Combined inhibition of vascular endothelial growth factor and platelet-derived growth factor signaling: effects on the angiogenesis, microcirculation, and growth of orthotopic malignant gliomas

MOHAMMAD REZA FARIHADI, M.D., HANS HOLGER CABELLE, M.D., RALF ERBER, AXEL ULLRICH, PH.D., AND PETER VAJKOCZY, M.D.

Department of Neurosurgery, University Hospital, Faculty of Clinical Medicine of the University of Heidelberg, Mannheim; and Department of Molecular Biology, Max-Planck-Institute for Biochemistry, Martinsried, Germany

Object. The goal of this study was to determine the effects of SU6668, a polyvalent receptor tyrosine kinase inhibitor against vascular endothelial growth factor receptor–2, platelet-derived growth factor receptor–β, and fibroblast growth factor–1 on tumor growth, angiogenesis, and microcirculation in an orthotopic malignant glioma model. Methods. Fluorescently labeled C6 malignant glioma cells were implanted into a long-term cranial window, which had been prepared in nude mice. The animals were treated with intraperitoneal injections of SU6668 (75 mg/kg/day) immediately (five animals) or 7 days (five animals) following tumor implantation. Control mice received intraperitoneal injections of vehicle (50 μl dimethylsulfoxide) immediately (five animals) or 7 days (four animals) after tumor implantation. Tumor growth, angiogenesis, and microcirculation were assessed by performing intravital fluorescence videomicroscopy over a 14-day observation period. To assess the effects of SU6668 on overall survival, C6 glioma cells were implanted stereotactically into the brains of 24 additional animals and treatment was initiated on Day 7. In both the immediate and delayed experimental setting, SU6668 treatment resulted in a significant reduction of total and functional tumor vessel densities (both p < 0.05), reflecting a suppression of angiogenesis and impairment of tumor perfusion. As a consequence, tumor growth was significantly inhibited (p < 0.05). Histological analysis demonstrated reduced tumor growth and less mass effect on the adjacent brain of treated animals. The survival experiments confirmed the importance of our results in that survival was significantly prolonged following SU6668 therapy (p < 0.05).

Conclusions. Targeting of multiple angiogenic signaling pathways by polyvalent tyrosine kinase inhibitors represents a promising strategy to interfere with the vascularization, microcirculation, and growth of angiogenesis-dependent tumors. This also applies to malignant gliomas, despite the uniqueness of the cerebral microenvironment and the singular pathobiology of this tumor entity.

Key Words • angiogenesis • experimental therapy • intravital fluorescence videomicroscopy • survival • tumor growth

Despite recent progress in the multimodality therapy of brain tumors including surgery, radiotherapy, and chemotherapy, malignant gliomas continue to remain resistant to today’s treatment strategies. This failure of current modes of treatment of malignant gliomas necessitates the development of alternative therapeutic approaches. Malignant gliomas are among the best vascularized tumors in humans. The fact that this microvascular proliferative activity cannot be observed in low-grade gliomas indicates that glioma-associated angiogenesis is crucial for the process of stepwise astroglial progression and the pathophysiology of this tumor entity.

Abbreviations used in this paper: ANOVA = analysis of variance; DMSO = dimethyl sulfoxide; FITC = fluorescein isothiocyanate; FGF = fibroblast growth factor; FGFR = FGF receptor; PDGF = platelet-derived growth factor; PDGFR = PDGF receptor; SD = standard deviation; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor.

Ported by the identification of tumor vessel density as an independent prognostic parameter for human astroglial tumors. Finally, experimental evidence supports the concept that the growth of malignant gliomas is dependent on tumor angiogenesis. As a consequence, antiangiogenic therapies represent one promising alternative for malignant gliomas.

A substantial body of evidence has led us to suspect that the VEGF and its cognate receptors represent the dominant signal transduction pathway regulating glioma angiogenesis and that targeting the VEGF system is the most promising antiangiogenic treatment strategy. Nevertheless, other angiogenic growth factors, such as PDGF, FGF, and the angiopoietins, have been shown to be expressed by glioma cells or glioma blood vessels, indicating that there is some molecular redundancy in the regulation of glioma angiogenesis. Consequently, inhibition of multiple angiogenic pathways may be superior to selective VEGF and VEGFR targeting in controlling malignant glioma angiogenesis.

The small-molecule tyrosine kinase inhibitor SU6668...
Inhibition of Vascular Permeability

SU6668, a polyvalent inhibitor of angiogenic signaling pathways, was shown to inhibit phosphorilation and activity of VEGFRs and PDGFRs. By doing this, SU6668 not only prevents tumor angiogenesis but also provides a potent antivascular efficacy, resulting in regression of selected experimental tumor types. Recently, we studied the effects of SU6668 on the angiogenesis, perfusion, and growth of experimental malignant gliomas by using intravital multifluorescence videomicroscopy. We were able to show that SU6668 exerts more potent antiangiogenic response and suppression of tumor oxygenation, and suppression of tumor growth. Nevertheless, the drawback of our proof-of-concept studies was that we studied the effect of SU6668 in a subcutaneous (ectopic) malignant glioma model. It remains unclear whether SU6668 is also effective in treating malignant gliomas that are growing in an orthotopic microenvironment and whether the agent is potent enough to affect survival when the tumor is growing intracerebrally.

The aim of the present study, therefore, was to assess the effect of SU6668 on the angiogenesis, microcirculation, and growth of malignant gliomas in an orthotopic tumor model. To achieve this we implanted malignant C6 glioma cells in a long-term cranial window preparation in nude mice and examined tumor vascularization and growth by performing intravital multifluorescence videomicroscopy. In addition, we implanted malignant C6 glioma cells intracerebrally by stereotactic means and assessed the effect of SU6668 on animal survival.

Materials and Methods

Cells and Cell Cultures

We cultivated C6 rat glioma cells in HAM F-10 culture medium in 12-well dishes at 37°C in a humidified atmosphere of 5% CO2/95% air. Two days after preparation of the cranial window, the coverslip was temporarily removed and a suspension of C6 cells (2.5 x 10⁶ tumor cells in 1.5 ml phosphate-buffered saline) was implanted. Animals Used for the Study

The experiments were performed in athymic, adult nude mice (nu/nu; weight range 28–32 g) that had been bred and maintained at the experiments authorities. The experiments were performed in athymic, adult nude mice by stereotactic means. The heads of the mice were fixed in a rodent stereotactic frame (David Kopf Instruments, Tujunga, CA). Implantation was performed by injecting 5 x 10⁶ cells into the left hemisphere of each animal. The animals were divided into three groups (A, B, and C) and treatment was initiated on Day 6 after glioma cell implantation, reflecting the treatment of already vascularized and progressing tumors. Control mice received the vehicle alone (DMSO) every 3 days (low dose), and Group C (eight mice) received 50 μl DMSO every day (high dose). The animals were killed as soon as one exhibited neurological signs that were considered to be the initial body weight. Survival data are presented in Kaplan–Meier survival curves. Histological Findings

At the end of the period, the tumors were removed and fixed in 10% formalin. The tumors were embedded in paraffin. Sections (3 μm) were cut and stained with hematoxylin.

Statistical Analysis

Quantitative data are expressed as mean values of the microvessels in each animal. Statistical analysis was performed using the pearson chi-squared test. Survival curves were compared using the log-rank test. Survival data are presented in Kaplan–Meyer survival curves. A probability of p < 0.05 was considered significant. Survival curves were compared using the log-rank test.
Inhibition of VEGF–PDGF signaling in an orthotopic glioma

In the present study, the effects of SU6668 on glioma vascular initiation were investigated. Intravital fluorescence videomicroscopic imaging of the C6 glioma microvasculature following treatment with DMSO (A–C) and SU6688 (D–F). A–C: The microvasculature of control animals was characterized by an early initiation of vessels on Day 3 and a dense network of perfused tumor blood vessels with a heterogeneous and chaotic angioarchitecture on Day 10 (B) and Day 14 (C). D–F: Tumors treated with SU6668 showed a markedly suppressed initiation of vessels with only a few vascular sprouts on Day 3 (arrows in D) and a retarded network of perfused tumor blood vessels on Day 10 (E) and Day 14 (F). Treatment of the animals began on the day of tumor implantation. Contrast enhancement with FITC–dextran-150 administered intravenously. Original magnification × 435.

Results

Antiangiogenic Effect of SU6668 During Vascular Initiation

In control tumors, the first signs of malignant glioma angiogenesis were observed by Day 3 after implantation (Fig. 1A). These signs included the formation of microvascular sprouts and sinusoidal vessel configurations originating from cerebral capillaries and postcapillary venules. The newly formed microvessels subsequently branched and interconnected, forming initial, red blood cell–perfused microvascular networks. By Day 10, the area of the glioma mass was completely vascularized (Fig. 1B). The microvasculature was characterized by a dense network of tumor blood vessels and a heterogeneous and chaotic angioarchitecture (Fig. 1B and C). In contrast, tumors treated with SU6668 from the time of their implantation displayed a markedly suppressed tumor angiogenic activity compared with controls. During the entire observation period, the microvasculature of SU6668-treated tumors was characterized by a retarded network of tumor blood vessels, most of which were not perfused (Fig. 1D–F).

The quantitative analysis of the total vascular density confirmed reduced angiogenic activity during SU6668 treatment. As illustrated in Fig. 2A, daily treatment of tumors resulted in a significantly reduced total vessel density throughout the 14-day observation period. This suppressed angiogenic activity translated into significantly impaired tumor perfusion, as indicated by the 70% reduction in functional vessel density (Fig. 2B). The SU6668 not only reduced the number of perfused vessels, but also negatively affected the diameter of functional tumor vessels, especially at the end of the observation period (Fig. 2C). A detailed assessment of antiangiogenic activity within the different

Statistical Analysis

Quantitative data are expressed as mean values ± SDs. The mean values of the microvascular density data were calculated from mean values in each animal. For an analysis of differences between groups, the post hoc unpaired Bonferroni t-test was used following a one-way ANOVA. Results in which the probability was less than 0.05 when compared with values in control animals were considered significant. Survival data are presented in Kaplan–Meier survival curves. For an analysis of differences in survival, the log rank test was used.
SU6668 treatment was begun 6 days after tumor implantation; that is, at a time when tumors had already established their microvasculature and had reached an average size of 20 mm². As demonstrated by sequential intravital microscopic studies of identical microvascular regions of interest, SU6668 not only inhibited angiogenesis, but also induced a massive regression of already existing tumor blood vessels (Fig. 4A and B). This antivascular activity of SU6668 was confirmed by the quantitative analysis of total and functional vessel densities (Fig. 4C and E). With therapy, SU6668 induced vessel regression by 44%, resulting in a significant failure of tumor vessel perfusion. This perfusion failure was further aggravated by the effect of SU6668 on tumor vessel diameters, which failed to enlarge any further during tumor growth after initiation of therapy (Fig. 4D). Interestingly, regression was not accompanied by an increase in tumor blood vessel permeability and cerebral edema formation, as judged by the extravasation kinetics of the fluorescent tracer (permeability index on Day 10 [mean ± SD]: 0.03 in control tumors and 1.10 ± 0.18 in SU6668-treated lesions—not a statistically significant difference). As a consequence of these microvascular changes, glioma growth was markedly suppressed. This was confirmed by the planimetric analysis of tumor growth, which revealed a 52% growth inhibition following daily treatment with SU6668 when begun at the vascular initiation phase (Fig. 2D). The histological analysis of tumors at the end of the observation period confirmed the antitumor activity of SU6668. As illustrated in Fig. 3, tumor volume and the related mass effect on the adjacent brain were markedly reduced in SU6668-treated tumors compared with control lesions (Fig. 3A–D). In addition, the immunohistochemical analysis involving the endothelial cell-specific marker CD31/PECAM confirmed the reduction in tumor blood vessel density in SU6668-treated tumors (Fig. 3E–F).

**Antivascular Effect of SU6668 on Established Tumor Blood Vessels**

So far we have shown that SU6668 inhibits angiogenesis in gliomas when administered at the time of tumor initiation. Next, we addressed the effects of SU6668 on an already established tumor microvasculature. For this purpose tumor compartments further revealed that it was more pronounced in peripheral areas than in the center of the tumor, that is, in regions where the highest angiogenic activity within the tumor microvasculature could be observed. In contrast to the inhibitory effect of SU6668 on tumor angiogenesis and tumor perfusion, a quantitative analysis of tumor vessel permeability revealed no significant difference between treated and control tumors (permeability index on Day 14 [mean ± SD]: 1.12 ± 0.18 in control tumors and 1.12 ± 0.03 in SU6668-treated lesions—not a statistically significant difference). As a consequence of these microvascular changes, glioma growth was markedly suppressed. This was confirmed by the planimetric analysis of tumor growth, which revealed a 52% growth inhibition following daily treatment with SU6668 when begun at the vascular initiation phase (Fig. 2D). The histological analysis of tumors at the end of the observation period confirmed the antitumor activity of SU6668. As illustrated in Fig. 3, tumor volume and the related mass effect on the adjacent brain were markedly reduced in SU6668-treated tumors compared with control lesions (Fig. 3A–D). In addition, the immunohistochemical analysis involving the endothelial cell-specific marker CD31/PECAM confirmed the reduction in tumor blood vessel density in SU6668-treated tumors (Fig. 3E–F).

M. R. Farhadi, et al.
Inhibition of VEGF–PDGF signaling in an orthotopic glioma

SU6668 treatment was begun 6 days after tumor implantation; that is, at a time when tumors had already established their microvasculature and had reached an average size of 20 mm². As demonstrated by sequential intravital microscopic studies of identical microvascular regions of interest, SU6668 not only inhibited angiogenesis, but also induced a massive regression of already existing tumor blood vessels (Fig. 4A and B). This antivascular activity of SU6668 was confirmed by the quantitative analysis of total and functional vessel densities (Fig. 4C and E). Within 4 days of therapy, SU6668 induced vessel regression by 44%, resulting in a significant failure of tumor vessel perfusion. This perfusion failure was further aggravated by the effect of SU6668 on tumor vessel diameters, which failed to enlarge any further during tumor growth after initiation of therapy (Fig. 4D). Interestingly, however, SU6668-induced vessel regression was not accompanied by an increase in tumor blood vessel permeability and cerebral edema formation, as judged by the extravasation kinetics of the fluorescent tracer (permeability index on Day 10 [mean ± SD]: 1.07 ± 0.03 in control tumors and 1.10 ± 0.05 in SU6668-treated lesions—not a statistically significant difference). Parallel to the microvascular changes, tumor growth persisted after initiation of SU6668 treatment, resulting in a size difference of 40% at the end of the observation period (Fig. 4F).

Discussion

Receptor tyrosine kinases have become an attractive target for pharmacological antitumor therapies during the past decade. Consequently, different small molecules have been developed, which now target individual or multiple receptor tyrosine kinases. At present, several of these inhibitors are under investigation in preclinical trials, whereas others will advance to Phase II and III trials in the near future. Among those, SU6668 represents a member of a novel family of polyvalent inhibitors that target multiple receptor tyrosine kinases implicated in glioma angiogenesis, such as VEGFR-2, PDGFR-β, and FGFR-1. In previous studies we have demonstrated a potent antiangiogenic and antivascular

![Image of figure 4](image-url)

**Fig. 4.** Effects of SU6668 on established glioma blood vessels. A and B: Intravital fluorescence videomicroscopic imaging of the same C6 glioma microvasculature before (A) and 4 days after (B) treatment with SU6668. Contrast enhancement with 2% FITC–dextran-150 administered intravenously. Original magnification ×435. C–F: Graphs demonstrating the results of a quantitative analysis of total vessel density (C), diameter of perfused blood vessels (D), functional vessel density (E), and tumor growth (F). Treatment of the animals was initiated on Day 6 after tumor implantation (indicated by arrows). Values are expressed as means ± SDs. Statistical analysis was performed using ANOVA followed by the post hoc unpaired Bonferroni t-test. *p < 0.05, **p < 0.01 compared with control.

J. Neurosurg. / Volume 102 / February, 2005

Page 367
efficacy for SU6668 in an ectopic malignant glioma model and we have been able to shed some light on the mechanisms of action of this kind of antiangiogenic compound. The principal novel finding of the present study is that SU6668 exerts both its antiangiogenic and antivascular effects in an orthotopic malignant glioma model. Furthermore, we have demonstrated that targeting multiple angiogenic receptor tyrosine kinases using SU6668 results in a significant prolongation of survival following orthotopic tumor implantation.

The C6 malignant glioma model has been extensively characterized with respect to tumor angiogenesis and tumor microcirculation. Recent studies have shown that the C6 malignant glioma is highly angiogenic and that its growth depends on the formation of new tumor blood vessels. Following orthotopic implantation, C6 malignant glioma cells induce vascularization via a sprouting angiogenesis within a few days and establish a functional microvasculature within 1 week after their implantation. Expression analyses have shown that C6 malignant glioma angiogenesis is primarily mediated by VEGF, which is expressed by the tumor cells and activates VEGFR-2 on the endothelial cells of host and tumor blood vessels. Simultaneously, in a coordinate fashion with VEGFR-2 expression, Ang-2 is upregulated by activated host and tumor endothelial cells, rendering the blood vessels in a state of proangiogenic plasticity and VEGF responsiveness. At the same time, Ang-1 expression is 11-fold lower than VEGF expression.

To assess the dynamic process of C6 malignant glioma growth, angiogenesis, and microcirculation in detail we observed the tumors via a long-term cranial window by performing intravital multi-photon fluorescence videomicroscopy. As previously shown, this experimental approach is advantageous over classic postmortem histological analyses in that it allows for a noninvasive and repetitive identification of individual glioma cells as well as the growing tumor mass, visualization of individual glioma microvessels, and a quantitative analysis of different microcirculatory parameters for a time period of up to 14 days.

We elected to study the effects of SU6668 on glioma growth and angiogenesis following two different protocols. First, we treated malignant gliomas immediately after their implantation. Following this protocol, SU6668 caused a significant reduction in tumor volume and an associated loss in immature vessels on therapy, and compensatorily expressed VEGF–VEGFR-2 signaling may be restricted to a fraction of immature vessels, whereas the majority of established glioma blood vessels to an interference with PDGF-B/PDGFR-β signaling seems to be detrimental for an angiogenic over classic postmortem histological analyses in that it is unlikely that these agents will be successful as a monotherapy, but may be of significant value as an adjunct to current multimodal treatments.

Inhibition of VEGF–PDGF signaling in an orthotopic glioma model

It is important to point out that SU6668 is neither toxic nor associated with side effects nor lesions of normal blood vessels. In line with previous studies, all animals remained undisturbed and showed no significant differences between the experimental groups. Moreover, previously, we have shown that the susceptibility of normal blood vessels to SU6668 treatment is higher compared to that of glioma vessels. As a result, SU6668 treatment was well tolerated, and in any of our experimental groups we did not detect a significant increase in glioma blood vessel permeability or extravasation of host cerebral tissue by activating proinvasive signaling pathways.
Inhibition of VEGF–PDGF signaling in an orthotopic glioma

It is important to note that the extended target profile of SU6668 is neither associated with an increase in drug-related side effects nor negatively affects physiological blood vessels. In line with this, our present intravital microscopic analyses have demonstrated that, in contrast to the glioma vasculature, the integrity and function of cerebral blood vessels remained unaffected by treatment with SU6668. This can be explained on the one hand by fundamental differences between the tumor and normal blood vessels. Accordingly, previous studies have shown that in contrast to normal blood vessels, pericytes of tumor vessels are only loosely associated with endothelial cells, therefore potentially failing to provide vessel maturation. On the other hand, it has been shown that tumor vessels and normal blood vessels are characterized by different gene expression patterns, indicating a distinct target profile when considering antiangiogenic therapies.

Initially, we believed that the acute and rapid destabilization of glioma blood vessels may be an important caveat to using similar polyvalent inhibitors in a clinical setting because it may provide the basis for cerebral edema formation and intracerebral hemorrhage. This would be especially detrimental in combining these inhibitors with radiation therapy, which already leads to transient vessel destabilization per se. Our intravital microscopic studies following SU6668 treatment of orthotopic glioma, however, failed to detect a significant increase in glioma blood vessel permeability. Also, we did not observe neurological deterioration in any of our experimental animals following initiation of SU6668 therapy.

Our survival experiments have demonstrated that the potent antiangiogenic, antiangiogenic, and antitumor effects of SU6668 translate into a significant prolongation of survival following intracerebral implantation of C6 malignant glioma cells. Nevertheless, the experiments have also shown that, unfortunately, a monotherapy consisting of SU6668 fails to cure these animals. This may be simply attributable to the aggressiveness of the C6 malignant glioma. Alternatively, this ultimate treatment failure may also be due to the fact that antiangiogenic therapies do not affect another major hallmark of the pathological characteristics of the glioma, that is, tumor invasion. Instead, it has been recently suggested that antiangiogenic therapies that increase tumor hypoxia may increase glioma cell invasion into surrounding cerebral tissue by activating proinvasive signaling pathways. Unfortunately, the reason for the ultimate escape of our C6 tumors from SU6668 therapy has to remain speculative at the present time because the histological workup of tumor tissue at the time the animals were killed failed to reveal a significant difference between treated and control tumors (data not shown). Nevertheless, the results of this study help define the potential role of antiangiogenic–antivascular therapies in the treatment of malignant glioma in vivo. Still, the authors wish that these agents will be successful as a monotherapy, but may be of significant value as an adjunct to current multimodality treatments.

Conclusions

In summary, we have shown that SU6668 treatment of orthotopic malignant gliomas potently inhibits tumor angiogenesis, impairs tumor microcirculation, induces regression of existing tumor blood vessels, and, thereby, suppresses tumor growth and prolongs survival. In conclusion, we suggest that targeting multiple angiogenic growth factor receptors with compounds such as SU6668 represents a promising strategy to interfere with the growth and progression of human malignant gliomas. An antiangiogenic–antivasculogenic monotherapy may not be sufficient to control this aggressive tumor entity, however, and thus multimodality therapeutic strategies that combine conventional therapies (resection, radiotherapy, and chemotherapy) with experimental therapies (antiangiogenic–antivasculogenic and antinvase treatments) should be considered for use in malignant gliomas in the future.

Acknowledgments

We thank S. Mohr and V. Powajo for their excellent technical assistance. We appreciate the help of Joachim Brade in the biometric analyses. We are grateful to Peter Hirth, Laura Shawver, Gerald McMahon, and Julie Cherrington (all from SUGEN, Inc.) for providing us with the tyrosine kinase inhibitors.

References


J. Neurosurg. Volume 102 / February, 2005

369
Rhabdoid meningioma is a rare and aggressive subtypes of meningiomas. It is characterized by a rhabdoid structure, which is a distinct histological pattern that resembles the cells of the rhabdoid tumor of infancy. This feature is identified by immunohistochemical staining, which typically shows strong positivity for vimentin, S100 protein, actin, HMB-45, and glial fibrillary acidic protein. The inclusions are composed of whorls of intermediate filaments, most commonly vimentin, and prominent nucleoli. The term “rhabdoid” refers to the rhabdoid tumor of infancy, a pediatric malignancy that shares similar morphological and immunohistochemical features.

Several recent reports have identified rhabdoid meningioma as a subtype of meningioma with a poor prognosis and aggressive behavior. It is characterized by a high rate of local recurrence after surgery, rapid progression, and high risk of extracranial metastases. The median time to recurrence after surgery for rhabdoid meningioma is less than 1 year, which is significantly shorter than other subtypes of meningioma.

The presence of rhabdoid features in meningiomas has been associated with a poor prognosis. Several studies have shown that the presence of rhabdoid features in meningiomas is an independent predictor of a worse outcome, including increased local recurrence rate and shorter survival.

Several recent reports have identified rhabdoid meningioma as a subtype of meningioma with a poor prognosis and aggressive behavior. It is characterized by a high rate of local recurrence after surgery, rapid progression, and high risk of extracranial metastases. The median time to recurrence after surgery for rhabdoid meningioma is less than 1 year, which is significantly shorter than other subtypes of meningioma.

The presence of rhabdoid features in meningiomas has been associated with a poor prognosis. Several studies have shown that the presence of rhabdoid features in meningiomas is an independent predictor of a worse outcome, including increased local recurrence rate and shorter survival.

Abbreviations used in this paper:

- CT = computerized tomography
- MR = magnetic resonance
- RM = rhabdoid meningioma
- VEGF = vascular endothelial growth factor
- VEGF R-2 = VEGF receptor-2
- Angiopoietin-2 = angiopoietin-2

Address reprint requests to: Peter Vajkoczy, M.D., Department of Neurosurgery, Klinikum Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim, Germany. email: peter.vajkoczy@nch.ma.uni-heidelberg.de.