was done 72 h after infection. To determine the effect of rapamycin on viral replication, cells were pretreated for 2 h with rapamycin (100 nM), infected with vvDD-EGFP (MOI, 0.01), incubated for 48 h, and lysed using three rounds of freeze thawing to extract viral particles. The viral titers of samples were determined using a standard plaque titration assay on CV1 cells.

Fig. 2. Systemic (i.p.) administration of vvDD-EGFP on growth of s.c. models of malignant glioma in nude mice. Female nude mice were injected with $1 \times 10^7$ C6, U87, and U118 cells s.c. in their right flanks. Seven days later, when tumor volumes reached $0.10 \text{ cm}^3$ for C6, $0.13 \text{ cm}^3$ for U87, and $0.06 \text{ cm}^3$ for U118, a blinded experimenter administered the treatment group with $10^7$ particle-forming units of vvDD-EGFP in 2 mL of HBSS i.p. and the control group with 2 mL of HBSS i.p. Tumors were then measured twice a week. A, C6 malignant gliomas in the treatment group had significantly ($P = 0.033$) smaller tumor volumes than the control group. B, the volume of U87 gliomas in the treatment group remained stable, whereas the tumor volume of the control group increased significantly ($P = 0.01$) with time. C, the volume of U118 malignant glioma also remained relatively stable over time in the treatment group, whereas the tumor volume of the control group increased significantly ($P = 0.03$).
Immunohistochemistry. Paraffin embedded sections of rat brain were deparaffinized and rehydrated with 1× PBS after blocking, then exposed to primary antibody (murine monoclonal vaccinia virus antibody, Abcam, Inc.) at a 1:10 dilution in PBS overnight at 4°C. Biotinylated donkey anti-mouse IgG (1:2,000, Vector Laboratories) was used as the secondary antibody. Sections were then incubated with avidin conjugated to horseradish peroxidase (Vectastain ABC immunohistochemistry kit, Vector Laboratories), and staining was visualized by the addition of 3,3'-diaminobenzidine substrate with hematoxylin counterstaining. Sections were mounted and viewed with a Zeiss inverted microscope (Axiovert 200M) and a Carl Zeiss camera (AxioCam MRc) to obtain images.

For the CD68/CD163 staining, frozen sections of the brains were fixed with 4% paraformaldehyde for 15 to 20 mins, followed by two washes with PBS (for paraffin embedded sections were deparaffinized and rehydrated with 1× PBS). The sections were incubated with primary antibody [mouse anti-rat CD68 (1:500; Serotec; Cat MCA 341R), CD163 (1:300; Serotec; Cat MCA 342R)] for 1 h at room temperature after blocking. Biotinylated anti-mouse IgG (1:500; Vector Laboratories; CatBA-2000) was used as a secondary antibody.

Virus recovery assays. Rats were sacrificed; saline was immediately infused; and the tissues were extracted and homogenized in HBSS using a Pellet Pestles Kit (VWR International), followed by repeated freeze thawing to release virus from the cells. Supernatants were plaque titrated on CV1 cells, as previously described (29). Viral plaques were counted, and particle-forming unit were calculated by the number of plaques multiplied by the dilution factor and normalized to the weight of the tissues.

Primary human glioma culture. Short-term cultures were established from patient samples of human gliomas (n = 8) obtained following brain tumor surgery at the Foothills Hospital (Calgary, Alberta, Canada). This study was approved by the Conjoint Medical Ethics Committee. Briefly, each patient specimen was split into two pieces for fixation in 10% formalin and short-term culture. For short-term cultures, the tissue was washed several times in sterile saline, cut into small pieces (~ 0.5-1 mm in diameter), and dissociated with trypsin (0.25%) and 50 μg/mL DNase (Roche Diagnostics) for 30 mins at 37°C. After filtering and washing with DMEM/F12 (containing 20% FBS), cells were resuspended in 20% FBS in DMEM/F12 and plated (at 10,000-100,000 cells per well) in 96-well plates. Cells were infected

Fig. 3. Systemic (i.v.) administration of vvDD-EGFP prolonged survival of immunocompetent rats bearing i.c. F98 and RG2 gliomas. A, F98 tumor model. Kaplan-Meier survival analysis of rats implanted with F98 (1 × 10^5 cells/rat) and treated with either HBSS (n = 6) or vvDD-EGFP (n = 6; 1 × 10^9 particle-forming units/rat single i.v. injection on day 7 after tumor implantation). All Fs are two sided. Arrow, virus injections. B, representative immunohistochemistry of vvDD analysis in F98 tumors and normal brain 7 d after single administration of virus. Left column, mock control. Original magnification, ×10 (top) and ×40 (bottom). C, RG2 tumor model. Kaplan-Meier plot showing survival curves of rats harboring i.c. RG2 tumor treated with dead virus (n = 5) or a single i.v. administration of vvDD-EGFP (live virus single; n = 5; 1 × 10^9 particle-forming units/rat) or multiple i.v. administration of vvDD-EGFP (live virus multiple; n = 6; 1 × 10^9/rat every 2 d for three injections). All Fs are two sided. Arrows, day of virus administration. D, histologic analysis showed that all of the dead virus – treated mice and live virus – treated mice had large tumors in the brain. Most of animals treated with i.v. administration of vvDD-EGFP showed area of tumor necrosis. Original magnification, × 25 (top) and × 400 (bottom).
The following day with vvDD-EGFP virus, live and UV inactivated, at MOIs of 0.1, 1, and 10. Cell viability was measured 96 h postinfection by MTT assay. Viral titers were obtained from patient short-term cultures after infection. Dissociated tumor cells from surgical samples of malignant gliomas were plated in six-well plates and infected the next day with vvDD-EGFP at an MOI of 0.1. Cells were collected at 0, 24, and 72 h after infection. These were then lysed using three rounds of freeze thawing to extract viral particles. U87 and NIH3T3 were used as positive and negative controls, respectively. The viral titers of samples were determined using a standard plaque titration assay on CV1 cells.

**Statistics.** All statistics were generated using StatView Software (Abacus Concepts, Inc.) and GraphPad Prism (version 4; GraphPad Software, Inc.). Survival curves were generated by the Kaplan-Meier method. The log-rank test and ANOVA for repeated measures were used to compare the effect of different forms of treatment. The Student’s t test was used when appropriate. Data was expressed as means ± SD. All Ps <0.05 were considered significant.

**Results**

**Vaccinia virus (vvDD-EGFP) infects and kills malignant glioma in vitro.** Malignant glioma cell lines were tested for susceptibility to infection by vvDD-EGFP using FACS analysis to quantitate the percentage of EGFP-positive cells. Malignant glioma cell lines (C6, A172, RG2, U87MG, and F98) were infected with vvDD-EGFP at an MOI of 1; the number of EGFP-positive cells was quantified 24, 48, and 72 hours after infection. All 5 malignant glioma cell lines were efficiently infected by vvDD-EGFP, with 70% to 99% of all cell lines infected by 24 hours (Fig. 1A).

To confirm whether infection was leading to oncolysis and cell death, MTT and cytopathic effect assays were done. All six cell lines (U87, U118, A172, C6, RG2, and F98) were susceptible to killing, and extensive cell death was observed 72 h after infection with an MOI of 1 (<40% of cells still viable compared with uninfected controls; Fig. 1B). Similar results were obtained with the cytopathic effect assay. There was no evidence of cytopathic effect in untreated (control) or dead virus–treated (UV vvDD) cells at an MOI of 1 at 48 hours postinfection (Fig. 1C).

**Antitumor effect of systemic administration of vvDD-EGFP on s.c. models of malignant glioma.** We next investigated if vvDD-EGFP would infect and kill malignant glioma in a s.c. tumor model in nude mice. Nude mice bearing s.c. xenografts of U87MG, U118, or C6 malignant glioma cell lines were infected systemically (i.p.) with 10^9 particle-forming unit of vvDD-EGFP or HBSS control. We observed a statistically significant inhibition of tumor growth in virus-treated mice compared with the HBSS-treated control mice bearing either C6 (ANOVA; P = 0.03; Fig. 2A) or U87 (ANOVA; P = 0.01) tumors (Fig. 2B). The volume of U118 malignant glioma remained relatively stable over time in the treatment group, whereas the tumor volume of the control group increased significantly (Fig. 2C; ANOVA; P = 0.03).

**Toxicity of intracerebral and i.v. administration of vvDD-EGFP.** We evaluated the toxicity of vvDD-EGFP in non-tumor-bearing immunocompetent rats when administered i.c. or i.v. A single dose of 1 × 10^8 particle-forming unit i.c. or 1 × 10^9 particle-forming unit i.v. (the highest feasible dose we can prepare) of vvDD-EGFP was safe in normal non-tumor-bearing rats. At the highest i.c. dose administered, we saw some weight loss, which quickly recovered (Supplementary Fig. 1). There were no deaths and no neurologic symptoms noted. Surprisingly, when tumor-bearing rats were subsequently treated, all i.c. doses were extremely toxic in a non-dose-dependent manner. Necropsy showed gross hemorrhage and swelling of the tumor, presumably because of the rapid oncolysis by the virus (data not shown). I.v. administration of 1 × 10^9 particle-forming units to tumor-bearing rats was nontoxic and was the dose/route used in subsequent studies.

**Survival following systemic administration of vvDD-EGFP in immunocompetent rats bearing i.c. malignant glioma.** To determine the efficacy of vvDD-EGFP oncolysis in immunocompetent models of i.c. malignant glioma, we implanted F98...
or RG2 cells into the brain of F344 rats to establish i.c. tumors. Rats were treated i.v. with single or multiple doses of vvDD-EGFP after tumor implantation. Animals were monitored daily. A single i.v. administration of vvDD-EGFP prolonged survival (Fig. 3A; long-rank test; \(P = 0.0475\)) of rats bearing i.c. F98 tumors. The median survival of vvDD-EGFP–treated animals was 24.5 days [95% confidence interval (95% CI), 20-28 days], whereas control treated animals died with a shorter median survival (mean, 21.5 days; 95% CI, 15-25 days; \(P = 0.0475\)). Histologic analysis showed that all of the control-treated and virus-treated rats had large i.c. tumors (data not shown).

Immunohistochemical staining for viral antigen was positive within the tumors of the vvDD-EGFP–treated rats but negative within the control-treated tumors (Fig. 3B).

This experiment was repeated with the rat RG2 i.c. model, and similar results were obtained. Rats bearing RG2 tumors treated with live vvDD-EGFP administered i.v. survived significantly longer [whether single or multiple injection(s) of virus; Fig. 3C; mean, 18 days (single) or 22.8 days (multiple); 95% CI, 17-19 days (single) or 18-20 days (multiple)] than those treated with dead virus (Fig. 3C; mean, 13.6 days; 95% CI, 13-15 days; long-rank test; \(P = 0.0009\)).

![Fig. 5. Combination therapy with rapamycin or cyclophosphamide enhanced vvDD replication and increased survival in immunocompetent rats bearing i.c. malignant gliomas. A, single i.v. administration of vvDD-EGFP. Rats bearing bilateral i.c. RG2 tumors were divided into three groups, virus only (left column), virus plus cyclophosphamide (middle column), or virus plus rapamycin (right column), and then treated i.v. with vvDD-EGFP at a dose of \(1 \times 10^9\) particle-forming units/rat 8 d after tumor cell implantation. One day before virus administration, combination treatment group animals were treated i.p. with rapamycin (5 mg/kg/d for 5 d) or cyclophosphamide (60 mg/kg). Animals were sacrificed 7 d after the viral administration. Photomicrographs of green fluorescent protein–labeled virus (green) in tumor (n = 2 rats/group) 7 d after virus administration. Original magnification, \(\times 12.5\). B, multiple i.v. administration of vvDD. Rats bearing bilateral i.c. RG2 tumors were treated with i.v. vvDD-EGFP (i.e., green) at a dose of \(1 \times 10^9\) particle-forming units/rat 8 d after tumor cell implantation. Then animals were divided into three groups: virus treatment only, virus plus rapamycin, or virus plus cyclophosphamide. Two days after the first virus administration, combination treatment group animals were treated i.p. with rapamycin (5 mg/kg/d for 5 d) or cyclophosphamide (60 mg/kg). The next day, all animals were treated again i.v. with vvDD-RFPLuc (i.e., red) at a dose of \(1 \times 10^9\) particle-forming units/rat. Animals were sacrificed 72 h after the last viral administration. Top row, photomicrograph of green fluorescent protein–labeled virus (green) in tumor (n = 2 rats/group). Original magnification, \(\times 12.5\). Middle and bottom rows, red fluorescent protein–labeled virus (red) in tumor (n = 2 rats/group). Original magnification, \(\times 12.5\) (middle row, axial view) and \(\times 20\) (bottom row, coronal view). C, Kaplan-Meier plot showing the survival of bilateral i.c. RG2 tumor–bearing rats after treatment with dead virus (\(n = 5\), vvDD-EGFP alone (\(n = 6\)), rapamycin alone (\(n = 5\)), or a combination of vvDD-EGFP and rapamycin (\(n = 5\); log-rank test; \(P < 0.0001\) compared with dead virus). D, Kaplan-Meier plot showing the survival of bilateral i.c. RG2 tumor–bearing rats after treatment with dead virus (\(n = 5\)), vvDD-EGFP alone (\(n = 6\)), cyclophosphamide alone (\(n = 5\)), or a combination of vvDD-EGFP and cyclophosphamide (\(n = 6\); log-rank test; \(P = 0.0011\) compared with dead virus). All \(P\)s are two sided.](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-08-2601)
Histologic analysis showed that all of the dead virus–treated rats and live virus–treated rats had large tumors in the brain, although most of the animals treated with vvDD-EGFP showed areas of tumor necrosis (Fig. 3D).

**Distribution of i.v. administered vvDD-EGFP in immunocompetent rats bearing bilateral i.c. malignant gliomas.** We next characterized the distribution of virus in the tumors. Rats bearing bilateral i.c. RG2 tumors (to mimic multifocal malignant gliomas) were treated with i.v. vvDD-EGFP 10 days after tumor implantation. Animals were sacrificed at different timepoints (1, 3, 5, 7 days) and 14 days (data not shown) after viral administration. EGFP-expressing virus, as visualized under fluorescence microscopy, was confined to the tumor only and was not found in the normal brain (Fig. 4A). Lung, kidney, liver, spleen, or heart (data not shown). Viral expression of EGFP began 1 day after infection, increased up to 3 days postinfection, was still detected by day 5, and was undetectable by 7 days after viral administration. The viral titers from the tumor and normal tissues, as determined by virus recovery assays on CV1 cells, confirmed the results from the fluorescence microscopy (Fig. 4B), but there was still virus replication in the tumor on day 7. This was presumably at a level that was not detectable using the less sensitive technique of fluorescent microscopy. At days 1 and 3, low levels of replicating virus were found in non-tumor-containing brain tissue, but this was not measurable after 3 days. All other normal tissues had no detectable virus after day 1 except the heart and ovaries, which were positive for virus until days 3 and 7, respectively (Fig. 4C). No virus was detected in blood cultures at all time points, including day 1 (data not shown).

**Combination therapy with rapamycin promotes vvDD-mediated oncolysis in rat cell lines** in vitro. To determine whether...
pretreatment with rapamycin promotes vvDD-mediated oncology of rat cell lines (F98, RG2) in vitro, viral EGFP expression and cell viability were assessed 48 or 72 hours postinfection in the presence or absence of rapamycin. We found that pretreatment with rapamycin increased EGFP expression more than vvDD-EGFP treatment alone in both cell lines (Supplementary Fig. 2A). We also found that treatment with vvDD-EGFP and rapamycin resulted in greater cell killing than either treatment alone (Supplementary Fig. 2B). To evaluate whether the increased cell killing was due to increased viral replication, viral titers were measured over time with or without rapamycin. As seen in Supplementary Fig. 2C, rapamycin significantly (P < 0.01) enhanced viral replication in both cell lines compared with media alone.

**Combination therapy with rapamycin or cyclophosphamide enhances vvDD-EGFP replication and further prolongs survival in immunocompetent rats bearing i.c. malignant glioma.** To determine if combined therapy with rapamycin or cyclophosphamide enhanced vvDD-EGFP replication in RG2 tumors in vivo, we used the rat bilateral i.c. RG2 tumor model and treated the rats with vvDD-EGFP combined with rapamycin or cyclophosphamide. EGFP expression was assessed 7 days after virus administration because it was previously undetectable at this time point after virus alone (Fig. 4A), although viral replication still occurred as detected by plaque assays (Fig. 4B).

Two animals in each group were sacrificed 7 days after virus treatment, and EGFP expression and whole brain images were obtained (Fig. 5A). Not surprisingly, we found that two animals treated with vvDD-EGFP alone had no detectable EGFP expression 7 days after viral administration (Fig. 5A, left column). In contrast, animals treated with a combination of vvDD-EGFP and either cyclophosphamide or rapamycin had visible EGFP expression evident (Fig. 5A, middle and right columns). Approximately 10% of the tumor cells expressed EGFP after vvDD + cyclophosphamide combined treatment, whereas 6% to 8% expressed EGFP after vvDD + rapamycin combination treatment.

To determine whether combination therapy with cyclophosphamide or rapamycin allowed a second i.v. administration (repeat) treatment and lead to more virus replication in vivo, we used the same bilateral tumor models as above and treated the rats first with vvDD-EGFP (i.e., appears green) and vvDD-RFPLuc (i.e., appears red) combined with either rapamycin or cyclophosphamide. To differentiate viral delivery/replication of two sequential i.v. administrations of vvDD, the vvDD-EGFP was administered first on day 8 (after tumor implantation), followed by a second dose of vaccinia (vvDD-RFPLuc) administered on day 11, and then, the animals were sacrificed 3 days later (on day 14). The brains were either examined using fluorescence stereomicroscopy or were frozen and used for viral plaque titering. Treatment with cyclophosphamide 24 hours before injection (day 7) of vvDD-EGFP and vvDD-RFPLuc markedly increased the anatomic area of viral EGFP and RFP expression (~8-10% of tumor cells expressed EGFP and 50-55% of tumor cells expressed RFP; Fig. 5B, middle row) compared with treatment with vvDD-EGFP and vvDD-RFPLuc alone (no EGFP or RFP expression; Fig. 5B, left row). Similarly, treatment with rapamycin beginning 24 hours before injection of vvDD-EGFP and vvDD-RFPLuc markedly increased the anatomic area of viral EGFP and RFP expression (~5-6% of tumor cells EGFP and 45-50% of tumor cells expressed RFP; Fig. 5B, right row) compared with treatment with vvDD-EGFP and vvDD-RFPLuc alone. The viral titers from the tumor tissues, as determined by virus recovery assays on CV1 cells (Supplementary Fig. 3A), confirmed that more viral replication occurred in tumors treated with combination therapy (either rapamycin or cyclophosphamide), and this correlated with fluorescence microscopy. Combination therapy with rapamycin and cyclophosphamide resulted in a 1.36- and 1.54-fold increase of viral replication compared with virus only (Supplementary Fig. 3A; P = 0.0013 and P = 0.0007, respectively; Student's t test).

To determine if combination therapy resulted in a significant prolongation of survival in this model, we treated animals with combination therapy using the doses and injection schedules described above. Treatment of animals with live vvDD-EGFP alone (Fig. 5C; mean, 21.5 days; 95% CI, 20-23 days; log-rank test; P = 0.0031) or rapamycin alone (mean, 31.4 days; 95% CI, 30-33 days; log-rank test; P = 0.015) significantly prolonged survival compared with dead virus–treated animals (mean, 17.7 days; 95% CI, 17-18 days). Rats treated with vvDD-EGFP plus rapamycin further prolonged survival (mean, 38 days; 95% CI, 31-43 days) compared with vvDD-EGFP alone (log-rank test; P = 0.0014), rapamycin alone (log-rank test; P = 0.0188), or dead virus (Fig. 5C; log-rank test; P < 0.0001, combination group compared with dead virus).

We saw similar results when vvDD-EGFP was combined with cyclophosphamide. Treatment of rats with cyclophosphamide alone (Fig. 5D; mean, 15 days; 95% CI, 14-17 days; log-rank test; P = 0.0889) did not prolong survival compared with dead virus–treated animals (mean, 14.2 days; 95% CI, 13-15 days). Rats treated with vvDD-EGFP alone (Fig. 5D; mean, 18 days; 95% CI, 18-20 days; log-rank test; P = 0.0026) significantly prolonged survival compared to dead virus–treated animals. Rats treated with vvDD-EGFP plus cyclophosphamide further prolonged survival (Fig. 5D; mean, 23.5 days; 95% CI, 18-25 days; log-rank test; P = 0.0011 compared to dead virus).

Because Fulci et al. (32, 33) provided mechanistic evidence that cyclophosphamide enhances the oncolytic effects of herpes simplex virus by inhibiting innate immune cells (CD68+ natural killer cells and CD163+ microglia/macrophages) in vivo, we asked whether or not the enhancement of the vvDD oncolytic effects by cyclophosphamide and rapamycin in our studies correlated with these innate immune cells in vivo. Immunohistochemistry staining for markers of natural killer cells/microglia/macrophages (CD68/CD163) 72 hours after i.v. administration of vvDD to rats pretreated with either rapamycin/cyclophosphamide or vehicle was done. CD68+ staining was notably reduced after either rapamycin or cyclophosphamide pretreatment compared with vvDD alone (Supplementary Fig. 3B and C). No change in CD163+ staining was observed.

**Infection and oncosis of primary human malignant gliomas from surgical specimens.** We next questioned whether the in vitro cell line results would also apply to primary glioma samples from patients. We examined the susceptibility to vvDD-EGFP of eight ex vivo brain tumor surgical specimens derived from four glioblastomas, one oligodendroglioma, two astrocytomas, and one gliosarcoma. Six of eight specimens tested were killed by vvDD-EGFP infection (Fig. 6A and B), similar to the level of infection and killing we found in U87. Viral-mediated EGFP expression was seen in live virus–treated glioma cells. In contrast, short-term cultures treated with dead
virus showed no viral-mediated EGFP expression (data not shown). We then quantified the viral titers on primary glioma cultures (Fig. 6C) and found that viral titers varied between different samples and mirrored their susceptibility to viral infection as found in Fig. 6A. Viral titers were similar to that recovered from the U87 cell line and much higher than that from the NIH3T3 cell line.

Discussion

We have presented the first evidence that an oncolytic vvDD is able to infect and kill malignant gliomas in vitro and in vivo. Vaccinia virus was able to infect all malignant glioma cell lines tested, including those such as U118, which have been notably resistant to other oncolytic viruses such as a reovirus (4). Normal NIH3T3 cells were not infected. In vivo, we saw dramatic responses in immunocompromised animals bearing human GBM xenografts treated with systemic virus, including some complete regressions. Most importantly, we were able to improve the survival of i.c. malignant gliomas in two immunocompetent rat models treated with oncolytic vvDD after systemic administration. Although we did not see any long-term cures, it is possible that some deaths occurring in the treatment group were actually due to tumor swelling after viral infection and cell death. In clinical trials, this would be mitigated by the use of corticosteroids.

The advantage of using an oncolytic virus for cancer therapy lies in its ability to specifically kill cancer cells while leaving normal cells intact. Depending on the virus, the mechanism by which these viruses are cancer specific varies. For example, reovirus and vesicular stomatitis virus can only replicate within cancer cells harboring defects in the ras or IFN pathways, respectively (34, 35). Measles virus can only infect cells that express the appropriate receptor such as CD46 in multiple myeloma (36). Herpes simplex virus and vaccinia virus have been genetically altered to replicate preferentially in dividing cancer cells (2, 14).

We have shown that the tumor specificity of a vvDD is maintained in models of malignant glioma. The mechanism of this tumor specificity is thought to be due to three factors. First, vvDD requires the substrates (such as TTP) present in dividing cancer cells because it lacks the thymidine kinase gene and vaccinia growth factor genes (14). In addition, gliomas are known to frequently contain mutations in the epidermal growth factor receptor pathway (37), and this may lead to complementation of the vaccinia growth factor (an epidermal growth factor homologue) deletion. Thirdly, vvDD is a large (∼300 nm) virus. It is difficult for it to extravasate from normal blood vessels in the absence of “leaky” vasculature such as that present in solid tumors and ovaries (38, 39). Presumably the s.c. xenograft model was nutritionally supported by a newly formed leaky tumor vasculature (39), and the i.c. tumors likely are accessed via a leaky blood–brain barrier (40).

Although the double-deleted virus is highly tumor specific, we previously recovered low levels of virus from normal tissues (14), and this corresponds with the pattern of viral replication seen here. Although some virus was recovered from the heart and normal brain, this was 6 to 8 logs lower than that recovered from the tumor, and these organs were structurally normal. This suggested that either the levels of infection were very low or the assay was contaminated by virally infected blood inadequately cleared by perfusion at the time of sacrifice. In any event, this provides us with a very large therapeutic window with which to avoid toxicity to these normal tissues. There was also an impressive differential infection between the human primary malignant gliomas and normal NIH3T3 cells in vitro. Most malignant gloma patient samples were easily infected by vvDD-EGFP, with >80% of cells expressing viral genes (EGFP). Vaccinia was able to replicate in and kill 75% of the primary malignant gliomas but not the normal cells.

One potential disadvantage of oncolytic viral therapy in general is the immune response to the virus. In this report, we were able to overcome this by combining the vaccinia viral therapy with the immunosuppressive agents rapamycin or cyclophosphamide. This is the first paper to show the enhanced effects of transient immunosuppression on vaccinia replication in immunocompetent animals. These drugs were able to significantly reverse the block to replication that occurred after the first dose of virus and allowed viral replication to occur following a second systemic administration of virus. The mechanism of action of cyclophosphamide has been described when herpes simplex virus is inoculated directly into the tumor in the brain (12, 32, 33). Cyclophosphamide increases viral replication and spread of herpes simplex virus in brain tumors by reducing the recruitment of natural killer (CD68+) and microglia/macrophage (CD163+) cells and the expression of IFN-γ (32, 33). We observed only a reduction in CD68+, but not CD163+, cells following treatment with rapamycin or cyclophosphamide, which could reflect the poor sensitivity of immunohistochemistry in detecting microglia/macrophage recruitment. The mechanisms whereby rapamycin affects innate immune responses are becoming clearer but remain largely unknown in malignant gliomas. At least in murine embryonic fibroblasts (41) and plasmacytoid dendritic cells (42), the mechanisms have been recently described and a model proposed (43). In murine embryonic fibroblasts, rapamycin inactivates translation of IFN regulatory factor 7 through effects of rapamycin on the translational repressors 4E-BPs. Therefore, treatment with rapamycin is expected to reduce IFN responses by reducing translation of IFN regulatory factor 7 with the concomitant reduction in the cascade of IFN responses to viral infection. In plasmacytoid dendritic cells, which recognize viral nucleic acids via toll-like receptors 7 and 9, the activity of IFN regulatory factor 7 is inhibited by rapamycin by blocking phosphorylation of S6K1,2. In turn, blocking activity of S6K1,2 precludes the formation of the toll-like receptor 9–MyD88 complex and downstream activation of IFN regulatory factor 7–mediated production of IFN. Which of these two mechanisms mediates the enhanced vaccinia virus infection in malignant glioma cells is currently under investigation in our laboratories. In addition to its effects on innate immunity in vitro, rapamycin affects immunity in vivo in a number of ways. In vivo rapamycin is a well-known immunosuppressive agent that prevents T-cell activation and proliferation (44). Rapamycin may expand the population of T-regulatory cells in mice, (45) but in man, the rapamycin-induced suppressor functions on the total population of CD4+ cells (46) rather than having a selective effect on T-regulatory cells. Hence, rapamycin may also improve oncolytic viral infection in vivo.
by suppressing T-cell-mediated viral responses. Because the predominant antivaccinia immune response is due to cellular immunity (47), the enhanced viral replication seen in our experiments may be due to its innate immune effects in vitro and its anti-T-cell effects in vivo. Finally, we chose rapamycin because it has activity as monotherapy in clinical trials in glioma patients (48, 49) and the doses achieved in patient tumor specimens are comparable to the doses used in this study (49).

This article provides strong evidence for the safety and efficacy of vaccinia virotherapy as a treatment for malignant glioma. It provides several distinct advantages over other viruses in that it is easy to engineer to large titers, it can infect and kill a wide variety of cancer types, and it is not dependent on specific cellular receptors or genetic alterations within tumor cells, giving it broad applicability. This suggests that vaccinia virus therapy of malignant glioma should be considered for further clinical development.

Disclosure of Potential Conflict of Interest

D.F. Stojdl and J.C. Bell are employed and have ownership interest in Jennerex, and J.A. McCarty is a patent coinventor for the vvDD.

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Efficacy of Systemically Administered Oncolytic Vaccinia Virotherapy for Malignant Gliomas Is Enhanced by Combination Therapy with Rapamycin or Cyclophosphamide

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