Bevacizumab has differential and dose-dependent effects on glioma blood vessels and tumor cells

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Abstract:

PURPOSE: Bevacizumab targets VEGF-A and has proved beneficial in glioma patients, improving clinical symptoms by the reduction of tumor edema. However, it remains controversial whether or not bevacizumab exerts anti-tumor effects in addition to (and potentially independent of) its effects on tumor vessels, and unknown what doses are needed to achieve this.

EXPERIMENTAL DESIGN: We established a novel orthotopic glioma mouse model which allowed us to simultaneously study the kinetics of the morphologic and functional vascular changes, tumor growth, and the viability of individual tumor cells during the course of anti-VEGF therapy in the same microscopic tumor region in real-time. Three doses of bevacizumab were compared: a subclinical dose, and two clinical doses (medium and high).

RESULTS: Low (subclinical) doses of bevacizumab led to a significant reduction of the total vascular volume without affecting tumor cell viability or the overall tumor growth rates. Medium and high doses triggered a similar degree of vascular regression, but significantly decreased tumor growth and prolonged survival. Remaining vessels revealed morphological features of vascular normalization, reduced permeability, and an increase in blood flow velocity; the latter was dose-dependent. We observed an uncoupling of the anti-tumoral and the anti-vascular effects of bevacizumab with the high dose only, which showed the potential to cause microregional glioma cell regression. In some tumor regions, pronounced glioma cell regression occurred even without vascular regression. In vitro, there was no effect of bevacizumab on glioma cell proliferation.

CONCLUSIONS: Regression of glioma cells can occur independently from vascular regression, suggesting that high doses of bevacizumab have indirect anti-cancer cell properties in vivo.

Key words: glioma, VEGF, bevacizumab, tumor growth, in vivo MPLSM, angiogenesis, normalization, U87-RFP, red blood cell velocity
Translational relevance:

Controversy exists whether the clinical benefit of patients treated with bevacizumab results exclusively from its effect on tumor vessels, or whether any additional anti-tumor cell activity might contribute to its clinical efficacy, and what doses are needed to achieve this. In glioblastoma, bevacizumab is most often used at a dose of 10 mg/kg every two weeks, either as addendum to chemotherapy or as monotherapy; however, its optimal dose has not been established yet.

This study demonstrates that VEGF inhibition by bevacizumab as a monotherapy induces normalization and regression of tumor vasculature in vivo even at low (subclinical) doses but has no anti-tumor effect by itself. Yet, higher doses (e.g., doses equivalent to 15 mg/kg every two weeks in patients) lead to a relevant tumor cell regression in addition to the vascular actions of the drug. Hence, despite increased costs and potentially toxicity, higher doses might be most efficient in monotherapeutic approaches.
Introduction:

Glioblastoma multiforme is an invariably fatal brain tumor accounting for approximately 40% of all primary malignant brain tumors. At present, the standard therapy is a multimodal regimen consisting of surgical resection combined with daily temozolomide and radiation, followed by 6 monthly cycles of temozolomide alone. Despite this treatment, the mean overall survival from time of diagnosis has been only moderately increased from 12.1 to 14.6 months in the selected study population [1]. The development of novel, more effective strategies to treat malignant gliomas therefore remains an unmet medical need.

Gliomas are highly vascularized tumors, and preclinical data have suggested that glioma growth critically depends on the generation of tumor-associated blood vessels [2]. Among multiple factors controlling the complex process of angiogenesis, VEGF and its associated signaling cascade are considered of central importance [3]. Glioma cells are a major source of VEGF, and high levels of VEGF generation have been reported to correlate with high grade malignancy and poor prognosis [4, 5]. Consequently, novel therapeutic strategies targeting VEGF or its downstream signaling pathways have yielded promising results as an addendum to standard therapy [6].

Currently, the most prominent drug targeting VEGF is bevacizumab, a recombinant humanized monoclonal antibody that binds to human VEGF-A. Encouraging results have been obtained in patients with malignant gliomas treated with bevacizumab, when used in combination with irinotecan [7] and as monotherapy [8]. Because the progression free and overall survival data from the phase II trials on recurrent glioblastomas where bevacizumab was tested as a single agent [8-11] do not seem to differ much from the survival data obtained with combinations of bevacizumab with chemotherapy [8, 12], bevacizumab monotherapy may gain further clinical importance in the future. Since the vast majority of clinical studies showed high radiographic response rates, promising progression free survival rates, and a low overall toxicity, bevacizumab was approved for therapy of recurrent glioma by the US Food and Drug Administration in May 2009 [13].

While bevacizumab is most often used at a dose of 10 mg/kg every two weeks in glioblastoma, its optimal dose has not been established yet [14]. No sound data on bevacizumab dosing exists: bevacizumab has been given to glioblastoma patients with a dose of 15 mg/kg every three weeks [7,
10], at 10 mg every two weeks [6, 9, 12], and at 5 mg/kg every two weeks [15]. A recent meta-analysis of phase II studies for recurrent glioblastoma could not detect a significant difference between the 5 mg/kg and the 10 mg/kg groups [16]. Still, the inherent limitation of this kind of analysis calls for a formal and prospective comparison of the doses. Outside the brain, there is at least one recent phase III study showing that bevacizumab 15 mg/kg every three weeks is more efficient than 7.5 mg/kg in breast cancer [17], and one phase II study showing the same for NSCLC [18]. However, these dose-response relationships could not be recapitulated for other tumor types, and sometimes appear to be even reversed [19]. Moreover, the question has been raised whether targeted therapies might have a different dose-response relationship [20, 21] than conventional cytotoxic therapies, which show increasing efficacy at higher doses [22].

The mode of action of bevacizumab that accounts for its clinical benefits is still not fully understood and might not be attributed to one mechanism alone; even off tumor targets have been suggested recently [23]. Initially, prevention of tumor angiogenesis and subsequent starvation of the tumor from oxygen and nutrients was considered the major mechanism of action of antiangiogenic therapy [24]. However, preclinical studies suggested that antiangiogenic therapies induce vascular normalization, thereby improving glioma oxygenation and subsequent response to radiation therapy [25]. This could explain the positive synergistic effects of VEGF-targeted therapies when used in a multimodal approach [26]. Normalization of tumor associated vessels and reduction of peritumoral edema could be demonstrated for glioma patients receiving an anti-VEGF tyrosine kinase inhibitor [27]. Finally, the results of a recent preclinical study suggest that the anti-edema effect of anti-VEGF monotherapy is the major reason for its clinical efficiency [28]. Likewise, the clinical impression that malignant gliomas continue to grow diffusely in the brain under bevacizumab therapy [29, 30] rose the question whether bevacizumab has any anti-tumor cell activity whatsoever. Contrast enhanced MRI imaging is used to assess the therapeutic response to anti-VEGF-A treatment, but its anti-edema and anti-permeability effects make it difficult to assess its effects on tumor burden [31]. Taken together, the exact role of vascular normalization vs. vascular regression, the existence of direct or indirect anti-tumor actions, and the optimum dose for each of these effects during anti-VEGF therapy are still unresolved but important questions [32].
In the present study, we established a mouse glioma model that made it possible to assess how vascular changes dynamically interact with tumor cell proliferation or regression during bevacizumab therapy. This allowed novel insights into the relevant modes of action, and their dose-dependencies.
Materials and Methods:

Cell culture and gene transfection of cancer cells

The human glioblastoma cell line U87MG was obtained from the American type culture collection (ATCC) in 2006. To determine the influence of therapy on tumor cells in real time, the red fluorescent protein (RFP) DsRed was stably transfected into U87MG cells. Briefly, the plasmid pEGFP-C3 (Clontech Laboratories, Inc., Palo Alto, CA) was cut using enzymes Eco47III and Scal. For generation of DsRed under the control of the CMV promoter, the HindIII/Apal fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA), containing the full-length DsRed2 cDNA, was inserted into the Hind III/NotI site of pC3-cloning. The resulting construct was transfected into cancer cells using lipofectamine. Clones of cancer cells expressing DsRed were isolated, cultured in selective medium for three weeks, and subsequently sorted using FACS Vantage (Becton Dickinson, Heidelberg, Germany).

Animal model

Cranial window preparation and stereotactic cortical tumor cell injection was performed as previously described (Winkler et al, 2009). Briefly, 8-10 week old male NMRI nu/nu nude mice (Charles River Laboratories, Sulzfeld, Germany) were anesthetized with an intraperitoneal injection of ketamine/xylazine (100mg/kg ketamine/10mg/kg xylazine). Craniectomy was performed and the dura mater was removed. The brain surface was covered with physiological sodium chloride solution and a custom-made round cover glass (6 mm diameter) was glued to the skull with dental acrylic glue. 3 weeks after cranial window preparation, the cover glass was removed, and 3 μl of a viscous suspension containing 1x10^5 U87-RFP cells was injected stereotactically into the mouse brain using a 10 μl Hamilton microsyringe and a 2 pt style needle (Hamilton, Reno, NV, USA). Cells were injected 1 mm lateral to the sagital sinus into a depth of 1 mm. All animal experiments were performed according to the Bavarian state regulations for animal experimentation and approved by the state authorities.

Application of Bevacizumab:

Bevacizumab (Avastin®, Roche, Switzerland) was used to inhibit tumor-cell derived human VEGF-A and was administered i.p. at doses of 25, 5 and 0.5 mg/kg bodyweight (n=5 per group) every second day for 12 days. A control group (n=5) with size-matched tumors received human polyclonal IgG (Intratect®, Biotest, Germany) at a dose of 25 mg/kg. To measure bevacizumab plasma...
concentrations, plasma samples from 3-4 animals per treatment group were collected after the i.p. application of 2 and 8 subsequent doses every other day, with the plasma obtained two days after last application. The plasma concentration of bevacizumab was determined using an ELISA for the quantitative detection of human antibodies in animal plasma. Briefly, a biotinylated monoclonal antibody against human Fcγ was bound to a streptavidin coated microtiterplate in the first step. By incubating plasma samples and reference standard, bevacizumab binds to the immobilized mAb<hFcγ>. The bound construct can be detected by digoxigenylated monoclonal antibody against human Fcγ (mAb<hFcγ>-Dig) followed by anti-Dig-horseradish-peroxidase antibody-conjugate (FAb<Dig>-HRP). ABTS-solution is used as the substrate for HRP.

Measurement of overall tumor growth
Tumor growth was monitored by in vivo epifluorescence microscopy, and as soon as the RFP signal reached a diameter of approximately 1.5 mm (range, 1.2 to 1.9 mm) usually around day 14 to 20 after single cell implantation, anti-VEGF treatment with bevacizumab was started. This was designated as day 0.
Tumor volume was calculated based on the equation: \( \text{Tumor size (mm}^2) = \text{(long axis)} \times \text{(short axis)} \times \pi \)
As shown by us and by others, this methods allows to approximate overall tumor growth [25, 28].

Survival analysis
For the survival study, the stereotactic injection of 5 x 10^5 U87-RFP cells was performed after drilling a borehole. Cells were injected 1 mm lateral to the sagital sinus into a depth of 2 mm, no cranial window was prepared. Treatment (n=6 animals per treatment group) was initiated 5 days after tumor implantation. Experiments were terminated when mice became moribund or died.

Dynamic in vivo Multiphoton Laser Scanning Microscopy (MPLSM)
A MaiTai Titanium Sapphire laser (690-1040 nm, 14 W pump laser; Newport Spectra Physics, Darmstadt, Germany) equipped with a two-photon microscope (LSM 5 MP; Zeiss, Jena, Germany) was used for in vivo MPLSM. A custom made microscopy stage allowing for stereotactic fixation of the cranium was used (Narishige, Tokyo, Japan). Mice were anesthetized by a mixture of O_2 and 1% isoflurane and body temperature of mice was held constant using a heating pad. Images were acquired at an excitation wavelength of 750 nm. Laser power was limited to a minimum, not exceeding
3% of total power at the surface, and 50% in 350 μm depth. At this power, no phototoxic damage to brain tissue was detectable over the time course of investigations. For each tumor, three adjacent regions were imaged through the cranial window and 133 images spared 3 microns apart were collected from the brain surface producing 650 x 650 x 400 μm volume stacks with a resolution of 0,64 x 0,64 x 3 μm per pixel. These regions were revisited and recorded on day 3, 6, 9 and 12.

In order to make sure that corresponding tumor regions were followed up over time we used 2 different strategies: (i.) all mice were mounted on a computerized stereotactic stage, allowing for relocation of identical regions in x, y and z dimension. (ii) to compensate for minor shifts due to tumor growth and due to cerebral edema, landmark vessels of the normal brain and the tumor were identified and used for proper alignment of the regions of interest. MPLSM angiography of glioblastoma vessels was performed after intravenous injection of 100 μl of FITC-labeled dextran (10mg/ml, 2M Da MW, green). In a group of 4 animals the observation period was extended to 25 days.

**Analysis of tumor vasculature**

3 regions showing both tumor cells and tumor blood vessels were randomly selected at day 0 for every tumor. The 2 regions that could be followed over time with the best image quality were subjected to 3D-reconstruction of the tumor vessels as well as analysis of the vascular network using Imaris 6.1 (Bitplane scientific software, Zurich, Switzerland). Briefly, 3D reconstructions of 100 images representing the region from 0 to 300 μm under the brain surface were created. In corresponding regions of interest (400 x 400 x 300 μm), a semi-automated quantification of vascular parameters (number of branchpoints, number of vessel segments, vessel diameter, intravascular volume and vascular surface) was performed for all time points using the filament tracer function of the software. Briefly, a wizard based interface guides the automatic creation of a tree-like filament based on the local intensity contrast. Tracking proceeds from start points to endpoints and a vectorized backbone of the vascular bed is proposed. In a last step, the radius at each point is defined according to the local contrast ridge (for representative images see Supplementary Fig. 1).

**Dynamic interdependence of blood vessels and tumor cells**

For the detailed analysis of regional tumor volume and regional vascular volume, a random tumor region of 200 x 100 x 33 μm was chosen on day 9. According to its vascular branching pattern, the exact corresponding region was identified for day 6 and day 0. Due to tumor growth in three
dimensions, these regions were recovered at varying Z-levels during the observation period. The volume of FITC-dextran-labeled vessels and the total volume of RFP-expressing tumor cells was determined in identical tumor regions using Imaris 6.1 (Bitplane scientific software, Zürich, Switzerland).

**Red blood cell velocity**

The motion of red blood cells was inferred from line-scan measurements as described previously [33]. Briefly, repetitive line scans of typically 15 μm along the central axis of a vessel were performed with a spatial resolution of 0.23 μm per pixel, a pixel dwell time of 10.2 μs and a record length of 3 s (for representative images see Supplementary Fig. 2). In a depth between 100 and 150 μm from the brain surface, RBC-velocity was determined in 12 vessels in 3 corresponding tumor regions before the onset and on day 6 of the treatment. In addition, in 4 animals bearing a cranial window but no tumor, RBC velocities were measured 3 weeks after cranial window implantation.

**Microregional vascular permeability**

Vascular permeability to bovine serum albumin (BSA) was determined after i.v. injection of 0.1 mL FITC-BSA (Invitrogen). Immediately after i.v. injection of BSA, Z stack images of 100 μm with 3 μm intervals were collected in a depth of -75 to -175 μm from the surface in 2 adjacent areas of interest (650 x 650 x 100 μm) every 4 minutes for 20 minutes. The increase in extravasated fluorescence dye intensity measured using Imaris 6.1 (bitplane scientific software, Zürich, Switzerland) and was normalized by blood vessel surface area using the equitation as described previously [25, 34].

**In vitro proliferation assay**

*In vitro* cell proliferation was determined using a cell proliferation assay according to the manufacturer's instructions (CellTiter 96 AQueous non-radioactive cell proliferation Assay, Promega, Madison, USA). Briefly, cells were incubated with different concentrations (5–1000 ng/ml) of bevacizumab or control IgG in the same volume for 48 hours. After adding the Cell titer reagent, absorbance was measured at 490 nm, using an ELISA-reader. Results are presented as mean of 4 replicate measurements. To determine the growth kinetics of U87 and U87-RFP (Supplemental Fig.1A) 10^4 cells per well were seeded in the same volume and were allowed to grow for different time
intervals (12 hours, 2, 4, 6 and 8 days) under standard conditions. After adding the Cell titer reagent, absorbance was measured as described above.

**Western blot Analysis**

Western blot analysis was carried out using a standard protocol. Briefly 50 μg Protein were electroblotted on a PVDF-membran. VEGF was detected using a rabbit polyclonal antibody directed against the N-terminus of human VEGF-A (sc-152, Santa Cruz technologies, California, USA, 1:50) and a secondary, alkaline phosphatase-conjugated goat-anti-Rabbit antibody (D0487, DAKO, Denmark, 1:2000). As a separate loading control, we used a monoclonal mouse antibody directed against α-Tubulin (T6199, Sigma Aldrich, Germany, 1:2200) and a secondary goat-anti mouse antibody conjugated to alkaline phosphatase (31320, Thermo Scientific, Germany, 1:2000). Quantification of the integrated grey values of the VEGF band and the α-Tubulin band was performed using ImageJ software.

**Quantification of the RFP signal in vitro**

In order to determine whether a correlation exists between U87-RFP cell numbers and the fluorescence intensity of the RFP-signal, we seeded U87-RFP cells at different numbers in clear 96-well plates (Biosearch, Bedford, MA). Cells were allowed to adhere for 12 hours, afterwards fluorescence determinations were made using a Fluorescence Microplate reader and a 565 excitation filter and a 590 nm emission filter (FLUOStar optima, BMG technologies, Offenburg, Germany). In a second subset of experiments, 5000 U87-RFP cells were incubated with different concentrations (5–1000 ng/ml) of bevacizumab or control IgG in the same volume for 48 hours. Subsequently fluorescence was determined as described above.

**Correlation of cell death and RFP fluorescence intensity**

In order to determine whether a loss of RFP-fluorescence correlates with cell death, U78-RFP cells growing under standard conditions were heated at 67°C for 10 min, irradiated with 130 Gy or left untreated. 36 hours later, U87-RFP cells were harvested and diluted in PBS. 10^5 cells of each group were incubated for 10 minutes with SYTOX® Blue Dead Cell Stain (Molecular probes, # S34857, final concentration 2 μM) according to the manufacture’s instruction. Systox is a high affinity blue fluorescing nucleic acid stain which penetrates cells with compromised plasma membranes.
Subsequently FACS analysis was performed to determine red fluorescence (RFP-Signal) and blue fluorescence (cell death stain) using a flow cytometer (MoFLo XDP cell sorter, Beckman Coulter).

**Statistics**

Data are expressed as mean ± SEM. To test for differences between groups, the Kruskal-Wallis One Way Analysis of Variance and Student Newman Keuls as post hoc test were used. For repeated measurements, Friedman Repeated Measures Analysis of Variance on Ranks or the Wilcoxon signed rank test was applied (SigmaStat 3.5 software). Survival was assessed by plotting survival curves according to the Kaplan-Meier method; comparisons were performed using the log-rank test. P < 0.05 was considered to be statistically significant.
Results:

**Bevacizumab dose-dependently inhibits tumor growth and increases survival**

First, we examined the kinetics of tumor growth and tumor angiogenesis after cerebral implantation of $10^5$ RFP expressing human U87-glioma cells. Initially, implanted U87-Glioma cells proliferated slowly. Within this period, the tumor vasculature was comprised mostly of preexisting brain capillaries, many of them undergoing vascular remodeling with enlargement (days 6 and 12 in Fig. 1A). The first new blood vessels occurred from day 12 on. Once the tumor has reached a threshold diameter of 900-1200 μm, tumor growth was paralleled by extensive vascular remodeling and by the formation of new vessels (days 19 - 30 in Fig. 1A). The onset of tumor angiogenesis was followed by a more rapid tumor growth. The ability to follow individual blood vessels in tumor microregions over time thus enabled us to clearly distinguish between new blood vessel formation (angiogenesis) and vascular remodeling. Animals became moribund 2-5 weeks after implantation of tumor cells when tumors reach a mean diameter of 3.5 to 4.5 mm (9.6 – 15.8 mm²). Importantly, *in vitro* growth rates of wild type and DsRed2-transfected cancer cell lines as well as VEGF-expression were equal (Supplementary Fig. 3 A-C).

Next, we sought to compare the effects of three different and clinically meaningful doses of bevacizumab (which inhibits human = tumor-cell derived VEGF-A). By giving 0.5, 5 and 25 mg/kg bevacizumab i.p. every two days to mice, plasma concentration of 4.9 +/- 2.01 μg/ml, 22.7 +/- 8.25 μg/ml and 196.89 +/- 35.32 μg/ml were reached after 2 applications. After 8 applications plasma levels of 11.4 μg/ml +/- 8.3 μg/ml, 76.3 +/- 13.1 μg/ml and 341.3 +/- 99.4 μg/ml, were obtained,(n= 3-4 animals per group). When compared to published pharmacokinetic data from humans [35], the application of 0.5 mg/kg i.p. in mice results in plasma concentrations that are achieved when humans are given 0.3 to 1 mg/kg bevacizumab i.v.; the 5 mg/kg mouse dose corresponds to a 3 mg/kg human dose; and the 25 mg/kg mouse dose corresponds to a 15 mg/kg human dose, approximately. Higher doses are required in mice since the half-life of bevacizumab is 3.5 - fold lower in mice (6 days in mice vs. 20 days in humans) [36, 37], and the route of administration was different (i.p. vs. i.v.). In conclusion, the selected doses represent a subclinical dose and two doses in the range that is used in the clinic.
A survival analysis showed that the mean survival after inoculation of 5 x 10^5 tumor cells is 21 +/- 2 days in the control group, 32 +/- 5 days, 44 +/- 6 and 55 +/- 8 in animals receiving 0.5, 5 and 25 mg/kg eod respectively. Survival dose-dependently increased, reaching significance in the medium and high dose groups (p< 0,05 vs. control respectively).

In order to determine whether the survival benefit resulted from a reduced tumor growth, we determined the size of the overall tumor as identified by measurement of the total RFP signal over time. Bevacizumab at a dose of 5 mg and 25 mg/kg eod lead to a significant reduction of glioma growth, when compared to animals receiving control IgG (Fig. 1C). On treatment days 6 and 12, mean tumor size was reduced by 74 and 78% in animals receiving high-dose bevacizumab and by 58 and 64% in mice receiving an intermediate dose of 5 mg/kg. In contrast, treatment with at low doses (0.5 mg/kg) of bevacizumab did not significantly alter tumor growth (Fig. 1C). Importantly, even high doses of bevacizumab did not inhibit U87 proliferation (Fig. 1D) or alter the RFP-signal in vitro (Supplementary Fig. 4A).

**Bevacizumab fails to induce glioma cell invasion in the U87 model within a treatment period of 25 days**

Some clinical and experimental evidence suggests that malignant gliomas continue to grow diffusely in the brain under bevacizumab therapy [30, 38], including in U87 xenograft models [39, 40]. In our model, no aggressive growth pattern occurred during a treatment period of 12 days in U87 xenografts which normally grow with sharp borders to the normal brain (Fig. 1A). Even when treatment was prolonged to 25 days, a true induction of local invasion by bevacizumab treatment could not be detected. However, we did observe regions of the tumor border that appeared to be less well defined due to the spread of single tumor cells close to the main tumor mass (Fig 1E, black arrows). The ability to monitor individual tumor microregions over time allowed us to clarify that these cells are remnants of regressing areas of the main tumor, and that they themselves tend to regress over time.

Importantly, extensive characterization of the fluorescence signal of U87-RPF in vitro confirms that (I) fluorescence intensity indeed correlates with the number of cells observed and is not affected by bevacizumab per se (see Supplementary Fig 4A, B) and that (II) the loss of RFP-fluorescence signal in vitro corresponds to cell death (Supplementary Fig.5). The latter has also been demonstrated by us for other red fluorescent cancer cells in vivo [41].
**Bevacizumab effects on tumor blood vessels**

To gain insight into the dynamic effects of different doses of bevacizumab on the tumor vasculature, we performed an in-depth analysis of the tumor vascularization using intravital multi-photon microscopy of identical tumor regions over time. Prior to treatment, vessel morphology in gliomas was highly heterogeneous (day 0 in Fig. 2A). In contrast to the normal cerebral vasculature, untreated tumors displayed a chaotic network of tortuous blood vessels with fluctuating, often large diameters, a high vascular density, and lack of a hierarchical network (Fig. 2A). In control animals, tumor angiograms of corresponding tumor regions showed a high vascular turnover (Fig. 2A, first panel): during an observation period of 12 days, the formation of new tumor vessels and the regression of established ones happened continuously. Due to three-dimensional tumor growth, vessels were recovered at a varying depth over the observation period and in some cases eventually escaped evaluation. Blood vessel diameter increased and at late stages of tumor growth, vessels appeared dilated and sinusoidal; the vascular density remained high throughout the observation period.

Bevacizumab inhibited the formation of new tumor vessels, which resulted in a marked reduction of vascular turnover, leading to a steady decrease of overall tumor vessel density in all doses given (Fig. 2A). Compared to the vascular network in control tumors, the vascular network that persisted despite treatment showed hierarchical and much less excessive and chaotic branching patterns (Fig. 2A-C).

A quantification of the tumor angiograms confirmed that bevacizumab induced a significant reduction of the vascular volume which reached a minimum at day 12 (Fig. 2C). The maximum extent of the reduction of vascular volume was similar for the different doses of bevacizumab; however, with a high dose of the drug the inhibitory effect occurred more rapidly after onset of treatment (Fig. 2C). The reduction in overall vascular volume was not due to a decrease in vascular diameter, as this parameter increased in all groups during the experiment and was only affected at late stages by the high dose of bevacizumab (Fig. 2D). Bevacizumab therapy rather preferentially pruned the small blood vessels of the tumor (Fig. 2a), which caused both a decrease in vascular volume and an increase in the mean diameter. Consequently, bevacizumab rapidly reduced the number of vascular branching points, and this was again independent of the dose (Fig. 2E). Extravasation of molecules from the bloodstream by diffusion critically depends on the ratio of vessel surface per vessel volume [42]. In mice treated with a high dose of bevacizumab, the vessel surface per vessel volume remained low over the entire course of the experiment (Fig. 2F), indicating a retention of a better vascular function over time.
Bevacizumab treatment increases blood flow velocity and reduces vascular permeability:

In order to determine the effects of bevacizumab on vascular physiology, we determined blood flow velocity and vascular permeability in tumor vessels by intravital multiphoton microscopy. In tumor microvessels, red blood cell velocities (RBC) were very low compared to normal brain, which can contribute to hypoxia and difficulties in drug delivery within tumors [43]. RBC velocities in arterioles and veins increased as a function of the vascular diameter in normal brain; this physiological dependency of blood flow velocity on vessel diameter was completely disrupted in the tumor vasculature (Fig. 3A and B). This is in line with the concept that abnormalities in both vasculature and blood viscosity increase the resistance to blood flow in tumors and override the physiological interrelationship between RBC velocities and vessel geometry [44]. Remarkably, treatment with bevacizumab dose-dependently increased RBC velocities in gliomas (Fig. 3C), and partially re-established the interdependence of blood flow and vessel diameter (data not shown).

In control tumors, tumor vessels are abnormally permeable to albumin (Fig. 3C, D). Treatment with bevacizumab leads to a significant reduction of vascular permeability in all three treatment doses (Fig. 3 C)

Dynamic analysis of the interdependence of tumor cell and blood vessel regression

Finally, we aimed to take advantage of our novel animal model to address the question whether or not bevacizumab can cause glioma cell regression in vivo, and whether this is due to its effects on tumor vasculature, or independent thereof. Therefore, a tumor region of limited depth (33 μm) was identified in all three dimensions at days 0, 6 and 9, and changes of blood vessel and tumor cell volume were tracked over time in all 4 treatment groups (Fig. 4A-D). In contrast to Fig 1B, where the total tumor volume (including tumor cells, non-tumor cells, vasculature and intratumoral edema) was determined, we now measured the tumor cell volume only. While both the intermediate and the high dose of bevacizumab induced a similar net decrease in vascular volume in these regions over time (Fig. 4E), only the high dose induced a rapid and pronounced reduction of tumor cell volume (Fig. 4F). No significant reductions of tumor cell volumes where detected in control animals (Fig.4A and F) and in animals treated with a low dose of bevacizumab (Fig. 4D and F). This was a first indication of an anti-tumor effect in addition to the vascular actions of bevacizumab when used in high doses. To analyze this discrepancy of anti-tumoral and anti-vascular effects in more detail, we performed an analysis of
the spatio-temporal correlation of both tumor compartments in mice treated with the same high dose of bevacizumab (Fig. 5). On day 9 of treatment, in 6 out of 10 tumor regions, the reduction of viable tumor cells in response to bevacizumab was preceded, at least paralleled by a decrease also in the vascular volume (Fig. 5A,B), suggesting that the anti-vascular action of the drug contributed to the reduction of viable tumor cells in these microregions. Notably though, in 4 out of ten tumor regions the anti-vascular and antitumor effect of bevacizumab appeared to be uncoupled: In two regions, bevacizumab triggered a substantial reduction in viable tumor cells without affecting overall vascularization (Fig. 5A,D), whereas there was no effect on tumor cell viability despite a substantial effect on tumor vascularization in another set of 2 micro regions (Fig. 5A,C).

These findings have three major implications: (i) The anti-vascular effect of bevacizumab is most often paralleled by anti-tumoral effects, which supports the classical concept of tumor starvation by anti-angiogenic therapy and proofs that bevacizumab does have the potency to reduce the number of cancer cells in a glioma; (ii) Some microregions within the tumor manage to escape the anti-angiogenic actions of anti-VEGF treatment and continue to grow despite disintegration of the local microvascular network. (iii) Tumor cell viability can be impaired even without concomitant changes of the local tumor vasculature morphology, suggesting that – in addition to its anti-vascular properties – bevacizumab-based anti-VEGF therapy also modulates tumor cell biology by mechanisms independent of vascular regression.
Discussion:

In the present study, we have directly addressed the effects of different doses of bevacizumab on tumor vessels, tumor growth and tumor cell viability using in vivo multi-photon microscopy of mouse glioma. Thus, for the first time, we were able to study dynamic interactions of blood vessels and tumor cells in vivo and in real time during anti-VEGF therapy. We show here that the anti-angiogenic effect of VEGF inhibition by bevacizumab occurs across the range of doses tested. Furthermore, normalization of vascular permeability was observed with all doses; only normalization of blood flow velocity was dose-dependent. However, only higher, but not sub-clinical doses of bevacizumab also reduced overall tumor growth, tumor cell viability, and prolonged survival. This indicates that blood vessel effects including vascular normalization occur with much smaller doses than the effects on tumor cells, which has considerable importance for future dosing considerations when bevacizumab is used as monotherapy, or in combination with cytotoxic therapies for glioblastomas.

Until recently, anti-angiogenic and anti-vascular effects were considered the most prominent mechanism of action of bevacizumab [45-48]. Only with the introduction of novel imaging technologies including epifluorescence and multi-photon microscopy in combination with chronic window models it has become obvious that the effects of anti-VEGF therapy are likely to be more complex than initially anticipated [34, 41]. Due to the previous inability to visualize of the dynamic events involved in tumor growth and angiogenesis, another question remained unsolved: does anti-angiogenic treatment with bevacizumab also exert anti-tumor cell effects, because of or independent from its anti-vascular properties? Using intravital multi-photon microscopy we show here that anti-VEGF treatment substantially affects not only tumor vasculature but also glioma growth; high doses even lead to regression of tumor cells in distinct microregions. This is in contrast to recent animal studies demonstrating an exclusive anti-edema (but not anti-tumor) effect of anti-VEGF tyrosine kinase therapy [28]. However, it is in line with the marked inhibition of tumor growth seen with viral anti-sense approaches and the use of antibodies against endogenous VEGF [34, 48-52].

While these findings indicate that anti-VEGF may have indirect anti-tumor potential in addition to its vascular effects, the interdependence of the vascular and anti-tumor effects of anti-VEGF strategies still is a matter of debate. In fact, there is increasing evidence suggesting that anti-tumoral and anti-vascular effects might occur independent from each other. A recent study demonstrated that the
vascular growth factor receptor-targeted kinase inhibitor cediranib induced rapid vascular normalization with a significant reduction of microvascular density and of vascular permeability leading to edema alleviation. The substantial effects on tumor vasculature were associated with prolonged survival; however, at the dose tested tumor growth remained unaffected [28]. This would fit to our finding for the low dose of bevacizumab, which significantly reduced tumor vasculature without affecting tumor growth and tumor cell viability. In contrast, animals receiving clinical doses of bevacizumab showed a decrease of tumor growth and tumor cell viability as well as a reduction in tumor vessels. The diversity which was observed in different glioma microregions reflects the well-known morphological, physiological and molecular heterogeneity of glioblastoma [53]. In most of the tumor microregions studied, tumor cell regression followed vascular regression, which is in accordance with the original hypothesis how antiangiogenic therapy should exert its effect [24]. However, it is remarkable that a similar extent of vascular regression in the low dose group did not result in significant tumor cell regression. Moreover, even with the high dose, we still observed distinct microregional uncoupling of its anti-tumoral and -vascular effects. While we cannot complete rule out that hypoxia occurs in areas without vascular regression, our present findings suggest that (i) the vascular effects of anti-VEGF treatment do not necessarily translate into anti-tumor cell effect and that (ii) bevacizumab can cause tumor cell regression despite a lack of vascular regression in some microregions.

The mechanisms of the vessel-independent anti-tumor cell effects of bevacizumab in vivo remain elusive. Some human glioma cell lines (including U87), and cells isolated from biopsy specimens express VEGF-receptors, including VEGFR-1/Flt-1, VEGFR-2/KDR, Neuropilin-1 (NRP-1) and Neuropilin-2 (NRP-2) [5, 54, 55]. However, both the ligand VEGF-A and VEGFR2 neutralizing antibodies did not influence cell proliferation or invasion in in vitro assays (including in this study), which argues against a relevant direct effect of bevacizumab on tumor cells [56]. Therefore, it seems most likely that anti-VEGF therapy modulates paracrine factors of endothelial cells (or other cells of the brain parenchyma, or progenitor cells [57, 58]) in vivo. Correspondingly, studies have shown that VEGF-A inhibitors can induce tumor-cell apoptosis by decreasing the levels of endothelial-cell-derived paracrine factors promoting cell survival in vitro [59]. Those possible paracrine factors may for example include tissue factor, Bcl-2 and VEGF-A itself [60] [59]. Likewise, it has been shown for several other tumor entities that modulation of VEGF receptor signalling blocks important functions of tumor cells in vivo despite the lack of an effect on tumor cell proliferation in vitro, e.g. in human
leukaemias [61, 62], and in human breast carcinoma [63]. Furthermore, glioma stem cells reside in a “perivascular niche” [64] and rely on this local microenvironment providing survival signals [65]. Interestingly, a recent report identified endothelial nitric oxide as an important factor for glioma stem cell characteristic in the perivascular niche [66]. This is of importance since bevacizumab has been shown to reduce nitric oxide production of endothelial cells [60]. Taken together, bevacizumab-induced decrease of nitric oxide production might target glioma stem cells and thereby inhibit the ability of certain tumor regions to maintain a proliferative state.

Derived from in vivo microscopical observations, Rakesh Jain suggested the concept of “vascular normalization” according to which judicious application of anti-angiogenic therapy could transiently normalize the structurally and functionally abnormal tumor vasculature [26]. This can lead to improved oxygenation of experimental gliomas, ultimately resulting in improved response to radiation therapy, but only during a time window [25]. However, it remained unclear how different doses of an anti-angiogenic agents influence the normalization phenomenon. It was hypothesized that there might be a “too little” and a “too much” of an agent when it comes to vascular normalization [26]. Nevertheless, one can speculate that the doses of anti-angiogenic agents that exert maximum anti-tumor effects are not the same that exert maximum normalization effects. Our results show that mice treated with bevacizumab reveal a morphological and functional normalization of tumor vessels, which is evident even with a very low dose of the drug. A decrease in vascular permeability was noted in all treatment groups. However, the abnormally low blood flow velocity of control tumors was increased only with higher doses, suggesting that some features of vascular normalization are indeed dose-dependent.

In conclusion, we show here that VEGF inhibition by bevacizumab induces regression and normalization of tumor vasculature, but tumor cell regression becomes relevant only at higher doses. The anti-tumor cell effects occur both dependent and independent of vascular regression of the drug, suggesting a bevacizumab-induced modulation of paracrine factors from the vasculature in some tumor microregions. These findings are clinically relevant: it is not clear yet whether bevacizumab should be used as a single agent or in combination therapies in glioblastoma [8, 9, 11], and what doses are optimal for both treatment approaches [14]. Our results suggest that using lower doses of bevacizumab can be sufficient to induce some extent of vascular normalization in gliomas, but have no anti-tumor effect by itself. In contrast, with the caveat regarding a higher toxicity that might occur,
higher doses, (e.g., 15 mg/kg every two weeks in patients) might be most efficient, at least for monotherapy approaches, by adding anti-tumor cell effects.

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Figure legends:

Fig. 1 (A) Characteristic pattern of glioma growth and angiogenesis imaged by in vivo multiphoton microscopy. Cortical vessels are highlighted in green after iv-injection of FITC-dextran; U87-RFP cells are displayed in red. Images represent maximum intensity projections of the mouse cortex from 0 to -350 μm at different time points after single cell implantation of $10^5$ U87-RFP cells (scale bars 200 μm). (B) Treatment with bevacizumab dose-dependently increased survival, reaching significance in the medium (5 mg/kg eod, blue graph) and in the high (25 mg/kg eod.) but not at the low dose group (0.5 mg/kg eod, grey graph), *p< 0.05 vs. control (black graph) , n = 6/group. (C) Bevacizumab dose-dependently inhibits glioma growth in vivo (*p< 0.05 vs. control, n = 5/group). Tumor size of animals treated with control antibodies (black graphs), bevacizumab 25 mg/kg eod (red graphs), 5 mg/kg eod (blue graphs) and 0.5 mg/kg eod (gray graphs). (D) Bevacizumab does not affect in vitro proliferation of U87 cells. (E) shows a tumor border region over a time period of 25 days. Over the time, the tumor border appears to be less well defined due to the presence of vital single tumor cells (arrows) within areas where the main tumor mass has regressed. Images represent 3D-reconstructions (650 x 650 x 450 μm) acquired by MPLSM. Respective upper panels show both, tumor cells (red) and tumor vessels (green), lower panels show tumor vessels only (scale bar 100 μm).

Fig. 2 (A) The effect of different doses of bevacizumab on the vascular phenotype of established U87-gliomas as assessed by in vivo MPLSM. Note the dynamic alteration of the vascular bed in control tumors by high vascular turnover during the observation period. Bevacizumab treatment leads to a stabilization of the vascular bed by inhibition of new blood vessel formation. Images represent 3D-reconstructions (600 x 600 x 350 μm) of corresponding tumor angiograms after iv-injection of FITC-dextran before (day 0) and on day 3, 6, 9 and 12 of treatment (scale bar 100 μm). (B) Representative angiogram of the normal brain. (C) Quantification of vascular volume, (D) mean vessel diameter, (E) number of vessel branches (*p<0.05 vs. control, 2 regions per animal, 5 animals per group) and (F) vessel surface per vessel volume (# p < 0.05 day 0 vs. day 12, 2 regions per animal, 5 animals per group).

Fig. 3 Red blood cell velocities ($V_{RBC}$) in relation to the microvascular diameter in the normal brain and established U87 gliomas (A). (B) $V_{RBC}$ before and on day 6 of treatment in control-treated (black graphs) and in bevacizumab-treated animals (red graphs: 25 mg/kg eod, blue graphs: 5 mg/kg eod
and gray graphs: 0.5 mg/kg eod). Note the dose-dependent increase of red blood cell velocities by bevacizumab (# p < 0.05 day 0 vs. day 6; day 6: * p < 0.05 vs. control; n= 12 measurements in 5 animals per groups).

(C) Quantification of the vascular permeability in control (black graphs) and in bevacizumab-treated animals (red graphs: 25 mg/kg eod, blue graphs: 5 mg/kg eod and grey graphs: 0.5 mg/kg eod). The increased permeability of tumor vessels is reduced by all three doses of bevacizumab (# p < 0.05 day 0 vs. day 6; day 6: * p < 0.05 vs. control; n= 2 measurements in 5 animals per group). (D) Vascular permeability as assessed by the extravasation of FITC-BSA. Images represent maximum intensity projections of -75 to -150 μm at day 6 after treatment with bevacizumab. Note the dose-independent reduction of extravascular fluorescence 20 minutes after i.v. injection of BSA.

**Fig. 4** Effect of different doses of bevacizumab on tumor cells within established U87-Gliomas. (A-D) Images represent 3D-reconstructions (600 x 600 x 33 μm) of corresponding tumor regions before (day 0) and on day 6 and 9 of treatment with different doses of bevacizumab and control IgG acquired by MPLSM. Respective upper panels show both tumor cells (red) and tumor vessels (green), lower panels show tumor vessels only (scale bar 100 μm). Quantification of (E) relative vascular volume and (F) relative regional tumor volume. Note that tumor cell regression, but not anti-vascular effects are limited to higher doses of bevacizumab (*p < 0.05 vs. control; # p<0.05 Bevacizumab 25 mg/kg vs. 5 mg/kg; n= 2 regions in 5 animals per group).

**Fig. 5** (A) Quantification of relative changes in micro-regional vascular volume and tumor cell viability at day 9 of treatment. In 6 out of 10 glioma regions (A; green ellipse) the decrease in the regional vascular volume is paralleled or preceded by a reduction of viable tumor cells in response to bevacizumab treatment, whereas in 4 out of 10 glioma regions the effect on tumor cells and on the regional vascular volume is uncoupled (A, black ellipse: vascular regression but no tumor cell regression; A, red ellipse: tumor cell regression but no vascular regression). (B-D) show tumor regions representing the three types. Note the synchronous blood vessel and tumor cell regression in (B), whereas (C) shows vascular regression without tumor cell regression, and (D) tumor cell regression without blood vessel regression in glioma microregions. Images represent 3D-reconstructions (600 x 600 x 33 μm) of corresponding tumor regions acquired by MPLSM. Respective upper panels show
both, tumor cells (red) and tumor vessels (green), lower panels show tumor vessels only (scale bar 100 μm).
Reference List


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Figure

A

Bevacizumab 25 mg/kg

B

C

D

E

Bevacizumab 25 mg/kg
Figure 3

A

(normal brain) vs. (U87-glioma)

B

Day 0 vs. Day 6

VRBC (μm/μm)

C

Day 0 vs. Day 6

Permeability (cm/s)

D

Control

Bevacizumab 0.5 mg/kg

Bevacizumab 5 mg/kg

Bevacizumab 25 mg/kg

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Figure 4

A

Control

B

Bevacizumab 25 mg/kg

C

Bevacizumab 5 mg/kg

D

Bevacizumab 0.5 mg/kg

E

Relative regional vascular volume (%)

F

Relative regional tumor volume (%)

Duration of treatment (days)
Figure 5

A
Day 9
Bevacizumab 25 mg/kg

B

C

D

Relative regional vascular volume (%)

Relative regional tumor volume (%)

day 0
day 6
day 9

day 0
day 6
day 9

day 0
day 6
day 9

day 0
day 6
day 9

100 μm

100 μm

100 μm
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