Oncolytic measles virus strains have significant antitumor activity against glioma stem cells

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INTRODUCTION
Glioblastoma (GBM) is the most common primary brain tumor in adults and has a dismal prognosis despite multimodality treatment including surgery, chemotherapy and radiation therapy.¹ One potentially significant hurdle to overcome in the treatment of GBM pertains to eradication of glioma cells with stem-cell-like properties (GSC); these are cells with self-renewing properties, able to differentiate into neurons, oligodendrocytes and astrocytes, and can initiate brain tumors in vivo. In addition, they have been associated with the promotion of angiogenesis, and development of resistance to radiation and chemotherapy.²–⁴ Our laboratory has previously demonstrated that derivatives of the measles virus (MV) Edmonston’s strain have significant activity against glioma lines and orthotopic xenografts;⁵,⁶ and a phase I trial of the measles derivative MV-CEA is currently ongoing in recurrent GBM patients. Assessing the ability of MV-strains to infect and eradicate glioma cells with stem-cell-like properties represents an important parameter, which could potentially affect the antitumor activity of this novel virotherapy platform and impact on the development of combinatorial strategies. Here, we show that GSC overexpress the MV receptor CD46. Furthermore, MV-strains of the Edmonston vaccine lineage have significant antitumor activity against GSCs in vitro and in vivo, and can decrease their tumorigenicity; these findings can have important translational implications in glioma treatment.

RESULTS
Viral strains
Antitumor activity of the MV-strains MV-CEA, MV-NIS and MV-GFP against GSC was examined. Their construction has been previously described.⁸–¹⁰ A schematic representation of the strains used is included in Figure 1.

Characterization of stem-cell marker expression in neurosphere lines
GBM xenografts were developed in BALB/c nude mice from GBM patients as previously described.¹¹,¹² Tumors were excised from mice bearing the GBM xenografts GBM6, 10, 12, 14, 23, 34, 38, 39, 43 and 44, mechanically disrupted and grown in NeuroCult medium: formation of neurospheres was observed within 2–6 weeks depending on the cell line. Neurosphere lines used in vitro and in vivo experiments were further characterized for the expression of the glioma stem-cell markers CD133, Nestin, Sox2, ATF5 and OLIG2. All neurosphere lines tested expressed at least 3/5 stem-cell markers. Representative results for three of the neurosphere lines are shown in Figure 2.

Expression of MV receptor CD46 in neurosphere lines
CD46 expression in neurosphere lines was tested by fluorescence-activated cell sorting (FACS) and immunohistochemistry overexpression was confirmed in all 10 lines tested. Representative results are shown for GBM34 and GBM22 (Figure 3).

Assessment of cytopathic effect of MV-strains against GSC
In order to access antitumor activity of measles virus strains against GSCs, neurosphere lines were infected with the MV-strains, MV-NIS, MV-GFP and MV-CEA at multiplicity of infection (MOI) of 0.1 or 1.0 and trypan blue exclusion assays were performed at multiple time points. Results were presented as percentage of the uninfected controls. MV derivatives had significant antitumor
activity against GSCs in vitro, which was dependent on MV-strain and MOI (Figure 4).

Assessment of viral replication
Virus replication was assessed using one-step viral growth curves as previously described.7 Neurosphere cultures supported robust growth of the MV-NIS, MV-GFP and MV-CEA virus (Figure 5).

Assessment of tumorigenicity of infected neurospheres
GSC cells obtained from GBM44 xenografts were infected with either MV-NIS (MOI of 10) and 3 x 10^5 cells were implanted into the right caudate nucleus of BALB/C nude mice. Mock infected GBM44 GSC were used as controls. There was significant prolongation of survival in mice implanted with infected neurospheres as compared with mice implanted with mock-infected neurospheres (P = 0.04), indicating that infection of GSC in vitro can modify their aggressive biologic behavior following orthotopic implantation (Figure 6).

As Figure 6 illustrates some of the mice implanted with infected neurospheres developed tumors. Examination of these tumors at the time of euthanasia showed lack of syncytia, indicating that the tumors likely derived from non-infected cells. Immunofluorescence for the natural killer cell lectin-like receptor and the macrophage marker CD68, showed lack of a significant immune infiltrate (Supplementary Figure 1), suggesting a low likelihood that innate immune response driven viral inactivation was responsible for tumor development in this model.

Assessment of therapeutic efficacy in vivo
In order to assess the antitumor activity of MV-strains against neurosphere-derived xenografts, GBM6 or GBM12 derived GSCs were orthotopically implanted into the right caudate nucleus of nude mice. Animals were treated with a total dose of either 9 x 10^5 TCID50 (GBM12) or 1.8 x 10^6 TCID (GBM6) of MV-GFP. Control animals received UV-inactivated virus. Significant prolongation of survival of the treated animals was observed in both models (P = 0.0416 and P = 0.0021, respectively) (Figure 7). In a parallel experiment, treated and control animals bearing orthotopic GBM6 GSC xenografts (a CD133-positive neurosphere line) were euthanized either on day 3 post-treatment completion or when moribund. Abundant syncytia were observed in MV-GFP-treated mice. Syncytia expressed CD133 (Figure 7d) confirming activity of the MV derivatives against GSC-derived tumors in vivo.

In situ hybridization for the MV N (nucleoprotein) -mRNA was

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**Figure 1.** Schematic representation of the viral strains: MV-CEA: MV-strain expressing the human carcinoembryonic antigen; MV-NIS: MV-strain expressing the sodium iodine symporter. N, nucleoprotein gene; MV-GFP, MV-strain expressing the enhanced green fluorescent protein; L, large protein gene; P, phosphoprotein gene; M, matrix protein gene; F, fusion protein gene; H, hemagglutinin gene; CEA, human carcinoembryonic antigen gene; NIS, sodium iodine symporter gene.

**Figure 2.** Neurospheres derived from GSC xenograft cultures express stem-cell markers (CD133, Nestin, SOX2, ATF5 and OLIG2). Representative data for the GBM6, GBM12 and GBM38 GSC lines are shown.

**Figure 3.** Expression of MV receptor CD46 was examined by IHC and flow cytometry. Significant expression of CD46 was observed in all neurosphere lines tested. Representative ICH and FACS data in GBM34 and GBM22 GSC lines are presented.

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strongly positive (Figure 7f), indicating viral replication in the GSC-derived syncytia.

**DISCUSSION**

GBM remains incurable with a median survival of 15–16 months, despite the use of multimodality treatments including surgery, chemotherapy and radiation therapy.13 Brain tumor stem cells are believed to play a key role in mediating glioma resistance to chemotherapy and radiation therapy, in part due to their ability to more efficiently repair damaged DNA 3,14–16 as compared with tumor cells without stem-cell properties. Treatment of GBM with temozolomide, an agent commonly used in glioma treatment, leads to preferential survival of a cell population, enriched in the stem-cell marker16 CD133, and GBMs that recur following chemoradiation are characterized by a stem-cell-related gene signature.17 Stem-cell resistance to chemotherapy agents such as temozolomide, carboplatin, paclitaxel and etoposide appears to be induced through elevated expression of antiapoptotic genes and O6-methylguanine-methyltransferase, a DNA repair enzyme that inhibits the activity of DNA methylating agents such as nitrosoureas and temozolomide.18 These cells have the capacity for self-renewal and multilineage differentiation and efficiently initiate tumor xenografts in vivo.7

Development of approaches that can effectively eradicate stem cells has therefore the potential to significantly improve outcome for GBM patients. Although small molecule inhibitors of key pathways in GSCs survival, such as the notch signaling pathway,
are currently in early clinical testing\(^1\) their clinical efficacy to date has been modest and the development of additional approaches targeting glioma cells with stem-like properties is needed. Oncolytic viruses might represent such an approach. DNA viruses such as the Delta-24-RGD adenovirus\(^1\) and oncolytic HSV-strains\(^2,3\) have been reported to have antitumor activity against GSCs. There is no information to date, however, regarding activity of oncolytic RNA viruses against GSC.

We have demonstrated that engineered strains of MV, a negative strain RNA virus of the paramyxovirus family, deriving from the Edmonston vaccine lineage have significant activity against glioma cells with stem-like properties. These cells allow efficient viral propagation as indicated by one-step viral growth curves and were effectively killed even in MOIs as low as 0.1. In addition, infection before orthotopic implantation of human GBM xenograft derived GSCs modified their aggressive biologic behavior, as demonstrated by the significant prolongation of survival of animals treated with infected cells. Treatment of orthotopic xenografts derived from GBM6 and GBM12 GSC, also led to therapeutic benefit and significant prolongation of median survival of virus treated animals. The characteristic cytopathic effect with formation of syncytia was observed in these CD133-positive orthotopic xenografts. In situ hybridization for MV mRNA confirmed in vivo replication of the virus.

Sensitivity of glioma cells to measles infection can at least in part be explained by overexpression of MV receptor CD46 in neurosphere lines. In contrast to the wild-type MV-strains that enter the cells predominantly via the SLAM receptor (expressed in B-, T-cells and macrophages),\(^2,3\) MV oncolytic vaccine strains of the Edmonston vaccine lineage have been shown to enter cells predominantly via the ubiquitous expressor CD46.\(^2,4\) CD46 or membrane cofactor protein is overexpressed in different tumors, including gliomas\(^2\) and protects them from complement-mediated lysis.\(^27-29\) We have tested the levels of CD46 expression by both immunohistochemistry and FACS in 10 different neurosphere lines deriving from patient originated xenografts and observed high expression in all 10 neurosphere lines tested, indicating that the GSCs represent a good target for CD46 targeted therapeutics such as MV-strains. In an ongoing trial of intratumoral and resection cavity administration of the measles derivative MV-CEA in recurrent GBM patients, we are in the process of investigating the correlation between CD46 expression in tumors and viral replication/response to oncolytic measles virotherapy.\(^29\)

It is of note that recently a third measles receptor, the adherens junction protein nectin-4, has been identified.\(^31\) In addition to the respiratory epithelium,\(^31\) this receptor is also expressed in ovarian, breast and lung cancer.\(^32-34\) Although members of the nectin family, such as nectin-1 and -3 appear to play an important role in the function of brain synapses,\(^35\) the role of nectin-4 is not well characterized in brain tumors or normal brain. FACS analysis in four different measles permissive GSC lines (GBM6, GBM12, GBM102, GBM143) showed no nectin-4 expression in GSC (Allen and Galanis, unpublished data), indicating a low likelihood that nectin-4 expression represents an important parameter determining the efficacy of MV-based therapeutics against GSC. We are in

![Figure 6. GBM44 GSC were either infected with MV-NIS (MOI 10) or mock-infected, and 3 x 10⁵ GSC cells were implanted into the right caudate nucleus of nude mice. Animals were followed for survival. There was significant prolongation of survival in mice which were implanted with cells infected with MV-NIS (P = 0.04) with median survival of 68 days in the control group versus not having been reached at 100 days in mice implanted with infected GSC.](image)

![Figure 7. GBM6 (a) or GBM12 (b) derived GSC were orthotopically implanted into the right caudate nucleus of nude mice. Animals were treated with a total dose of 1.8 x 10⁸ or 9 x 10⁷ TCID50 MV-GFP, respectively, or UV-inactivated virus. In both orthotopic treatment models, significant prolongation in survival of MV treated mice was observed (P = 0.0021 and P = 0.0416, respectively). Abundant syncytia were observed in MV-GFP-treated mice in H and E stain (c, e). Syncytia expressed the stem-cell marker CD133 (d). Viral replication was confirmed by in situ hybridization for MV N mRNA (f).](image)
the process of expanding this analysis in a larger panel of GSC, however.

Our results demonstrate the efficacy of MV-strains against glioma cells with stem-cell-like properties in vitro and in vivo. They also suggest that combinatorial strategies of oncolytic measles virus strains with agents representing the standard of care in GBM treatment, such as RT or temozolomide, represent a direction worth exploring: MV virotherapy has the potential to improve outcome in response to these conventional treatment strategies by eradicating resistance mediating cells with stem-cell-like properties.

**MATERIALS AND METHODS**

**Viral construction**

The construction of recombinant MV strains that contain the soluble extracellular domain of human CEACAM5 (MV-CEA), green fluorescent protein-eGFP (MV-GFP) or the sodium-iodide symporter gene (NIS, MV-NIS, Figure 1) has been described elsewhere.6–10

**Cell lines**

Subcutaneous xenografts were developed in BALB/c nude mice from GBM patients as previously described.36 When orthotopically implanted, these xenografts have been demonstrated to maintain the histologic characteristics and invasiveness of the primary tumor they derived from. Tumors were excised from mice bearing the GBM xenografts GBM6, 10, 12, 14, 22, 34, 38, 39, 43 and 44 and each was mechanically disrupted with a sterile blade and grown at 37°C in a humidified environment and 5% CO2 in either regular media (Dulbecco’s minimum essential medium (DMEM) with 10% fetal bovine serum, 1 × Penn/Strep) or Neuro Cult media (Stemcell Technologies, Vancouver, BC, CA, USA).

**Immunohistochemistry to assess expression of GSC markers**, CD133, SOX2, Nestin, ATFS, OLG2 and the MV receptor CD46 in neurospheres

Neurospheres from primary GBM xenograft cultures were harvested, expanded in vitro, fixed in acetone and examined by immunohistochemistry for the expression of: (a) GSC markers, CD133, SOX2, Nestin, ATFS, OLG2 and MV receptor CD46. Primary antibodies to test marker expression were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and immunohistochemistry was performed as per the manufacturer’s instructions. In summary, slides were blocked in phosphate-buffered saline (PBS) containing 10% goat or donkey serum, 1% bovine serum albumin and 0.025% Triton-X-100 for 1 h and incubated overnight at 4°C with 1:100 dilution of primary antibody. Primary antibody was omitted in negative controls. The slides were rinsed thrice for 5 min in PBS and exposed to a 1:500 dilution of either biotinylated goat anti-mouse or donkey anti-goat secondary antibody, followed by incubation with avidin peroxidase or biotinylated horseradish peroxidase (for visualisation with DAB substrate staining) followed by VECTASTAIN Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained with ACCUSTAIN (Sigma-Aldrich Inc., St Louis, MO, USA).

**CD46 flow cytometry**

Primary GSC cultures deriving from GBM6, 10, 12, 14, 22, 34, 38, 39, 43 and 44 xenografts (5 × 106 cells), were incubated for 45 min on ice with 10 μg of fluorescein isothiocyanate-labeled mouse anti-human CD46 (BD Pharmingen, San Jose, CA, USA) in Dulbecco’s phosphate-buffered saline containing 0.5% bovine serum albumin, fluorescein isothiocyanate-labeled mouse IgG2a, κ serving as the isotype control. The cells were washed twice, fixed in Dulbecco’s phosphate-buffered saline containing 0.5% paraformaldehyde and were analyzed using a Becton Dickinson FACSscan Plus cytometer (Franklin Lakes, NJ, USA) with Cell Quest software.

**Assessment of cytotoxicity of MV-strains against GSC**

Neurospheres were enzymatically and mechanically disrupted in TrypLE (Invitrogen, Carlsbad, CA, USA) with a 10-m pipette; 5 × 106 cells were plated into six-well dishes and infected 24 h later at an MOI of 0.1 or 1.0 with MV-GFP, MV-CEA or MV-NIS, Uninfected cells were used as controls. Cytotoxicity of MV-strains against GSC was evaluated by counting the number of viable cells by trypsin blue exclusion assay as previously described18 and compared against uninfected controls.

**Assessment of viral growth in GSC**

Viral growth was assessed by one-step viral growth curves as previously described.18 In summary, neurospheres were infected with MV-NIS, MV-GFP or MV-CEA at an MOI of 1. Cells were harvested at different time points and viral titer was determined by 50% end point dilution assay on Vero cells, as previously described.57

**Tumorigenicity experiments**

Neurospheres derived from GBM44 xenografts were infected with MV-NIS in vitro at a MOI of 10 TCID50. Mock-infected neurospheres were used as controls. Six hours later, the cells were harvested, mechanically disrupted and subsequently 3 × 105 cells were orthotopically implanted into the right caudate nucleus of 5- to 6-week-old BALB/c mice (n = 5 per group). Mice were followed daily and euthanized when neurologic symptomatology or >10% weight loss was observed. All animal experimental protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

**Assessment of therapeutic efficacy in vivo**

GBM6 and GBM12 orthotopic models were established by implantation of 3 × 106 GSC into the right caudate nucleus of 5-week-old BALB/c mice, using the small animal stereotactic frame (ASI Instruments, Warren, MI, USA) and a 26-gauge Hamilton syringe. Treatment was initiated at either 1 or 2 weeks post-implantation, respectively. Mice received either MV-GFP (N = 5) or UV-inactivated virus as control (N = 5) using the same coordinates as for implantation. MV-GFP-treated mice received 1 × 105 (GBM12 GSC model) or 2 × 105 (GBM6 GSC model) TCID50 per dose three times per week over a 3-week period for a total dose of 9 × 105 or 1.8 × 106 TCID50, respectively. Mice were observed daily and were euthanized when neurologic symptomatology or >10% weight loss was observed.

In a parallel experiment, designed to incorporate correlative studies in treated tumors, 3 × 106 GBM6 derived GSC (a line expressing the stem-cell marker CD133) were implanted into the right caudate nucleus of 5- to 6-week-old BALB/c mice as previously described. One group of animals (n = 10) received MV-GFP, 1.5 × 105 TCID50 per dose every second day for a total of three doses starting at 2 weeks following implantation and a second group treated with UV-inactivated virus served as the control (n = 5). Treated mice and corresponding controls were euthanized either at 3 days following completion of viral treatment (n = 5) or when mice became moribund (n = 5). Animal brains were formalin-fixed, stained with H and E and examined by immunofluorescence for CD133 expression and in situ hybridization, for viral N-mRNA.

**Immunofluorescence for CD133 expression in vivo**

Formalin-fixed brains were examined for CD133 expression by immunofluorescence: following heat-mediated sodium citrate antigen retrieval, slides were blocked in PBS containing 10% goat serum, 1% bovine serum albumin and 0.025% Triton-X-100 for 1 h and incubated overnight at 4°C with 0.1% DTT followed by primary antibody (AbCam Inc., Cambridge, MA, USA; 1:200 dilution) or without primary antibody control. The slides were rinsed three times for 5 min in PBS and exposed to Alexa-Fluor 647 goat anti-rabbit (Invitrogen, Eugene, OR, USA). Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and examined with a Zeiss LSM510 (Maple Grove, MN, USA) confocal microscope.

**In situ hybridization for detection of MV N (nucleoprotein)-mRNA**

A custom-designed DIG (digoxigenin)-labeled oligonucleotide probe (GeneDetect) was used for ISH. The samples were treated with Proteinase K (20 mg ml−1) for 20 min at 37°C, washed twice with PBS/Glycine and prehybridized for 2 h at 39.4°C. The sections were then washed and incubated overnight with the DIG-labeled oligonucleotide probe (250 ng) at 39.4°C in a humidified chamber. The next day the slides were washed in SSC/DTT followed by the detection step performed using anti-DIG-AP. Fab fragments (Roche Diagnostics, Indianapolis, IN, USA) at 4°C in a humidified chamber, followed by serial washes, NBT/BCIP detection and counterstaining with Nuclear Fast red (Sigma).

**Immunofluorescence for natural killer cell lectin-like receptor G1 and CD68**

In all, 5 μm sections were cut from formalin-fixed, paraffin-embedded mouse brains, deparaffinized with CitriSox (ThermoFisher Scientific, Waltham, MA, USA) and then rehydrated in graded ethanol baths with a
CONFLICT OF INTEREST

The authors declare no conflict of interest.

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