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Molecular ablation of tumor blood vessels inhibits therapeutic effects of radiation and bevacizumab

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Abstract

Background. Glioblastoma (GBM) is an aggressive and highly vascular tumor with median survival below 2 years. Despite advances in surgery, radiotherapy, and chemotherapy, survival has improved modestly. To combat glioma vascular proliferation, anti-angiogenic agents targeting vascular endothelial growth factor (VEGF) were introduced. Preclinically these agents were effective, yet they did not improve overall survival in phase III trials. We tested the hypothesis that ganciclovir (GCV)-mediated killing of proliferating endothelial cells expressing herpes simplex virus type 1 thymidine kinase (HSV1-TK) would have direct antitumor effects, and whether vessel ablation would affect the antitumor activity of anti-VEGF antibodies and radiotherapy.

Methods. Proliferating endothelial cells were eliminated using GCV-mediated killing of proliferating endothelial cells expressing HSV1-TK (in Tie2-TK-IRES-GFP mice). Syngeneic NRAS/p53 (NP) gliomas were implanted into the brains of Tie2-TK-IRES-GFP mice. Endothelial proliferation activates the Tie2 promoter and HSV1-TK expression. Administration of GCV kills proliferating tumor endothelial cells and slows tumor growth. The effects of endothelial cell ablation on anti-angiogenic therapy were examined using anti-VEGF antibodies or irradiation.

Results. GCV administration reduced tumor growth and vascular density, increased tumor apoptosis, and prolonged survival. Anti-VEGF antibodies or irradiation also prolonged survival. Surprisingly, combining GCV with irradiation, or with anti-VEGF antibodies, reduced their individual therapeutic effects.

Conclusion. GCV-mediated killing of proliferating endothelial cells expressing HSV1-TK, anti-VEGF antibodies, or irradiation all reduced growth of a murine glioma. However, elimination of microvascular proliferation decreased the efficacy of anti-VEGF or irradiation therapy. We conclude that, in our model, the integrity of proliferating vessels is necessary for the antiglioma effects of anti-VEGF and radiation therapy.

Keywords

bevacizumab | glioma angiogenesis | radiotherapy | Tie2-TK | tumor vessels

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults. Median survival is less than 2 years from diagnosis.[1] Standard therapy includes maximal safe surgical resection, chemotherapy, and radiotherapy. Molecular diagnosis is becoming integrated into the management of glioma tumors along with the classical histological findings of necrosis, pseudopalisading, and microvascular proliferation.[2] Gliomas are a highly vascular tumor. Increased vascular density is associated with glioma aggressiveness and correlates with worse patient prognosis.[3] There are a number of mechanisms by which gliomas acquire a blood supply.[4] Tumor cell migration initially

Importance of the study

This study addresses the role of proliferating vessel endothelial cells in glioma growth using a transgenic mouse model in which we can ablate proliferating endothelial cells. This model allowed us to test the role of endothelial proliferation on tumor growth, on its own and in combination with other treatments. Elimination of endothelial proliferation, radiation, or bevacizumab increased animal survival. To our surprise, by combining the elimination of endothelial proliferation with either of the other two treatments, we obtained a reduced survival response. Bevacizumab is thought to act through vessel normalization, and glioma irradiation requires adequate tissue oxygenation, to reduce tumor growth. Our results show formally that, in our model, intact tumor blood vessels are necessary for the antitumor effects of bevacizumab and radiotherapy. The clinical implications of our results suggest that the status of tumor vascularization is an important parameter to consider when evaluating tumor responses to potentially vessel-disrupting agents.

occurs along existing blood vessels through vessel co-option.[5,6] The existing vasculature is eventually unable to support tumor growth resulting in hypoxia and expression of hypoxia inducible factor 1α (HIF-1α).[7,8] In response to HIF-1α, gliomas undergo an "angiogenic switch" and increase secretion of pro-angiogenic growth factors. The most important growth factor induced by HIF-1α is vascular endothelial growth factor (VEGF), the expression of which has been shown to correlate with tumor grade and patient survival.[9] Due to its central role in angiogenesis, inhibition of VEGF has been a general strategy for the treatment of glioma. Bevacizumab is a humanized monoclonal antibody that neutralizes VEGF, thus preventing its interaction with VEGF receptors and subsequent angiogenesis.[10] Bevacizumab was approved for recurrent glioma in 2009 after demonstrating increased progression-free survival (PFS) in clinical trials.[11] Despite early promise, 2 large phase III trials involving bevacizumab failed to demonstrate an overall survival (OS) benefit in unselected patient populations compared with standard combination temozolomide and radiation followed by cyclic temozolomide.[12–14] The impact of bevacizumab on PFS indicates that anti-VEGF therapy is biologically active; however, tumor escape mechanisms may prevent a durable treatment response.

Cellular and molecular mechanisms of resistance to anti-angiogenics are not well understood. VEGF-independent mechanisms of angiogenesis may be important contributors to treatment resistance.[15] Glioma patients resistant to anti-VEGF therapy have increased plasma levels of alternative pro-angiogenic factors such as fibroblast growth factor 2, stromal cell derived factor 1, platelet derived growth factor, angiopoietin 1 and 2, and c-Met.[16] Additionally, dual inhibition of VEGF and angiopoietin 2 improved OS in a murine glioma model.[17] Angiogenesis may also occur through migration of bone marrow–derived endothelial precursor cells to the tumor, such as Tie2 positive monocytes and macrophages.[18] Simultaneous targeting of multiple angiogenesis pathways may overcome anti-VEGF treatment resistance.

To understand the role of tumor blood vessel integrity on glioma progression, we studied the effect of inducible genetic ablation of proliferating vascular endothelium on OS in a murine glioma model. We tested the hypothesis that the therapeutic effect of anti-VEGF and radiation therapy in glioma would be potentiated by eliminating cells that contribute to the repair and growth of blood vessels in the brain (ie, vascular endothelial cells and bone marrow–derived monocytes).

Our results indicate that killing of cells that contribute to the repair and growth of glioma blood vessels prolonged the survival of Tie2-TK mice implanted with syngeneic glioma cells. To our surprise, however, in our model, disruption of the glioma microvasculature inhibited the antitumor effect of anti-VEGF or radiation. We conclude that, in our model, intact tumor blood vessels are necessary for the long-term effectiveness of antiglioma therapy such as anti-VEGF or irradiation. Clinically, our results suggest that careful consideration ought to be given to the effects of antiglioma drugs, especially when administered concomitantly with irradiation and/or bevacizumab.

Materials and Methods

Animal Strains

Six- to 8-week-old transgenic Tie2p/e LV [Tie2-TK-IRIS-GFP] Friend virus B mice were genetically engineered to conditionally express thymidine kinase specifically within Tie2-expressing cells. These mice were a kind gift from Dr Luigi Naldini (see Supplementary material for details). All animals were maintained and experiments conducted in accordance with the Unit for Laboratory Animal Medicine at the University of Michigan.

Glioblastoma Cell Lines

A primary glioma cell line expressing NRAS and reduced levels of p53 (NP) was generated from a mouse bearing an NP-glial tumor induced by the Sleeping Beauty system[19] (see Supplementary material).

Stereotactic Tumor Implantation and Processing of Tissue Samples

NP neurospheres (1.0×10^3 cells in 1 μL) were stereotactically injected into the right striatum of Tie2-TK adult mice, as previously described[20]; see Supplementary material for details.

Immunohistochemistry and Hematoxylin-Eosin Staining

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining was performed on vibratome and paraffin brain sections of mice treated with phosphate buffered saline (PBS), ganciclovir (GCV), ionizing radiation (IR), IR+GCV, VEGF, and VEGF+GCV. IHC and H&E protocols can be found in the Supplementary material.

Ganciclovir Administration

NP tumor bearing Tie2-TK mice were treated twice daily with 25 mg/kg of the prodrug, GCV (Boitang, cat # RG001) or PBS via intraperitoneal injection.[21] Injections were performed from day 4 to 11 post tumor implantation.

Anti-VEGF Therapy

NP tumor implanted mice were treated with mouse anti-VEGF antibody (Genentech #5563) on days −1, 2, 5, 8, 11, 14, and 17 at a dose of 40 mg/kg i.p.

Radiation Treatment

Five days post implantation, Tie2-TK mice were irradiated with 20 Gy IR (2 Gy/day for 10 days).

Quantification of Immune Infiltration

The following groups were quantified for the detection of immune infiltration: PBS, GCV, IR, IR+GCV. Immune infiltration was made up of neutrophil nests, which were composed of >90% neutrophils, as determined by a pathologist (H.A.). Immune infiltration (ie, neutrophil nests) were quantified using ImageJ from images taken randomly from each tumor. A mean of 13 images per tumor were used, and we analyzed 2–5 tumors per group.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7. Data were analyzed with a Student's *t*-test or ANOVA as specified in Fig. legends. Data are represented as mean ± SEM. Values were considered significant at $P \leq 0.05$. Kaplan–Meier survival curves were analyzed using the Mantel–Cox log-rank method.

Results

Characterization of Anti-Angiogenesis in a Tie2-TK Glioma Model

Tie2-TK mice were genetically engineered to express the conditionally cytotoxic gene thymidine kinase from herpes simplex virus type 1 thymidine kinase (HSV1-TK) under the transcriptional control of the endothelial Tie2 promoter. This promoter was also linked to green fluorescent protein (GFP) by an internal ribosomal entry site (IRES) element (Fig. 1A). In these mice, Tie2-positive TK-expressing proliferative endothelial cells were selectively killed by the administration of GCV (Fig. 1B). TK converts GCV to GCV triphosphate, a purine analog that inhibits DNA replication selectively in proliferating Tie2-positive cells and leads to their apoptosis (Fig. 1B). IHC demonstrates that tumor vessels from Tie2-TK mice are positive for GFP and express cluster of differentiation (CD)31 (Fig. 1C). Co-localization confirms TK expression specifically in endothelial cells. Endogenous GFP expression from the Tie2 promoter and IRES is not strong enough alone to be detected without IHC (Fig. 1C3). GFP expression within the blood vessels co-localized with CD31 expression demonstrated at low-power (Fig. 1C4) and high-power (Fig. 1C5) merged images. We also examined the invasion pattern of NP tumors in an intracranial orthotopic model established in Tie2-TK mice. Confocal imaging of CD31 expression revealed that NP tumor cells (shown in green) were associated with the blood vessels (shown in red) at the invasive tumor border, as indicated by white arrowheads (Fig. 1D1 and D2).

GCV Treatment Impairs Tumor Induced Angiogenesis in Tie2-TK Mice

We hypothesized that GCV treatment of Tie2-TK mice bearing NP tumors would result in reduction of neoangiogenesis. We analyzed blood vessel morphology and density across the tumors among 4 treatment groups (IR alone, GCV alone, IR+GCV, and PBS). Microscopic analysis revealed the presence of avascular regions within the center of GCV treated tumors (Fig. 2A5, A6, and A7). We also observed reduced blood vessel density in the IR+GCV treated group (Fig. 2A13, A14, and A15). Blood vessel density within the center of the tumor in PBS and IR treated mice was not affected (Fig. 2A1, A2, A3, and A9, A10, A11). No differences were observed in vessel density at the tumor periphery (Fig. 2A4, A8, A12, and A16). ImageJ quantification of CD31 expression demonstrated a statistically significant reduction in blood vessel density at the tumor core in GCV treated ($^{**}P < 0.048$) and IR+GCV ($^{**}P < 0.05$) treated mice (Fig. 2B2 and B4). However, there was no significant difference in blood vessel density from tumor core to tumor border in PBS or IR treated mice (Fig. 2B1 and B3).

Direct Blood Vessel Killing Prolongs Survival of Glioma-Bearing Tie2-TK Mice

Inhibition of tumor angiogenesis with anti-VEGF therapy has failed to increase glioma patient survival in phase III trials.[13,22] Therefore, we wanted to determine if disrupting the proliferation of tumor vasculature through direct vessel cytotoxicity (ie, Tie2-HSV1-TK mice + GCV), alone or in combination with radiotherapy or anti-VEGF therapy, prolongs survival (Fig. 3A). Kaplan–Meier survival analysis was performed for 4 treatment groups: PBS, GCV, IR, and GCV+IR (Fig. 3B). The mice in the GCV treatment group exhibited increased median survival compared with the PBS group (OS: PBS = 12 vs GCV = 18 days, $^{**}P \leq 0.001$) (Fig. 3B). IR treatment alone prolonged median survival compared with PBS treated mice (OS: IR = 25 vs

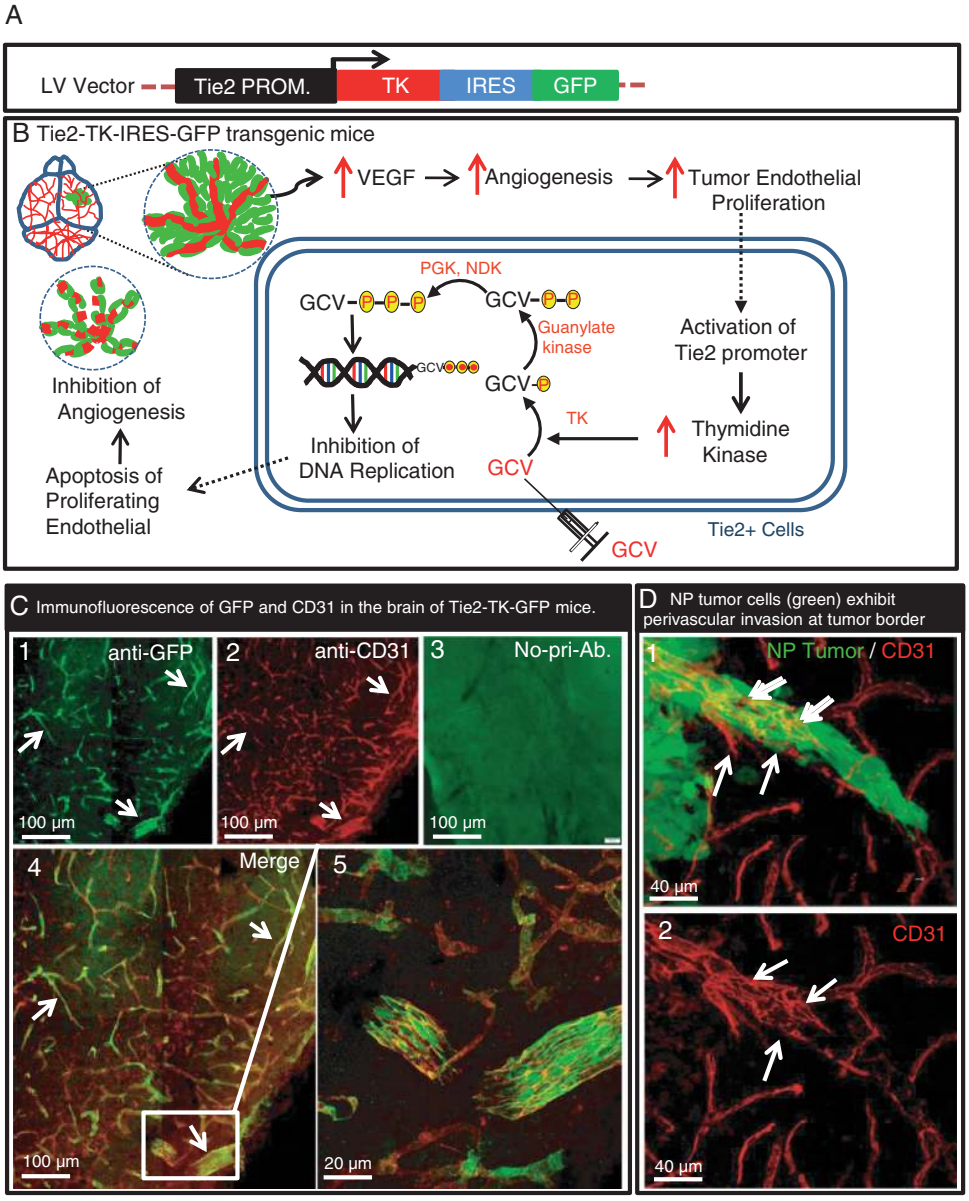

Fig. 1 TK-GCV mediated killing of proliferating endothelial cells in Tie2p/e LV [TgN(Tie2-TK-IRES-GFP)] mice. (A) Schematic representation of transgenic vector encoding herpes simplex virus for thymidine kinase, and GFP with an IRES element under endothelial specific promoter Tie2. (B) TK-GCV mediated killing of proliferating endothelial cells in the presence of GCV. GCV is monophosphorylated by HSV-TK, and further phosphorylated by guanylate kinase, phosphoglycerate kinase (PGK), and nucleoside diphosphate kinase (NDK). The triphosphate form of GCV (deoxythymidine triphosphate) is a purine analog that inhibits DNA replication and leads to apoptosis of dividing endothelial cells. (C) IHC of Tie2-TK mouse brain against GFP and CD31. (C1) Fluorescence confocal imaging of Tie2-TK mouse brain sections depicting GFP expression in brain microvessels. (C2) Brain microvessels from the same mouse immuno-labeled with endothelial-specific anti-CD31; white arrowheads illustrate GFP expression in endothelial cells. (C3) GFP expression was not detected in sections without primary anti-GFP antibody. (C4) Low and (C5) high magnification merged images confirm expression of GFP in brain microvasculature of Tie2-TK mice. (D) GFP expressing green NRAS/sh-P53 (NP) neurosphere tumor cells exhibit perivascular invasion. Fluorescence confocal image of GFP expressing NP tumor cells within the Tie2-TK mouse brain (left) and CD31 expressing microvasculature in red (right). White arrowheads indicate microvasculature-associated tumor invasion.

PBS = 12 days, $***P \leq 0.0001$). However, the combination IR+GCV treatment resulted in lower median survival compared with IR alone (OS: IR = 25 vs IR+GCV = 19 days; Fig. 3B). These results demonstrate that (i) GCV-mediated killing of proliferating endothelial cells in the tumor correlated with significantly prolonged survival of Tie2-TK mice and (ii) OS was reduced in IR+GCV treated mice compared with IR alone (OS: IR = 25 vs IR+GCV = 19 days). This

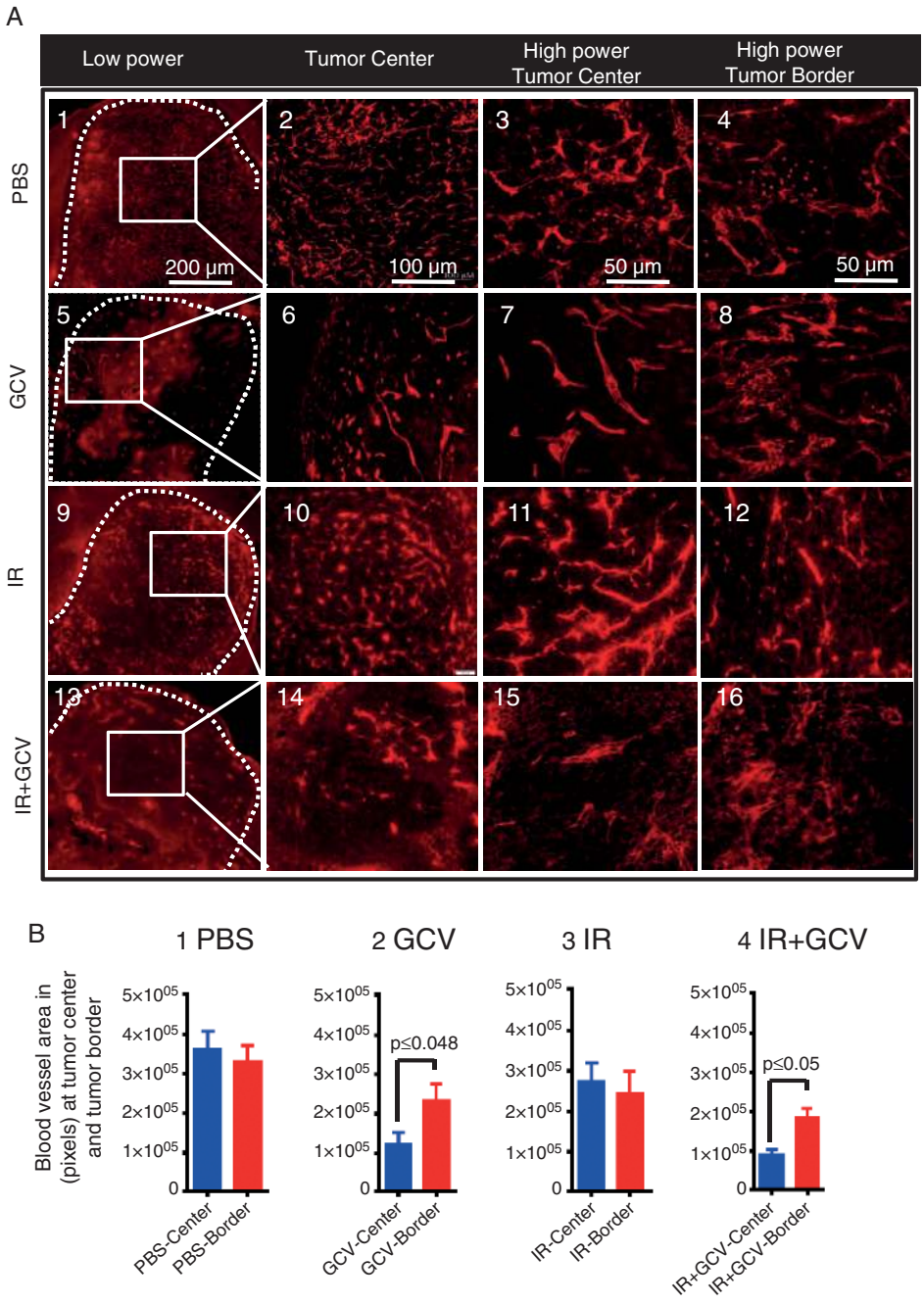

Fig. 2 GCV treatment effects microvasculature area in the tumor core. (A) Immunohistochemistry on sections of NP tumor-bearing Tie2-TK mice treated with PBS, GCV, IR, or IR+GCV with blood vessel specific anti-CD31 antibody. Significantly lower density of CD31-positive vessels was observed in GCV and GCV+IR treated mice compared with PBS treated mice. Higher magnification shows that GCV and GCV+IR treated mice have decreased blood vessels in the tumor core compared with the PBS treated group. Blood vessel density in the tumor core in IR treated mice was not disrupted. Blood vessel density at tumor border in all 4 four groups was unaffected. (B) Representation of total blood vessel area at tumor center in (PBS-C, GCV-C, IR-C, and IR+GCV-C) and at the tumor border (PBS-B, GCV-B, IR-B, and IR+GCV-B). Blood vessel area was significantly lower in the tumor center in GCV (**$P < 0.048$) and GCV+IR (**$P < 0.05$) treated mice compared with PBS-treated mice. Unpaired *t*-test. Nonsignificant = n.s. Error bars = mean ± SEM.

indicates that GCV-mediated killing of proliferating endothelial cells attenuates the efficacy of IR sensitivity on NP tumor progression in Tie2-TK mice.

Histopathologic analysis of tumors from these treatment groups revealed that tumors from GCV treated mice had large necrotic regions with invasion of polymorphonuclear

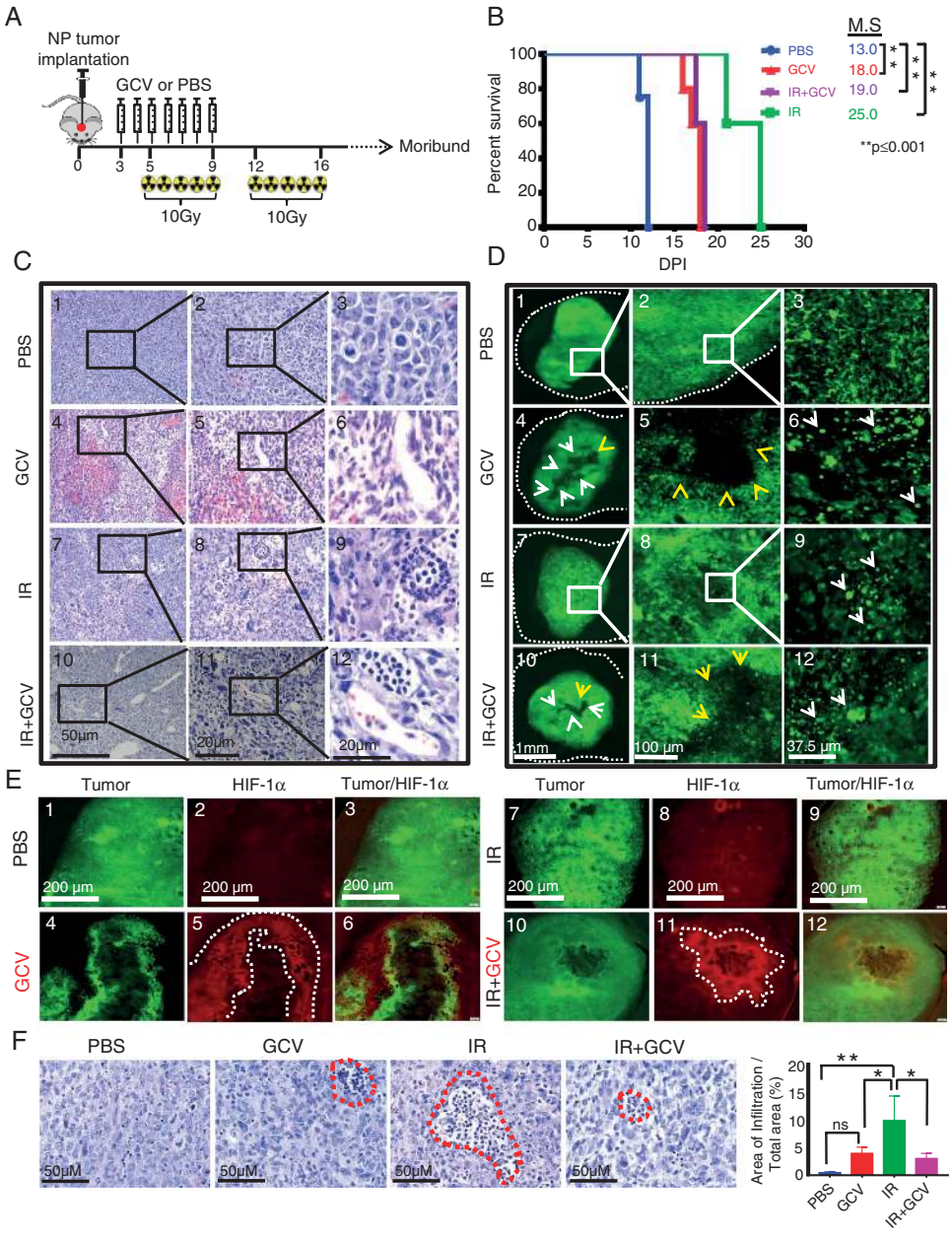

Fig. 3 Effect of GCV and IR treatment on NP tumor bearing Tie2-TK mice. (A) Experimental design for testing GCV treatment alone or in combination with radiation. (B) GCV and IR treatment alone significantly increases survival relative to PBS control. Median survival for PBS treated mice was 12 days, and for GCV and radiation treated mice 18 (**$P < 0.001$) and 25 (**$P < 0.001$) days, respectively. Median survival for GCV plus radiation treated mice was 18 days (**$P < 0.001$). (C) Histology is H&E staining of paraffin embedded sections from PBS, GCV, radiation, and GCV+radiation treated mice. Vessels with enlarged lumen and endothelial thinning were present in the GCV and IR+GCV groups. (D) Fluorescence scanning confocal micrographs of vibratome-cut brain sections of GFP expressing NP tumor cells (in green) implanted into mice treated with PBS, GCV, radiation, or GCV+ radiation. Sections from GCV treated mice have more necrosis relative to the PBS treated (white and yellow arrowheads). At higher magnification, there are a greater number of rounds, dying tumor cells indicated with thick white arrowheads. (E) Immunohistochemistry on sections of NP tumor-bearing Tie2-TK mice treated with PBS, GCV, IR, or IR+GCV with an antibody against hypoxia marker, HIF-1α. HIF-1α-positive hypoxic areas were marked with dotted white line in GCV and IR+GCV treated groups. (F) Inflammation (leukocyte infiltration) was evaluated in H&E stained sections from PBS, GCV, IR, and IR+GCV treated mice. The area occupied by neutrophil and leukocyte nests in IR and IR+GCV are indicated by red dotted lines in Fig. 3F. Areas occupied by infiltrating leukocytes were quantified using ImageJ software. Bar graphs (Fig. 3F, right-hand side) represent the total area of infiltrating leukocytes in each group. The area occupied by infiltrating leukocyte nests was highest in IR (**$P < 0.0016$, IR vs PBS; *$P < 0.024$, IR vs GCV; *$P < 0.024$ IR vs IR+GCV). Statistical evaluation was performed using a one-way ANOVA, followed by a posterior multiple comparison test, corrected for multiple comparisons by controlling the false discovery rate using the 2-stage step-up method of Benjamin, Krieger, and Yekutieli using the PRISM software. Nonsignificant = n.s. Error bars = mean ± SEM.

cells compared with PBS treatment (Fig. 3C4 and C5). Furthermore, blood vessels in GCV or GCV+IR treated mice displayed enlarged lumina and thinning of endothelial cells, likely leading to vessel leakiness. Blood vessels in PBS and IR treated groups appeared intact (Fig. 3C6 and C12). Tumors of GCV treated mice displayed extensive necrotic areas, mainly within the tumor centers (Fig. 3D). High power images of the necrotic areas demonstrated an abundance of dying round-shaped tumor cells, as indicated by white arrowheads in (Fig. 3D6 and D12). Tumors from PBS treated mice or IR alone did not exhibit necrosis (Fig. 3D1, D3, and D7). We next compared the pattern of HIF-1α immunoreactivity in each group by IHC. GCV and IR+GCV treated groups showed highest levels of HIF-1α immunoreactivity; in the GCV group the distribution was centered around the tumor periphery, while in the GCV+IR group it was centrally located (Fig. 3E). Higher expression of HIF-1α in the IR+GCV group compared with IR alone suggests that activation of the HSV1-TK system disrupts the tumor blood supply, thereby reducing tumor oxygenation, stimulating expression of HIF-1α, and thus reducing sensitivity to radiation. We further investigated the extent of inflammation (mostly polymorphonuclear infiltration) in brain sections from moribund mice (Fig. 3F). ImageJ analysis revealed that polymorphonuclear cells were significantly increased in the IR group compared with all other groups. Mice treated with GCV or IR+GCV had significantly reduced numbers of infiltrating polymorphonuclear cells compared with IR treated mice. Lower infiltration of inflammatory cells in the IR+GCV group suggests that GCV-mediated killing of proliferating endothelial cells reduces inflammatory infiltration, which could contribute to reducing the effects of IR therapy.

Endothelial Cell Death Increases Tumor Cell Apoptosis and Reduces Tumor Proliferation

GCV treatment blocked angiogenesis by disrupting reactive blood vessels, as shown in Fig. 3. We further investigated the effect of GCV treatment on the proliferation of tumor cells. Ki67 expression was assessed in PBS, GCV, IR, and GCV+IR treatment groups. Analysis revealed a lower Ki67 index in GCV (Fig. 4A4 to A6), IR (Fig. 4A7 to A9), and IR+GCV (Fig. 4A10 to A12) treatment groups within the tumor core compared with the PBS group (Fig. 4A1 and A3), as indicated by the white arrowheads. Tumor cell proliferation at the tumor border was not affected by GCV treatment (data not shown). Total Ki67-positive cells were significantly lower in GCV (***$P < 0.0003$), IR (***$P < 0.0002$), and IR+GCV (***$P < 0.0027$) groups relative to the PBS treatment group (Fig. 4B). Decreased Ki67-positive cells in GCV and GCV+IR treatment groups correlated with increased survival of Tie2-TK mice implanted with NP tumors.

GCV-Mediated Killing of Proliferating Endothelial Cells Enhances Cleaved Caspase-3 Immunoreactivity

We analyzed brains from moribund mice treated with PBS, GCV, IR, or GCV+IR for expression of cleaved caspase-3 (CC3), a marker of apoptosis. The tumor cells from animals treated with GCV (Fig. 5A3 to A6), IR (Fig. 5A7 to A9), or GCV+IR (Fig. 5A10 to A12) exhibited extensive apoptosis within the center of the tumor compared with PBS treatment (Fig. 5A1 to A3). The tumor border for all 4 treatment conditions did not demonstrate CC3 positivity (data not shown). Quantification of the total number of CC3-positive cells in each treatment group revealed that tumors from mice treated with either GCV (***$P < 0.0001$), IR (***$P < 0.0001$), or GCV+IR (***$P < 0.0005$) had significantly higher CC3-positive tumor cells compared with the PBS control (Fig. 5B). However, NP tumor bearing mice treated with IR+GCV had a lower number of CC3-positive cells compared with IR alone (Fig. 5B). The increased number of CC3-positive cells in the GCV and IR treatment groups correlates with increased survival.

GCV-Mediated Killing of Proliferating Endothelial Cells Attenuates the Efficacy of Anti-VEGF Therapy

To determine whether inhibition of proliferating vascular endothelium can increase the efficacy of anti-VEGF therapy, we studied the effect of combination anti-VEGF and GCV treatments on the OS of NP tumor bearing Tie2-TK mice. We treated 4 groups ($n = 4$/group) of NP tumor implanted Tie2-TK mice with (i) PBS (control), (ii) GCV, (iii) anti-VEGF antibodies, and (iv) GCV+anti-VEGF antibody and monitored tumor progression (Fig. 6A). GCV therapy alone significantly increased the survival of tumor bearing Tie2-TK mice (OS: GCV = 18 days) (Fig. 3B). Treatment with anti-VEGF antibodies alone extended the median OS of NP tumor bearing mice (OS: anti-VEGF = 25 days) compared with control (OS: PBS = 13 days) (Fig. 6B). However, mice treated with combined anti-VEGF plus GCV resulted in lower median survival compared with anti-VEGF antibody alone (OS: anti-VEGF = 25 vs anti-VEGF+GCV = 19 days) (Fig. 6B). Given the significant improvement in survival observed in mice treated with anti-VEGF therapy alone, but not with dual anti-VEGF plus GCV therapy, this prompted us to investigate the tumor vascular integrity and normalization in these treatment groups. CD31 IHC staining was performed for each treatment group. Microscopic analysis revealed that PBS treated tumors had increased angiogenesis. As previously demonstrated, GCV treated mice had a significant decrease in vessel density, with the most pronounced effect at the center of the tumor (Fig. 6C4 to C6). Tumors from mice treated with anti-VEGF therapy displayed a high density of intact and pathological blood vessels, suggesting a greater extent of vessel normalization after anti-VEGF therapy (Fig. 6C7 to C9). Blood vessel morphology in dual GCV and anti-VEGF therapy treated mice was disrupted and vessel normalization was not observed (Fig. 6C10 to C12). Low blood vessel density was observed in tumors of mice that received GCV treatment alone or in combination with anti-VEGF therapy. These data suggest that GCV-mediated killing of proliferating endothelial cells limits the efficacy of anti-VEGF treatment.

Discussion

Neoangiogenesis is a central biological process for glioma progression. Inhibiting the tumor blood supply is an

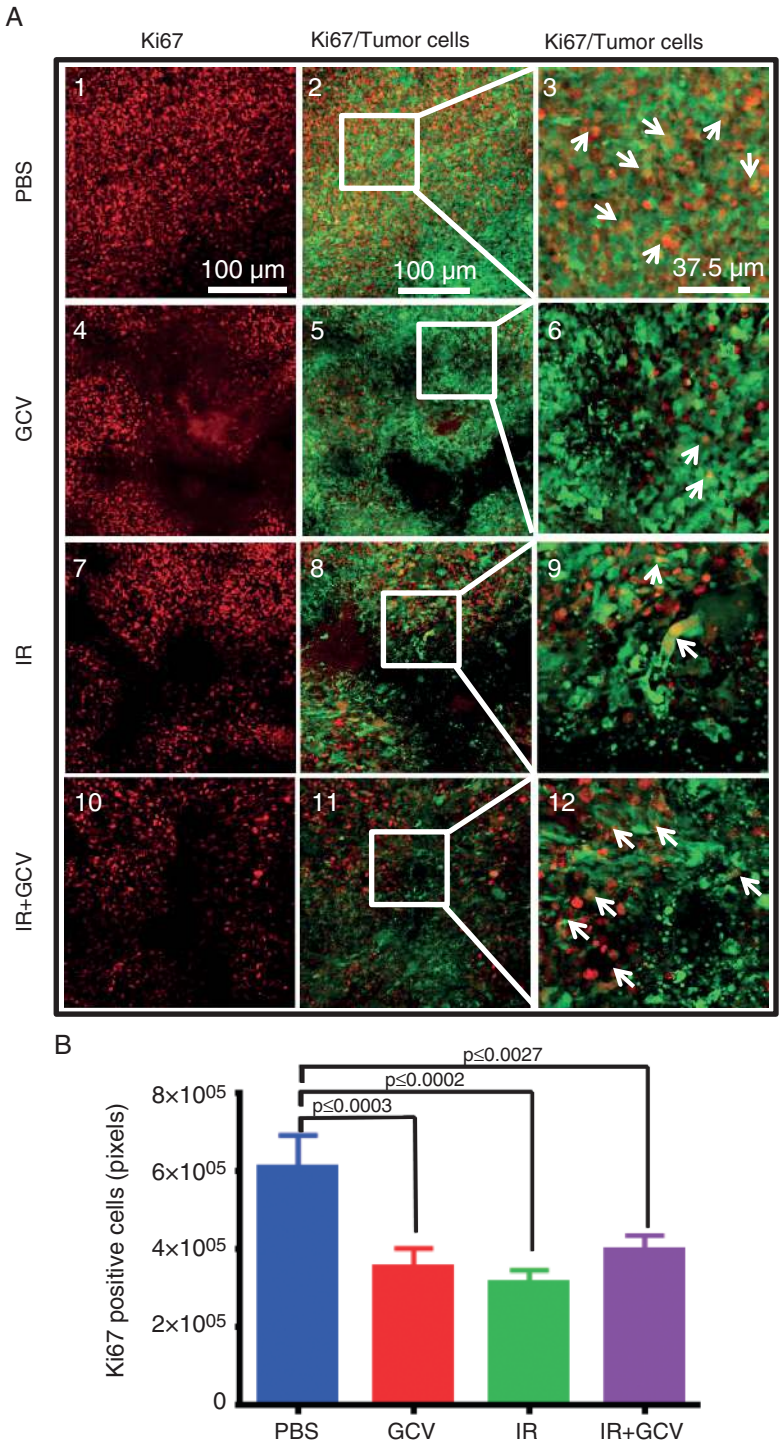

Fig. 4 GCV treatment reduces proliferation of NP tumor cells in Tie2-TK mice (A) Confocal images of Ki67 IHC demonstrating reduced proliferation with GCV, IR, and IR+GCV treatment relative to PBS treatment. (B) Bar graphs represent total Ki67-positive tumor cells in PBS, GCV, IR, and IR+GCV treated mice (***$P < 0.0003$ in all groups). Unpaired *t*-test. Nonsignificant = n.s. Error bars = mean ± SEM.

attractive therapeutic strategy. Bevacizumab is a monoclonal antibody that binds VEGF and is the most comprehensively studied anti-angiogenic therapy for the treatment of glioma. Clinical trials indicate that bevacizumab extends PFS in patients with primary gliomas.[13,14] However, multiple phase III randomized controlled trials failed to demonstrate improved OS.[12–14,23,24] The basis of bevacizumab's mechanism of action, the role of tumor neoangiogenesis

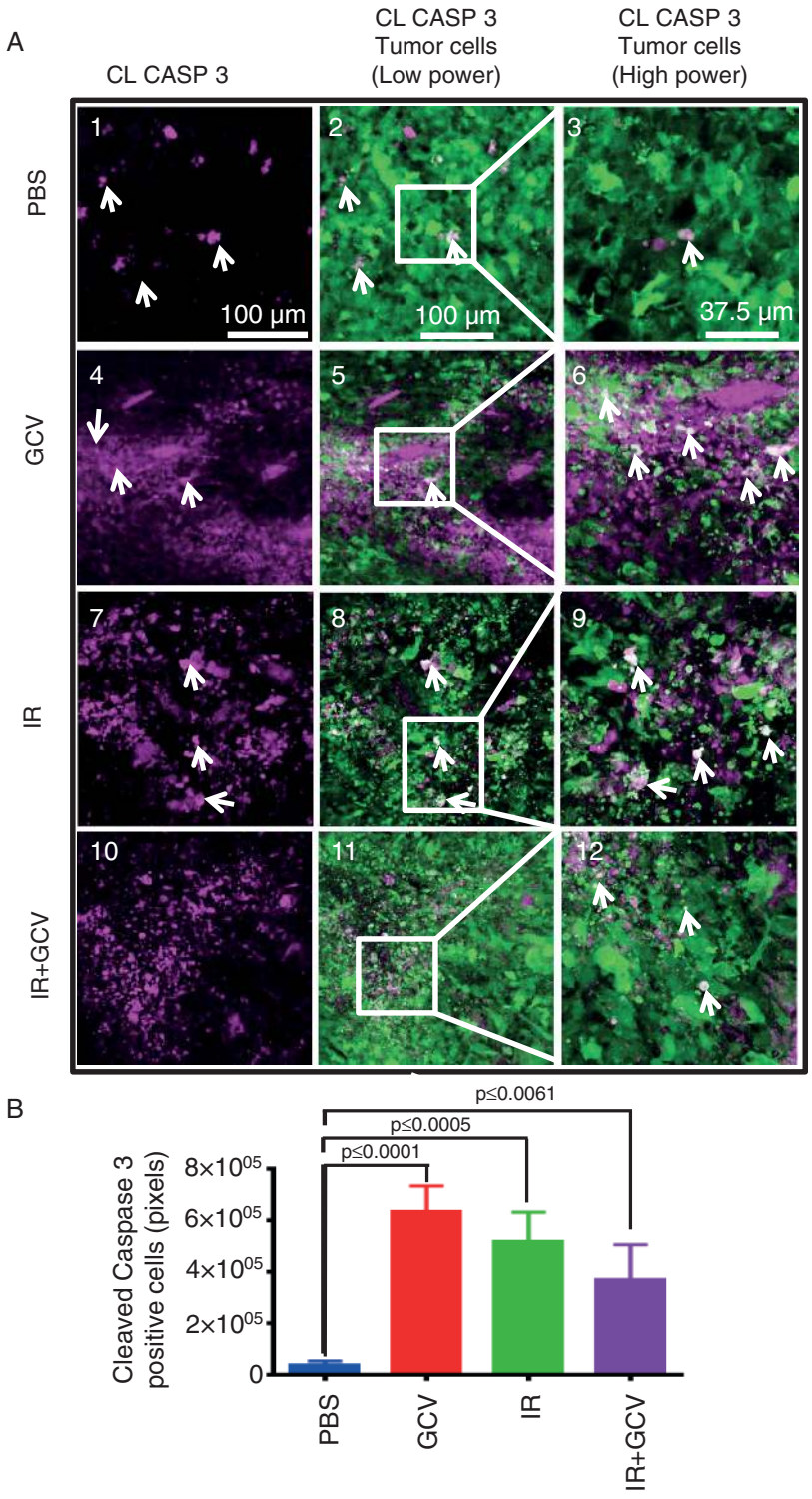

Fig. 5 GCV treatment induces cleaved caspase-3 (CC3) mediated NP tumor cell death in Tie2-TK mice. (A) Immunohistochemistry on sections of NP tumor-bearing Tie2-TK mice treated with PBS, GCV, IR, or IR+GCV with an antibody against apoptosis marker, CC3. Confocal imaging demonstrating a higher number of CC3-positive tumor cells in GCV, IR, and IR+GCV treated groups compared with PBS treatment. (B) Bar graphs represent total CC3-positive tumor cells in PBS, GCV, IR, and IR+GCV treated mice (***$P < 0.0061$ in all groups). Unpaired *t*-test. Nonsignificant = n.s. Error bars = mean ± SEM.

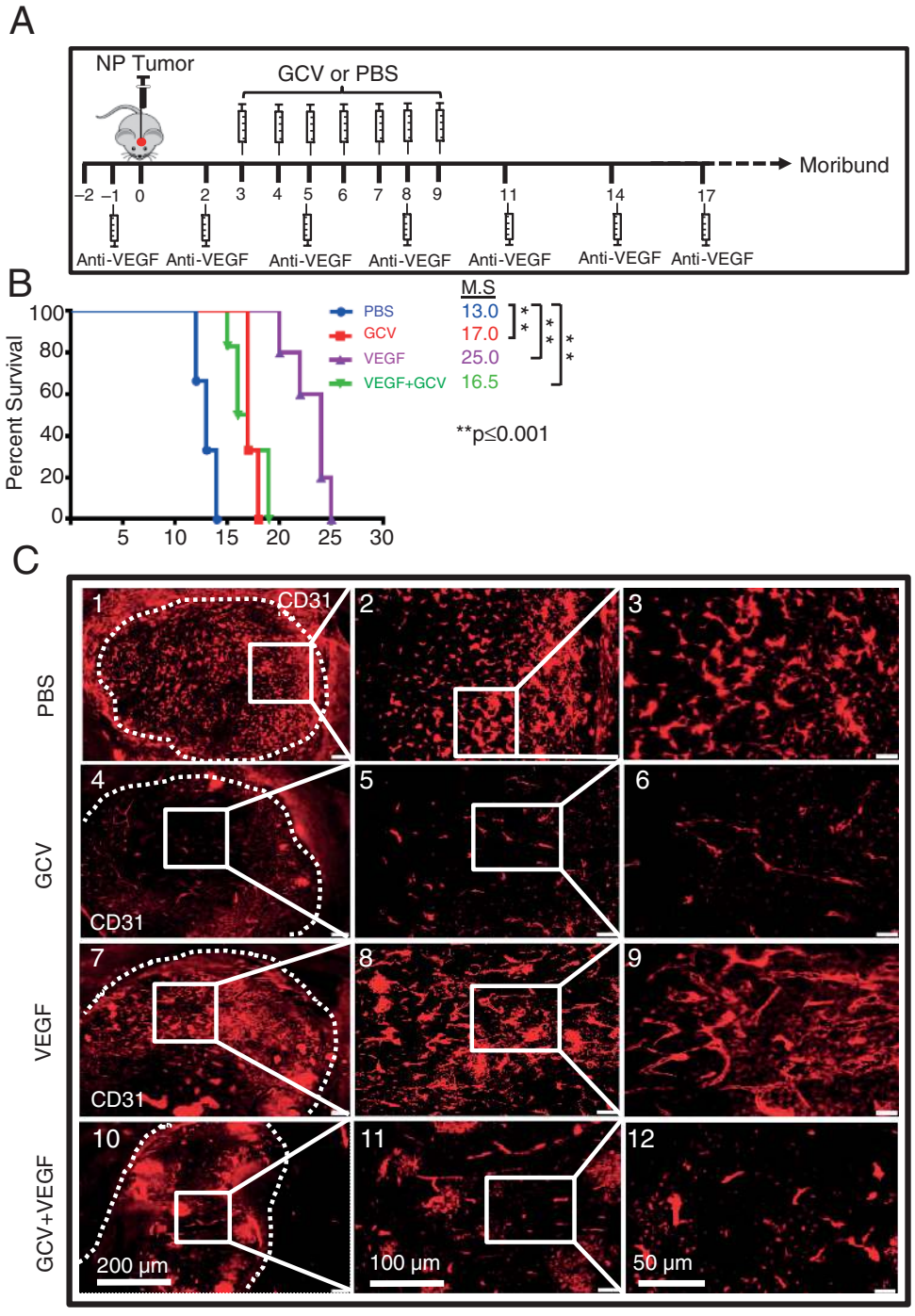

Fig. 6 GCV-mediated killing of proliferating endothelial cells in Tie2-TK mice reduces the efficacy of anti-VEGF therapy. (A) GCV treatment and anti-VEGF treatment schedule in Tie2-TK mice (n = 4/group) implanted with NP tumor cells. (B) GCV and anti-VEGF treated groups have prolonged survival compared with the PBS treated group. Median survival for PBS treated mice was 13 days and for GCV and anti-VEGF antibody treated mice 17 (**P < 0.001) and 25 (**P < 0.001) days, respectively. Median survival for GCV plus anti-VEGF treated mice was 16.5 days (**P < 0.001). Combined anti-VEGF antibody and GCV treatment reduced median survival of mice compared with anti-VEGF antibody treatment alone. (C) Brain microvasculature analysis of Tie2-TK mice bearing NP tumors, treated with PBS, GCV, anti-VEGF antibody, and GCV+anti-VEGF antibody. Fluorescence scanning confocal micrograph of brain microvessels labeled with anti-CD31 antibody at low and high magnification. CD31-positive blood vessels were more disrupted in GCV and GCV+anti-VEGF antibody treated mice compared with PBS and anti-VEGF antibody treated mice.

on tumor progression, and the mechanisms of resistance to bevacizumab are only partially understood. We examined the effects of bevacizumab and irradiation in a transgenic model of inducible elimination of proliferating endothelial cells. In this model, elimination of proliferating endothelial cells reduced the efficacy of both treatments.

Several mechanisms have been proposed to explain the resistance to anti-VEGF therapy in glioma patients. Recruitment of vascular progenitors such as Tie2-positive monocytes from the bone marrow and activation of Tie2/angiopoietin 2[25] pathways are associated with resistance to bevacizumab.[26] We have targeted VEGF independent mechanisms of angiogenesis by using a transgenic GBM mouse model with inducible, selective ablation of proliferating vascular endothelial cells. In this model, HSV1-TK is selectively expressed in vascular endothelium under transcriptional control of the Tie2 promoter. Administration of GCV leads to cytotoxic activity of TK and selective killing of proliferating endothelial cells.

Our data demonstrate that elimination of neoangiogenesis decreases tumor growth and prolongs animal survival. In these mice, tumor necrosis is more abundant in the center of the tumor, likely due to hypoxia and impaired angiogenesis caused by HSV1-TK. Further, our data demonstrate that there is a reduction in tumor cell proliferation and an increase in caspase-3 mediated apoptosis. A study by De Palma et al[18] demonstrates that GCV-mediated elimination of Tie2 expressing monocytes reduced tumor angiogenesis and tumor growth in N202 tumors implanted subcutaneously in mice.[18] However, this study did not evaluate the effect of TK-GCV on the antitumor efficacy of radiation and anti-VEGF therapy.

We also demonstrate that targeting Tie2-TK expressing cells preferentially eliminates endothelial cells from the center of the tumor. Hypoxic conditions are more prevalent in the tumor center, leading to increased activation and proliferation of endothelial cells and increased cell death upon administration of GCV. As per our[6] and others'[27] previous data, tumor cells toward the periphery are mainly located along remaining intact blood vessels. Radiosensitivity of tumor cells is determined in part by the concentration of dissolved tissue oxygen and reduction reactive oxygen species.[28] In our experiments, radiation therapy alone improved survival. However, activation of the HSV1-TK system counteracted the therapeutic effects of radiation. We demonstrate that activation of the HSV1-TK system disrupts the tumor blood supply, thereby reducing tumor oxygenation and sensitivity to radiation. This is consistent with recent phase III trials (AVAglio and RTOG 0825) in which no increased OS was observed when bevacizumab was combined with radiation therapy.[13,14]

Although the direct inhibition of proliferating endothelial cells prolongs survival in this in vivo glioma model, it fails to induce complete growth arrest and tumor regression. This suggests that tumor progression occurs independently of angiogenesis. We and others have previously demonstrated that glioma cell growth and invasion occur by vessel co-option and perivascular invasion.[6,29] The subjugation of the native blood supply of adjacent normal parenchyma may also be an escape mechanism by which a growing tumor evades anti-angiogenic therapy. This mechanism is independent of the inhibition of neoangiogenesis attributed to anti-VEGF therapy or by selective ablation of proliferating vascular endothelium as we have done in the Tie2-TK mice. In our model, the elimination of reactive blood vessels, contrary to our predictions, reduced the efficacy of anti-VEGF therapy. This may indicate that intact blood vessels and the induction of vessel normalization are necessary conditions for bevacizumab's therapeutic effect. Irradiation is known to increase the influx of inflammatory cells into tumors.[17,30]

Stimulation of inflammatory cell influx is likely to have an inhibitory effect on tumor growth. It is thus possible that GCV-mediated killing of proliferating endothelial cells may reduce the entry of inflammatory cells into tumors. Indeed, IR treated mice have the highest amount of infiltrating polymorphonuclear cells, and the highest survival. Treating these animals with GCV reduces both inflammatory infiltration and survival. GCV-mediated killing of proliferating endothelial cells may, at least in part, inhibit the therapeutic effect of radiation therapy. In summary, treatment of a murine glioma implanted into transgenic mice (expressing HSV1-TK in endothelial cells and precursors) with (i) GCV, (ii) anti-VEGF antibodies, or (iii) radiation prolongs animal survival. If, however, GCV and bevacizumab, or GCV and radiation, are administered concurrently, GCV-mediated killing of proliferating endothelial cells reduces the efficacy (ie, survival) of anti-VEGF therapy or radiation. These findings suggest that in our model intact blood vessels are necessary for full therapeutic efficacy of anti-VEGF and radiation. Whether in human patients the efficacy of anti-VEGF antibodies or radiation treatment is also dependent on the presence of intact blood vessels remains to be determined. Equally, whether any treatment (ie, radiation or temozolomide) that induces devascularization can inhibit later treatment with bevacizumab also requires further investigation.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

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References

1. Zhang AS, Ostrom QT, Kruchko C, Rogers L, Peereboom DM, Barnholtz-Sloan JS. Complete prevalence of malignant primary brain tumors registry data in the United States compared with other common cancers, 2010. *Neuro Oncol*. 2017; 19(5):726–735.
2. Scherer HJ. The forms of growth in gliomas and their practical significance. *Brain*. 1940;63:1–35.
3. Leon SP, Folkerth RD, Black PM. Microvessel density is a prognostic indicator for patients with astroglial brain tumors. *Cancer*. 1996;77(2):362–372.
4. Holash J, Maisonpierre PC, Compton D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science*. 1999;284(5422):1994–1998.
5. Yadav VN, Zamler D, Baker GJ, et al. CXCR4 increases in-vivo glioma perivascular invasion, and reduces radiation induced apoptosis: A genetic knockdown study. *Oncotarget*. 2016;7(50):83701–83719.
6. Baker GJ, Yadav VN, Motsch S, et al. Mechanisms of glioma formation: iterative perivascular glioma growth and invasion leads to tumor progression, VEGF-independent vascularization, and resistance to antiangiogenic therapy. *Neoplasia*. 2014;16(7):543–561.
7. Du R, Lu KV, Petritsch C, et al. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell*. 2008;13(3):206–220.
8. Calinescu AA, Yadav VN, Carballo E, et al. Survival and proliferation of neural progenitor-derived glioblastomas under hypoxic stress is controlled by a CXCL12/CXCR4 autocrine-positive feedback mechanism. *Clin Cancer Res*. 2017;23(5):1250–1262.
9. Chen W, He D, Li Z, Zhang X, Pan D, Chen G. Overexpression of vascular endothelial growth factor indicates poor outcomes of glioma: a systematic review and meta-analysis. *Int J Clin Exp Med*. 2015;8(6):8709–8719.
10. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun*. 2005;333(2):328–335.
11. Cohen MH, Shen YL, Keegan P, Pazdur R. FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme. *Oncologist*. 2009;14(11):1131–1138.
12. Stupp R, Mason WP, van den Bent MJ, et al; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352(10):987–996.
13. Gilbert MR, Dignam JJ, Armstrong TS, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med*. 2014;370(8):699–708.
14. Chinot OL, Wick W, Mason W, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. *N Engl J Med*. 2014;370(8):709–722.
15. Soda Y, Myskiw C, Rommel A, Verma IM. Mechanisms of neovascularization and resistance to anti-angiogenic therapies in glioblastoma multiforme. *J Mol Med (Berl)*. 2013;91(4):439–448.
16. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer*. 2008;8(8):592–603.
17. Peterson TE, Kirkpatrick ND, Huang Y, et al. Dual inhibition of Ang-2 and VEGF receptors normalizes tumor vasculature and prolongs survival in glioblastoma by altering macrophages. *Proc Natl Acad Sci U S A*. 2016;113(16):4470–4475.
18. De Palma M, Venneri MA, Galli R, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*. 2005;8(3):211–226.
19. Koschmann C, Calinescu AA, Nunez FJ, et al. ATRX loss promotes tumor growth and impairs nonhomologous end joining DNA repair in glioma. *Sci Transl Med*. 2016;8(328):328ra328.
20. Baker GJ, Chockley P, Yadav VN, et al. Natural killer cells eradicate galectin-1-deficient glioma in the absence of adaptive immunity. *Cancer Res*. 2014;74(18):5079–5090.
21. Sandmann T, Bourgon R, Garcia J, et al. Patients with proneural glioblastoma may derive overall survival benefit from the addition of bevacizumab to first-line radiotherapy and temozolomide: retrospective analysis of the AVAglio trial. *J Clin Oncol*. 2015;33(25):2735–2744.
22. Stupp R, Hegi ME, Gorlia T, et al; European Organisation for Research and Treatment of Cancer (EORTC); Canadian Brain Tumor Consortium; CENTRIC study team. Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071-22072 study): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2014;15(10):1100–1108.
23. Chinot OL, Nishikawa R, Mason W, et al. Upfront bevacizumab may extend survival for glioblastoma patients who do not receive second-line therapy: an exploratory analysis of AVAglio. *Neuro Oncol*. 2016;18(9):1313–1318.
24. Diaz RJ, Ali S, Qadir MG, De La Fuente MI, Ivan ME, Komotar RJ. The role of bevacizumab in the treatment of glioblastoma. *J Neurooncol*. 2017;133(3):455–467.
25. Cascone T, Heymach JV. Targeting the angiopoietin/Tie2 pathway: cutting tumor vessels with a double-edged sword? *J Clin Oncol*. 2012;30(4):441–444.
26. Choe G, Horvath S, Cloughesy TF, et al. Analysis of the phosphatidylinositol 3′-kinase signaling pathway in glioblastoma patients in vivo. *Cancer Res*. 2003;63(11):2742–2746.
27. Watkins S, Robel S, Kimbrough IF, Robert SM, Ellis-Davies G, Sontheimer H. Disruption of astrocyte-vascular coupling and the blood-brain barrier by invading glioma cells. *Nat Commun*. 2014;5:4196.
28. Dayal R, Singh A, Pandey A, Mishra KP. Reactive oxygen species as mediator of tumor radiosensitivity. *J Cancer Res Ther*. 2014;10(4):811–818.
29. Rubenstein JL, Kim J, Ozawa T, et al. Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. *Neoplasia*. 2000;2(4):306–314.
30. Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer*. 2015;15(7):409–425.