

Angiogenic Response Caused by Oncolytic Herpes Simplex Virus–Induced Reduced Thrombospondin Expression Can Be Prevented by Specific Viral Mutations or by Administering a Thrombospondin-Derived Peptide

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Abstract

Wild-type (WT) herpes simplex virus (HSV) causes some pathology, such as ocular keratitis, by increasing infected tissue vascularity, possibly reflecting altered angiogenic factor expression in infected cells. Oncolytic HSVs possess specific mutations enabling selective replication in tumor cells. We investigated whether this ability to enhance infected tissue vascularity is retained in oncolytic HSV, which could be an undesirable effect of oncolytic HSVs that may need to be addressed when treating tumors with oncolytic HSVs. s.c. tumors derived from U87 human glioma cells in athymic mice were treated with oncolytic HSVs G207 or G47Δ in the presence or absence of a recombinant protein composed of the three type-1 repeats (3TSR) of thrombospondin-1 (TSP-1). Real-time reverse transcription-PCR and Western blot of infected cultured cells measured angiogenic factor expression. Microvessel density was assessed using immunofluorescence. G207-treated U87 s.c. tumors had elevated microvessel densities compared with saline- and G47Δ-treated tumors, and G207 treatment caused delayed tumor growth resumption. G207-infected U87 and U373 cells exhibited reduced protein, not mRNA, expression of angiogenesis inhibitors TSP-1 and thrombospondin-2 (TSP-2). 3TSR restored the G207-treated tumor microvessel density to the low level of G47Δ-treated tumors and prevented delayed growth resumption. Oncolytic HSV G207 thus retains the ability of WT HSV to increase infected tissue vascularity. In infected tumors, this increased vascularity is mediated by reduced TSP-1 and TSP-2 levels and causes delayed tumor growth resumption. Incorporating viral mutations, such as those seen in G47Δ or administering thrombospondin-derived peptides, counteracts the angiogenic effect of oncolytic HSV and should be considered when designing oncolytic HSV therapies. [Cancer Res 2007;67(2):440–4]

Introduction

Wild-type (WT) herpes simplex virus (HSV) type 1 (HSV-1) causes some of its pathology by increasing the vascularity of infected tissue, as occurs during herpetic ocular keratitis. HSV-1 can specifically inhibit the ability of infected smooth muscle, endothelial cells, and keratinocytes to secrete extracellular matrix

proteins, such as fibronectin (1–3), collagen types I and III (1, 3), type IV procollagen (1, 3), and thrombospondin-1 (TSP-1) and thrombospondin-2 (TSP-2; refs. 1–4), with the degree of suppression dependent on the dose of the viral inoculum. TSP-1 and TSP-2 are major naturally occurring antiangiogenic proteins whose expression is post-transcriptionally reduced in keratinocytes infected with WT HSV-1, a likely cause of the neovascularization seen during herpetic ocular keratitis (4). In addition to inhibiting expression of antiangiogenic proteins TSP-1 and TSP-2, HSV-1 can also up-regulate expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9), by certain cell types (5, 6), which may be another contributing factor to the increased vascularization seen in herpetic keratitis.

HSV has also undergone extensive laboratory investigation and early clinical trials (7, 8) as an oncolytic virus engineered to selectively replicate in tumor cells due to specific viral mutations. Although some have suggested that oncolytic HSV has antiangiogenic activity based on its ability to infect endothelium and cause endothelial damage (9), particularly when inoculated intravascularly (10), this effect may be counteracted if oncolytic HSV retains the ability of WT HSV to inhibit TSP-1 and TSP-2 expression or stimulate VEGF and MMP-9 expression by infected cells. Indeed, if, after infecting tumor cells, oncolytic HSV retains the ability to increase vascularity similar to WT HSV-1 infection of certain tissues, the resulting increase in tumor vascularity could lead to tumor growth and may need to be addressed when using oncolytic HSV as a therapeutic agent. We therefore investigated the effect of oncolytic HSV infection on the expression of these factors and the resultant tumor microvessel density. We found that the oncolytic HSV G207 infection of U87 glioma cells reduced TSP-1 and TSP-2 levels without affecting VEGF and MMP-9 levels and this caused an increase in microvessel density *in vivo*, with an associated delayed resumption of tumor growth. This effect of viral infection on TSP-1 and TSP-2 levels was not seen when using the oncolytic HSV G47Δ, which is derived from G207 by deleting the viral gene *α47* and the promoter region of viral gene *US11*, leading to inhibition of host protein shutoff (11), nor by combining G207 with an exogenously administered TSP-1–derived peptide.

Materials and Methods

Cell culture. Human U87 and U373 glioma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO₂.

Viruses. HSV-1 strains G207 (γ34.5⁺ICP6[−]LacZ⁺; ref. 12) and G47Δ, derived from G207 by deleting the *α47* gene and the promoter region of the *US11* gene (11), were grown, purified, and titered by plaque assay on Vero cells as described (12).

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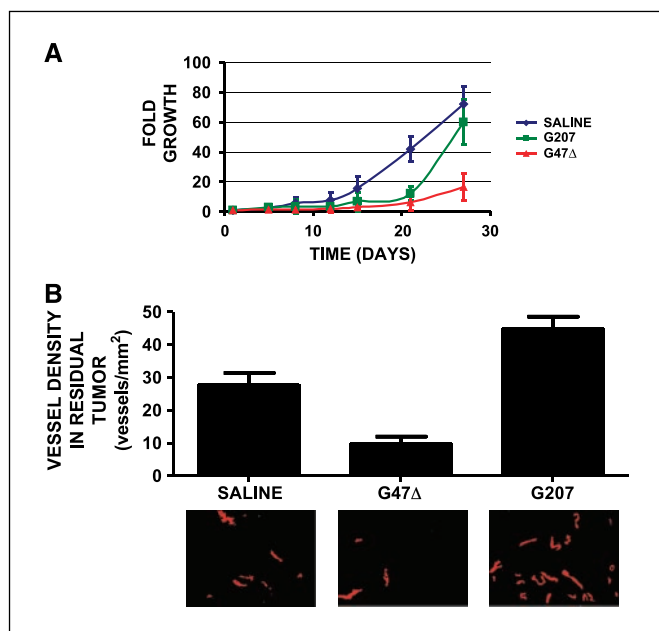


Figure 1. Treatment of s.c. U87 gliomas. **A**, 2 wks after s.c. U87 tumor cell implantation, when tumors had achieved volumes of 80 to 120 mm³, biweekly tumor measurements began. Two and 5 d after measurements began (treatment days 2 and 5; X axis), treatment with 5×10^6 pfu G207, G47Δ, or saline intratumorally occurred. Fold growth of s.c. U87 tumors over time. Initially, the two viruses exhibited comparable efficacy, with 13-fold growth in G207-treated tumors and 6-fold growth in G47Δ-treated tumors after 21 d, both significant reductions compared with the 42-fold growth seen in saline-treated tumors at this time ($P = 0.0003$). However, after 27 d, G47Δ treatment was more effective than G207, resulting in 15-fold growth, compared with 60-fold growth with G207 ($P = 0.0002$) and 80-fold growth with saline-treated tumors. There were five mice per treatment group. Bars, SD. **B**, vessel density of residual s.c. U87 tumors after treatment day 27. The microvessel density of G207-treated tumors was significantly higher than that of saline- or G47Δ-treated tumors ($P = 0.00007$). Columns, upper panel, vessel density in residual tumor; bars, SD; lower panel, representative immunofluorescence anti- ν WF–stained microvessels (red).

Quantitative real-time reverse transcription-PCR. U87 and U373 cells (2.5×10^5) in 12-well plates were infected with G207 or G47Δ at a multiplicity of infection (MOI) of 3 for 6 and 24 h. RNA was extracted from cells with Trizol (Invitrogen, Carlsbad, CA). A high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) was used to generate cDNA. Real-time reverse transcription-PCR (RT-PCR) was done on an ABI Prism 7000 (Applied Biosystems) machine using human TSP-1, 5'-AACATGCCACGGC-CAACAAA-3' (forward) and 5'-TGCACTTGGCGTCTCTGTGGC-3' (reverse); TSP-2, 5'-AAGGATAACTGCCCCATCT-3' (forward) and 5'-CCGTCATTGT-CATCGTCATC-3' (reverse); VEGF, 5'-ACTGCCATCCAATCGAGACC-3' (forward) and 5'-GATGGCTTGAAGATGACTCGATCT-3' (reverse); and MMP-9, 5'-TGGCACCACCACAACATCAC-3' (forward) and 5'-GCAAAGGCGTCGT-CAATCA-3' (reverse) primers (Invitrogen) combined with SYBR Green Master Mix (Applied Biosystems) or primer-probe combinations for 18S rRNA (Applied Biosystems part no. 4308329) combined with Taqman Master Mix (Applied Biosystems). Relative quantification was done using 18S rRNA as an endogenous control. All reactions began with 10 min at 95°C for AmpliTaq Gold activation followed by 40 cycles at 95°C for 15 s for denaturation then 60°C for 1 min for annealing/extension.

Western blots. Thirty micrograms of protein extracted in radio-immunoprecipitation assay buffer from U87 and U373 cells (2.5×10^5) in 12-well plates that were uninfected or infected for 6 and 24 h at MOI of 3 were separated on a 10% SDS-PAGE gel, transferred to polyvinylidene difluoride membrane, and incubated with antibodies to TSP-1 (1:200 rabbit anti-human/mouse, Lab Vision, Fremont, CA), TSP-2 (1:100 goat anti-human/mouse, Santa Cruz Biotechnology, Santa Cruz, CA), VEGF (1:200 chicken anti-human/mouse, Abcam; Cambridge, MA), MMP-9 (1:5,000

rabbit anti-human/mouse, Affinity BioReagents, Golden, CO), and actin (rabbit anti-human/mouse, Sigma, St. Louis, MO) at 4°C overnight. Membranes were then incubated with peroxidase-conjugated secondary antibodies for 40 min the next day. Protein was visualized using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

In vivo studies. A suspension of 10^6 U87 cells was implanted s.c. into athymic mice. i.p. treatment with saline or 3 mg/kg/d recombinant protein composed of the three type-I repeats (3TSR) of thrombospondin-1 (kindly provided by J. Lawler, Beth Israel Deaconess Medical Center, Boston, MA), the antiangiogenic domain of TSP-1 (13), and initiated 2 weeks after tumor cell implantation, when tumors had achieved volumes of 80 to 120 μ L mm³, and was continued until euthanasia. Intratumoral injections of 5×10^6 plaque-forming units (pfu) G207 or G47Δ in 30 μ L were administered 17 and 20 days after tumor cell implantation. Mock-treated mice received equivalent i.p. or intratumoral volumes of saline. There were five mice per treatment group. Tumors were measured biweekly using calipers to calculate length, width, and height, with the measurer blinded to each treatment group of the animal. Tumor volume was the product of these dimensions, and fold growth was calculated relative to treatment day one. Measurement of the tumor of a mouse continued until the mouse had to be sacrificed due to excessive tumor burden (2.1 cm maximal dimension).

Immunofluorescence. Tumors were removed, frozen in liquid nitrogen-cooled *N*-methylbutane, and sectioned coronally by cryostat to 8- μ m thickness. Tumor-containing slides were immunostained for vWF (rabbit anti-mouse, DAKO, Carpinteria, CA). Secondary staining used Jackson ImmunoResearch antibodies. A Nikon Eclipse TE2000-U inverted microscope imaged five fields from five animals per treatment group. Microvessel density was counted by an observer blinded to treatment group.

Statistical analysis. Descriptive statistics were generated for all quantitative data with presentation of means \pm SDs. Significance of comparisons between experimental groups was tested using the Student's *t* test.

Results

In vivo infection with the oncolytic HSV G207 leaves behind residual tumor with delayed growth resumption and elevated microvessel density compared with tumors infected with oncolytic HSV G47Δ. In a human xenograft model, athymic mice harboring s.c. tumors derived from U87 glioma cells were treated with two intratumoral inoculations of G207 or G47Δ spaced 3 days apart. Initially, the two viruses exhibited comparable efficacy, with 13-fold growth in G207-treated tumors and 6-fold growth in G47Δ-treated tumors (Fig. 1A) after 21 days, both significant reductions compared with the 42-fold growth seen in saline-treated tumors at this time ($P = 0.0003$). However, after 27 days, G47Δ treatment was

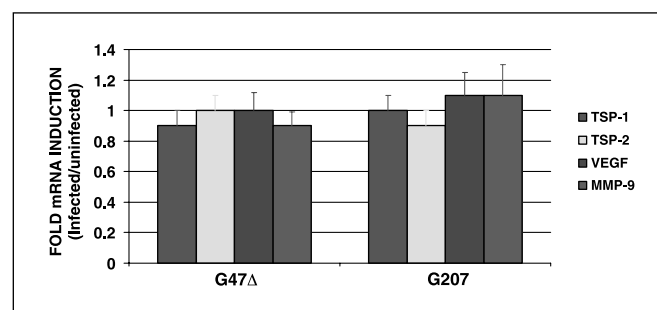


Figure 2. Assessing the effect of oncolytic HSV infection on the transcripts for TSP-1, TSP-2, VEGF, and MMP-9. mRNA was isolated from cultured U87 cells 6 h after infection with G47Δ or G207 at MOI of 3, and transcript levels were determined by real-time RT-PCR using 18S rRNA as a control. There was no significant change in the mRNA levels of the assessed transcripts ($P > 0.5$). Similar results were obtained 24 h after infection (data not shown).

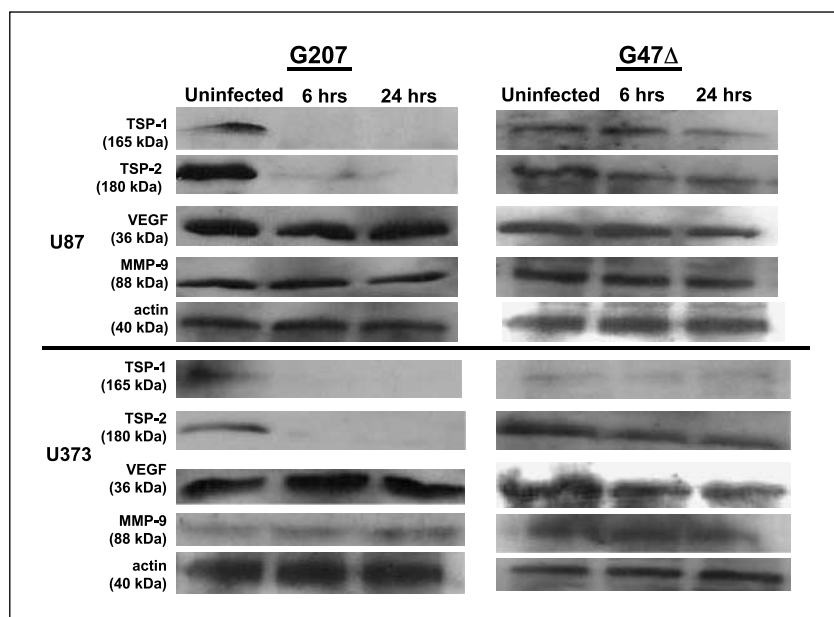


Figure 3. Assessing the effect of oncolytic HSV infection on protein levels of TSP-1, TSP-2, VEGF, and MMP-9. Western blots revealed protein expression of TSP-1, TSP-2, VEGF, and MMP-9 (with actin as a control) 6 and 24 h after infecting cultured U87 (*top five rows*) and U373 (*bottom five rows*) cells with G207 (*left*) and G47 Δ (*right*) at MOI of 3. Expression of both TSP-1 and TSP-2 was reduced after G207 infection of both cell lines to undetectable levels, whereas VEGF and MMP-9 levels were unchanged in either cell line with either virus. G47 Δ caused only a slight reduction of TSP-1 levels at the 24-h time point in U87 but otherwise did not affect the levels of the assessed factors in either cell line.

more effective than G207, resulting in 15-fold growth compared with 60-fold growth with G207 ($P = 0.0002$) and 80-fold growth with saline-treated tumors (Fig. 1A), a difference that has been reported previously at a comparable extended time point using other doses of these viruses (11).

To determine whether differing tumor growth in the different treatment groups reflected differences in post-treatment angiogenesis, microvessel densities were determined in all tumors after 27 days by immunohistochemical analysis. Interestingly, the microvessel density of G207-treated tumors was significantly higher (45 vessels/mm²) than that of saline-treated tumors (28 vessels/mm²), whereas G47 Δ treatment lowered microvessel density (10 vessels/mm²) compared with saline treatment ($P = 0.00007$; Fig. 1B). The elevated microvessel density in G207-treated tumors was one possible explanation for the delayed resumption of growth in G207-treated tumors at a rate comparable with the growth rate seen throughout the observation period in saline-treated tumors.

Infecting cultured cells with G207, not G47 Δ , reduces tumoral protein, not mRNA, expression of TSP-1 and TSP-2.

We then used cultured U87 cells, along with cultured U373 human glioma cells, which have a 50% higher microvessel density than U87 (14), to investigate whether the elevated microvessel density seen in G207-treated tumors occurred because infected tumor cells exhibited reduced expression of antiangiogenic proteins TSP-1 and TSP-2 or increased expression of angiogenic factors VEGF and MMP-9, responses that have been observed after WT HSV infection of nontumor cells. Real-time RT-PCR of infected cells revealed no change in the mRNA expression of TSP-1, TSP-2, VEGF, or MMP-9 in G207- and G47 Δ -infected U87 (Fig. 2) or U373 (data not shown) cells compared with uninfected cells. Because HSV is capable of altering protein expression without affecting expression of the corresponding transcripts, we investigated protein expression of these factors. Western blots of protein from infected cells revealed reduced protein expression of TSP-1 and TSP-2 in G207-infected U87 or U373 cells compared with G47 Δ -infected and uninfected U87 or U373 cells (Fig. 3). This suggested that reduced TSP-1 and TSP-2 protein expression in

G207-infected tumor cells could explain the elevated microvessel density and associated delayed resumption of growth seen in G207-infected tumors.

Reduced microvessel density and prevention of tumor growth resumption when G207 is combined with 3TSR to treat s.c. tumors. To determine if exogenous administration of the thrombospondin whose expression was reduced by G207 infection could prevent the delayed resumption of growth seen in

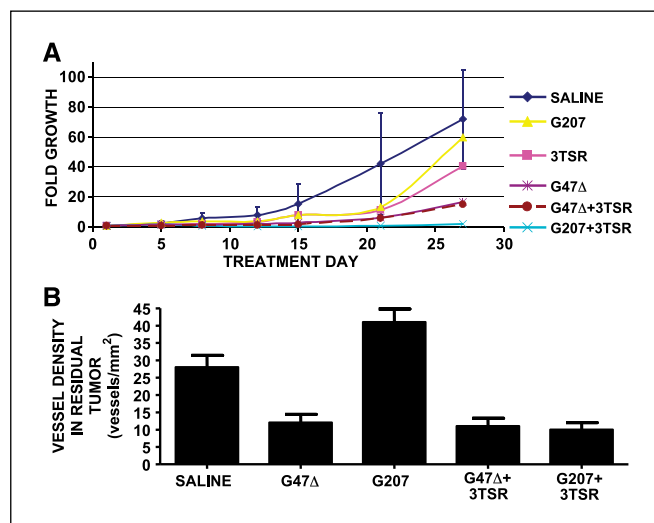


Figure 4. TSP-1–derived peptide 3TSR in combination with G207 synergistically inhibits tumor growth and decreases vessel density. *A*, s.c. U87 tumors were treated with 5×10^6 pfu G207, G47 Δ , or saline intratumorally on treatment days 2 and 5, along with 3 mg/kg/d 3TSR or saline i.p. on treatment days 1 to 27. 3TSR alone did not inhibit tumor growth ($P = 0.1$ compared with saline-treated tumors) and in combination with G47 Δ was no better than G47 Δ alone ($P = 0.4$). Combining 3TSR with G207 was significantly more effective than G207 alone ($P = 0.04$). *B*, microvessel density of residual s.c. U87 tumors after 27 d of treatment with saline, G47 Δ , G207, G47 Δ + 3TSR, and G207 + 3TSR was determined by staining tumor sections with anti-vWF antibodies. Addition of 3TSR did not alter the microvessel density of G47 Δ -treated tumors (11 vessels/mm²) but did lower that of G207-treated tumors to 10 vessels/mm² ($P = 0.0006$).

G207-infected tumors, we treated mice bearing s.c. U87 tumors with G207 combined with 3TSR, a peptide containing the antiangiogenic domain of TSP-1. After 28 treatment days, G207-treated mice exhibited 60-fold tumor growth, whereas G47 Δ -treated mice exhibited 17-fold growth, a significant difference ($P = 0.04$; Fig. 4A), similar to the one seen in Fig. 1A. When a 3TSR dose that alone caused a small, but not significant, decrease in tumor growth compared with saline-treated tumors ($P = 0.1$) was combined with G207, tumor growth was significantly reduced by 30-fold compared with G207 alone ($P = 0.04$; Fig. 4A). In addition, two of five combination treatment mice were cured of tumors and a third exhibited tumor regression. Combining 3TSR with G47 Δ had no effect on tumor growth compared with G47 Δ alone ($P = 0.4$; Fig. 4A).

To confirm if the significant impairment in tumor growth seen when G207 was combined with 3TSR reflected an inhibition of the previously seen G207-induced increase in tumor vascularity, we analyzed the microvessel densities of residual tumors after these treatment regimens. Similar to the results in Fig. 1B, G207-treated tumors had a microvessel density of 41 vessels/mm², which was higher than the 28 vessels/mm² seen in saline-treated tumors or the 12 vessels/mm² seen in G47 Δ -treated tumors (Fig. 4B). Addition of 3TSR did not alter the microvessel density of G47 Δ -treated tumors (11 vessels/mm²) but significantly lowered that of G207-treated tumors to 10 vessels/mm² ($P = 0.0006$), a level comparable with that seen with G47 Δ treatment (Fig. 4B). This reduction in microvessel density seen in tumors treated with G207 plus 3TSR may have caused the lack of delayed growth resumption in these tumors.

Discussion

Oncolytic HSVs have undergone clinical trials in the treatment of various tumors, including glioblastoma, hepatic colorectal metastases, melanoma, and cutaneous and s.c. metastases (8). Although some encouraging results have arisen from these studies, the considerable tumor-related mortality in these trials may reflect limitations that have been suggested previously, such as viral distribution (15) or the immune response (16). Our findings suggest that oncolytic HSV-induced angiogenesis in residual tumors may be an additional potential limitation that will need to be addressed when using oncolytic HSV as a therapeutic agent. In particular, we found delayed resumption of growth due to a proangiogenic effect potentially mediated by reduced TSP-1 and TSP-2 expression in tumor cells infected with oncolytic HSV G207. This proangiogenic effect and delayed resumption of growth was not seen when G207 was combined with a TSP-1-derived angiogenesis inhibitor or when oncolytic HSV G47 Δ was used instead of G207.

Numerous cell types have been shown to produce TSP-1 and incorporate it into the extracellular matrix, including human tumors, such as squamous carcinoma, melanoma, glioma, osteosarcoma, and breast adenocarcinoma (17). TSP-1, the first naturally

occurring protein shown to be antiangiogenic, inhibits migration and proliferation of vascular endothelial cells, induces endothelial apoptosis, and blocks capillary tube formation (18). TSP-1 and TSP-2 bind and inhibit the activity of proangiogenic factors, such as basic fibroblast growth factor, VEGF, and MMP-9 (19).

The mechanism by which the oncolytic HSV inhibits tumoral TSP-1 and TSP-2 expression remains unclear. Although productive HSV infection eventually eliminates most host cell protein synthesis (11), the effects on TSP-1 and TSP-2 were specific compared with other angiogenic factors. Furthermore, the lack of an effect of G47 Δ infection on TSP-1 and TSP-2 expression suggests that viral gene $\alpha 47$ or the promoter region of viral gene *US11* may play a role in the ability of G207 to specifically alter infected tumor cell expression of the TSP-1 and TSP-2 proteins. G47 Δ has been shown previously to exhibit enhanced replication, oncolytic activity, and greater induction of antitumor immunity than G207 (11). The antiangiogenic effect of G47 Δ represents a further advantage of G47 Δ over G207, which elevated microvessel density by lowering thrombospondin levels. In addition, the effect of oncolytic HSV on tumor vascularity is likely multifactorial. Oncolytic HSV 1716, which carries a $\gamma 34.5$ deletion like G207 but lacks the ribonucleotide reductase mutation seen in G207, has been reported to significantly reduce tumor vessel density in treated murine ovarian tumors overexpressing VEGF (10). This difference from our results with G207 may reflect VEGF tumor overexpression rendering the endothelium more proliferative and therefore more infectable by oncolytic HSV, or it might reflect differences in the abilities of the two viruses to infect and lyse endothelial cells.

Our results suggest that combined treatment of tumors with G207 and angiogenesis inhibitors derived from TSP-1 and TSP-2 might allow tumor cell death from viral replication, whereas inhibiting G207 induced increased vascularity and delayed tumor growth resumption. This therapeutic strategy might be readily implemented because G207 has already been proven safe and moderately effective in early phase I clinical trials in glioma patients (7), and several TSP-1- and TSP-2-derived angiogenesis inhibitors have already undergone phase I clinical trials in patients with advanced cancer (20). Alternatively, oncolytic HSV G47 Δ , once its clinical safety is confirmed, could be as effective as the combination of G207 with thrombospondin-1- and TSP-2-derived angiogenesis inhibitors due to the lack of TSP-1 and TSP-2 reduction and associated angiogenesis seen with G47 Δ .

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