MRI assessment of hemodynamic effects of angiopoietin-2 overexpression in a brain tumor model

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Despite treatment efforts, the median survival in patients with glioblastoma multiforme, the most aggressive form of glioma, does not extend beyond 12–15 months. One of the major pathophysiological characteristics of these tumors is their ability to induce active angiogenesis. Thus, based on the lack of efficient therapies, agents that inhibit angiogenesis are particularly attractive as a therapeutic option. However, it has been recently proposed that although specifically targeting vascular endothelial growth factor, the main angiogenic factor, certainly leads to significant tumor regression, it could also be followed by tumor relapses. In this case, angiogenesis is driven by alternate pathways that include other angiogenic factors. One possible strategy to overcome this therapeutic obstacle is to overexpress antivascular factors such as angiopoietin-2 (Ang2). Here, by using MRI and histological analysis, we studied the vascular events involved in glioma growth impairment induced by Ang2 overexpression. Our results show that an increase in Ang2 expression, during the tumor growth, leads to a significant decrease in tumor growth (~86%) along with an increase in the survival median (~70%) but does not modify the tumor vascular area or cerebral blood volume. However, tumor Ang2-derived blood vessels display an abnormal, enlarged morphology. We show that

the presence of Ang2 leads to an enhancement of tumor perfusion and a decrease in tumor vessel permeability. Based on our MR image evaluations of hemodynamic tumor vessel changes, we propose that Ang2-derived tumor vessels lead to an inadequate oxygenation of the tumor tissue, leading to impairment in tumor growth. *Neuro-Oncology* 11, 488–502, 2009 (Posted to *Neuro-Oncology* [serial online], Doc. D08-00046, January 23, 2009. URL <http://neuro-oncology.dukejournals.org>; DOI: 10.1215/15228517-2008-117)

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Glioblastoma (GBM) is one of the most frequent malignant brain tumors in adults and has a poor response to chemotherapy and radiation. GBM is a highly vascularized tumor with atypical, disorganized, and leaky neovessels known to be driven by hypoxia and angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) and Ang2.¹ Thus, agents that inhibit angiogenesis are particularly attractive as a therapeutic option.² To date, major anti-angiogenesis strategies have focused on inhibition of individual proangiogenic factors such as VEGF. Anti-VEGF strategies that sequester VEGF,^{3,4} block the interaction of VEGF with VEGF receptor 2 (VEGFR-2),⁵ or inhibit VEGFR-2 tyrosine kinase activity^{6,7} have shown significant efficacy in preclinical tumor models when administered chronically. However, although disrupting a single proangiogenic signal has shown some success, an increasing body of evidence shows that this strategy

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is challenged by the existence of multiple proangiogenic factors. Moreover, it has recently been proposed that specifically targeting the function of tumor-associated VEGF leads primarily to significant tumor regression but could also be followed by tumor relapses in which angiogenesis is no longer driven by VEGF but by alternate proangiogenic pathways, including basic fibroblast growth factor and Ang1.^{8,9} Rather than inhibiting the formation of new vessels, one possible strategy to overcome this therapeutic obstacle is to overexpress antivascular factors to destroy tumor vasculature. If the rate of tumor vessel destruction exceeds the rate of angiogenesis, the overall tumor vasculature will regress, and vessel involution may impair tumor growth.¹⁰

Among the angiopoietin family, Ang1 and Ang2 play an important role in regulating vessel stability.¹¹ Ang1 and Ang2 are described as agonist and antagonist of the Tie-2 receptor, respectively. Ang1 promotes angiogenesis and stabilizes vessels,¹² whereas Ang2 shows context-dependent pro- or antiangiogenic activities.¹³ Importantly, Ang2 is upregulated only at sites of active vascular remodeling, which involves vessel destabilization and regression, especially in tumor growth. There is now evidence that tumors could initially grow by co-opting existing host vessels.¹⁴ This co-opted host vasculature does not immediately undergo angiogenesis to support the tumor but instead regresses, leading secondarily to an avascular tumor and massive tumor cell loss. Ultimately, however, the remaining tumor is rescued by active angiogenesis at the tumor margin. The expression patterns of the angiogenic antagonist Ang2 and of proangiogenic VEGF suggest that these proteins may be critical regulators of the balance between vascular regression and growth. Ang2 expression, in particular, has been detected in these co-opted tumor vessels and in areas of vessel regression during angiogenesis.^{14,15} Therefore, Ang2 might be considered as a marker of the vessels that are under active remodeling.

Based on these observations, it is of interest to investigate whether Ang2 overexpression may impair tumor growth. To date, several studies have used this strategy in different tumor models either by tumoral overexpression or by systemic overexpression of Ang2 in solid peripheral tumors as well as in brain tumors.^{10,16,17} In glioma models, according to its vascular destabilizing effect, a beneficial effect of an Ang2 overexpression has been reported. Ang2 overexpression prolongs the survival of tumor-bearing animals¹⁶ and leads to smaller tumors in which vessels display an aberrant structure and a lower density than in control tumors.¹⁷ However, although these studies suggest that the beneficial effect of Ang2 on tumor growth is due to a disruption in tumor angiogenesis, no studies to our knowledge have evaluated the influence of sustained Ang2 overexpression on the hemodynamic properties of the tumor vessels in relation to their structure and to glioma growth.

The evaluation of brain tumor response to therapy depends on both appropriate criteria and appropriate methodologies to detect the efficacy and mechanisms of treatment, in particular for antivascular therapy. Promising techniques include atraumatic approaches such as

MRI. With respect to the improvement of these techniques to measure the spatial and temporal changes in tumor blood flow and vascular permeability with higher resolution, it might be now possible to assess precisely the effects of antivascular therapy. We used dynamic susceptibility contrast (DSC) and T1-enhanced MRI methods to assess hemodynamic changes and vessel permeability in the tumors. With respect to the perturbations observed in the recirculation phase of the first passage of the contrast agent (CA) associated with the chaotic tumor vasculature, which prevents an accurate cerebral blood volume (CBV) computation,¹⁸ we also used a steady-state MRI method by measuring the transverse relaxation times prior to and after injection of an intravascular iron-based CA, which yields an absolute estimation of CBV.¹⁹ All MR estimates were also compared with a quantitative histological analysis of tumor vessels. This study contributes to the enhancement of data concerning morphological and functional vessel changes that occur in response to an antivascular therapy. Based on Ang2 overexpression in a glioma model, we studied vessel structure, permeability, hemodynamic changes, tumor growth, and survival median.

Materials and Methods

Cells and Cell Culture

The 9L glioma cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum and glutamine (2 mM) in RPMI medium (Sigma-Aldrich Chimie S.a.r.l., Saint Quentin Fallavier, France) at 37°C in 5% CO₂, 95% air.

Generation of Glioma Cells That Stably Express Ang2

A eukaryotic expression vector driven by the cytomegalovirus promoter incorporating a neomycin resistance gene was used. This construct vector containing human Ang2 (hAng2) or the vector alone (pcDNA3.1) was transfected into 9L cells by lipofection according to the manufacturer's protocol (Transfast, Promega, Charbonnières, France). Selective medium containing neomycin (G418) at 200 µg/ml was added 5 days later, and viable cells were selected and cloned into 96-well plates. Cells from subconfluent cultures were then harvested and used for reverse transcriptase (RT)-PCR, Western blot analyses, and in vivo animal experiments, as described below. The selected clone is named 9L-Ang2. The corresponding control cells transfected with the vector alone are named 9L-control.

Quantitative Real-Time RT-PCR

Total RNA (1 µg) from cells was reverse transcribed using the Promega RT system at 42°C for 1 h. RT products (50 ng) were then used for PCR amplification in a total volume of 25 µl.²⁰ Forward (F) and reverse

(R) primers were designed for each gene using Beacon Designer software (Bio-Rad, Marnes La Coquette, France): rat actin F: 5'-TTCAACACCCAGCCTGT-3' and R: 5'-GCAACAGGGACAACAAGCC-3'; human Ang2 F: 5'-CCTCCTGCCAGAGATGG'CAAC-3' and R: 5'-CCTCTGCACCGAGTCATCG-3'.

Assays were run in duplicate on the iCycler iQ real-time PCR detection system (Bio-Rad). The amplification profile was as follows: Hot Goldstar enzyme activation, 95°C for 3 min; 50 cycles at 95°C, 15 s; and 60°C, 1 min. Quantitative PCR was done according to the manufacturer's protocol using the qPCR Core kit for Sybr Green I No ROX (Eurogentec, Angers, France). The amount of target amplicon is given by the formula 2^{-C_t} , where C_t represents the fractional cycle number at which the amount of amplified target reaches a fixed threshold.

For brain tissues, total RNA was extracted from tumors of the ipsilateral striatum and from corresponding healthy contralateral striatum for animals of the 9L-control group and of the 9L-Ang2 group (three animals/group) at 18 days after implantation of the tumor cells. Quantitative RT-PCR were performed to quantify rat mRNA levels of Ang1, Ang2, Tie-2, VEGF, VEGF receptor-2 (VEGFR-2), platelet-derived growth factor-B (PDGF-B), PDGF receptor- β (PDGFR- β), and cyclophilin with rat primers and hAng2 with human primers. Final quantification of each sample was relative to rat cyclophilin as a house-keeping gene. The primers sequences were as follows: Ang1 F: 5'-AAAGGTCAGAAGAGAGGAGCAAG-3' and R: 5'-AAGGAAAAGTGTCTTGTACTGCC-3'; Ang2 F: 5'-GCTGAAGGACTGGGAAGGCA-3' and R: 5'-CTGGTTGGCTGATGCTACTGATT-3'; Tie-2 F: 5'-TGCGTAAGAGCCGAGTG-3' and R: 5'-TTTTGGCTCAAGTAGTCCATCCC-3'; VEGF F: 5'-CGTCTACCAGCGCAGCTATTG-3' and R: 5'-GCACTCCAGGGCTTCATCATT-3'; VEGFR-2 F: 5'-AGTGGCTGTCAAGATGTTGAAAGA-3' and R: 5'-ATGTGGATGAGGATCTTGAGTTTCG-3'; PDGF-B F: 5'-CGGTGCAGGTGAGAAAGAT-3' and R: 5'-CCGAGTTTAGGTGTCTTG-3'; PDGFR- β F: 5'-TGTGCAGCCTAATGAGACT-3' and R: 5'-AGGAGATGGTGAAGAAGTG-3'; Cyclophilin F: 5'-GACCAAACACAAATGGTTCCAG-3' and R: 5'-TTCGTGCCGTCCTTCACCTT-3'.

Western Blot Analyses

After separation by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, proteins (20 μ g) were transferred to polyvinylidene difluoride membranes (Perkin Elmer-NEN, Courtaboeuf, France) as previously described.²⁰ Membranes were then blocked for 1 h in 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (T-TBS) and incubated with antibodies (goat antihuman Ang2, 1 μ g/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; rabbit anti-actin, 0.8 μ g/ml, Sigma-Aldrich) overnight at 4°C. After washing in T-TBS, membranes were incubated for 1 h at room temperature with appropriate peroxidase-labeled secondary antibodies. The immunoreactive bands were

made visible by using enhanced chemiluminescence (Perkin Elmer-NEN). The blots were then incubated in stripping buffer (62 mM Tris-HCl [pH 6.8], 2% SDS, and 100 mM β -mercaptoethanol) for 30 min at 50°C. After washing, membranes were reprobed with actin antibodies. Human recombinant Ang2 (R&D Systems, Lille, France) was used as a positive control.

In Vitro Cell Proliferation Assay

Cells (10^4) were plated onto 96-well plates. Each day from day 1 to day 5, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was done as follows: 100 μ l of 0.5 mg/ml solution of MTT was added to wells and incubated 30 min at 37°C. The supernatant was removed, and the reaction was stopped with dimethyl sulfoxide (100 μ l/well). The plates were placed on a shaker for 5 min, and the absorbance was determined on a plate reader at 550 nm. Each assay performed on three distinct wells was repeated from three different cultures.

Intracerebral Glioma Model

All procedures with animals were performed according to institutional guidelines for use of laboratory animals. Experiments were performed under permit 14-55 (S.V.) and animal care facility B14118001 from the French Ministry of Agriculture (experiment authorization 0507-02). Syngenic male rats (Fischer 344) weighing between 200 and 250 g were purchased from Charles River (L'Arbresle, France). All experiments were performed under anesthesia: isoflurane at 5% for induction and 2% for maintenance, in 70% N₂O/30% O₂. Rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$ throughout the experiments. Cells were trypsinized, washed twice, and resuspended in phosphate-buffered saline (PBS)/1 mM glutamine. Cells ($5 \times 10^4/3 \mu\text{l}$) were stereotactically inoculated into the right striatum as previously described.²¹ For determination of Kaplan-Meier survival curves, animals were maintained until death ($n = 10$ animals in each group). A time-course study of the tumor volumes was determined from T2-weighted MR images on these corresponding animals (study 1). In addition, separate studies were performed 18 days after tumoral cell implantation. Study 2 included tumor volume and gadolinium first-pass determination using MRI and vessel analysis by immunohistochemistry on 9L-Ang2 ($n = 7$) and 9L-control rats ($n = 6$). Study 3 was undertaken on another series of animals to determine steady-state MRI parameters (CBV) on 9L-Ang2 ($n = 6$) and 9L-control rats ($n = 6$).

Immunohistochemistry

Anesthetized rats were perfused transcardially with saline followed by 4% paraformaldehyde (Sigma-Aldrich) 18 days after tumor cell implantation. Brains were removed and placed in a 30% sucrose solution for 3 days, and 50- μ m coronal sections were cut on a freezing microtome (Microm International GmbH,

Walldorf, Germany). The slices were kept at -20°C in a cryoprotectant mixture (30% glycerol, 20% ethylene glycol, and 50% PBS). Immunohistochemical staining for rat endothelial cell adhesion-1 (RECA-1) was used to quantify tumor vessels. After blocking the nonspecific binding in 0.3% PBS/Triton X-100/3% bovine serum albumin (BSA) for 1 h at room temperature, slices were incubated overnight with monoclonal mouse anti-RECA-1 (1:100; AbD Serotec USA, Raleigh, NC, USA) at 4°C in 3% PBS/Triton X-100/0.3% BSA, and vessels were revealed using a Cy3-linked goat antimouse IgG (1:1,000; Jackson Immunoresearch, West Grove, PA, USA). Pimonidazole was used as a hypoxia marker. Animals ($n = 3$ per group) were injected with pimonidazole solution (Hypoxyprobe-1; Chemicon, Millipore, Molsheim, France; 80 mg/kg i.p.) 60 min before the animals were sacrificed. Brains were collected and fixed in formalin, and immunohistochemical analysis was performed on paraffin-embedded 5- μm -thick sections. To visualize pimonidazole staining, deparaffinized sections were immunostained with a Hypoxyprobe-1 antibody following manufacturer's instructions (Chemicon).

Tumor Vessel Analysis

Tissue sections were examined at $\times 10$ magnification with a Leica DM6000 fluorescence microscope. For assessment of the tumor vessels, two fields in each section were photographed at 1- μm resolution: one in the healthy striatum (contralateral) and the other one in the tumor core. Four sections per animal were examined, with a total of four rats in the 9L-control group and five rats in the 9L-Ang2 group. Analysis of blood vessels was performed automatically using NIH ImageJ software, version 1.41 for Windows (<http://rsb.info.nih.gov/ij/>). The photographs of blood vessels were binarized by local thresholding at the average between the minimum and maximum intensity of the neighborhood, thus segmenting vessels close to half-height.

Vessel area was computed as the surface occupied by vessels; vessel length was derived from skeletonization. The vessel diameter at each pixel location along the skeleton and the quantification of distance of parenchymal pixels to the closest vessel were determined using distance maps.

Magnetic Resonance Imaging

MRI Experiments. Experiments were performed in a Pharmascan horizontal-bore magnet operating at 7 T (16-cm bore diameter; Bruker, Ettlingen, Germany) and equipped with actively shielded magnetic field gradient coils. Imaging was performed using a linear volume coil (32-mm bore diameter; Bruker). All experiments were performed under anesthesia: isoflurane at 5% for induction and 2% for maintenance in 70% N_2O /30% O_2 . Rectal temperature was maintained at $37.0 \pm 0.5^{\circ}\text{C}$ throughout the experiments. The rat was lying prone, its head secured via ear and tooth bars. Respiration was monitored using a pressure-sensitive balloon adhered to the abdomen of the rat. Rapid imaging (three images in

axial, coronal, and sagittal orientations, fast low-angle-shot sequence; repetition time [TR], 100 ms; echo time [TE], 4 ms; nominal resolution, $0.39 \times 0.39 \times 3 \text{ mm}^3$; acquisition time, 12 s) was performed for subsequent slice positioning.

For studies 1, 2, and 3, tumor-associated edema was detected using a fast T2-weighted sequence (rapid acquisition with relaxation enhancement [RARE], factor of 8; TR, 5,000 ms; TE [effective], 62.5 ms; four experiments, 10 slices; nominal resolution, $0.15 \times 0.15 \times 0.75 \text{ mm}^3$; acquisition time, 8 min).

Apparent diffusion coefficient (ADC) of water was computed from diffusion-weighted spin-echo planar images (five shots with navigator echoes; 30 diffusion directions; TR, 2,000 ms; TE, 38.97 ms; 10 slices; nominal resolution, $0.175 \times 0.300 \times 1.5 \text{ mm}^3$; acquisition time, 5 min 50 s) with b-value = 1,000 s/mm^2 and five reference images (b-value $\approx 0 \text{ s/mm}^2$). These images were acquired in all animals (studies 2 and 3).

Precontrast T1-weighted images (study 2) were acquired using an accelerated T1-weighted sequence (RARE factor of 4; TR, 1,300 ms; TE [effective], 7.3 ms; one experiment, 10 slices; nominal resolution, $0.15 \times 0.15 \times 1.5 \text{ mm}^3$; acquisition time, 2 min). Single-shot gradient-echo (GE) echo-planar images were then acquired for 15 s before and 1 min 45 s after a 0.2-mmol/kg bolus injection of the gadolinium chelate gadoterate meglumine (Gd-DOTA [Dotarem]; Guerbet LLC, Roissy, Charles de Gaulle, France); TR, 500 ms; TE [effective], 10.32 ms; one experiment, 10 slices; nominal resolution, $0.15 \times 0.15 \times 1.5 \text{ mm}^3$; acquisition time, 2 min; saturation slices at the edges of the field of view). Immediately thereafter, postcontrast T1-weighted images were acquired with the same imaging parameters as the precontrast acquisition.

For CBV measurements (study 3) with the CA in a steady state, multi-GE images (TR, 1,500 ms; TE, 3.1 ms; 12 equally spaced positive readout GEs, 3.01–58.78 ms; one experiment; nominal resolution, $0.15 \times 0.15 \times 0.75 \text{ mm}^3$; acquisition time, 5 min) were acquired just prior to and 4 min after administration of ferumoxtran-10 (Sinerem, Guerbet LLC; Combix, AMAG Pharmaceuticals, Lexington, MA, USA; 0.2 mmol Fe/kg via the tail vein in about 20 s). Subsequently, T1-weighted images (TR, 500 ms; TE [effective], 10.32 ms; one experiment, 10 slices; nominal resolution, $0.15 \times 0.15 \times 1.5 \text{ mm}^3$; acquisition time, 2 min) were acquired prior to and following an intravenous injection of Gd-DOTA.

MR Image Postprocessing. Image analysis was performed with ImageJ software.

Tumor Volume. Tumor delineation was performed manually both on the T2-weighted and on difference T1-weighted images (i.e., postcontrast minus precontrast).

Regions of Interest. The above delineations of tumors on difference T1-weighted images were used as tumor regions of interest (ROIs). Corresponding contralateral ROIs were also manually drawn. These ROIs were

used for Gd-DOTA first-pass, T1 enhancement, and CBV analyses. In addition, hemorrhagic areas, identified by histology and visualized as a signal decrease on GE images, were delineated. For tumor ROIs to analyze volume transfer coefficient (Ktrans) maps, we used the ROIs derived from difference T1-weighted images with hemorrhagic areas excluded. These excluded pixels represent about 5% of the tumor ROI.

Hemodynamic Parameters. For DSC MRI, signal intensity in each ROI was extracted from the baseline corrected T2-weighted images (>2 min). Data from all slices in each animal were averaged, after weighting by the size of each ROI. A temporal registration to the arrival time of the bolus in each rat was performed to allow calculation of a mean $\pm$ SEM signal over all animals in each group.

Ktrans maps were computed using the DSCoMAN ImageJ plug-in, which implements the Boxerman-Weisskoff algorithm.²²

For steady-state imaging, percent CBV maps were computed from $\Delta R2^*$ maps and $\Delta\chi$:¹⁹

$$\text{CBV (\%)} = \frac{3}{4\pi} \left(\frac{\Delta R2^*}{\gamma \cdot \Delta\chi \cdot B_0} \right)$$

$\Delta\chi$ is the increase in the magnetic susceptibility difference between the extra- and intravascular compartments (nonrationalized units) induced by the presence of the CA in the vasculature, B_0 is the main magnetic field (T), and γ is the gyromagnetic ratio of protons.

Statistical Analyses

Statistical analyses were performed using Statview SE software 5.0 (SAS Institute Inc., Cary, NC, USA). The different test programs 5.0 used are detailed in each figure caption. The Mann-Whitney rank test was used for survival analyses.

Results

Expression of Ang2 in Rat 9L Glioma Cells

Among the eight clones we obtained from 9L-Ang2-transfected cells, we selected clone 8, which expressed the highest quantity of Ang2 at the mRNA level, and we verified the corresponding protein expression (Fig.

Fig. 1. Overexpression of angiopoietin-2 (Ang2) in 9L-transfected cells. (A) Clone 8 had the highest Ang2 mRNA expression. For this clone, the corresponding Ang2 protein expression was verified by Western blotting (inset; rH.Ang2, recombinant human Ang2). (B) No difference in cell proliferation was observed between 9L parental cells and 9L-control (9L-empty plasmid) or 9L-Ang2-transfected cells (mean $\pm$ SD).

Fig. 2. Angiopoietin-2 (Ang2) overexpression increases life span of rats bearing brain tumors: Kaplan-Meier survival curves for 9L-control rats ($n = 9$) and 9L-Ang2 rats ($n = 9$). Ang2 overexpression increased the survival median by 14 days (9L-control group, 20 days; 9L-Ang2 group, 34 days; $p < 0.001$, Mann-Whitney test).

1A). To confirm that Ang2 overexpression in tumor cells would display a paracrine effect on tumor vessels *in vivo*, we first verified that this overexpression had no effect on tumor cell proliferation. We thus compared the growth curve of the transfected selected clone (9L-Ang2), 9L-empty vector cells (9L-control), and parental 9L cell line *in vitro*. No significant difference in growth rate was observed between the three cell lines (Fig. 1B).

Chronic Overexpression of Ang2 Increases Life Span of Rats Bearing Brain Tumors

We then determined whether Ang2 overexpression by tumor cells might prolong survival in animals with

Fig. 3. Angiopoietin-2 (Ang2) overexpression delays tumor growth. Tumor volumes were determined from T2-weighted MR images over time (mean $\pm$ SD). ** $p < 0.005$; *** $p < 0.0001$, versus 9L-control group, Bonferroni/Dunn test after a significant two-way analysis of variance.

tumors (study 1). Animals were maintained until death for Kaplan-Meier analysis. As depicted in Fig. 2, chronic Ang2 overexpression enhanced rat survival by 70%. Median survival for animals of the 9L-Ang2 group was 34 days versus 20 days for animals of the 9L-control group ($p < 0.001$).

Chronic Overexpression of Ang2 Delays Tumor Growth

To establish the influence of Ang2 overexpression on tumor growth, we performed a longitudinal study on the animals of the survival study, using T2-weighted MR images (Fig. 3). Rather than tumor regression, we observed that Ang2 overexpression delayed tumor growth. We observed a significant reduction of tumor volume around 83% at day 14 and 77% at day 20 for the rats of the 9L-Ang2 group compared with the rats of the 9L-control group. Based on the time-course curve presented in Fig. 3, a volume similar to that obtained at 30 days after tumor cell implantation in the 9L-Ang2 rats ($341 \pm 114 \text{ mm}^3$, $n = 9$) was reached at 20 days for 9L-control animals ($398 \pm 114 \text{ mm}^3$, $n = 7$).

We next focused our study at 18 days after glioma cell implantation, just prior to the survival median into the 9L-control group, on the mechanisms of the reduction of the tumor growth, which could be due to vascular changes induced by Ang2 overexpression (study 2). Representative examples of T2-weighted and T1-weighted images after contrast injection are presented in Fig. 4 for rats that received implantation of 9L-control or 9L-Ang2 cells. Based on difference T1-weighted images, at 18 days, overexpression of Ang2 led to drastic reduction in glioma volume by around 86% (control, $277 \pm 121 \text{ mm}^3$; Ang2, $39 \pm 17 \text{ mm}^3$; $p < 0.001$; Fig. 4A).

Similar results were obtained using delineation of tumor volume from T2-weighted MR images (control, $267 \pm 119 \text{ mm}^3$; Ang2, $39 \pm 14 \text{ mm}^3$; $p < 0.001$; Fig. 4B).

Ang2 Does Not Modify Water Mobility

In vivo MR image assessment of ADC within the tumor may reflect 9L cell changes.^{23,24} At 18 days after cell implantation, although an increase in ADC values was observed within the 9L-control tumor compared with the contralateral striatum (contralateral, $779 \pm 29 \mu\text{m}^2/\text{s}$; 9L-control, $949 \pm 24 \mu\text{m}^2/\text{s}$; $p < 0.0001$), no difference was observed between 9L-control and 9L-Ang2-expressing tumors (9L-control, $949 \pm 24 \mu\text{m}^2/\text{s}$; 9L-Ang2, $929 \pm 48 \mu\text{m}^2/\text{s}$). These results suggest a similar cellularity in both tumor groups.

Effects of Ang2 Overexpression on Tumor Vasculature

We next studied whether the reduction of tumor growth was related to a vascular effect of Ang2 overexpression (study 2). At 18 days, we quantitatively analyzed tumor-associated vasculature detected with an antibody directed against RECA-1. Fig. 5A depicts typical vessels present in tumors derived from 9L-Ang2 cells and from 9L-control cells in a healthy contralateral striatum. As

Fig. 4. Angiopoietin-2 (Ang2) overexpression reduces tumor growth. (A) Tumor volumes (mean $\pm$ SD) were determined on the difference T1-weighted images (post-contrast minus precontrast with gadolinium). At 18 days, overexpression of Ang2 led to a reduction in glioma volume (9L-control, $n = 6$; 9L-Ang2, $n = 7$). $*p < 0.0001$, Student's t -test. (B) Similar results were obtained by delineation of tumor volume from T2-weighted images (mean $\pm$ SD). $*p < 0.001$ versus 9L-control, Student's t -test. Difference T1-weighted and T2-weighted images of representative animals of the 9L-control and 9L-Ang2 groups are shown to the right.

expected, the quantitative analysis revealed that the vessel area of the 9L-control-derived tumor is larger than that of control healthy tissue (Fig. 5B) and characteristic of tumor vessels: tortuous and dilated (Fig. 5A). In contrast, although 9L-Ang2-derived tumors showed an increase in tumor vessel area, these vessels display less branching or sprouting (Fig. 5A) and are also enlarged (mean diameter, $12.6 \pm 0.8 \mu\text{m}$ vs. control, $8.5 \pm 0.4 \mu\text{m}$; $p < 0.0001$). Morphometric analysis (Fig. 5D) revealed an increased number of large vessels (13–60 μm diameter) and a decrease in small vessels (1–8 μm diameter) in Ang2-overexpressing tumors compared with 9L-control tumors. In addition, a decrease in total vessel length (Fig. 5C) without any difference in vascular area (Fig. 5B) was observed for the Ang2-overexpressing tumors compared with control tumors. An increase in distances of parenchymal pixels to the closest vessel was also observed for the two tumor groups, with a higher effect in the Ang2-overexpressing tumors (Fig. 5E).

Ang2 Influences Tumor Perfusion and Vessel Permeability

We then determined whether the overexpression of Ang2 modifies tumor perfusion measured using MR imaging (studies 2 and 3). Perfusion analysis performed at 18 days in the contralateral healthy hemisphere showed a typical first pass of the CA with a rapid loss of signal and rapid return to basal value (Fig. 6A, gray curve).

The reduction of signal in the 9L-control tumors was less pronounced (Fig. 6B), with no obvious separation between first and subsequent passes of CA (Fig. 6A, black curve, open circles). At the end of the analyzed period, the signal within the tumor also increased (Fig. 6A, gray curve). In contrast, 9L-Ang2 tumors exhibited a signal loss larger than in the contralateral side (Fig. 6A, black curve; Fig. 6B). Moreover, the first pass of CA was clearly observable with a signal shape close to that of the contralateral side. Bare T1 enhancement was observed for the 9L-Ang2 tumors (Fig. 6A, black curve; Fig. 6B). To investigate whether the hemody-

Fig. 5. Angiopoietin-2 (Ang2) overexpression modifies glioma vasculature. Blood vessels revealed by rat endothelial cell adhesion-1 (RECA-1) immunohistochemistry were quantified in healthy contralateral striatum and in tumors of 9L-control and 9L-Ang2 groups. (A) Representative photographs of vessels revealed by RECA immunodetection. Scale bar, 100 μm. Compared with vessels of control healthy tissue, tumor vessels in the 9L-control-derived tumor are tortuous and dilated, with very heterogeneous diameters. In contrast, in 9L-Ang2-derived tumors, vessels display less branching and a regular diameter. (B) Vessel area of the 9L-control-derived tumors is larger than that of control healthy tissue. This increase in tumor vessel area is also observed in 9L-Ang2-derived tumors to a similar extent as in 9L-control-derived tumors. (C) A decrease in total vessel length is observed for the Ang2-overexpressing tumors compared with control tumors. (D) Histograms of vessel diameters. Morphometric analysis revealed an increased number of large vessels (13–60 μm diameter) and a decrease in small vessels (1–8 μm diameter) in Ang2-overexpressing tumors compared with 9L-control tumors. (E) Histograms of vessel domains. An increase in distance of parenchymal pixels to the closest vessel is observed for the 9L-control and 9L-Ang2 tumor groups, with a higher effect in the Ang2-overexpressing tumors. All data are mean + SD. * $p < 0.05$ versus contralateral; # $p < 0.05$ versus 9L-control group, Fisher protected least significant difference following a significant analysis of variance.

Fig. 6. 9L-angiopoietin-2 (9L-Ang2) tumors display an increase in perfusion compared with 9L-control tumors. (A) Average signal of DSC MRI over 2 min, before and after a bolus injection of the gadolinium chelate gadoterate meglumine in contralateral healthy striatum, 9L-control tumor, and 9L-Ang2 tumor regions of interest (mean + SEM). Perfusion analysis performed at 18 days in the contralateral healthy hemisphere shows a typical first pass of the contrast agent with a rapid loss of signal and rapid return to basal value (gray curve). In 9L-control tumors, the signal loss decreased with obvious abnormal recirculation. In contrast, 9L-Ang2 tumors exhibited a signal loss larger than in the contralateral side with a qualitatively normal recirculation. At the end of the analyzed period, the signal within the 9L-control tumors increased (black curve with open circles). The 9L-Ang2 tumors showed bare enhancement (black curve with solid circles). (B) T2*-weighted images from a representative animal of the 9L-control and 9L-Ang2 groups at three time points, as indicated in A: basal period (a), signal at peak (b), and last time point (c).

dynamic changes observed between both groups reflect a difference in tumor volume rather than a vascular effect specific to Ang2, we compared the reduction of signal in 9L-Ang2 tumors at 18 days and in 9L-control tumors at 10 days after tumor cell implantation, when both groups display similar tumor volumes. The same differences as above were found when we compared the two groups (data not shown).

A semiquantitative analysis of vessel permeability was performed from Ktrans maps computed from DSC MRI and from signal changes following T1 enhancement.²² Vessels present in the control tumors are highly permeable, resulting in an increase in Ktrans (2.6 ± 1.5) and also in T1 enhancement (18.6 ± 5.3 ; Fig. 7A, B). In contrast, in the presence of Ang2, vessels are significantly less permeable than in the control tumors, with a Ktrans of 1.6 ± 0.6 and a T1 enhancement of 10.7 ± 1.7 (Fig. 7A, B). Data are presented in arbitrary units, with normalization to an average contralateral ROI value of 1.

Ang2 Overexpression Does Not Modify CBV

The absence of a clear separation, with DSC MRI, between first and subsequent passes of CA, at least for 9L-control tumors, prevents any accurate CBV estimation. However, with respect to the drastic loss of signal observed for the tumor overexpressing Ang2, an increase in CBV may be hypothesized. CBV maps were therefore computed using an iron oxide CA-based steady-state method. As expected, CBV was increased in the 9L-control tumors ($7.99 \pm 0.50\%$) compared with the contralateral striatum ($4.67 \pm 0.19\%$; Fig. 8). However, no significant difference in CBV was observed between the 9L-control tumors and the 9L-Ang2 tumors ($8.53 \pm 1.04\%$; Fig. 8).

Fig. 7. Angiotensin-2 (Ang2) overexpression reduces tumor vessel permeability compared with vessels derived from 9L-control tumors: volume transfer coefficient (Ktrans; A) and T1 enhancement (B) in 9L-control and 9L-Ang2 tumors and contralateral regions of interest (ROIs). Vessels present in the 9L-control tumors are highly permeable, resulting in an increase in Ktrans and in T1 enhancement. In contrast, in the presence of Ang2, vessels are significantly less permeable than in the control tumors, leading to a decrease in Ktrans and T1 enhancement. Data are presented as mean + SD in arbitrary units (a.u.), with an average contralateral ROI value of 1. * $p < 0.0001$ versus contralateral; # $p < 0.0001$ versus 9L-control group, Fisher protected least significant difference following a significant analysis of variance. At the bottom, Ktrans maps and T1 enhanced images from representative animals of the 9L-control and 9L-Ang2 groups are presented.

Ang2 Overexpression Increases Tumor Hypoxia

Based on the above results of the vascular effects of Ang2, it could be proposed that the paracrine effect of Ang2 leading to enlarged vessels with an increase in perfusion could induce tumor hypoxia. Therefore, we further examined the effect of Ang2 overexpression on the tumor environment by pimonidazole staining. Pimonidazole is used to delineate the hypoxic site of the tumors due to its specific binding to cells exhibiting a loss of partial oxygen tension.^{25,26} Fig. 9 shows the immunohistochemical staining of pimonidazole of one representative tumor tissue per group. Cells in the tumor tissue from the 9L-Ang2 group (Fig. 9D) are strongly positive for this hypoxic marker, with clear cytoplasmic staining compared with cells of the 9L-control tumor (Fig. 9B).

Sustained Tumor Ang2 Overexpression Does Not Modify the Endogenous Expression of Tumor Angiogenic Factors

To gain insight into the signaling mechanisms underlying tumor blood vessel changes induced by the sustained Ang2 overexpression, the expression of the major angiogenic factors and their receptors was analyzed in the tumor tissues of animals of the 9L-control and

9L-Ang2 groups by RT-PCR, 18 days after implantation of hAng2-transfected 9L cells, and compared with the expression of these genes in the corresponding healthy tissue. Using human-specific primers, we measured mRNA expression of hAng2 in the tumor at around 35-fold greater than that of endogenous mRNA expression of rat Ang2 (data not shown). However, although Ang2 is expressed in a large excess, the expression of all the studied rat genes remained unchanged when Ang2 is overexpressed compared with control tumors (Fig. 10). In both groups, compared with contralateral tissue, although a slight increase in VEGF and Ang2 mRNA expression is observed, Ang1 mRNA expression decreased significantly. In addition, at this time, the mRNA expression of the corresponding angiogenic factor receptors VEGFR-2 and Tie-2 decreased, although PDGFR- β mRNA slightly increased but to a similar extent for the two tumor groups compared with healthy tissue (Fig. 10).

Prolonged Ang2 Overexpression Leads to Regression of Tumor Vasculature

We were interested to study whether the vascular effects of Ang2 overexpression were sustained during the course of tumor growth. By an immunohistochemical analysis, we showed that, compared with vessels detected at

Fig. 8. Cerebral blood volume (CBV) determined using a steady-state method is similar in 9L-angiopoietin-2 (9L-Ang2) and 9L-control tumors. (A) CBV is increased in the 9L-control tumors compared with the contralateral striatum. However, no significant difference in CBV in the tumor is observed between 9L-control and 9L-Ang2 tumors. Data are mean $\pm$ SD. * $p < 0.0001$ versus contralateral, Fisher protected least significant difference following a significant analysis of variance. (B) T2-weighted (T2w) and T1-weighted (T1w) images and CBV maps of the 9L-control and 9L-Ang2 tumors from representative animals.

18 days, at day 34 (which corresponds to the survival median of 9L-Ang2 animals), the vasculature tends to regress in the tumor (Fig. 11).

Discussion

A possible strategy for blocking tumor growth could consist of overexpressing antivascular factors in order to destroy tumor vasculature rather than inhibiting angiogenesis. Accordingly, overexpression of Ang2 in brain tumors leading to an impairment in tumor growth has previously been described in rodent orthotopic glioma models.^{16,17} However, the hemodynamic effects of such Ang2 overexpression remain to be clarified since only

Fig. 9. Angiopoietin-2 (Ang2) overexpression leads to tumor hypoxia: detection of hypoxic cells in 9L-control (a and b) and 9L-Ang2 (c and d) tumors on a representative tumor tissue section for each group. Immunohistochemical detection of pimonidazole shows a more pronounced cytoplasmic staining in cells of the 9L-Ang2 tumors (c and d) compared with 9L-control tumors (a and b) at low (a and c) and at higher (b and d) magnification. Scale bars, 50 μ m.

the morphological vascular effects induced by this strategy have been assessed.^{16,17}

Our results present, for the first time, evidence that overexpression of Ang2 by glioma cells promotes drastic tumor vascular changes accompanied by a reduction in tumor growth. We provide data that characterize the hemodynamic effect of Ang2 overexpression as well as the functionality of glioma vessels. We show that 9L-Ang2-derived tumors display an increase in tumor perfusion with a CBV identical to that of untreated tumors. We also give direct proof of an antipermeability effect of Ang2 on tumor vessels.

Our results show that overexpression of Ang2 is associated with a drastic reduction in tumor growth (~86% at 18 days after 9L-Ang2 cell implantation) and a drastic increase in the life span of rats bearing 9L-Ang2 tumors (~70% compared with 9L-control animals). We verified that this autocrine Ang2 overexpression has no effect on glioma cell proliferation in vitro and no effect on ADC values, which has been suggested to reflect cellularity;^{23,24} the effects of Ang2 on tumor growth might be attributable to vascular effects.¹⁷

Based on two different MRI modalities (T1 enhancement and Ktrans), we provide direct proof of an antipermeability effect of Ang2 on tumor vessels. Our results are in line with those of Machein et al.¹⁷ but differ from those showing that endogenous Ang2 expression is detected in tumor vessels that are under destabilization¹⁴ and is negatively correlated with pericyte coverage both in humans and in glioma models.^{1,27} The discrepancy between the effects observed with overexpression of exogenous Ang2 and endogenous Ang2 could be explained by the different levels of Ang2, which could lead to very different outcomes.²⁸

Fig. 10. Sustained angiopoietin-2 (Ang2) overexpression does not modify expression of tumor endogenous angiogenic factors: angiogenic expression profile of 9L-Ang2 (black bars) and 9L-control tumors (white bars) relative to corresponding healthy contralateral tissue as assessed by RT-PCR using rat primers (mean + SD; $n = 3$ per group). VEGF, vascular endothelial growth factor; PDGF-B, platelet-derived growth factor-B; VEGFR-2, VEGF receptor-2; Tie-2, Ang1 receptor; PDGFR-β, PDGF receptor-β. * $p < 0.001$ versus contralateral healthy tissue, Fisher protected least significant difference following a significant analysis of variance.

Employing DSC MRI,^{29,30} the most widely used method to analyze vascular functions, we clearly observed a drastic loss of signal within the 9L-Ang2 tumors, which may be attributed to an increase in tumor perfusion or an increase in CBV. Due to the chaotic tumor vasculature, CBV cannot be reliably derived from DSC MR images since signal changes are affected by potential broadening of the arterial input function and by alteration of blood–brain barrier permeability and abnormalities in the recirculation of the CA,^{18,31,32} as observed in the present study. As a consequence, absolute CBV maps were computed from a steady-state MR method. By this means, we showed that the CBV remains stable between both tumor types. Consequently, we postulate that the signal loss observed within the 9L-tumor is mainly due to an increase in perfusion.

The stability in CBV that we observed between the two tumor groups is consistent with the lack of Ang2 effect on tumor vascular area as assessed by immunohistochemistry. Based on this morphological study, in contrast to Machein et al.,¹⁷ we observed in gliomas derived

from Ang2-overexpressing cells that vessels displayed an apparently normalized morphology, except that they showed an increased diameter not only compared with normal nontumoral vessels but also compared with 9L-control tumor vessels. The Ang2-derived vasculature might, paradoxically, be considered a functional vascular structure rather than a vascular structure in regression. These results could be compared with recent concepts concerning antiangiogenesis-targeted strategies that, paradoxically, might promote maturation of unstable vessels, which could be efficient for delivering chemotherapeutic agents and oxygen.^{33,34} However, in contrast to the vasculature of the healthy tissue, these Ang2-derived vessels are enlarged and exhibit increased perfusion, suggesting that the effects of Ang2 cannot be depicted as a normalization. Furthermore, in the Ang2-derived tumor, these enlarged vessels impair tumor growth rather than contribute to its growth.

We hypothesize that these vessels render tumor perfusion inefficient for appropriate oxygen and nutrient exchange, leading to tumor asphyxia. Their enlarged

Fig. 11. Prolonged angiopoietin-2 (Ang2) overexpression leads to a regression of tumor vasculature: detection of blood vessels in 9L-control and 9L-Ang2 tumors on a representative tumor tissue section for each group. Blood vessels are revealed by rat endothelial cell adhesion-1 immunohistochemistry in 9L-control tumors (9L-Ct) and contralateral healthy tissue (inset) at 18 days and in the 9L-Ang2 group at 18 days and 34 days after tumor cell implantation ($n = 3$ per group).

lumen, together with the decrease in total length, leads to a decrease in the exchange surface between blood and parenchyma. Furthermore, the high velocity of blood in the 9L-Ang2 tumor vessels may also contribute to decreased oxygen exchange compared with 9L-control vessels, in which circulation impairment may lead to blood stagnation and therefore better oxygen delivery. In addition, the increase in the distance of cells to the closest vessel observed in the 9L-Ang2 tumors might also account for a decrease in tissue oxygenation. This hypothesis is supported by the stronger immunostaining with a hypoxic marker (pimonidazole) in tumor cells of the 9L-Ang2 group compared with tumor cells of the 9L-control group.

In the present study, instead of an antivasular effect of Ang2, our MR results showed, for the first time, that Ang2 overexpression could lead, paradoxically, to a morphologically normalized vasculature but with strongly modified perfusion, which would not be suspected with the exclusive use of histological vascular characterization. These results demonstrate the importance of assessing different independent vascular parameters by complementary methods to get a clear picture of the action mechanisms of vascular targeting strategies. In particular, the use of MRI enables identification of the hemodynamic effects of vascular remodeling, which cannot be deduced from morphological data. Although vessels present in 9L-control tumors and 9L-Ang2 tumors display very distinct morphology and hemodynamic properties, their profiles of angiogenic factor expression, analyzed at 18 days after tumor cell implantation, are very similar. In both tumors, a slight increase in rat VEGF and Ang2 expression but a decrease in Ang1 expression is observed. This change in the angiogenic expression profile is also reflected in the Ang1:Ang2 ratio, which is around 1:8 in 9L-control and 9L-Ang2 tumors, according to the tumor angiogenic plasticity.³⁵ This ratio is sustained even in the presence of hAng2, which is around 35-fold greater than that of endogenous Ang2. Thus, we speculate that high amounts of Ang2, even in the presence of VEGF, neither stimulate tumor angiogenesis nor induce vessel regression at this time. Paradoxically, in presence of high amounts of Ang2, tumor vessels are also less leaky, although no changes in gene expression of PDGF-B and PDGFR- β is observed. Our results suggest that in our experimental conditions, the Ang2-induced vessel stabilization might be independent of pericyte recruitment. Altogether, the present data revealed that elevated Ang2 levels might have profound pleiotropic effects on tumor vessel structure, perfusion, hemodynamic properties, and oxygenation, independent of any expression changes in the other related angiogenic factors. However, additional studies are required to understand and clarify the molecular mechanisms by which Ang2 alters tumor vessels and perfusion. According to Kim et al.,³⁶ high amounts of Ang2 might act as a survival factor for endothelial cells or induce or modify the localization of platelet endothelial cell adhesion molecule (PECAM)³⁷ or vascular endothelial cadherin³⁸ into junctions between endothelial cells, strengthening these junctions. In this study, the provoked imbalance between

Ang2 secreted by tumor cells and endogenous VEGF is in favor of an excess of Ang2 (35-fold greater in tumors of the 9L-Ang2 group compared with tumors of the 9L group). In response to this sustained Ang2 overexpression, the vascular remodeling induced by Ang2 seems to be transient, as at day 34 (which corresponds to the survival median of 9L-Ang2 animals), the vasculature finally regresses in the tumor. This result, showing a biphasic effect of Ang2 overexpression as an antivasular strategy, might be compared with the normalization window of the tumor vessels observed with antiangiogenic therapy.³³

As a whole, our data reinforce the interest in antivasular strategies for glioma treatment in parallel with antiangiogenic strategies, which can be limited by tumor relapse. Based on our results, overexpression of Ang2 might be an attractive strategy. However, because we used *in situ* Ang2 delivery in the tumor by genetic modification of tumor cells themselves, an approach more appropriate to the clinical context would be to deliver Ang2 systemically in its proteic form. To date, no data indicate that Ang2 crosses the blood-brain barrier, although it has been demonstrated for Ang1.³⁹ It would be interesting to verify this point for Ang2 in another preclinical study and then to test the effect of chronic Ang2 administration to treat brain tumors and establish the best schedule of administration.

Nevertheless, antivasular therapy alone may not be a sufficient strategy to treat tumors. Presently, the major antiangiogenic strategy targets VEGF. There are now promising clinical data showing that when this therapy is given in conjunction with cytotoxic therapy, it can improve overall patient survival in colorectal and lung cancer.⁴⁰ In brain tumors, the early trials with the first generation of antiangiogenic agents conducted in GBM patients yielded disappointing results. However, interest in anti-VEGF therapies was rekindled by MRI clinical data showing that these agents can transiently normalize the tumor vasculature in GBM patients, enhancing the penetration of chemotherapeutics.⁴¹ Because Ang2 alters tumor vessels and perfusion, it would be important to characterize the time course of morphological, functional, and molecular changes in the vasculature of glioma in response to Ang2 overexpression and the relationship between these vascular changes, tumor hypoxia, and response to radiation or chemotherapy. Beside molecular biomarkers, noninvasive imaging techniques, which have the potential to measure functional parameters, might be considered as surrogate markers for the evaluation of the efficacy and mechanisms of therapy of tumors.

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