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Targeting HSV-1 virions for specific binding to epidermal growth factor receptor-vIII bearing tumor cells

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Abstract

Oncolytic herpes simplex virus (HSV) vectors have been used in early phase human clinical trials as a therapy for recurrent malignant glioblastoma. This treatment proved safe but limited improvements in patient survival were observed. The potency of these vectors might be enhanced by targeting vector infectivity to tumor cells. Glioma tumors often express a mutant form (vIII) of the epidermal growth factor receptor (EGFR) resulting in the presence of a novel epitope on the cell surface. This epitope is specifically recognized by a single chain antibody designated MR1-1. HSV-1 infection involves initial binding to heparan sulfate (HS) on the cell surface mediated primarily by the viral envelope, glycoprotein C (gC). Here we joined the MR1-1 single chain antibody (scFv) to the gC sequence deleted for the HS binding domain (HSBD) as a means of targeting viral attachment to EGFRvIII on glial tumor cells. Virions bearing MR1-1-modified-gC had 5-fold increased infectivity for EGFRvIII-bearing human glioma U87 cells compared to mutant receptor-deficient cells. Further, MR1-1/EGFRvIII mediated infection was more efficient for EGFRvIII-positive cells than was wild-type virus for either positive or negative cells. Sustained infection of EGFRvIII+ glioma cells by MR1-1-modified-gC bearing oncolytic virus, as compared to wild-type gC oncolytic virus, was also shown in subcutaneous tumors *in vivo* using firefly luciferase as a reporter of infection. These data demonstrate that HSV tropism can be manipulated so that virions recognize a cell specific binding site with increased infectivity for the target cell. The retargeting of HSV infection to tumor cells should enhance vector specificity, tumor cell killing and vector safety.

Keywords

EGFR; oncolytic; HSV; glioma; tropism; glycoprotein C; mouse tumor model; HSV vector retargeting

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Introduction

Malignant gliomas are the most common primary brain tumors and are almost universally fatal despite aggressive therapies, including surgery, radiotherapy, and chemotherapy.[1] Patients with glioblastoma multiforme (GBM) have a median survival of 12-18 months from initial diagnosis in spite of current treatments. Efforts to improve survival have included a variety of biologic therapies, such as viral vectors, monoclonal antibodies coupled to toxins and immunotherapy.[1-5] Several types of viral vectors derived from herpes simplex virus (HSV) are under development: attenuated replication-competent (replication-conditional) oncolytic recombinant virus (RV), replication-deficient RV and amplicon vectors.[6] Thus far, oncolytic HSV has been used in early phase clinical trials for a variety of tumors, including GBM.[7,8,1,9-11] Genetically engineered oncolytic HSV, such as G207 and 1716, have been found to be relatively non-toxic for normal cells with some evidence of virus replication in tumors. These oncolytic viruses are designed to target cells based on rapid cell division or alterations in signal-transduction pathways that promote tumorigenesis, such as Ras activation.[12] They selectively replicate in dividing tumor cells so as to generate new vectors on-site and to “leap frog” some distance from the main tumor mass by replication and infection of tumor foci in the brain parenchyma.[13]

Some progress has been made in targeting HSV infection selectively to tumor cells by using tumor-specific promoters to control viral genes critical to replication or by increasing infection of tumor cells.[14] Targeting of virion infection has the potential advantages of increasing infection of the appropriate target cell relative to normal cells, thereby increasing vector safety and potentially decreasing the effective vector dose. To accomplish targeted infection, virions must be modified to carry engineered ligands that bind specifically to tumor cells based on recognition of surface receptors. This depends on the identification of cell-specific surface receptor(s) that are more abundant on, or unique to target cells, and on modification of virions for recognition of these receptors without compromising infectivity. Successful targeting of vector infection to tumor cells has been achieved for other virus vectors, including adenovirus,[15,16] adeno-associated virus[17] and retrovirus/lentivirus.[18]

Infection by HSV involves initial binding of gC and gB envelope glycoproteins to heparan sulfate (HS) on the cell surface followed by binding of gD to natural receptors for HSV, HveA or HveC/nectin-1, and fusion between the virion envelope and cell plasma membrane.[19] Several attempts to alter the host range of HSV have been reported involving deletion of all of gC or its HS binding domain (HSBD) and/or the HSBD of gB.[20,21] Modified HSV-1 virions were targeted to cells expressing IL-13 receptors when the IL-13 ligand was introduced into gC or gD,[22] and virions with the his-tag preferentially infected cells expressing a pseudo-his-tag receptor.[23] HSV-1 binding has also been altered by the introduction of ligands into gC for the pre-SI peptide from hepatitis B virus which targets hepatocytes[21] and erythropoietin which targets its cognate receptor implicated in angiogenesis.[24] An alternative approach to introducing novel ligands into viral glycoproteins for targeted HSV entry involves the development of soluble adapter molecules designed to mediate both cell-specific binding and the gD-dependent fusion. The V-domain of HveC, which binds gD, was fused to a single-chain antibody (scFv) to the epidermal growth factor

receptor (EGFR) in an effort to target EGFR-bearing cells.[25] These reports demonstrate the feasibility of modifying the HSV virion to achieve specific cell targeting.

For cancer therapy, cell surface receptors for virus targeting should include ones enriched or mutated on tumor cells. A specific mutant form of EGFR receptor, EGFRvIII, is highly expressed on many glioblastomas, breast carcinomas, and other tumors[26] and is associated with increased invasiveness and growth rate of tumors.[27] Several antibodies have been described that are specific to EGFRvIII and do not cross-react with wild-type EGFR. One of these, Mutant Receptor 1 (MR1), was isolated by the Bigner laboratory from a scFv phage display library by panning with successively decreasing amounts of EGFRvIII mutant-specific peptide.[4] By mutagenesis and selection using a phage display library a variant of MR1 was identified, MR1-1, which has an approximately 15-fold higher binding affinity to the EGFRvIII extracellular domain as compared to MR1.[28] This group also generated a mutated form of MR1-1, MRB, with no affinity for EGFRvIII.

Here we report the generation of an oncolytic HSV vector targeted to glial tumor cells expressing EGFRvIII. The coding sequence for scFv MR1-1 was fused in-frame with gC lacking the HSBD (gCΔ). This recombinant gC molecule was designed to replace native HS-binding with EGFRvIII-specific binding. Virions containing only MR1-1-gCΔ (and no wild-type gC) were shown to infect EGFRvIII-expressing glioma cells with a 5-fold increase in efficiency as compared to wild-type gC virions in culture. Injection of MR1-1-gCΔ and gC wild-type virions encoding firefly luciferase (Fluc) into subcutaneous human glioma U87 tumors expressing EGFRvIII in nude mice revealed more sustained infection by MR1-1-gCΔ virions over the first week after infection, as compared to gC wild-type virions. These data demonstrate that HSV tropism can be shifted to recognition of tumor-specific receptors with apparent enhancement tumor-specific vector replication.

Results

Constructs and virus used to generate and monitor targeted virions

gC fusion proteins were generated in which full-length scFv sequences for antibodies with varying affinity for EGFRvIII were used to replace amino acids (a.a.) 33 to 174 of wild-type gC, thus eliminating the HS binding domain.[29] Antibody sequences for the extracellular EGFRvIII domain included those for the very high affinity scFv, MR1-1, and the mutated scFv, MRB with little-to-no affinity[4],[28] (kindly provided by Dr. Darell Bigner, Duke University Medical Center). Expression constructs for MR1-1-gCΔ, MRB-gCΔ, and gCΔ, all under the gC promoter, were inserted in the pCONG amplicon which also contains an expression cassette for green fluorescent protein (GFP) to monitor amplicon vector infection[30],[23],[31] (Fig. 1). To create stocks in which virions carried only modified gC in their envelope, cells were transfected with one of the scFv amplicons and then infected with a mutant HSV, gCΔ2-3, in which the gC gene is deleted and replaced with a *lacZ* expression cassette.[32] Controls included virions generated by transfection with pCONG amplicon carrying the wild-type gC gene and infection with gCΔ2-3, or a virus stock of the HSV mutant, hrR3, which encodes wild-type gC and has a *lacZ* expression cassette in place of the gene encoding the large subunit of ribonucleotide reductase.[33]

Characterization of targeted virions

To analyze incorporation of modified gC into virions, virus stocks including: hrR3 virus carrying wild-type gC; gCΔ2-3 virus lacking gC (gC-minus); and gCΔ2-3 virus packaged in the presence of the MR1-1-gCΔ amplicon were purified by banding on sucrose gradients to remove cellular proteins. Then the equivalent of 10^7 plaque forming units (pfu) per stock was left untreated or deglycosylated with endoglycosidase F (endo F) and resolved by SDS-PAGE with western blotting carried out using antibodies to envelope proteins, gC and gD, and to the tegument protein, VP16 (Fig. 2). Wild-type gC contains both N-linked and O-linked glycosyl residues, with only the N-linked side chains being sensitive to endo F. The N-glycosidase-treated gC wild-type virions showed a reduction in molecular weight with different sized bands representing the extent of remaining O-glycosylation. No gC immunoreactive band was seen for gCΔ2-3 virus. MR1-1-gCΔ virus revealed one gC band of 64 kD, which is consistent with the predicted single (glycosylation) event based on its sequence (Fig. 2A). Staining with antibodies to gD revealed a single band resulting from deglycosylation (Fig. 2B). Interestingly, there appeared to be somewhat more gD per infectious virion for MR1-1-gCΔ, and somewhat less for gC-minus virus, as compared to the wild-type virus for the same pfu. VP16 was also substantially increased in the MR1-1-gCΔ virions as compared to wild-type and gCΔ2-3 virions (Fig. 2C). Thus, the relative ratio of gD in infectious virions appeared to vary with the presence and type of gC. In the absence of gC, infectious virions had reduced levels of gD as compared to wild-type virions, while in the presence of modified gC there was an apparent increase in gD. Because a similar pattern of altered VP16 levels was observed, it is likely that these changes reflect an increase in particle to PFU ratios rather than gC-dependent alterations in gD incorporation into the vector envelope.

Binding and infection efficiency of MR1-1 modified virions for glioma cells with and without EGFRvIII receptors

In order to evaluate the differential infectivity of glioma cells expressing and not expressing EGFRvIII receptors, U87 and U87ΔEGFR (stably transduced with an expression cassette for EGFRvIII) cells were incubated with vectors for 60 min at 4°C, then cells were washed and incubated at 37°C for 24 hrs, and transgene expression (*lacZ*) was evaluated (Fig. 3). Infections employed similar transducing units (tu) (*lacZ*) of gC-minus (gCΔ2-3), and gC-minus virus packaged during transfection with MR1-1-gCΔ, MRB-gCΔ or gC amplicon DNA, as well as wild-type gC (hrR3). At low multiplicity of infection (M.O.I.) (0.015 pfu/cell) virions with gC bearing the high affinity antibody, MR1-1 showed increased infectivity (> 5-fold) for U87ΔEGFR cells as compared to U87 cells (Fig. 3). This phenomenon was less pronounced at higher M.O.I. (0.15), but still showed a 2-fold increase for MR1-1-gCΔ infection of U87ΔEGFR versus U87 cells (data not shown). Remarkably, the infectivity of the MR1-1-gCΔ modified virions was greater than that of wild-type virions for glioma cells expressing EGFRvIII by about 6-fold at M.O.I = 0.015 and a little less than 2-fold at M.O.I. = 0.15 (data not shown). No significant difference in binding/infection between U87 and U87ΔEGFR cells was seen for any of the other vector stocks, including gC-minus, MRB-gCΔ, and hrR3. Virions lacking gC had similar infectivity to those with wild-type gC at M.O.I. = 0.015. Further, virions prepared by transfection with an amplicon encoding gC and

infection with gC-minus virus, behaved like a gC wild-type virus (hrR3). These results indicate that MR1-1 in the context of gC deleted for the HSBD can increase infectivity for cells expressing the EGFRvIII, even beyond that of wild-type virus.

Relative efficiency of targeting of gC and MR1-1-gCΔ modified virions to U87ΔEGFR tumors in vivo

To monitor infection of tumors *in vivo* by bioluminescence imaging, amplicons were generated which encoded Fluc under the CMV promoter, termed HRCFluc. This amplicon was then co-transfected with pCONG amplicons (gC or MR1-1-gCΔ) and packaged with gC-minus helper virus.

U87ΔEGFR tumor cells (5×10^5) were implanted subcutaneously in nude mice and given 2 weeks to form small unilateral tumor. Two vector stocks were compared both generated using HRCFluc amplicon and gCΔ2-3 virus - one with MR1-1-gCΔ CONG and the other with gC CONG amplicon transfection. Tumors were injected directly with virus stocks normalized to 5×10^5 tu HRCFluc amplicon vector (containing $1.5\text{-}2.5 \times 10^7$ gC-minus helper virus and about 2×10^4 tu CONG amplicon vectors). Bioluminescence of tumors was tracked over a 1 week period (Fig. 4). Twenty-four hrs after infection a very strong bioluminescence signal was seen in all tumors. Forty-eight hrs after vector injection this diminished, but a robust signal was still seen in tumors infected with both vector types. After 1 week, however, a markedly stronger bioluminescence signal was seen in tumors infected with virions incorporating MR1-1-gCΔ versus gC. Since Fluc is expressed by the amplicon vector and equal numbers of TU were introduced into the tumor for amplicons containing either gC or MR1-1-gCΔ, the MR1-1-gCΔ vector provided a higher level of initial infection than the gC vector. This signal may have been amplified by possible intratumoral replication and packaging of the HRCFluc amplicon vector supported by gCΔ2-3 viral replication. This *in vivo* experiment supports the ability of MR1-1-gCΔ virions to increase infectivity of tumor cells expressing EGFRvIII, as compared to gC virions.

Discussion

This study demonstrates that infectivity of HSV virions can be directed to specific cell surface receptors by fusion of receptor-specific scFv antibody sequences to the N-terminus of the gC envelope glycoprotein, lacking the HSBD. In this case MR1-1 antibody sequences were used, which bind with very high affinity to mutant EGFR (EGFRvIII) found on many tumor cells.[4] Infectivity of MR1-1-gCΔ virions was increased over 5-fold for human glioblastoma cells expressing this mutant receptor as compared to virions bearing wild-type gC at low M.O.I.. This low M.O.I. mimics the relatively low ratio of vector to tumor cells expected in therapeutic paradigms *in vivo*. Further, the targeted virions achieved markedly higher infectivity for cells expressing EGFRvIII as compared to wild-type virus, indicating that the infection efficiency of HSV can be selectively increased for specific cell types above natural levels of infectivity by genetic manipulation of the virion surface. This targeted virion design was also found to be effective in prolonging expression of a luciferase reporter gene in subcutaneous glioma tumors following direct injection of vector incorporating MR1-1-gCΔ fusion protein as compared to gC. Further studies are in progress

to increase the specificity of this HSV oncolytic vector by introducing the MR1-1 binding ligand into additional HSV glycoprotein envelope molecules.

Similar vector retargeting studies were recently reported by Paraskevakou et al.[34] using replication competent measles virus. They generated a retargeted virus by recombining a single chain antibody specific for EGFR into the virus hemagglutinin deleted for the natural CD46 or SLAM receptor recognition sites. The measles vector differed from our MR1-1-gCΔ HSV vector in as much HSV was shown to recognize with enhanced binding activity only the mutant form of the EGFR receptor found on glioblastoma cells and infection was more efficient than wild-type HSV. The measles vector, however, bound both the mutant and wild-type form of EGFR. This difference may impart a measure of enhanced safety for the HSV RV since wild-type EGFR is expressed in normal brain cells.[35]

To evaluate infection efficiency of different virions, amplicon vectors expressing gC or modified gC were produced together with replication competent helper virus that was gC deficient. In this way gC or modified gC was incorporated into the envelope of both the amplicon and helper virus virions in the same stock. This strategy relies on plasmid-based amplicons to supply and screen targeted glycoproteins and avoids the more labor-intensive process of generating recombinant viruses encoding various modifications of envelope proteins. This same approach could be used to modify gB and/or gC using gC-minus and gB-minus virus which are replication competent. By using amplicons to produce modified glycoproteins one simplifies constructions by plasmid based recombination and transfection of cells. Thus, by infecting amplicon-transfected cells with replication competent virus deleted or mutated in viral glycoprotein genes, modifications which enhance selected infection can be readily screened. This screening is further simplified by monitoring infection using fluorescent or *lacZ* reporter genes in culture or bioluminescent enzymes *in vivo* incorporated into amplicon vectors or virus.[36-39] This strategy should also be effective in combination with an oncolytic virus vector with wild-type gC, as the overexpression of the targeted form of gC can result in greater levels of modified gC incorporated into virions than wild-type gC. For example, we previously observed that infection by the oncolytic virus, hrR3 along with transfection of an amplicon plasmid encoding his-tagged gCΔ resulted in enveloped virus particles that contained a high ratio of gC-his-tag compared to wild-type gC.[23] Treatment of engineered target cells bearing a pseudo his-tag receptor with anti-his-tag antibodies reduced the efficiency of infection and plaque formation by these his-tagged virions by 1-2 logs.

HSV recombinant and amplicon vectors have been used separately and in combination for gene therapy for brain tumors.[6,40] Therapeutic genes can be incorporated into both replicative RV and amplicon vectors and co-produced, allowing the use of amplicons for both virion targeting and for expression of other genes which may increase tumor killing and oncolytic vector effectiveness.[41-43]

This study demonstrates the ability to increase infectivity of HSV virions for specific cell types by modification of envelope glycoproteins. In this case a high affinity antibody expressed on the virion to the mutant EGFR present on glioma cells was able to increase infection efficiency in culture by 5-fold at low M.O.I and to sustain transgene expression in

tumors *in vivo*. The enhancement of targeted infection at lower M.O.I. corresponds to decreasing concentrations of oncolytic virus during propagation and spread within the tumor *in vivo*. Vector targeting also provides an added safety advantage in that targeted vectors can selectively infect tumor cells as compared to normal cells, thus reducing toxicity to normal cells. Given that glioblastomas are heterogeneous and continually undergoing changes in tumor characteristics and cell surface markers, virion predicted to target different tumor cell phenotypes might be achieved by tailoring the specificity of infection to different tumor surface receptors.

Materials and Methods

Cell culture

Vero 2-2 (2-2) cells stably transfected with the HSV gene for ICP27 (from Dr. Rozanne Sandri-Goldin, University of California at Irvine, CA),[44] U87MG cells (from the American Type Culture Collection) and U87MGEGFR (here termed U87ΔEGFR, expressing the mutant EGFRvIII receptor from Dr. Web Cavenee, Ludwig Cancer Inst., CA) were maintained in DMEM growth medium supplemented with 200 μM L-glutamine (Invitrogen, Carlsbad, CA), 100 units/ml penicillin and 100 g/ml streptomycin (Sigma, St. Louis, MO), and 10% fetal bovine serum (Sigma). The U87ΔEGFR cell line was generated by transfecting U87MG cells with as expression cassette for EGFRvIII and neoR[45] and expresses 4-13 × 10^5 EGFRvIII receptors per cell in culture.[46] Expression of EGFRvIII on U87ΔEGFR and lack of expression on U87 cells was confirmed by SDS-PAGE and western blot analysis of cell lysates using L8A4 antibody (kindly provided by Dr. Darrell Bigner, Duke Univ. Med. Ctr.) (data not shown). Geneticin (G418; Invitrogen) was added to 2-2 and U87ΔEGFR cells at 400 g/ml to select for continued transgene expression.

Helper virus stock

The HSV recombinant viruses used in this study were hrR3 (ICP6-minus, *lacZ*$^+$ mutant from Dr. Sandra Weller, Univ. Conn.)[33] and gCΔ2-3 (gC-minus, *lacZ*$^+$; from Dr. Curtis Brandt, Univ. Wisc.),[32] both derivatives of HSV strain KOS.

hrR3 and gCΔ2-3 stocks were prepared by infecting 2-2 cells at an M.O.I. of 1 tu/cell. Cells and medium were harvested after approximately 16-24 hrs when the cells displayed 100% cytopathic effect and subjected to three cycles of freeze-thawing (alternating between −80°C and 37°C baths). The virus were then separated from cell debris by low speed centrifugation (500 g × 10 min at room temperature) and the supernatant concentrated by centrifugation at 72,000 x g for 3 hrs at 4°C through a 25% sucrose cushion.[47] Virus stocks were resuspended in phosphate buffer saline (PBS), pH 7.4 and aliquoted into 2 ml cryogenic tubes (Corning Glass Works, NY), stored at −80°C and titered by transduction assay. This virus was titered either as tu (*lacZ*+ cells; see below) or plaque forming units (pfu) on Vero cells.[23]

Titering by transduction

2-2 cells (1.5 × 10^6 per well in 6-well plates) were infected in a total volume of 1 ml DMEM with dilutions of virus stocks (10^2 to 10^{11}) in duplicate. After incubation at 37°C for 2 hrs, the viral solution was aspirated, the cells washed twice with PBS and replaced with normal

growth medium. For *lacZ*+ virus, cells were fixed 24 hrs later in 4% paraformaldehyde (PFA; EMS, Fort Washington, PA) in PBS for 10 min at room temperature. Cells were washed twice with PBS and stained with X-galactosidase (X-gal) solution, pH 7.4, overnight at 37°C.[48] For amplicon vectors, cells were visualized for expression of enhanced green fluorescent protein (GFP) for CONG vectors or red fluorescent protein (RFP) for HRCFluc vectors 24 hrs post-infection by fluorescence microscopy.

Amplicon construction

The full-length HSV-1 gC gene with its own promoter was subcloned between the *Pst*I and *Hind*III sites in an amplicon plasmid (pCONG) bearing an expression cassettes for GFP and neomycin resistance (Neo^r) (from pcDNA3.1 (−) Invitrogen) inserted into a unique XhoI site downstream of the gC gene.[30] The HS binding domain of gC was deleted by digestion with AscI and EcoNI, corresponding to removal of a.a. 33 to 174 (423 bp), and replaced with in-frame sequences for MR1-1, the single chain antibody able to bind specifically and with high affinity to the EGFRvIII or with a mutated form of that antibody, MRB, that no longer binds to the receptor (from Dr. Bigner). MR1-1 and MRB sequences were amplified by PCR from plasmids pHRST MR1-1 and PHRST MRB[28] using the following primers: 5′ primer: 5′-CATGGCGCGCCCGATGGACTGGATTTGGCGC-3′ and 3′ primer: 5′-CAGGACCTCCTCTAGGTCTAGTTTTTCAAGCTTGGT-3′.

The firefly luciferase cDNA (Promega, Madison, WI) was subcloned into the *Nhe*I and *XbaI* restriction sites of the amplicon plasmid pHRCX, derived from pHCGX[49] with the eGFP expression cassette replaced by one for *Discosoma* red fluorescent protein (DsRed2; Clontech).[50]

Generation and titering of amplicon vector and helper virus

To generate vector stocks, amplicon plasmids, pCONG, pCONG-MR1-1-gCΔ, pCONG-MRB-gCΔ and pHRCFluc were transfected separately into 2-2 cells (6×10^6 per 100-mm plate) using LipofectAmine (Invitrogen) according to manufacturer's protocol. Twenty-four hrs post-transfection, the cell monolayers were infected at an M.O.I. of 1 with gCΔ2-3 virus. Virus was harvested when 100% cytopathic effect was evident and concentrated as described above. Titers of amplicon vector and helper virus were determined by transduction assay counting GFP-positive or RFP-positive cells for amplicon vectors and *lacZ*-positive cells for helper viruses.

Typically titers were $1 – 5 \times 10^9$ tu/ml for helper virus and $1 – 10 \times 10^7$ tu/ml for amplicon vectors. For *in vivo* experiments vector stocks were generated which contained gCΔ2-3 helper virus and two amplicon vectors: HRCFluc expressing RFP/Fluc and either the CONG-gC encoding wild-type gC or CONG-MR1-1-gCΔ (or CONG-MRB-gCΔ) encoding a recombinant protein including scFv MR1-1 (or MRB) and gC deleted for HSBD. Virus titers for stocks used in *in vivo* experiments were as follows: CONG-MR1-1-gCΔ/HRCFluc/gCΔ2-3 contained CONG amplicon (GFP+) = 5×10^7 tu/ml, HRCFluc amplicon (RFP+) = 1×10^8 tu/ml and gCΔ2-3virus (*lacZ*+) = 3.3×10^9 tu/ml; and CONG-gC/HRCFluc/gCΔ2-3 contained CONG amplicon (GFP+) = 1.7×10^7 tu/ml, HRCFluc amplicon (RFP+) 5×10^8 tu/ml and gCΔ2-3 virus (*lacZ*+) = 5×10^9 tu/ml.

Western blot analysis of modified gC in virions

Amplicon vector and virus stocks were prepared as above. Stocks were spun at 1500 RPM in IECCentra-8R tabletop centrifuge for 5 min and supernatants (approximately 30 mL) were layered on sucrose gradients consisting of 3 ml 10% sucrose, 7 ml 30% sucrose and 7 ml of 60% sucrose in PBS and centrifuged at 25,000 rpm in an SW28 rotor at 4°C for 1 hr. Pelleted virus was resuspended in PBS and centrifuged repeated. Then virion pellets (equivalent to about 10^7 pfu each) were resuspended in lysis buffer [50 mM Tris, pH 8, 150 mM NaCl, 0.02% Na azide, 0.1% SDS, 1% Triton X100, 1x Protease Inhibitor cocktail (Roche, Indianapolis, IN)]. Half of samples were treated with N-glycosidase F according to manufacturer's instructions (New England Biolabs, Beverly, MA), which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins. Untreated and glycosidase-treated samples were resolved by electrophoresis in a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with antibodies specific for gC (1:10000; rabbit 47 polyclonal antibody; kindly provided by Drs. G.H. Cohen and R.J. Eisenberg, Univ. Penn.)[51], gD (1:1000 mouse monoclonal; Goodwin Labs., Plantation, FL) and VP16 (1:500 mouse monoclonal LP1, kindly provided by Dr. Tony Minson, Division of Virology, Department of Pathology, Cambridge University, United Kingdom) in 2% milk, 1X TBS 0.05% Tween 20 (TBS-T). The membranes were then washed 3 times with TBS-T and incubated for 1 hr at room temperature with anti-mouse or anti-rabbit HRP-conjugated antibody (Sigma) in 5% milk TBS-T solution. After incubation, the blots were washed again and antibody signal revealed by ECL (Pierce, Rockford, IL).

Binding and penetration assay

Monolayers of confluent U87 and U87ΔEGFR cells (1.5×10^6 per well in 6-well plates) were incubated at 4°C for 30 min, washed twice with cold PBS and incubated with different virus stocks [gCΔ2-3 (gC^-), CONG-MR1-1-gCΔ, gC^-, CONG-MRB-gCΔ', CONG-gC^+ gC^- and hrR3] at identical titers (10^5 tu *lacZ*/well, 6-well plate) in triplicate. The viruses were allowed to bind for 60 min at 4°C, after which the unbound virus was removed by washing the cells 3 X with cold PBS. Cells were then shifted to 37°C to allow virus penetration. Twenty-four hrs post-infection, cells were stained for *lacZ* to determine the number of cells infected.

In vivo studies

Athymic nude mice (nu/nu; 8 weeks old, females; Mass. General Hospital Colony) were implanted with U87ΔEGFR tumor cells (5×10^6 in 100 ml DMEM) subcutaneously into one flank of each mouse. Two weeks later tumors were injected with 5×10^5 tu (HRCFluc) vector stocks in 5 in l using MR1-1-gCΔ CONG + HRCFluc + gCΔ2-3 or gC CONG + HRCFluc + gCΔ2-3 stocks. At 24 hrs, 48 hrs and 1 week after vector injection mice were imaged for Fluc activity by intraperitoneal injection of D-luciferin (4.5 mg/animal in 150 l saline) and photon counts recorded 10 min later using a cryogenically cooled high efficiency change-coupled device camera system (Roper Scientific, Trenton, NJ). Post-processing and visualization was performed as described.[37]

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Abbreviations

GBM	glioblastoma multiforme
HSV	herpes simplex virus
EGFR	epidermal growth factor receptor
HS	heparan sulfate
gC	glycoprotein
MR1	antibody to mutant EGFRvIII
GBM	glioblastoma multiforme
RV	recombinant virus
HSBD	HS binding domain
Fluc	firefly luciferase
a.a.	amino acid
pfu	plaque forming units
PBS	phosphate buffer saline
PFA	paraformaldehyde
X-gal	5-bromo-4-chloro-3-indolye-β-D-galactopyranoside
endo F	endoglycosidase F
M.O.I.	multiplicity of infection
GFP	green fluorescent protein
RFP	red fluorescent protein
tu	transforming units

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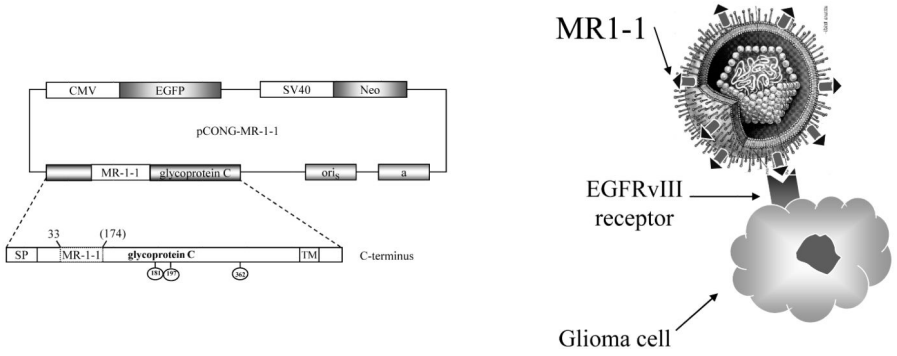

Fig. 1. MR1-1-gCΔ construct

On the let panel, structure of HSV-amplicon vector pCONG-MR1-1 encoding MR1-1-gCΔ. The MR1-1 sequence was inserted in-frame within gC replacing the a.a. residues 33 to 174 with MR1-1 to create the recombinant fusion protein MR1-1-gCΔ under the control of the gC promoter. This amplicon also bears the transgene cassette for GFP under the CMV promoter and the *neo*^{R} under the SV40 promoter. Abbreviations: SP, signal peptide; TM transmembrane domain; Circles, N-linked glycosylation site; a.a., position number. On the right panel, schematic representation of amplicon bearing MR1-1-gCΔ infecting glioma cells by specific binding between MR1-1 and EGFRvIII.

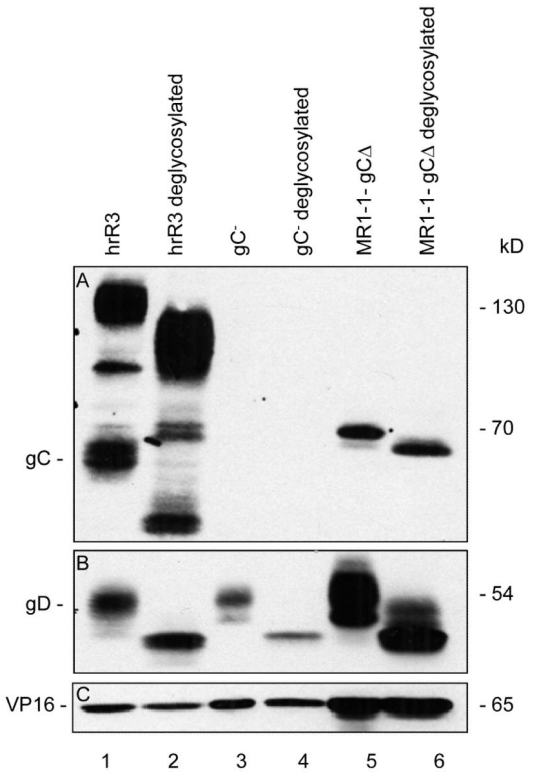

Fig. 2. Western blot of virions

293 cells were infected with hrR3 virus (lanes 1 and 2) or gC-minus virus (lanes 3 and 4), or were first transfected with pCONG-MR1-1 plasmid and then 24 hrs later infected with gC-minus virus (lanes 5 and 6) Virions were harvested and purified by density gradient centrifugation, and then virion proteins were resolved by SDS-PAGE and western blot analysis carried out with antibodies to gC (Panel A), gD (Panel B) or VP16 (Panel C) with stripping of signal between antibodies. In lanes 2, 4 and 6 virion proteins were treated with endo F prior to SDS-PAGE.

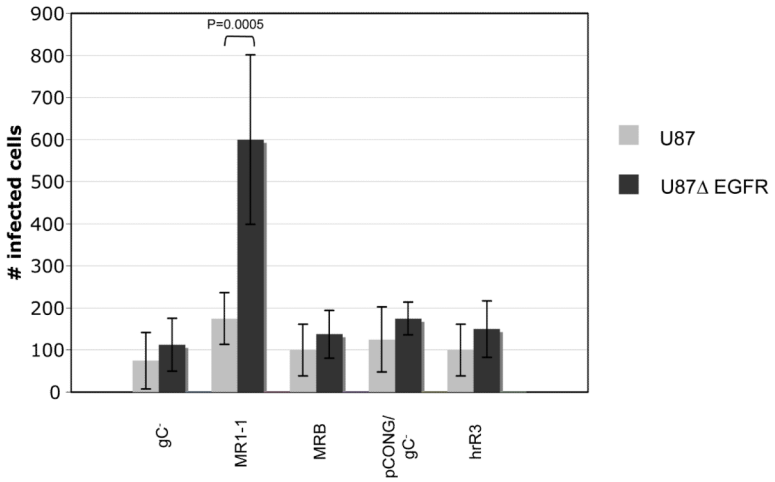

Fig. 3. Binding and infection efficiency of virions containing wild type and modified gC for U87 and U87ΔEGFR cells

Monolayers of U87 (solid) and U87ΔEGFR (open) glioma cells (1.5×10^6 per well in 6-well plates) were incubated with different concentrations of virus stocks, including lane: 1) gCΔ2-3(gC-minus, *lacZ*+); 2) CONG-MR1-1 (gC modified to include MR1-1 in gCΔ2-3); 3) CONG-MRB in gCΔ2-3; 4) CONG-gC in gCΔ2-3 and hrR3 (gC+). Cells were infected with: 10^3 tu/well corresponding to *lacZ* – in gCΔ2-3 or hrR3 (equivalent to M.O.I. = 0.015); in triplicate. Results represent the mean ± standard deviation of three independent experiments.

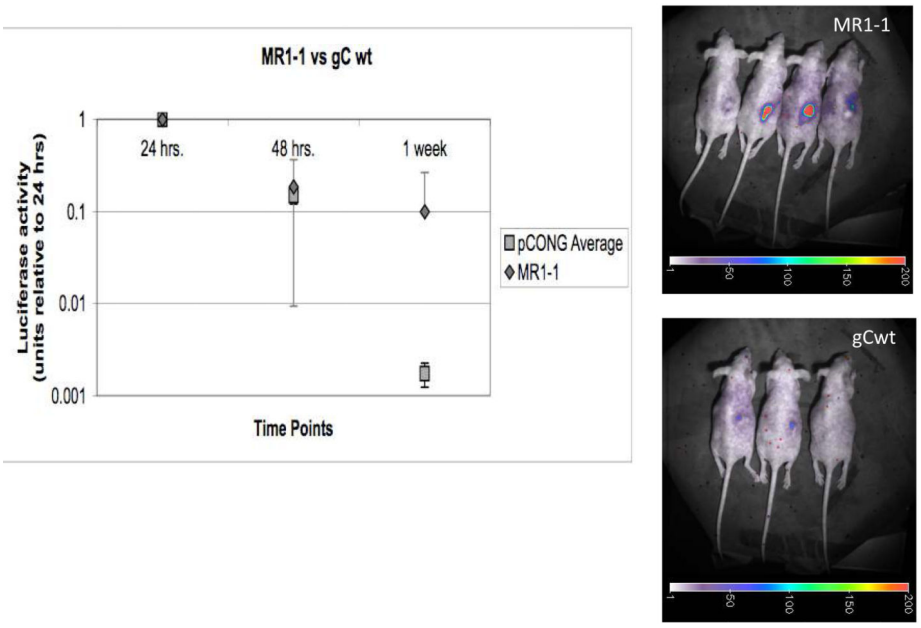

Fig. 4. Bioluminescence in tumors in vivo

Subcutaneous U87ΔEGFR tumors were injected with a combination of either MR1-gCΔ CONG + HRCFluc + gCΔ2-3 or gC CONG + HRCFluc + gCΔ2-3 vector stock and Luciferase activity was measured at 24 hrs, 48 hrs and 1 week after vector injection using bioluminescence imaging system. Left panel: The plotted values represent the average ratio between the photon counts per second measured that day and the photon counts measured on day 1 for each animal in tumor area ± S.D. Right panel: Representative pictures of bioluminescence emission from the U87ΔEGFR tumor of mice injected with amplicon vector carrying MR1-1-gCΔ (upper panel) or wild type gC (lower panel).