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Human Cytomegalovirus US28 Found in Glioblastoma Promotes an Invasive and Angiogenic Phenotype

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Abstract

Human cytomegalovirus (HCMV) infections are seen often in glioblastoma multiforme (GBM) tumors, but whether the virus contributes to GBM pathogenesis is unclear. In this study, we explored an oncogenic role for the G protein-coupled receptor-like protein US28 encoded by HCMV that we found to be expressed widely in human GBMs. Immunohistochemical and RT-PCR approaches established that US28 was expressed in ~60% of human GBM tissues and primary cultures examined. In either uninfected GBM cells or neural progenitor cells, thought to be the GBM precursor cells, HCMV infection or US28 overexpression was sufficient to promote secretion of biologically active VEGF and to activate multiple cellular kinases which promote glioma growth and invasion, including phosphorylated STAT3 and e-NOS. Consistent with these findings, US28 overexpression increased primary GBM cell invasion in Matrigel. Notably, this invasive phenotype was further enhanced by exposure to RANTES/CCL5, a US28 ligand, associated with poor patient outcome in GBM. Conversely, RNAi-mediated knockdown of US28 in human glioma cells persistently infected with HCMV led to an inhibition in VEGF expression and glioma cell invasion in response to CCL5 stimulation. Analysis of clinical GBM specimens further revealed that US28 co-localized in situ with several markers of angiogenesis and inflammation, including VEGF, p-STAT3, COX2 and e-NOS. Taken together, our results indicate that US28 expression from HCMV contributes to GBM pathogenesis by inducing an invasive, angiogenic phenotype. Additionally, these findings argue that US28-CCL5 paracrine signaling may contribute to glioma progression and they suggest that targeting US28 may provide therapeutic benefits in GBM treatment.

Keywords

Cytomegalovirus; US28; Glioblastoma; Invasion; Angiogenesis

INTRODUCTION

Human cytomegalovirus (HCMV) is a beta-herpesvirus that can cause life threatening infections in human fetuses and immunocompromised individuals. HCMV is a major cause of congenital brain infection and disability in humans. Our laboratory first reported the

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association of HCMV infection with human glioblastomas (GBMs) (1). These findings have been confirmed by other groups (2) and clinical trials are currently underway for the treatment of GBM with anti-HCMV agents and immunotherapy approaches. A growing body of evidence suggests that HCMV infection of malignant glioma does not simply represent an epiphenomena, but rather expression of HCMV gene products in tumor cells and the tumor microenvironment directly impacts tumor progression. Several HCMV gene products have been found to have mutagenic and transforming potential (3). We previously demonstrated that the HCMV IE1 gene product promotes GBM mitogenicity by interfering with Rb, p53, and AKT signaling pathways (4) and that HCMV envelope glycoprotein B can directly activate oncogenic signaling pathways through activation of the PDGFR- α receptor tyrosine kinase (5).

US28 is a HCMV encoded G-protein coupled receptor which is a homologue of the human CCR1 chemokine receptor. US28 is constitutively active and may be further activated by binding of several ligands: SDF-1, CCL2/MCP-1, CCL5/RANTES, and CX3CL1/Fractalkine (6). US28 has properties of a viral oncogene, since ectopic expression of US28 can induce a pro-angiogenic and transformed phenotype *in vivo* via activation of the NF- κ B and COX2 signaling pathways (7). A recent report demonstrated that US28 induces IL-6 and VEGF through NF- κ B activation, resulting in potent activation of the STAT-3 transcriptional activator in NIH 3T3 mouse fibroblasts (8).

We sought to determine whether US28 could a) influence early events that lead to gliomagenesis in neural precursor cells, and b) promote key oncogenic features in established glioblastoma cells. We hypothesized that US28 expression in NPCs (possibly the glioma “cell of origin”) may promote the pathogenesis of GBM. We evaluated changes that occur in gene expression patterns and in the angiogenic and invasion pathways in adult NPCs, when infected by HCMV or overexpressing US28. Next, we assessed the changes in the invasive and angiogenic phenotypes of primary GBM cultures induced by US28 overexpression. Lastly, we performed loss of function studies in human GBMs persistently infected with HCMV, to demonstrate the specificity of US28-induced pathogenesis.

MATERIALS AND METHODS

Cell culture

U251 and U87 cell lines were obtained from ATCC and grown in DMEM/F12+10% FBS. Primary glioblastoma/neural precursor cell- derived cultures were generated using tissue from surgical resections at CPMC obtained according to the IRB approved protocol. Tissues were dissociated using enzymatic and mechanical dissociation as previously described. Single cell suspensions were cultured using neural basal medium+ N2 supplement, 20 ng/ml EGF, 20 ng/ml bFGF, and 1 μ g/ml laminin as previously described (9). For ELISA for VEGF experiments and tube formation assays, cells were cultured in the absence of FBS or growth factors at least 48 hours prior to media collection. The NPC cell line was derived from the hippocampus tissue removed from a patient with intractable epilepsy. Cells were characterized by immunofluorescence and found positive for Nestin, GFAP, Tuj1, and Olig 2. All experiments were performed on passages 2–5 from the NPC culture. HUVECs were obtained from Invitrogen and grown in the complete endothelial cell growth media recommended by the manufacturer.

US28 Expression Vectors

The Ad-US28 and Ad-Control adenoviruses were a gift from Dr. Dan Streblow, OHSU. The pcDEF-US28 plasmid was a gift from Dr. Martine Smit. The US28 insert was excised from

the pcDEF plasmid and cloned into the pLXSN vector (4). Retroviruses were produced and used to infect glioma cells as previously described by our laboratory (4).

Viruses

The Towne and AD169 HCMV strains were obtained from ATCC and grown in human embryonic fibroblasts (HEL), as previously described (10). The TR virus strain was a gift from Dr. Lee Fortunato, University of Idaho.

Knockdown experiments using siRNA to US28

US28 knockdown was achieved using a duplex of two siRNA oligonucleotide duplexes custom synthesized by Dharmacon. The sense sequences for the two siRNAs are as follows: CGACGGAGUUUGACUACGAUU(1) and CUCACAAAUUACCGUAUU(2). Experiments were performed with each siRNA individually and the two duplexes combined. As a negative control, non-targeting control pool from Dharmacon (D-001810-10-05) was used. Effective protein knockdown was verified at 48 and 72 h post transfection and prior to functional assays, as described below.

Fluorescence Measurements to quantify US28 expression levels

Images were taken at fixed exposures with an Axio Image Z2 microscope (Zeiss). The fluorescence intensities, from at least 100 cells, were quantified using ImageJ software; plots representing cumulative distribution of mean pixel intensity for various conditions are shown. Kolmogorov-Smirnov test was used to determine whether the measured differences were statistically significant.

Expression profiling using the HCMV DNA array and the Affymetrix Gene ST1 array

Total RNA was isolated and the quality verified as described below for RT-PCR. The RNA was processed for microarray hybridization at the Center for Applied Genomics, UMDNJ-New Jersey Medical School. The HCMV arrays were printed and processed as described previously (11). Briefly, the array contains 65-mer oligonucleotides representing 194 predicted open reading frames of the HCMV strain AD169, 19 oligonucleotides for ORFs in the Toledo strain that are not found in AD169 and 44 human genes as controls. Total RNA (3 ug) was reversed transcribed to cDNA using Superscript II RT in the presence of Cyanine-3 or Cyanine-5 dUTP. The labeled cDNA was purified and hybridized to the arrays at 58°C for 16 hours. The slides were scanned using an Axon 4200AL scanner and the images were processed using GenePix Pro 6.1. A normalization factor was calculated using 36 human control genes (11) by dividing the median intensity of the Cy5 signal by the median intensity of Cy3 signal of the controls. The data were normalized by multiplying the Cy3 signal of each spot by the normalization factor. The ratio of the Cy5 median intensity over the Cy3 median intensity was determined for each spot and the average ratio determined for the replicate spots. The accession number for data from both Affymetrix and HCMV platforms is **GSE31142**.

HUVEC Tube Formation Assay

Geltrex (Invitrogen #12760-013) was obtained from Invitrogen and thawed overnight at 4C. 100µl Geltrex/well was placed on the bottom of 24 well culture dishes and allow to solidify at 37C for 30 min. HUVECs were detached using EDTA and resuspended in endothelial cell medium supplemented with various growth factors or conditioned media at 40,000 cells/200µl/well. Tubes were allowed to form for 8–10 hrs and cells were visualized using a Nikon Inverted Eclipse TE-2000E microscope, fitted with a CCD Cascade II camera. NIS Elements AR3.0 was used to acquire images, which were further processed in Photoshop.

Statistical Data Analysis

Significant differences were determined using ANOVA or the unpaired Student's t-test, where suitable. Bonferroni-Dunn post-hoc analyses were conducted when appropriate. P values <0.05 defined statistical significance.

Additional methods are available as supplementary information.

RESULTS

US28 is endogenously expressed in human GBMs

US28 protein expression in human glioblastomas was assessed using immunofluorescence analysis of primary glioblastoma-derived cultures and immunohistochemical analysis of paraffin embedded tissues from several GBM specimens, including some that were used to generate the primary cultures. RT-PCR for US28, HCMV UL56 (a DNA packaging essential viral gene), and Rab14 (human house keeping gene) was performed using RNA isolated from snap-frozen tissues from the same cases. Fig 1A shows an example of immunofluorescence analysis of primary GBM cells which exhibit cytoplasmic and membrane staining for the US28 antigen. Pre-incubation of the primary antibody with excess US28 blocking peptide demonstrated specificity of immunostaining (Fig 1B).

As shown in Fig 1C, US28 expression was detected GBM biopsy paraffin embedded specimens. Fig 1D demonstrates specificity of staining, using the US28 blocking peptide in excess, as described above. Sections from the same sample show abundant staining for VEGF (Fig 1E) and COX2 (Fig 1F) suggesting the presence of enhanced angiogenesis and inflammation in and around the US28 positive tumor cells. Co-localization of US28 and VEGF in another case of primary glioblastoma is shown in Supplementary Fig 1. The specificity of the US28 antibody was established by comparing immunostaining of cells that were mock-infected, HCMV-infected, or ectopically expressing US28 (Supplementary Fig 2). To confirm that HCMV US28 mRNA was likewise expressed in human GBM specimens, we performed RT-PCR on RNA extracted from GBM biopsy specimens from several different patients. Uninfected NPCs showed no evidence of the amplified US28 gene product, or another conserved HCMV gene product-UL56 (Fig 1G). In contrast, we detected amplified US28 RNA transcripts in the primary GBM biopsy specimens from several patients, including a case found positive by immunohistochemistry (shown in panels C–F). All amplified US28 RT-PCR products were sequenced to confirm specificity to HCMV, and unique gene polymorphisms were identified in several specimens indicating that no laboratory or cross specimen PCR contamination occurred (C-terminal sequences alignment is provided in the supplementary information). Additional GBM and control brain tissues were immunostained for US28, COX2, VEGF, p-STAT3, and e-NOS (Supplementary Table 1). Of the 35 different brain tissues screened 53% were positive for US28 by RT-PCR and 65% were positive by immunohistochemistry; there was > 90% concordance in the results showing US28 detection, when both approaches were used (Supplementary Table 1).

HCMV infection of NPCs induces expression of US28 and CCL5, which together promote glioma invasiveness

To understand the role HCMV US28 might play in gliomagenesis, we first wished to ascertain that US28 is expressed during HCMV infection of human adult NPCs, the purported cells of origin of adult GBM. NPCs were infected with HCMV (Towne and TR strains, 1MOI) - or mock infected. Total RNA was harvested at 72h and HCMV gene expression was assayed using a custom-made oligonucleotide microarray representing all the predicted open reading frames for HCMV (11). The same samples were profiled using human Affymetrix DNA arrays. As shown in Fig. 2.A, US28 was among the most

abundantly expressed HCMV transcripts following infection with either viral strain. Interestingly, one of most up-regulated human transcripts was the chemokine CCL5/RANTES (Fig 2B, arrow). While US28 can act as a constitutively active receptor, CCL5 is a *bona-fide* ligand for US28, and can further stimulate US28 signaling, suggesting that US28 and CCL5/RANTES co-expression might induce a potent autocrine signaling loop. To determine whether expression of CCL5 is a relevant biomarker for GBM, we analyzed the *Rembrandt* GBM data base. We determined that CCL5 expression levels were inversely correlated with survival in human glioblastomas (Fig. 2C). Analysis of previously characterized glioblastoma molecular subclasses (12) showed that CCL5 expression levels are elevated in the “mesenchymal” GBMs, characterized by poor patient outcome (reference 12 and Supplementary Fig 3).

To assess the effects of US28 expression on glioma invasiveness, we performed Matrigel invasion assays comparing LXS-N to US28-LXS-N transduced U251 and U87 glioma cells and two primary glioma cultures, which had no detectable HCMV transcripts. US28 over-expression resulted in a ~30% increase in the invasiveness of all glioma cell lines tested (Fig 2D). The presence of 50ng/ml recombinant human CCL5 in the lower chamber further enhanced invasiveness of glioma cells and primary GBM cultures by 50–60%, as shown in Fig 2D. These data demonstrate that CCL5, which is up-regulated by HCMV infection, can augment US28-induced glioma cell invasion.

To establish the specificity of US28 effects on glioma cell invasion, we used a siRNA approach to knockdown (KD) US28 expression in a well characterized human glioma cell line, U87 (13) persistently infected with HCMV (Supplementary Fig 4). US28 protein levels were measured using fluorescence intensity measurements of cells processed for US28 immunofluorescence. US28 siRNA 1 induced a ~40% US28 KD, while siRNA2 induced ~60% US28 KD (Supplementary Fig 5). When used together, siRNA1+2 induced a ~80% US28 KD (Supplementary Fig 5). We used a CCL5 neutralizing antibody to distinguish between US28 constitutive activity and the response to the CCL5 ligand secreted by human glioma cells. Fig 2E shows that CCL5 levels were significantly (~75%) inhibited in U87 cells by pre-incubation with a CCL5 neutralizing antibody (20 ng/ml, 12h), regardless of the presence of HCMV or US28. While US28 KD had no effect in uninfected U87 cells, Matrigel invasion of HCMV+ U87 cells was inhibited by ~20% by US28 siRNA1 or 2 used alone, and by 30% when the two siRNAs were used together (Fig 2F and supplementary Fig 5). Pretreatment with CCL5 neutralizing antibody inhibited glioma cell invasion by ~30–35% and the use of both US28 KD and CCL5 neutralization did not further increase this effect (Fig 2F and supplementary Fig 5). US28KD a primary GBM culture, confirmed to be HCMV positive, resulted in inhibition of tumor cell invasion by ~35%, both baseline and in response to CCL5 stimulation (Supplementary Fig 6).

US28 activates multiple oncogenic pathways in human NPCs

To determine additional oncogenic pathways activated by HCMV infection/US28 expression in NPCs, we used a phospho-kinase human array (R&D) embedded with antibodies specific for multiple phospho-proteins (Fig 3A, B). Pathways associated with glioma progression and invasion, including phospho-STAT3, AKT, ERK1/2, FAK, Src and eNOS were significantly activated by both whole virus infection and US28 overexpression in NPCs (Fig 3C). Immunofluorescence analyses of US28 over-expressing NPCs confirmed up-regulation of COX2, VEGF, p-STAT3, and e-NOS (Fig 3D). e-NOS levels, which are elevated in gliomas, correlate with increased tumor aggressiveness (14, 15). In addition to its pro-angiogenic role, e-NOS mediates production of nitric oxide, which was shown to induce the growth of glioma initiating cells (16). This is the first report documenting that HCMV US28 induces e-NOS activation, which contributes to glioma pathogenesis.

Using western blot and immunofluorescence, we confirmed that US28 induces p-STAT3 in neural precursor cells (Supplementary Fig 7). STAT3 activation is critical for NPC malignant transformation towards a mesenchymal GBM phenotype (17), suggesting that US28-induced activation of p-STAT3 may contribute to gliomagenesis. Consistent with a recent report, we also found that US28 and p-STAT3 co-localize in primary glioblastomas *in situ* (Supplementary Fig 8), which would explain why HCMV positive glioma cells exhibit activation of the STAT3 pathway, implicated in promoting inflammation, maintenance of glioma stem cells, and tumor progression (8, 18).

US28 promotes GBM angiogenesis

We next investigated whether US28 can modulate VEGF levels in neural precursor and glioma cells using immunofluorescence and ELISA. Fig 4A shows that VEGF is significantly up-regulated in US28 expressing NPCs. VEGF levels were measured in four different cell types (NPC, U251 and U87 glioma cell lines, and a primary GBM-derived line) using a highly sensitive ELISA. Seventy two hours following infection with either Towne or TR HCMV strain, or US28 overexpression, VEGF was induced > 2 fold in all cell types tested (Fig 4B). US28 overexpression alone was sufficient to induce equivalent levels of VEGF expression to those found after infection with whole HCMV, suggesting that US28 may play a predominant role in the HCMV-induced VEGF secretion. Remarkably, NPCs, which are non-malignant, were also induced to produce VEGF, suggesting that US28 expression may promote an angiogenic phenotype in normal adult neural cells. A human umbilical vein endothelial cell (HUVEC) tube formation assay was used to quantify angiogenesis. Fig. 4C shows that NPCs HCMV –infected or overexpressing US28 produced supernatant enriched in pro-angiogenic growth factors which induced a dramatic increase in HUVEC tube formation compared to mock infection or transduction with control vector (Fig 4D). These data indicate that US28 expression in a normal neural precursor cell could stimulate angiogenesis of neighboring endothelial cells. To demonstrate specificity of the US28 pro-angiogenic activity, we performed loss of function experiments, using siRNA to knock down US28 in persistently infected glioma lines. US28 KD inhibited VEGF production and glioma cell mediated angiogenesis as measured by HUVEC tube formation assays. Fig 5A illustrates US28 and VEGF detection in persistently infected U87 glioma cells before and after US28 KD. We used quantification of immunofluorescence signals to measure the extent of US28 protein knockdown (Fig 5B and supplementary Fig 5). Cumulative distribution of pixel intensity for immuno-positivity illustrates that ~80% of US28 positive cells lost their signal after treatment with targeting US28 siRNA 1+2, confirming effective protein knockdown (Fig 5B). A similar level of US28KD was achieved in the 4121-HCMV infected cells following 72h treatment with targeting siRNA1+2 (data not shown). VEGF secretion was inhibited by US28 KD (Fig 5B, lower panel). Using ELISA, we determined that VEGF levels (initially induced by HCMV) were inhibited by 35% in HCMV –infected U87 and primary glioma cells, following US28 KD using siRNA1+2 (Fig 5C). Each US28 siRNA used separately had a more modest effect in inhibiting VEGF secretion, while uninfected glioma cells did not show a change in VEGF levels, confirming specificity of the US28KD effect (Fig 5C). Supernatants from persistently infected glioma cells +/- US28 siRNA 1+2 were used in a HUVEC tube formation assay. As shown in Fig 5D– E, US28 KD significantly inhibited the pro-angiogenic activities of the HCMV+ glioma cell supernatants. US28 KD in an endogenously infected primary GBM-derived culture inhibited VEGF secretion by ~50% (Supplementary Fig 6), suggesting potential therapeutic benefits for targeting US28 in GBM patients.

Further analysis of primary GBM cells from patients identified several tumor cases in which US28 expression was significant and where VEGF expression had a high level of co-localization with US28 (Fig. 6A– C and Supplementary Table 1). Immunofluorescence

analysis of primary GBM cells for eNOS and US28 indicated that US28 also co-localized with eNOS (Fig. 6D–F). Using paraffin embedded tissue samples from the same patient, we found HCMV US28, VEGF, e-NOS, and COX2 co-expressed both in tumor cells and within the tumor microenvironment (Figure 6G–L), suggesting that pro-inflammatory and pro-angiogenic signaling is, at least in part, initiated and promoted by US28 expression in infected GBM cells. Together with the other already described mechanisms, such as activation of the IL6-p-STAT3 pathway (8), and induction of CCL5 (our data), HCMV US28 emerges as a key regulator of GBM progression, by enhancing tumor cell invasion and angiogenesis (Figure 6M- diagram).

DISCUSSION

Our laboratory first identified and reported the presence of various HCMV proteins in human glioblastoma. In the current study, we investigated the role of US28 in driving critical signaling pathways supporting glioma growth, such as invasion and angiogenesis.

We performed a systematic screening of human primary glioma tissues and controls using immunohistochemical and RT-PCR/sequencing, which indicate that ~ 60% of human GBMs are US28 positive by one or more techniques. Based on these findings, we hypothesized that US28 expression in normal neural precursor cells might promote gliomagenesis, and that US28 expression in established GBM cells could promote tumor angiogenesis and invasion.

The experimental findings we present here support our hypothesis. When we infected NPCs with either a laboratory or a clinical HCMV isolate, we detected US28 among the most highly expressed HCMV genes. Furthermore, HCMV infection of NPCs resulted in ~ 40 fold increase in CCL5 mRNA levels, which could further enhance US28-mediated signaling in an autocrine manner, since CCL5 binds and activates US28 (19). CCL5 overexpression has been previously associated with glioblastoma (20) and we determined that its expression levels correlate with poor GBM patient outcome, by interrogating a public data base.

Our data demonstrate for the first time the existence of an autocrine signaling loop in HCMV infected or US28 expressing glioma cells which respond to CCL5 stimulation with increased invasive behavior. Interestingly, this interaction appears to be cell type specific, as another study has shown that macrophages expressing US28 migrate towards Fraktalkine (another US28 ligand) rather than CCL5 (21). In the context of glioma-associated inflammation, US28 may therefore modulate multiple autocrine and paracrine loops promoting an oncogenic tumor microenvironment. US28 overexpression also induced ~ three fold increase in VEGF levels in both NPCs and GBM cells. Together, these data indicate that US28 induces a pro-angiogenic and invasive phenotype in malignant glioma cells and in NPCs. To ascertain specificity of the pro-invasive and pro-angiogenic activities of US28, we performed loss of function experiments in persistently infected glioma cells and a primary glioma culture. We used the U87 glioma cell line bearing well defined genomic alterations in conjunction with two custom made siRNA duplexes used alone and in combination to assess the specificity of US28 effects. Our data demonstrate that US28KD significantly inhibited GBM cell invasion and secretion of biologically active VEGF in HCMV positive gliomas. These results also suggest that targeting US28 may have therapeutic benefits for GBM patients.

Immunohistochemical analysis of glioma patient biopsy specimens demonstrating co-localization of US28 with VEGF, COX2, p-STAT3, and e-NOS *in situ* suggests once again that multiple US28-driven mechanisms contributing to the aggressiveness of primary GBMs may exist. Our data add to and corroborate recent reports indicating that HCMV US28 expression can promote oncogenesis. Maussang et al have shown that US28 expression can

induce oncogenic transformation of 3T3 fibroblasts, and this work was recently extended upon by the demonstration that a critical mediator of the US28 oncogenic signaling pathway is NF- κ B, whose activation drives expression of VEGF and COX2 (22). Our data indicate that biologically active VEGF is induced by US28 in neural precursors and glioma cells and that COX2 and VEGF are co-expressed with US28 in GBMs.

Slinger et al. recently showed that US28 expression could be detected in human GBM specimens, and that it activates the IL6-STAT3 signaling pathway in a fibroblast model system (8). Our data demonstrate for the first time that US28 induces p-STAT3 activation in NPCs and documents co-localization of US28 and p-STAT3 in primary GBM cultures. A recent report showed that expression of HCMV US28 in intestinal epithelial cells led to high penetrance of adenocarcinomas in a transgenic mouse model of intestinal neoplasia (23). These tumors could be accelerated by co-expression of an US28 ligand, CCL2. Consistent with these observations, our results demonstrate that US28 increased glioma cell invasion, which was further enhanced by the addition of another US28 ligand, CCL5, a pro-inflammatory cytokine associated with aggressive GBMs.

Taken together, our data suggest that HCMV US28 may be a critical factor in promoting the transformation of NPCs and the invasive and angiogenic properties of established glioblastomas. Our results suggest that targeting specific HCMV proteins (e.g., US28) in endogenously infected GBMs may disrupt critical pathways and constitute a novel anti-tumor approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. HCMV US28 transcript and protein are expressed in human GBMs

A–B. Primary GBM-derived cultures were processed for US28 immunofluorescence in the absence (A) or presence (B) of a blocking peptide. Nuclei are counterstained with propidium iodide. Bar= 100μm. **C–F.** Consecutive (5μm) paraffin sections obtained from a different GBM patient sample were processed for US28 (C, D), VEGF (E), and COX-2 (F) immunohistochemistry. Counterstaining, hematoxylin. Bar= 100μm. **G.** RT-PCR for US28 was performed using cDNA from several GBM cases. HCMV infected neural precursor cells (NPC+CMV) served as positive control. Several cases show a US28 band of the correct size. HCMV UL56 detection is also shown. Rab 14 was used to verify equal loading. NC= Negative control.

Figure 2. US28-CCL5 signaling promotes glioblastoma invasiveness

A. NPCs infected with HCMV Towne and TR (1MOI, 72 h) were profiled using a HCMV DNA microarray containing all predicted open reading frames for Ad169/Toledo strains. Expression levels of HCMV transcripts are displayed as fold increase over uninfected control. **B.** RNA from HCMV-treated and control NPCs were profiled using Affymetrix Gene 1.0 ST DNA arrays. The heatmap shows the 30 most upregulated and 30 most downregulated human transcripts in HCMV-infected NPCs vs mock. CCL5 was induced over 40 fold by HCMV treatment (arrow). **C.** Kaplan-Meier curves showing the relationship between levels of CCL5 transcript and survival probability in patients with glioblastoma (log-rank p-value upregulated vs all other samples, $p=0.001523$, REMBRANDT data base, NCI). **D.** Human glioma cells (U251 and U87) and two primary glioblastoma-derived cultures (designated GBM#1 and GBM #2) transfected with US28 or control vector were subjected to Matrigel invasion assays in the absence or presence of CCL5 (50ng/ml). ** $p<0.005$, ANOVA. **E.** CCL5 levels measured by ELISA in mock-treated U87 cells, or HCMV infected +/- CCL5 neutralizing antibody. **, $p<0.005$ ANOVA. **F.** Mock and HCMV-infected U87 cells were subjected to Matrigel invasion assays. Mean number of cells/filter are shown for each condition. ** $p<0.005$ ANOVA. US28 KD was achieved using two

siRNA duplexes in combination (siRNA1+2). Data from one representative experiment are shown. Each condition was performed in triplicate and experiments were repeated three times.

Figure 3. US28 induces activation of cellular kinases involved in glioma pathogenesis
AB. HCMV (Towne, 1MOI) and Mock- treated NPCs (**A**) and glioma cells (**B**) were profiled using a human phosphor-kinase antibody array. **C.** Densitometry measurements were performed per manufacturer's instructions. Percentage change in phosphorylation levels between HCMV/US28 treated and control cells are shown. One (out of two) representative experiment is shown. **D.** Double immunofluorescence for US28 and the indicated proteins in NPCs transduced with LXSN-US28 for 48h. Right panels represent IgG staining controls. Nuclei were counter-stained with propidium iodide. Bar = 50µm.

Figure 4. US28 promotes glioma angiogenesis

A. NPC transduced with either LXSN-HA-US28 or Ad-US28 and control LXSN/Mock treated cells were processed for immunofluorescence. Right panels- NPCs that express US28 (green) secrete VEGF (blue), as demonstrated by co-localization of the two markers. Nuclei are stained with propidium iodide. Bar= 100 μ m. **B.** NPC, U251, U87, and a primary GBM line (4121) were treated with HCMV (Towne, TR, 1 MOI), transduced with Ad-US28, or treated with EGF (50ng/ml) in serum free media. Supernatants were used in an ELISA for VEGF. Samples were assayed in quadruplicate and the experiment was repeated twice. Comparisons between treated and mock within the same cell line, were analyzed using ANOVA *, $p=0.02$, **, $p<0.002$. **C.** NPC-derived supernatants were tested in HUVEC tube formation assays. Complete endothelial cell growth media was used as a positive control. Representative photomicrographs are shown. Each condition was assayed in six wells of a 24 well plate and the experiment was repeated twice. Bar= 100 μ m. **D.** Average numbers of branch points and endothelial cell lumens are shown from one representative experiment. Comparisons were analyzed using ANOVA. *, $p<0.02$ in all cases.

Figure 5. US28 Knockdown in HCMV infected glioma cells inhibits VEGF secretion and subsequent angiogenesis

A. Immunofluorescence was used for detection of US28 (green) and VEGF (red) in U87 cells persistently infected with HCMV treated either with control siRNA (upper panels) or siRNAs1+2 targeting US28. Nuclei were counterstained with DAPI. Bar = 50 μ m. **B.** Cumulative distribution of mean pixel intensity per cell obtained from immunofluorescent detection of US28 and VEGF in U87 cells treated with either targeting (1+2) or control siRNAs. Kolmogorov-Smirnov test was used to determine significance of differences in the fluorescence intensity measured in > 100 cells/condition, $p=0.0001$. **C.** VEGF levels were measured using ELISA in U87 glioma cells and primary 4121 GBM cells uninfected or HCMV –infected in the presence of either control or US28 targeting siRNA1, siRNA2, or siRNA 1+2. Differences were significant, * $p=0.05$; ** $p=0.002$ student T-test. **D.** Quantification of HUVEC branches and lumens formed in each of the indicated conditions. **, $p<0.02$ ANOVA. **E.** Representative photomicrographs of HUVEC tube formation assay in the presence of various types of conditioned media, as indicated. Bar= 100 μ m. HUVEC tube formation assays were repeated three times, each condition was run in quadruplicate.

Figure 6. HCMV US28 co-localizes with markers of invasiveness and angiogenesis *in situ*
A–F. Primary glioblastoma derived cells were processed for immunofluorescence using antibodies against US28 (**A** and **D**), VEGF (**B**) and e-NOS (**E**). Co-localization of US28 and the two markers of angiogenesis is shown by the merged photo-micrographs in panels **C** and **F**. Nuclei are counterstained using propidium iodide. Bar, 100µm. **G–L.** Consecutive paraffin sections (5µm apart) from a glioblastoma specimen were stained for US28, VEGF, e-NOS, and COX2 and developed using HRP-DAB. Arrows indicate cells positive for several markers in the same area. Counterstaining, hematoxylin. Bar= 50µm. **M.** The diagram summarizes the autocrine and paracrine signaling pathways through which US28 promotes GBM growth, invasion, and angiogenesis.