Oncolytic Herpes Simplex Virus Type-1 Therapy in a Highly Infiltrative Animal Model of Human Glioblastoma

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Abstract

We have examined the spread and antitumor efficacy of an oncolytic herpes simplex virus-1–based vector (G207) in glioblastoma biopsy spheroids in vitro and in vivo after local delivery to corresponding intracranial xenografts. Spheroids from three patients were infected with increasing doses of G207 and transgene expression was quantified. Other infected spheroids were followed for 10 days to assess cytotoxic effects. For the in vivo study, spheroids were grafted intracerebrally into Rowett nude rats. The resulting highly infiltrative xenografts were injected with 3.4 × 106 plaque-forming units (penetration study) or 6.8 × 108 plaque-forming units (therapeutic study) of G207 using microprocessor-controlled stereotaxic delivery. Vector spread was tracked by histochecmical staining. In the therapeutic study, tumor volumes were monitored weekly by magnetic resonance imaging, and survival data were collected. In vitro, lacZ expression was seen at the spheroid surfaces 24 h postinfection, whereas the spheroid cores were transgene positive after 96 h. Cytotoxic susceptibility varied between the patients, showing a 36% to 95% lysis 10 days postinfection. Local delivery of G207 into intracranial xenografts resulted in extensive vector spread throughout the lesions. In the therapeutic study, G207 application reduced tumor volumes compared with controls, but did not significantly improve survival of the animals. Histologic analysis revealed infection of host structures such as the ventricular and choroid plexus ependyma. In conclusion, G207 replicates in patient-derived glioblastoma multiforme xenografts and tumor volumes are reduced after intratumoral delivery; however, the survival data suggest that the therapeutic effect could be improved by repeated vector application or through combination with other treatment modalities.

Glioblastoma multiforme, the most frequent primary brain tumor, is associated with a poor prognosis. Currently, the median survival of younger patients (<50 years) is 8.8 months, whereas those older than 80 years live only 1.6 months (1). Such disappointing figures underscore the need for improved therapeutic strategies.

The widespread use of cell line–based tumor models has compromised the clinical relevance of experimental data obtained from preclinical testing (2). The rationale is that established tumor cell lines go through an artificial selection process in culture, which reduces their genetic diversity. For instance, monolayer cell cultures derived from glioblastoma multiforme samples undergo a number of detectable phenotypic changes, characterized by a mesenchymal differentiation (3). On transplantation to rodents, glioma cell lines often grow as hypercellular lesions with demarcated borders toward the host brain tissue (4). Therefore, they do not reflect the highly infiltrative dissemination seen in glioblastoma multiformes in situ (4, 5). We have established a more representative xenograft model by transplanting multicellular tumor cell spheroids generated from patient biopsy samples without previous adaptation to monolayer (5, 6). Biopsy spheroids maintain several of the biological characteristics of the original lesion, such as the presence of preserved vessels, connective tissue, and macrophages (6). After intracerebral implantation into immunodeficient rodents, bona fide glioblastoma multiformes develop, which display central necrotic areas, endothelial proliferations, thrombosis (5), and extensive single-cell infiltration into the host brain tissue (7). We have previously applied this model to study viral gene transfer to glioblastoma multiforme (8–10) as well as in studies of tumor cell invasion (11).

In initial gene therapy trials for glioblastoma multiforme, replication-defective vectors carrying toxic or therapeutic transgenes have been delivered in situ to patients undergoing surgical resection (12–15). Unfortunately, these vectors failed to transduce sufficient quantities of tumor cells. Inefficient gene delivery has been attributed to poor penetration of the...
viral particles in solid tumor tissue as well as to the lack of infiltration by vector-producer cells (16, 17). To enhance penetration ability in solid tissues, tumor-selective, conditionally replicating viruses (oncolytic vectors) have been developed (18). After intratumoral inoculation, oncolytic vectors undergo continuous replication, resulting in an active spread of the cytotoxic effect. By deleting essential viral genes, oncolytic vectors have been restricted to cancer cells, in which the aberrant expression of oncoproteins complements their defects (18).

G207 is a multi-mutated, oncolytic vector derived from herpes simplex virus type 1 (HSV-1). It was generated by deleting both copies of the HSV-1 ICP6 gene as well as inserting the Escherichia coli lacZ sequence in the ICP6 gene (19). γ34.5 is a major determinant of neurovirulence; its mutants fail to interfere with the protein kinase R–mediated shutdown of protein synthesis that occurs on viral infection in host cells (20). Thus, G207 is generally precluded from replicating in nontransformed cells. By inactivating ICP6, which encodes the large subunit of ribonucleotide reductase, a key enzyme for DNA synthesis in nondividing cells, G207 has been further attenuated (21). Other safety features include hypersensitivity to acyclovir and temperature sensitivity, which terminates viral replication in case of encephalitis-associated fever. Intratumoral delivery of G207 resulted in significantly extended survival periods for animals challenged with U87 glioma xenografts (19). Currently, G207 is under clinical evaluation for malignant brain tumors. Early reports suggest a nontoxic profile in humans (22).

The current study was designed to evaluate the spread and oncolytic effect of G207 in spheroids generated from glioblastoma multiforme patient biopsy tissue and in corresponding xenografts in nude rats. We show that G207 infects and lysed primary glioblastoma cells in vitro and in vivo. Site-directed delivery of 6.8 × 10^6 plaque-forming units (pfu) of G207 into intracerebral xenografts in a therapeutic setting resulted in a significant reduction in tumor volumes up to day 29 after inoculation. The median survival of animals was prolonged by 10 days, but not found to be significant. Histologic analyses revealed that in single animals, G207 also infected host brain structures such as the ependymal lining and the choroid plexus. We believe that our data have direct clinical implications and suggest that site-directed delivery of G207 could be preferentially combined with other treatment modalities to obtain an enhanced therapeutic benefit.

### Materials and Methods

**Vector production.** G207 vector was obtained from MediGene AG. The vector was produced as described (19). The stock was filtrated, concentrated by size-exclusion chromatography, and tested for sterility and endotoxin. The infectious titer of the stock solution was 3.4 × 10^9 pfu/mL.

**Tissue culture.** Tumor fragments from six glioblastoma multiforme patients were obtained at surgery (GBM1-GBM6). The collection of biopsy tissue was approved by the regional ethical committee. Tissue specimens were taken from viable tumor areas that corresponded to regions with contrast enhancement on preoperative magnetic resonance imaging scans. The specimens were transferred to test tubes containing complete growth medium, and spheroids were prepared as previously described (6). Briefly, tissue samples were minced into ~0.5-mm fragments and placed into 80-cm^2 tissue culture flasks (Nunc) base-coated with 0.75% agar (Difco). The spheroids were maintained in a standard tissue culture incubator with 5% CO_2 and 100% relative humidity at 37°C. The medium was changed once a week. Spheroids with diameters between 400 and 600 μm were selected for in vitro experiments and for intracerebral implantation.

**Dose-finding study.** Glioblastoma multiforme biopsy spheroids (GBM1-GBM3) generated from three different patients were placed in two 24-well plates base-coated with agar. Ten spheroids were placed in each well in 1-mL complete medium. The G207 stock solution was diluted in complete medium to the following final concentrations: 3.4 × 10^3, 3.4 × 10^4, 3.4 × 10^5, 3.4 × 10^6, and 3.4 × 10^7 pfu/mL. Spheroids from each patient were treated with all the different vector concentrations. Two wells for each spheroid type were infected with the same vector dose (total of 120 spheroids). For one of the parallels, the experiment was terminated after 24 h; the remaining spheroids were treated for 96 h. The experiments were done twice. After termination, the spheroids were harvested, frozen in liquid N_2, and sections were cut on a cryomicrotome (Leica CM3050S, Nussloch).

**Cytotoxicity study.** Tumor spheroids from patients GBM1, GBM2, and GBM4 were placed in 96-well plates base-coated with agar. There was one spheroid in each well. The applied vector concentrations were 3.4 × 10^5 and 3.4 × 10^6 pfu/mL in complete medium. Control spheroids received complete medium only. Spheroid volumes and morphology were assessed daily. The spheroid surface changes (ruffling) indicating damage and preceding spheroid dissociation were noted.

**Tumor inoculation.** Twenty-nine rats were implanted with biopsy spheroids generated from three patients. Of these, 14 rats implanted with either GBM4 or GBM5 spheroids were used to study vector dissemination. Fifteen rats were implanted with GBM6 spheroids and used to assess therapeutic effect. Surgery was done as previously described (8). The handling of the animals and the surgical procedures were done in accordance with the Norwegian Animal Act and the local ethical committee approved the protocol.

**Vector infusion.** One month postimplantation, the animals were anesthetized and prepared for vector injection. The skin was withdrawn to reveal the location of the craniotomy. For the penetration/dissemination study, 10 μL of G207 vector stock (3.4 × 10^7 pfu) were delivered using a glass syringe (model 701, Hamilton) secured in a microprocessor-controlled infusion pump (UMP 2-1, World Precision Instruments). The coordinates of injection were the same as used for spheroid implantation (3 mm to the right and 1 mm behind the bregma, 2.5 mm deep). Vector infusion was done in the course of 45 min. After infusion, the needle was left in place for 5 min to avoid vector reflux. The needle was slowly retracted and the skinfolds were closed with polyamide surgical thread. Following surgery, rats were allowed to recover in an incubator set at 35°C before returning them to the cages. Rats were sacrificed either 10 days (n = 11) or 24 h (n = 3) after vector injection.

For the therapeutic study, the animals were treated with two subsequent intratumoral injections, for a total G207 dose of 6.8 × 10^6 pfu, delivered in the course of one surgical operation. The total duration of the infusion was 45 min.

Eight animals were injected with vector and seven animals with sterile saline (control group). The animals were sacrificed when neurologic symptoms of tumor growth were evident.

**Preparation of brain sections.** The brains were removed, embedded in Tissue-Tek optimum cutting temperature medium (Sakura Finetek), and frozen in liquid N_2. Coronal cryosections of 8 to 12 μm were prepared and collected on coated glass slides (Superfrost Plus, Menzel). The sections were stored at -80°C. For immunostaining, 6-μm paraffin-embedded sections were prepared following routine procedures.

**β-Galactosidase (lacZ) histochemistry.** Staining was done as previously described (8). Negative control slides, containing sections of a
xenograft generated from the same patient spheroids without G207 treatment, were routinely included. Transgene-positive sections were counterstained with H&E and mounted for microscopic examination. For quantification, sections were counterstained with eosin only to avoid the interference of hematoxylin-stained nuclei with transgene-positive fields.

**Evaluation and quantification of lacZ expression.** During sectioning of infected brains, the section number and thickness were noted, enabling us to establish the extent of transgene expression in the z (anterior-posterior) plane. For quantification of lacZ expression, images were captured at ×40 magnification and analyzed by morphometry (see below). A threshold of blue color intensity was set to distinguish transgene-positive tissue from nontransduced areas.

To quantify transgene positivity in the spheroid sections, the entire spheroid area was first selected and measured. Thereafter, the lacZ-stained area was calculated and the percentage of the area with positive staining was estimated. This procedure was repeated three to seven times for each spheroid type and vector dose to determine the percentage of transduction.

**Light microscopy.** For images with magnifications of ×40 to ×400, a Nikon Eclipse E600 light microscope was used with an attached Nikon DXM1200 digital camera and Nikon Plan Fluor objectives (×4, ×10, ×20, and ×40; Nikon Instruments Europe BV). Image capture and quantification were done using the Lucia G morphometry software (Lucia G, Laboratory Imaging Ltd.). The program created images at $1,232 \times 972$ pixels, which were later processed for illustrations. For $\times 1,000$ images and for overview images at ×25 magnification, we used a Leica DMLB.

**Fig. 1.** Penetration ability and oncolytic effect of G207 in organotypic spheroids derived from patient biopsies. Spheroids were infected with $3.4 \times 10^6$ pfu of G207 and analyzed for transgene expression or oncolytic effect. A. Photomicrographs showing lacZ expression in the spheroids. At 24 h postinfection, transgene-positive cells were observed at the spheroid surface, whereas penetration into the spheroid core occurred 96 h postinfection. Original magnifications, ×200 (GBM2, 96 h) or ×400. Bar, 100 μm. B. Assessment of lacZ expression in infected spheroids. Images were captured and the blue areas were compared with the total area of the spheroid sections using computer software. Similar low levels of transgene expression were observed after 24 h. After 96 h, more extensive transduction was seen, with GBM2 showing most robust transgene expression. For GBM1 spheroids, we did not quantify transgene expression at 96 h as most of the spheroids were already disintegrated (asterisk). C. Curve showing the percentage of nondisintegrated tumor spheroids, followed up to 10 d postinfection. GBM2 spheroids were lysed most rapidly (starting at day 2) and most efficiently (95% of the spheroids lysed 10 d postinfection). Spheroids from GBM2 and GBM3 started disintegration 1 wk postinfection, and 63% and 36% of the spheroids were lysed 10 d postinfection, respectively. Noninfected patient spheroids served as controls. D. Photomicrograph showing a noninfected spheroid (top) with even edges and compact sphere-like morphology. An infected spheroid (bottom) displays ruffled edges and a flattened morphology, indicating cell death and disintegration of the three-dimensional spheroid structure. Original magnification, ×100. Bar, 300 μm.
Table 1. Cytotoxic effect of G207 on primary glioblastoma spheroids

<table>
<thead>
<tr>
<th>GBM patient</th>
<th>Vector dose (pfu)</th>
<th>LacZ expression after 24 h</th>
<th>LacZ expression after 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>GBM1</td>
<td>3.4 × 10⁶</td>
<td>Surface</td>
<td>Surface and center*</td>
</tr>
<tr>
<td></td>
<td>3.4 × 10⁵</td>
<td>Surface</td>
<td>Surface and center</td>
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<tr>
<td></td>
<td>3.4 × 10⁴</td>
<td>Single cells on the spheroid surface</td>
<td>Surface and center</td>
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<tr>
<td></td>
<td>3.4 × 10³</td>
<td>None</td>
<td>None</td>
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<tr>
<td></td>
<td>3.4 × 10²</td>
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<td></td>
<td>3.4 × 10⁵</td>
<td>Single cells on the spheroid surface</td>
<td>Surface</td>
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<tr>
<td></td>
<td>3.4 × 10⁴</td>
<td>None</td>
<td>Single cells on the spheroid surface</td>
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<tr>
<td></td>
<td>3.4 × 10³</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*The center of the spheroid was necrotic after 96 h, but some lacZ-positive cells were detected.

light microscope with Leica N Plan objectives (Leica Microsystems). The images were captured with an Olympus Colorview soft imaging system camera and the Olympus DP-Soft 5.0 software (Olympus).

**Immunostaining.** Paraffin-embedded tissue sections were placed in xylene for 2 × 3 min, absolute ethanol for 2 × 3 min, 96% ethanol for 2 × 2 min, and finally in distilled water for 30 s. Epitope retrieval was done by heating the sections in a steam cooker at 99°C for 20 min in 10 mmol/L citrate buffer at pH 6.0. The sections were probed with polyclonal rabbit anti–herpes simplex virus type 1 (B0114, DAKO) or mouse anti-human vimentin (M7020, DAKO) antibodies, both at 1:100 dilution. The sections were developed using the EnVision+ or the Envision Doublestain system (DAKO), with 3,3′-diaminobenzidine or Fast Red substrate, following the manufacturer’s instructions.

**Magnetic resonance imaging.** Axial T1-weighted MSME sequences (repetition time, 1,000 ms; echo time, 8.7 ms; slice thickness, 1 mm; field of view, 3.2 cm; matrix size, 256 × 256; 20 slices) were acquired after s.c. administration of contrast agent (1.0 mL of 0.5 mmol/mL Omniscan; Amersham Health AS). Axial T2-weighted RARE sequences were also acquired (repetition time, 4,200 ms; echo time, 36 ms; slice thickness, 1 mm; field of view, 3.2 cm; matrix size, 256 × 256; 20 slices) using the same slice positioning as for the T1-weighted images. During scanning, the animals were kept in anesthesia with 1.5% isofluorane (Schering-Plough) mixed with 50% air and 50% O₂.

**Evaluation of tumor take and establishment of experimental groups.** For all of the animals in the therapeutic study, magnetic resonance imaging was done to verify tumor take before vector injection. We calculated the tumor volumes of the animals 1 month postimplantation. The rats were paired after finding the two lesions with approximately the same size and a similar location and appearance. One animal was then assigned to the treatment group and one animal to the control group. The animals were scanned at three different time intervals postinjection (days 15, 22, and 29). Tumor volumes were calculated from T1-weighted and T2-weighted images using standard viewing and reformating software (Osiris version 4.19, University Hospital of Geneva, Switzerland).

**Statistical analysis.** Statistical significances of the tumor volume differences between the two groups were compared by the Mann-Whitney test. To test for differences in survival, we generated Kaplan-Meier plots and analyzed the data using the log-rank test. The SPSS 14.0.2 software package for Windows (SPSS, Inc.) was used for calculation and plot generation.

**Results**

**G207 infection of biopsy spheroids.** Biopsy spheroids generated from three patients were infected with doses of G207 ranging from 3.4 × 10⁵ to 3.4 × 10⁶ pfu. Staining for lacZ revealed transgene expression exclusively in the spheroids treated with the three highest vector doses. After 24 h, expression was mainly detected at the spheroid surface, whereas after 96 h, it was also seen in the spheroid core (Fig. 1A; Table 1). Spheroids derived from GBM1 were most permissive to infection at 24 h (Fig. 1B) and to cytotoxicity (Fig. 1C). Spheroids from GBM2 and GBM3 were infected at similar low rates after 24 h. After 96 h, penetration into the core was observed in both spheroid types, with significantly higher transgene expression for GBM2. The vector dose that led to the penetration of viral particles to the core in all spheroids was 3.4 × 10⁶ pfu. The dose of 3.4 × 10⁵ pfu also led to transgene expression in all the spheroids; however, less penetration was observed. Thus, the two highest vector doses were chosen for in vitro cytotoxicity studies.

**In vitro cytotoxicity.** The spheroids were defined as intact if the surface was smooth with no ruffling (Fig. 1D, top). Initial damage was characterized by a ruffling of the spheroid surface accompanied by an increase in spheroid volume (data not shown). This process was followed by spheroid disintegration (Fig. 1D, bottom). The number of intact and damaged spheroids was counted daily. The number of intact spheroids as a function of time is shown in Fig. 1C. Our data indicate that biopsy spheroids obtained from different patients present different sensitivities to G207. Spheroids obtained from GBM1 were extremely sensitive to the damage caused by G207, whereas spheroids obtained from GBM3 were more resistant.

**Quantification of transduced tissue areas and evaluation of vector spread.** Site-directed delivery of G207 into growing xenografts resulted in several intratumoral clusters (foci) of viral replication with high concentrations of infected tumor xenografts. Axial T1-weighted MSME sequences (repetition time, 1,000 ms; echo time, 8.7 ms; slice thickness, 1 mm; field of view, 3.2 cm; matrix size, 256 × 256; 20 slices) were acquired after s.c. administration of contrast agent (1.0 mL of 0.5 mmol/mL Omniscan; Amersham Health AS). Axial T2-weighted RARE sequences were also acquired (repetition time, 4,200 ms; echo time, 36 ms; slice thickness, 1 mm; field of view, 3.2 cm; matrix size, 256 × 256; 20 slices) using the same slice positioning as for the T1-weighted images. During scanning, the animals were kept in anesthesia with 1.5% isofluorane (Schering-Plough) mixed with 50% air and 50% O₂.

To assess the volume of human glioblastoma tissue that could be transduced in an in vivo setting by site-directed delivery, we added the transgene-positive tissue volumes from 23 subsequent sections. We found that a single injection of 3.4 × 10⁶ pfu G207 transduced ~7 mm³ of glioblastoma multiforme tissue. The presence of HSV type 1–specific antigens was determined by immunostaining. LacZ expression and HSV-1–specific proteins colocalized and showed the same distribution when
evaluated from parallel cryosections (data not shown), indicating that both methods detected vector replication.

Twenty-four hours postinjection, immunostaining revealed infection around the needle track (Fig. 2B). In rats sacrificed 10 days postinjection, HSV antigens were typically seen clustered in several foci encompassing 50 to 100 cells throughout the central tumor mass (Fig. 2C).

In nonxenografted animals, site-directed delivery of G207 into the corpus callosum/cortex region did not yield β-galactosidase or HSV antigen staining (data not shown). Similarly, tumor-bearing rats injected with saline did not show β-galactosidase or HSV expression (Fig. 2D).

Evaluation of tumor histology. The histologic features of the lesions used both in the penetration and the therapeutic studies were evaluated by a neuropathologist (H.M.). GBM4, which was used for the penetration study, exhibited massive infiltration into both hemispheres to almost all brain regions, including the hippocampus and gray matter. No vascular

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**Fig. 2.** Spread of G207 after injection in intracranial xenografts (GBM5). A, photomicrograph of coronal cryosections stained for β-galactosidase prepared from the same brain injected with $3.4 \times 10^5$ pfu of G207, analyzed 10 d postinjection. The macroscopic tumor-brain borders are marked by lines. The numbers indicate the distances traveled in caudal (posterior) direction from the first tissue slice collected. Magnified view shows the distribution of lacZ-positive cells in foci of replication as well as scattered single cells in the tumor center. Original magnification, ×40. Bar, 1 mm. B to D, paraffin-embedded coronal tissue sections immunostained against HSV-1 structural (capsid and core) proteins. B, a rat injected with $3.4 \times 10^5$ pfu G207, sacrificed 24 h postinjection, shows viral protein expression limited to the site of injection. Arrowhead indicates the needle track. Original magnification, ×25. Bar, 1 mm. C, a rat injected with $3.4 \times 10^5$ pfu G207, sacrificed 10 d postinjection, showing more widespread distribution of viral gene expression throughout the lesion. In this section, the injection track was overgrown by the tumor. Original magnification, ×25. Bar, 1 mm. D, control xenograft injected with saline. No HSV expression was observed. Arrowhead indicates the needle track. Original magnification, ×25. Bar, 1 mm. E, immunostaining against human-specific vimentin reveals single-cell infiltration by individual glioma cells to the hippocampus as well as the corpus callosum and the contralateral hemisphere (arrowheads). The lesion also exhibited local expansive growth, causing midline shift in the cortex (arrow). ctx, cortex; cc, corpus callosum; CA1, CA1 field of Ammon’s horn (hippocampus); IBl, inner blade of dentate gyrus. Original magnification, ×40. Bar, 1 mm.
proliferations or necrotic areas were noted. GBM5 (penetration study) was also a highly infiltrative lesion, with extensive single-cell migration along white matter tracts. Staining for human-specific vimentin confirmed the invasive nature of GBM5 (Fig. 2E). In this lesion, we noted beginning vascular proliferations, but no glomeruloid structures or necroses were seen. GBM6, used for the therapeutic study, displayed all of the “classic” glioblastoma multiforme hallmarks. This lesion was a solid tumor containing single infiltrative tumor cells. Glomeruloid vascular proliferations, thrombotic vessels, and necrotic areas with palisading were evident in GBM6 xenografts.

The histologic features of infected xenografts were compared with control tumors by light microscopy. We observed cellular changes consistent with HSV infection and replication. H&E-stained sections revealed the presence of eosinophilic intranuclear inclusions in tumor cell nuclei (Fig. 3A). Evidence of more advanced stages of cellular degeneration was also observed, such as dissolution of the nuclear membranes and vacuolar cytoplasmic degenerations (Fig. 3A). Another prominent feature was the presence of HSV-immunopositive multinucleated cells (Fig. 3B). Multinucleated cells were not seen in the control lesions, indicating that they were generated by viral infection rather than being a feature of neoplasia. In infected xenografts, necroses were generally devoid of HSV signal (Fig. 3C). Still, a few infected cells were usually found in necrotic areas as well (Fig. 3D). Vascular endothelia were markedly negative for HSV signal even in tumor regions with extensive HSV expression (data not shown). Histologic changes associated with viral infection and replication were not seen in noninfected lesions or in saline-injected control tumors (data not shown).

**Tumor development.** For the therapeutic study, 15 rats were implanted with spheroids derived from patient 6 (GBM6). Four weeks after implantation, the presence of tumors (defined as day 1 of scanning) was verified for all animals (Fig. 4A, left). In the control group, tumor progression was visualized on T2-weighted images, which showed areas with altered intensity around the implantation site, indicating tumor necrosis (hypointense, dark areas) and edema (hyperintense, light areas; Fig. 4A, left). These areas were gradually spreading into cortical and subcortical areas (Figs 4A, left to right). As the tumors grew, the presence of large necrotic regions (dark areas) in the tumor center was evident. A midline shift was seen on day 22 and at day 29 due to extensive tumor growth.

**Tumor volumes.** Tumor volume calculations were done by evaluating the tumor areas on magnetic resonance images and multiplying with the interslice distance. At day 1, the mean tumor volumes were 17.0 ± 1.5 mm$^3$ (SE) in the saline group and 16.8 ± 2.2 mm$^3$ in the G207 group ($P = 0.77$; Fig. 4B, left). The tumor volumes at day 15 increased to 90.8 ± 24.0 mm$^3$ in the saline group and to 60.2 ± 9.7 mm$^3$ in the G207 group ($P = 0.52$). On day 22, the differences in tumor volumes were statistically significant (248.0 ± 43.3 mm$^3$ in the saline group and 111.0 ± 23.0 mm$^3$ in the G207 group; $P = 0.03$). On day 29, the tumor volumes were 370.8 ± 51.1 mm$^3$ in the saline group, versus 227.2 ± 52.2 mm$^3$ in the G207 group ($P = 0.05$).

**Vector spread.** To assess if G207 infection persisted 30 days postinjection, five of the rat brains injected with high-dose G207 were immunostained and vector spread was evaluated. Representative sections from three different animals in the treatment group are shown in Fig. 4C. Whereas extensive regions of tumor tissue were transduced, there were still large noninfected areas. In some cases, the tumor core was infected; in other lesions, a more peripheral localization was observed. Generally, invasive cells at the tumor border were not infected.

**Survival.** We injected eight rats with high-dose ($6.8 \times 10^6$ pfu) G207 vector and seven rats with sterile saline and followed...
the animals until symptoms occurred. Three of the rats in the treatment group experienced neurologic symptoms beginning from the day after vector injection. One rat had to be sacrificed 1 day postinjection and two rats had to be sacrificed 5 days postinjection. The data from these animals were not included in the survival analysis because the observed symptoms were most likely not related to tumor growth. The rest of the animals were followed until symptoms associated with excessive tumor burden appeared. Magnetic resonance imaging analysis confirmed the presence of full-sized lesions at the time of sacrifice. Median survival time lengths were: for the control group, 58 days (range, 54-68; mean, 61.2; SE, 5.2), and for the treatment group, 68 days (range, 63-70; mean, 67.0; SE, 2.0). Survival analysis revealed that G207 treatment did prolong median survival by 10 days. However, the difference in life span of the animals was not significant (log-rank test, \( P = 0.15 \)).

**Infection of nonneoplastic tissues.** In the rats that experienced neurologic symptoms after vector injection, histology showed that the xenografts grew adjacent to the right lateral ventricles (Fig. 5A and B). Immunostaining revealed transduction of the

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**Fig. 4.** Outcome of the therapeutic study. A, magnetic resonance images taken during a time period of 29 d, where day 1 was defined as the day when the first scans were done. Top, T2-weighted magnetic resonance images obtained from a G207-injected animal, after 1, 15, 22, and 29 d. Bottom, T2-weighted magnetic resonance images taken of a saline-injected animal, at similar time intervals. B, bar graphs comparing the mean tumor volumes in G207-treated animals and saline-injected animals, estimated from magnetic resonance imaging data; bars, SE. C, representative images of xenograft sections immunostained against HSV-1 from three rats in the treatment group. The pictures show extensive spread of the G207 vector in solid tumor tissue after \( \sim 1 \) mo postinjection. Original magnification, \( \times 25 \). Bar, 1 mm.
cells in both the left and right ventricular walls (Fig. 5C). Double immunostaining against HSV-1 and human-specific vimentin (which identified infiltrative tumor cells) revealed infection of ependymal cells (Fig. 5D, inset). Loss of single cells was observed in the ependymal lining, most likely related to cytolysis. Both the lateral and medial ependymal linings were infected, as well as the surface adjacent to the corpus callosum. In one animal, positive staining was observed in a few cells of the choroid plexus in the right lateral ventricle, adjacent to the tumor (data not shown). Transduction was also evident in the 3rd and 4th ventricle ependyma. Double staining confirmed that the infected cells were of host origin (Fig. 5E). In both ependymal and choroid plexus infections, several adjacent noninfected cells (not stained with anti-HSV antibody) exhibited nuclear fragmentation, a sign of apoptotic cell death (Fig. 5E). Infected regions of the xenografts displayed positive signals for both vimentin and HSV, confirming the specificity of the double staining procedure (Fig. 5F).

**Discussion**

Primary glioblastomas are associated with common genetic changes such as frequent EGF amplifications and PTEN mutations (1). These factors participate in mitogenic signaling and survival pathways that have been shown to be important for supporting the replication of mutant oncolytic HSVs (23–25). In our in vitro cytotoxicity study, interpatient variations may have resulted in discrepant activity of the critical pathways in our spheroids, yielding differences in permissiveness to G207. The penetration of viral particles in solid tissues is thought to depend on the particle size and surface charges, as well as on the density of the extracellular matrix (26). Differences in cellularity and pore sizes of the extracellular matrix between spheroids may well explain the observed differences in penetration and transduction ability of G207. HSV-based vectors are among the largest of viral particles used in gene therapy, implying that their penetration is relatively inefficient compared with other vectors. Nevertheless, G207 particles reached and infected the spheroid core after 96 h, revealing a transgene expression pattern similar to what has been observed for adeno-associated viruses, which are roughly one tenth in diameter (8, 26).

Immunostaining revealed that intraneoplastic inoculation of G207 in vivo resulted in foci of virus replication with dense concentrations of HSV-positive tumor cells. Notably, HSV-1–specific immunostaining detected the expression of viral envelope and core proteins, corresponding to advancement to viral replication, not solely an initial infection event. Expression of late viral genes was observed in vivo already after 24 h postinjection. This is consistent with the completion of one round of replication cycle, which
generally occurs within 12 to 18 h of infection (27). More robust transgene expression was seen after 10 days, indicating several replication cycles and possible spread by diffusion and intratumoral pressure gradients. There were no evident differences between the extents and patterns of HSV expression between the xenografts derived from the various patients, indicating that these lesions were similarly permissive to G207 infection and replication. However, applying an enhanced vector dose and incubating the vector for longer time periods (therapeutic study) versus 10 days (penetration study) did result in more robust transduction. In GBM6 xenografts, necrotic areas did not support vector replication, which is not surprising taking into account the paucity of viable cells in these regions. Most likely, necrotic areas represent barriers that reduce intratumoral vector dissemination and thereby limit transduction efficacy. This implies that multiple injections at the tumor site should be done in the clinical setting to ascertain sufficient vector dissemination. The finding that G207 may infect single glioma cells distant from the primary lesion, such as in the corpus callosum, suggests that HSV particles may be distributed to infiltrating glioma cells by interstitial fluid pressure gradients. Notably, no staining for β-galactosidase or HSV-1 was observed in neuronal or glial tissues after G207 delivery to the brain parenchyma, whereas site-directed delivery into tumors always produced transduction. This confirmed that G207 does not replicate in host neural or glial tissue after injection into nude rat brains.

The observed widespread transduction of glioblastoma multiforme xenografts after intratumoral injection of 6.8 × 10^6 pfu vector corresponded to a significant reduction of mean tumor sizes on day 22, whereas on day 29 the difference was exactly in the 5% cutoff value. Thus, the data show that G207 has noteworthy effects on the volume of intracerebral gliomas. Nevertheless, survival analysis did not reveal a significant benefit for the animals in the treatment group. Taken together, the data suggest that the penetration ability and replicative potential of the applied G207 load are not sufficient to eradicate or stabilize the growth of lesions generated from glioblastoma multiforme patient biopsy material. The underlying reason may be that the obtained transduction efficiency was insufficient. Of note, we used an animal model wherein the T-cell–mediated immune response is absent (28), and some data indicate that the contribution of the immune system may be essential to effective oncolytic HSV-therapy (29). Still, the intratumoral inoculation of 2 × 10^6 pfu of G207 resulted in a significant survival benefit in immunodeficient nude mice bearing U87 gliomas (19). The tumor volumes at the start of therapy and the vector doses applied are different between the original study (19) and the current data, which may, to some extent, explain the observed discrepancy. Furthermore, the two tumor models have different biological behaviors (5), underlining the necessity of using a clinically more relevant animal model to assess novel therapies.

Intratumoral and intracerebral injection of 3.4 × 10^6 pfu of G207 was tolerated in all of the injected rats, whereas intratumoral delivery of 6.8 × 10^6 pfu was followed by neurologic symptoms in three of eight animals. The sizes of the corresponding lesions were similar to those in the rest of the treatment group at this time point; therefore, excessive tumor burden could not explain the observations. For the animal that died 1 day postinjection, it is uncertain if the symptoms were related to vector replication. Previous data indicate that at least 2 days are necessary to produce HSV-related deaths (30). Histologic examination failed to show signs of encephalitis in any of these animals. Immunostaining against HSV-1 revealed infection of the ventricular ependyma and of cells of the choroid plexus. The presence of HSV-positive cells in the lateral, the 3rd, and the 4th ventricles suggests that viral particles may be distributed with the cerebrospinal fluid after escape into the right lateral ventricle and transduce the ependymal lining in more distant regions of the brain. It is not clear why host tissues were transduced because G207 replication in theory requires the activation of oncogenic pathways (23–25). The production of cerebrospinal fluid in choroid plexus epithelial implies that these cells are in a metabolically active state and may express factors that complement the defective vectors. In contrast, other host cells such as neurons and glia likely express molecular phenotypes that do not support the replication of attenuated HSV. Importantly: the neurologic symptoms observed in some animals indicate that it was not feasible to increase the vector dose in our setting to improve the therapeutic effect. Other investigators have similarly reported preferential infection of the ventricular ependyma by G207 (31); however, transgene expression was shown to be transient and without the loss of ependymal cells or neurologic deficit. After intracerebroventricular injection of increasing G207 doses in newborn mice (32), cases of ventriculomegaly were frequent; however, the changes did not produce symptoms or affect the mice in neurologic tests. In our study, we have seen a sporadic loss of ependymal cells, but we did not observe ventriculomegaly, possibly due to the short vector incubation time. It is not clear why we observed neurologic symptoms taking into account the subtle changes seen on histologic examination. Of note, intracerebroventricular delivery of the γ34.5-mutant HSV 1716 into BALB/c mice led to ependymal loss and hydrocephalus but no mortality (33), whereas intracerebroventricular injections into nude mice caused high mortality even at low doses (30). This suggests that immunocompromised animals such as the athymic nude rats used in our study may be more sensitive to oncolytic HSV, especially after intracerebroventricular injections. It is not likely that systemic virus dissemination caused the symptoms because previous studies indicate that the vast majority of an applied vector stock is retained in the tumor or in the adjacent brain tissue after intratumoral injection (34). Although an inadvertent ventricular injection of G207 in a patient was resolved quickly without serious symptoms (35), our observations suggest that escape of G207 to the cerebroventricular system should be avoided.

In conclusion, we have shown that G207 exhibits variable oncolytic potency in glioblastoma multiforme biopsy material derived from different patients. We have noted that its antitumor effects are retained in vivo; however, survival analysis suggests that attempts could be made to improve its efficacy by combining it with other treatment modalities to produce a clinically relevant therapeutic benefit.

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References


