Published in final edited form as:

Brain Res. 2009 September 8; 1288: 125–134. doi:10.1016/j.brainres.2009.06.095.

Glioma-induced Remodeling of the Neurovascular Unit

Jisook Lee¹, Caren Lund-Smith¹, Alexandra Borboa¹, Ana M. Gonzalez², Andrew Baird¹, and Brian P. Eliceiri¹

¹ Department of Surgery, University of California San Diego, CA

² Molecular Neuroscience Group, Division of Medical Sciences, University of Birmingham, UK.

Abstract

The normal BBB (blood-brain barrier) consists of a series of structures collectively known as neurovascular units, or NVU, that are composed of endothelial cells and astrocyte end feet separated by a basal lamina at their interface. The integrity of the BBB and specifically endothelial tight junctions is maintained by interactions between these different components and the local microenvironment of the NVU. Central nervous system cancers such as gliomas disrupt the integrity of the BBB and this compromise is associated with increased tumor growth and invasion of the surrounding brain parenchyma. Because the relationship between glioma-induced BBB breakdown and glioma invasion remains poorly understood, and the host microenvironment can influence tumor cell migration, we used immunohistochemical techniques to characterize tumor associated BBB remodeling. Using an orthotopic xenograft model of glioma, we demonstrate that tumor cells induce specific changes in the composition of the basal lamina and in astrocytic components of the NVU. We suggest that these changes may be essential to understand the capacity of gliomas to regulate BBB integrity and as such, glioma invasion into brain parenchyma.

Keywords

blood brain barrier; glioma; neurovascular unit; invasion

1. Introduction

Gliomas are associated with aggressive invasion of the surrounding brain parenchyma. While resection reduces the primary tumor burden, extensive migration of glioma cells away from the primary tumor mass prevents the complete removal of tumor cells and represents a significant challenge in the treatment of glioma. In vivo experimental models of glioma have been used to demonstrate that tagging tumor cells can be used to monitor the infiltration of tumor cells in the parenchyma and/or perivascular spaces even at a single cell level (Lampson et al., 1993; Lund et al., 2006; Winkler et al., 2009; Zhang et al., 2002). However, the relationship between glioma-induced BBB dysregulation and glioma invasion remains poorly understood.

Corresponding author : Brian Eliceiri Department of Surgery, Division of Trauma 212 Dickinson Street San Diego, CA 92103 FAX: (619) 543–2325 Office: (619) 543–2905 beliceiri@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Gliomas disrupt the integrity of the blood-brain barrier (BBB), and this compromise is associated with increased glioma tumor growth and infiltration in human and rodents (Lund et al., 2006; Neuwelt and Rapoport, 1984). Whereas normally, the BBB consists of endothelial cells held together by tight junctions, it also contains a basal lamina surrounding the capillary walls and adjacent astrocyte end feet that form a structure collectively known as the neurovascular unit, NVU (Iadecola, 2004; Simard and Nedergaard, 2004). The interactions between the different cell types of NVU are disrupted in the microenvironment of the glioma (Abbott et al., 2006; Abramsson et al., 2007; Virgintino et al., 2007).

While many studies with malignant glioma focus on the dysregulated signaling pathways in tumor cells as a potential target for therapeutics (Furnari et al., 2007), our previous studies have shown the importance of the host microenvironment to influence the migration of the tumor cells (Criscuoli et al., 2005; Lund et al., 2006). For example, the presence of specific host factors such as src in the brain endothelium is essential for tumor-mediated BBB breakdown and glioma invasion (Eliceiri et al., 1999; Lund et al., 2006). In this study, we compared tumor-induced changes in the NVU of primary vs secondary tumors (as defined by their distance from the primary tumor), and suggest that changes in basal lamina composition may mediate the capacity of gliomas to regulate BBB integrity.

2. Results

Immunostaining of the astrocyte-endothelial interface of the BBB

In the normal brain, a subset of astrocytes extends projections to the basal lamina that surrounds the microvascular endothelium. Immunohistochemical staining with anti- glial fibrillary acidic protein (GFAP) demonstrates the difference between arterioles/venules and the capillary bed and highlights the specific association of GFAP-positive astrocytes with larger vessels that have a minimal role in regulating vascular permeability (Fig 1A). Immunostaining of 60 μ m thick tissue sections with an anti-GFAP antibody reveals astrocytes along arterioles and venules (Fig 1A) while lesser staining for GFAP was observed on branching capillaries (arrow, Fig 1A). To define the endothelial-astrocyte junction in the normal mouse brain and characterize the specificity of the various astrocyte markers associated with blood vessels, we examined the distribution of astrocytes using three different astrocyte markers; 1) GFAP as a generic marker of astrocytes (Eng, 1985); 2) aquaporin-4 (AQ4), a water channel protein expressed in astrocyte endfeet (Warth et al., 2004), and 3) aldehyde dehydrogenase 1 family, member L1 (AldH1L1), a protoplasmic astrocyte marker (Cahoy et al., 2008; Oldham et al., 2008) (Fig 1B). As shown in Fig 1, astrocytes at the astrocyte-endothelial junction of the BBB are AQ4-positive but GFAP-negative. The anti-AQ4 antibody labels the astrocytic endfeet that surrounds the CD31-positive capillaries while GFAP-positive astrocytes are not associated with CD31-positive capillaries (Fig 1B and 1C). In contrast, the GFAP-positive astrocytes that are associated with blood vessels generally define arterioles or venules. In contrast, AldH1L1 immunoreactivity was detected in both subclasses of astrocytes and is associated with capillaries and arterioles/venules (Fig 1D). These studies define a subset of AQ4⁺, GFAP⁻ astrocytes that constitute the primary population of astrocytes that are associated with the normal brain capillary endothelium.

Orthotopic glioma model

Although cell culture studies have characterized the interactions between astrocytes and brain endothelial cells (Abbott et al., 2006), these in vitro approaches do not fully recapitulate the complexity of the brain microenvironment. Therefore, we developed an orthotopic xenograft model of glioma growth and invasion in an immuno-deficient background to assess the dysregulation of BBB induced by human malignant glioma. DBTRG malignant gliomas (Kruse et al., 1992) maintain the characteristic infiltrative and pseudopalisading phenotype of gliomas

and induce vascular permeability in the brain when injected in vivo into mice, thus serving as a pathophysiologically relevant model to study glioma (Lund et al., 2006). To facilitate the characterization of glioma-induced remodeling of astrocyte-basal lamina interface, we used reporter genes to enable noninvasive localization of DBTRG glioma cells in vivo. DBTRG-luc cells were used to localize tumors noninvasively and DBTRG-RFP cells were used for high resolution because of its compatibility with indirect immunofluorescence microscopy. These complementary labeling techniques enable the non-invasive quantitation of glioma growth while also facilitating localization of individual tumor cells by fluorescence. Furthermore, tumor growth throughout the cortex of the brain and extensive infiltration of the tumor cells can be readily detected ex vivo. A representative set of brain slices of these tumor bearing-mice is shown in Fig 2C and 2D to localize the tumor. Tumor progression was also monitored noninvasively at 0, 4, 7, 14, and 21 days after implantation (Fig 2A and 2B) by measuring luciferase activity in the tumor cells. Representative brain slices showing luciferase activity confirm the presence of tumor cells and were then evaluated by immunohistological analyses for markers of BBB integrity.

Glioma-induced remodeling of the extracellular matrix (ECM)

We used antibodies for specific ECM markers such as laminin, agrin and tenascin and an endothelial cell marker (CD31) to characterize glioma-induced remodeling of the basal lamina of the NVU. Tumor margins were identified by indirect fluorescence of the tumor cells and their characteristic dense nuclei pattern from DAPI staining. Ipsilateral and contralateral control areas (with no tumor) in tumor-bearing brains slices showed similar staining pattern to the normal brain, and were used as internal controls for immunostaining in this study. In this study, primary tumors are identified by its size, (i.e. the main bulk of the tumor) and tumors located at least 50 μm distal to the primary tumor margin that are smaller in size in the brain were defined as secondary invasive tumors. Using CD31 staining, intratumoral vasculature had decreased vessel density, higher intervascular distance, but increased microvascular diameter when compared to normal blood vessels (Fig 3A). In primary tumors, laminin staining was restricted to the basal lamina of the endothelial perivascular space (Fig 3B). However, in secondary tumors, laminin was observed in a strong, multilayered staining pattern at the leading edge of secondary tumors and not with any apparent association with endothelial cells (Fig 3B). Whether the laminin detected is tumor-derived or host-derived remains unknown. To evaluate the effects of tumor-induced remodeling on proteoglycans deposited into the basal lamina, we assessed agrin immunoreactivity in the tumor microenvironment. Agrin is an extracellular heparan sulfate proteoglycan that is expressed by both the astrocyte and the endothelial cells (Barber and Lieth, 1997; Rascher et al., 2002; Warth et al., 2004). As shown in Fig 3C, agrin staining was decreased in the primary tumor, and like laminin, was localized to the surface of endothelial cells, surrounds the leading edge of the secondary tumor, and is not associated with endothelial cells. In contrast, tenascin, a high molecular weight, multifunctional, extracellular matrix glycoprotein that is upregulated in malignant glioma in vivo and promotes migration of glioma cells in vitro (Deryugina and Bourdon, 1996), shows a significantly different pattern of staining. Tenascin staining was increased within the solid tumor mass and a wide spread network of tenascin immunoreactivity was observed in the tumor core but not in secondary tumors (Fig 3D). Although in vitro studies predict a role for tenascin in glioma-cell migration, tenascin staining was decreased at the tumor margins and in secondary tumors; regions where it was predicted to be elevated if it were involved in secondary glioma infiltration in vivo (Fig 3D).

Glioma-induced remodeling of astrocytes

Astrocytes are widely recognized components of the BBB that confer brain endothelial cells with their characteristic barrier tight junctions (Abbott et al., 2006), yet the capacity for astrocytes to undergo tumor-induced redistribution is poorly understood. To this end, we used

an anti-GFAP antibody as a general astrocyte marker, an anti-AQ4 antibody as a marker for the astrocyte-endothelial interface and an anti-Aldh1L1 antibody as a protoplasmic astrocyte marker (Cahoy et al., 2008; Oldham et al., 2008) to assess the effects of gliomas on BBB astrocytes. Elevated GFAP staining was found at the margin of the primary tumor, however, within the solid tumor mass, GFAP was decreased (Fig 4B). AQ4 immunoreactivity was increased in the primary tumor where astrocytic contact with endothelial is lost (Fig 4C). Secondary tumors showed elevated AQ4 immunoreactivity at the leading edge of the tumor (Fig 4C), but not in intratumoral blood vessels. This is in contrast to the localization of AQ4, which is observed at the glial endfeet around the vessels in normal brain (Fig 1B) and in control area in tumor-bearing brain (Fig 4C). Aldh1L1 staining was also increased at the margin of primary tumors but decreased in the tumor core (Fig 4D). These findings suggest that glioma induce differential remodeling in different subclasses of astrocytes.

3. Discussion

In this study, we show that tumor induces specific changes in endothelial cells, remodeling of the ECM and changes in endothelial-associated astrocytes. We observed a decrease in laminin and agrin, and an increase in tenascin in the primary tumor (Fig 3). In secondary tumors, both astrocytes and blood vessels remain separated by a basal lamina that contains laminin and agrin (Fig 3 and Fig 4). The changes in staining observed in the primary tumor are in general agreement with observations from rodent models and clinical samples of glioma (Chekhonin et al., 2007; Rascher et al., 2002; Warth et al., 2004; Warth et al., 2007). For example, tenascin upregulation is accompanied with loss of agrin expression in human glioma tissues (Rascher et al., 2002). Increased expression of laminin, fibronectin, and vitronectin was also shown at the infiltration zone of human glioma cells implanted into rat brain (Mahesparan et al., 2003). Here, we provide evidence for a differential glioma-mediated remodeling of the NVU in primary vs secondary tumors. Understanding the temporal-spatial control of these differences may be important in terms of identifying novel therapeutic targets.

Changes in ECM composition influence rigidity and structure (i.e., fibrillary collagen, fibronectin) and suggest that a malignant infiltration of glioma may stimulate the development of distinct provisional ECM. Indeed, tumor cells induce changes in ECM by secretion of growth factors and angiogenic factors such as VEGF, bFGF, PDGF, and TNF- α (Gesa Rascher-Eggstein, 2004). In turn, the different components of the provisional ECM influence tumor cells, either by changing the mechanical rigidity (i.e. stiffness) of the environment (Huang and Ingber, 2005; Paszek et al., 2005) or by modulating the activity of different growth factors and creating a positive-feedback loop in the tumor-host microenvironment.

The xenograft model described here is well suited to distinguish the role of host/tumor in tumor growth and progression and is associated with BBB breakdown: First, tumor margins can be easily defined using RFP-tagged tumor cells (Lund et al., 2006). Second, the leading edge of primary and secondary tumors are readily defined using confocal microscopy on thick brain sections. Finally, the cellular origin of various ECM components can be assessed using species-specific antibodies. For example, a mouse-specific antibody to tenascin can be used to demonstrate that the high levels of tenascin detected in primary tumors are produced by the host, even though the levels of immunoreactivity decrease at the leading edge of the tumor (Fig 3).

The findings described here also suggest that changes in the astrocytic endfeet (i.e. AQ4) of the NVU and/or changes in the basal lamina (i.e. agrin) in brain tumors may influence the function of astrocytes. These changes presumably lead to dysregulation of the BBB. Maintenance of their integrity is necessary in order to restrict ionic and fluid movements between the blood and the brain. The water channel protein AQ4 has a crucial role in

maintaining the integrity of BBB by forming a functional complex with dystrophin and dystroglycan to form orthogonal arrays of intramembranous particles at the astrocytic endfoot membranes (Wolburg et al., 2009). The stability of this complex is dependent on extracellular matrix components, for example agrin (Noell et al., 2007). Although the molecular mechanisms underlying the changes in the endothelial BBB remain unclear, understanding how the integrity of BBB is maintained in the normal brain has a wider impact on other pathologies of the central nervous system including stroke, traumatic brain injury, multiple sclerosis and neurodegenerative diseases (e.g. Alzheimer's disease) which like glioma are all associated with functional alterations of the BBB (Abbott et al., 2006).

4. Experimental Procedures

Mice

Rag2 knockout mice were used for xenograft studies based on previous studies using $\text{rag2}^{-/-}$, $\text{src}^{+/+}$ or $\text{rag2}^{-/-}$, $\text{src}^{+/-}$ hosts (Eliceiri et al., 1999; Lund et al., 2006). All animal handling procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Glioma tumor cells

Early passages of patient-derived human glioma cells, DBTRG (a kind gift from Dr. C. Kruse) were used for xenograft studies. Cells were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, nonessential amino acids, and glutamine in a humidified atmosphere containing 5% CO_2 at 37°C.

For in vivo bioluminescence imaging of orthotopic brain tumors, early passages of DBTRGs were transduced with lentivirus expressing firefly luciferase (DBTRG-luc) or red fluorescent protein (DBTRG-RFP). To engineer lentivirus expressing luciferase, the open reading frame of firefly luciferase was excised from pGL4.14 (Promega) and cloned into the *Xho* I site and *Xba* I sites of the lentiviral vector, pLVX (Clontech). Infectious lentiviral particles were prepared in a 293T packaging cells (ATCC) using the manufacturer's protocol (Lenti-X Lentiviral Expression Systems, Clontech). Transduced cells were selected by either flow cytometry (DBTRG-RFP) or by adding 2 $\mu\text{g}/\text{ml}$ puromycin in the culture medium for at least 2 weeks (DBTRG-luc).

Intracranial stereotactic injections

After immobilizing the mice in a rodent stereotactic frame, an incision was made in the skin, and a burr hole made in the skull. One million tumor cells or same volume of saline were injected at a rate of 1–2 microliter/minute using a microsyringe (Hamilton, Reno, NV) mounted on a stereotactic frame (Kopf Instruments, Tujunga, CA) using coordinates of 1 mm lateral and 1mm posterior to the Bregma and 1.5 mm below the dura. The incision was closed with veterinary adhesive and topical lidocaine administered. After 21 days, mice were subjected to systemic intracardiac perfusion with 1 USP unit/ml of heparin dissolved in saline, and serial-cryosections of brains were processed as indicated in the figure legends to yield 20 μm or 60 μm brain slices. Immunostaining of tissue sections were imaged with an Olympus Fluoview 1000 (ASW 1.7b) laser scanning confocal microscope (Olympus, Melville, NY) equipped with 20x/0.7N.A.(dry), or 860x/1.4N.A.(oil) objective lenses on a BX61 microscope (Olympus).

Immunohistochemistry

Three weeks after implantation, the animals were perfused with heparin/saline by intracardiac injection, and brains or 1mm sections of the brains were harvested and cryoembedded in OCT.

Standard immunohistochemistry was performed on cryosections (20 μ m) of tumor samples using the following primary antibodies at the following dilutions. Anti-CD31(553370, BD Biosciences, 1:100), rabbit polyclonal anti-laminin(L9393, Sigma, 1:1200), mouse monoclonal anti-GFAP (C9205, Sigma, 1:200), anti-aquaporin 4 (Chemicon,1:1000), monoclonal anti-mouse tenascin (T3413,Sigma,1:100), anti-Aldh1L1 (N103/39 NeuroMab, 1:100), anti-agrin (rabbit polyclonal antibody, a kind gift from Dr. M.A. Ruegg, 1:100). Alexa-fluor-conjugated secondary antibodies were used at a 1:200 dilutions and were purchased from Molecular Probes (Eugene, OR). All sections were counterstained with 4'-6-Diamidino-2-phenylindole (DAPI).

In vivo bioluminescent imaging

Hair was removed from mice with electric clippers and Nair before imaging at each time point. Mice were injected intraperitoneally with D-luciferin (150 μ l of 15mg/ml stock) to image tumor burden and monitor tumor growth immediately after and at 4, 7, 14, and 21 days after implantation. Bioluminescent signals were assessed 10 minutes after luciferin injection over an integration time of 10 seconds and 1 minute using a cooled charge-coupled device (CCD) camera (Lumina; Caliper Life Sciences, Hopkinton, MA) capable of in vivo imaging. Tumor growth was monitored by quantitation of light emission from a region of interest drawn over the tumor region at each time point. Pseudocolor images representing the intensity of the bioluminescent signal (blue is the least intense and red is the most intense) were superimposed over the grayscale reference image and displayed with the scales for the pseudocolor intensity (Unit = photons/sec/ cm²/steradian). Images were analyzed using Living Image software version 3.1 (Caliper Life Sciences). Following non-invasive imaging over 21 days, mice were sacrificed and images from 1mm sections of fresh mouse brain were collected to identify tumor-bearing sections and for immunohistochemical analyses.

Acknowledgements

We acknowledge the expert technical assistance of Montha Pao.

These studies were supported by grant from the NHLBI (B.E.).

References

- Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* 2006;7:41–53. [PubMed: 16371949]
- Abramsson A, Kurup S, Busse M, Yamada S, Lindblom P, Schallmeiner E, Stenzel D, Sauvaget D, Ledin J, Ringvall M, Landegren U, Kjellen L, Bondjers G, Li JP, Lindahl U, Spillmann D, Betsholtz C, Gerhardt H. Defective N-sulfation of heparan sulfate proteoglycans limits PDGF-BB binding and pericyte recruitment in vascular development. *Genes Dev* 2007;21:316–31. [PubMed: 17289920]
- Barber AJ, Lieth E. Agrin accumulates in the brain microvascular basal lamina during development of the blood-brain barrier. *Dev Dyn* 1997;208:62–74. [PubMed: 8989521]
- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci* 2008;28:264–78. [PubMed: 18171944]
- Chekhonin VP, Baklaushev VP, Yusubalieva GM, Pavlov KA, Ukhova OV, Gurina OI. Modeling and immunohistochemical analysis of C6 glioma in vivo. *Bull Exp Biol Med* 2007;143:501–9. [PubMed: 18214311]
- Criscuolo ML, Nguyen M, Eliceiri BP. Tumor metastasis but not tumor growth is dependent on Src-mediated vascular permeability. *Blood* 2005;105:1508–14. [PubMed: 15486073]
- Deryugina EI, Bourdon MA. Tenascin mediates human glioma cell migration and modulates cell migration on fibronectin. *J Cell Sci* 1996;109(Pt 3):643–52. [PubMed: 8907709]

- Eliceiri BP, Paul R, Schwartzberg PL, Hood JD, Leng J, Cheresh DA. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell* 1999;4:915–24. [PubMed: 10635317]
- Eng LF. Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. *J Neuroimmunol* 1985;8:203–14. [PubMed: 2409105]
- Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, DePinho RA, Cavenee WK. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev* 2007;21:2683–710. [PubMed: 17974913]
- Gesa Rascher-Eggstein, SL.; Wolburg, Hartwig. The blood-brain barrier in the human glioma.. In: Westman, H.S.S.a.J., editor. *Blood-Spinal Cord and Brain Barriers in Health and Disease*. Elsevier Inc; 2004. p. 561-576.
- Huang S, Ingber DE. Cell tension, matrix mechanics, and cancer development. *Cancer Cell* 2005;8:175–6. [PubMed: 16169461]
- Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat Rev Neurosci* 2004;5:347–60. [PubMed: 15100718]
- Kruse CA, Mitchell DH, Kleinschmidt-DeMasters BK, Franklin WA, Morse HG, Spector EB, Lillehei KO. Characterization of a continuous human glioma cell line DBTRG-05MG: growth kinetics, karyotype, receptor expression, and tumor suppressor gene analyses. *In Vitro Cell Dev Biol* 1992;28A:609–14. [PubMed: 1331021]
- Lampson LA, Lampson MA, Dunne AD. Exploiting the lacZ reporter gene for quantitative analysis of disseminated tumor growth within the brain: use of the lacZ gene product as a tumor antigen, for evaluation of antigenic modulation, and to facilitate image analysis of tumor growth in situ. *Cancer Res* 1993;53:176–82. [PubMed: 8416743]
- Lund CV, Nguyen MT, Owens GC, Pakchoian AJ, Shaterian A, Kruse CA, Eliceiri BP. Reduced glioma infiltration in Src-deficient mice. *J Neurooncol* 2006;78:19–29. [PubMed: 16552622]
- Mahesparan R, Read TA, Lund-Johansen M, Skaftnesmo KO, Bjerkvig R, Engebraaten O. Expression of extracellular matrix components in a highly infiltrative in vivo glioma model. *Acta Neuropathol* 2003;105:49–57. [PubMed: 12471461]
- Neuwelt EA, Rapoport SI. Modification of the blood-brain barrier in the chemotherapy of malignant brain tumors. *Fed Proc* 1984;43:214–9. [PubMed: 6692941]
- Noell S, Fallier-Becker P, Beyer C, Kroger S, Mack AF, Wolburg H. Effects of agrin on the expression and distribution of the water channel protein aquaporin-4 and volume regulation in cultured astrocytes. *Eur J Neurosci* 2007;26:2109–18. [PubMed: 17927773]
- Oldham MC, Konopka G, Iwamoto K, Langfelder P, Kato T, Horvath S, Geschwind DH. Functional organization of the transcriptome in human brain. *Nat Neurosci* 2008;11:1271–82. [PubMed: 18849986]
- Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM. Tensional homeostasis and the malignant phenotype. *Cancer Cell* 2005;8:241–54. [PubMed: 16169468]
- Rascher G, Fischmann A, Kroger S, Duffner F, Grote EH, Wolburg H. Extracellular matrix and the blood-brain barrier in glioblastoma multiforme: spatial segregation of tenascin and agrin. *Acta Neuropathol* 2002;104:85–91. [PubMed: 12070669]
- Simard M, Nedergaard M. The neurobiology of glia in the context of water and ion homeostasis. *Neuroscience* 2004;129:877–96. [PubMed: 15561405]
- Virgintino D, Girolamo F, Errede M, Capobianco C, Robertson D, Stallcup WB, Perris R, Roncali L. An intimate interplay between precocious, migrating pericytes and endothelial cells governs human fetal brain angiogenesis. *Angiogenesis* 2007;10:35–45. [PubMed: 17225955]
- Warth A, Kroger S, Wolburg H. Redistribution of aquaporin-4 in human glioblastoma correlates with loss of agrin immunoreactivity from brain capillary basal laminae. *Acta Neuropathol* 2004;107:311–8. [PubMed: 14735305]
- Warth A, Simon P, Capper D, Goepfert B, Tabatabai G, Herzog H, Dietz K, Stubenvoll F, Ajaaj R, Becker R, Weller M, Meyermann R, Wolburg H, Mittelbronn M. Expression pattern of the water channel aquaporin-4 in human gliomas is associated with blood-brain barrier disturbance but not with patient survival. *J Neurosci Res* 2007;85:1336–46. [PubMed: 17335082]

- Winkler F, Kienast Y, Fuhrmann M, Von Baumgarten L, Burgold S, Mitteregger G, Kretschmar H, Herms J. Imaging glioma cell invasion in vivo reveals mechanisms of dissemination and peritumoral angiogenesis. *Glia*. 2009
- Wolburg H, Noell S, Wolburg-Buchholz K, Mack A, Fallier-Becker P. Agrin, aquaporin-4, and astrocyte polarity as an important feature of the blood-brain barrier. *Neuroscientist* 2009;15:180–93. [PubMed: 19307424]
- Zhang X, Li X, Wu JW, Gao DK, Liang JW, Liu XZ. Experiment and observation on invasion of brain glioma in vivo. *J Clin Neurosci* 2002;9:668–71. [PubMed: 12604281]

Fig 1. Immunohistochemical analysis of neurovascular unit in the normal mouse brain

Antibodies directed to endothelial cells (CD31) and astrocytes (AQ4, GFAP or AldH1L1) were used to characterize the endothelial-astrocyte interface.

(A) Thick tissue sections of normal mouse brain were immunostained to detect GFAP-positive astrocytes and staining shows a distinction between arterioles/venules vs. capillaries. Lesser staining for GFAP was observed in branching capillaries (arrow). Size bars = 100 μ m

(B) AQ4-positive astrocytes and CD31-positive capillaries were examined by confocal microscopy and the merged images were used to localize the astrocyte-endothelial interface. Note the overlap of both proteins in most capillaries. Size bars = 100 μ m

(C) AQ4-positive astrocytes and GFAP-positive astrocytes were examined by confocal microscopy and the merged images were used to localize the astrocytic endfeet at the astrocyte-endothelial interface. Note the distinct pattern of staining for most blood vessels. Double-positive vessels are arterioles/venules. Size bars = 100 μ m

(D) AldH1L1-positive astrocyte and CD31-positive capillaries were examined by confocal microscopy and the merged images were used to localize the astrocyte-endothelial interface. Note the distinct staining of both proteins in capillaries vs. arterioles/venules. Size bars = 100 μ m

Fig 2. Tumor growth progression in an orthotopic glioma model

(A) Monitoring tumor growth progression in an orthotopic glioma model using bioluminescence.

DBTRG-luc cells injected into mice (N=8) were detected with administration of luciferin. Mice injected with equal volume of saline were used as negative controls (N=4). Ten minutes after a luciferin administration (i.p.), ten second exposure image was captured immediately after implantation and at day 4, 7, 14, and 21 after implantation. Tumor growth was quantitated by measuring light emission from a region of interest drawn over the tumor region at each time point.

(B) Non-invasive imaging of luciferase-labeled tumor cells xenografted into mice. Shown are exposure matched, representative images of bioluminescent activity of tumor cells of a single animal taken at immediately after implantation (day 0), or day 4,7,14 and 21 after implantation. Ten minutes after a luciferin administration (i.p.), series of ten second exposure image was captured. Scales for the pseudocolor intensity plots are displayed with the images. Units = photons/sec/cm² /steradian

(C) Detection of tumor cells in brain sections using fluorescence.

RFP-labeled DBTRG cells were injected into mice and sacrificed, and 1 mm brain sections imaged with a laser scanning confocal microscope as described in Materials and Methods. Anterior to posterior sections (numbered 1 to 6) through the region of brain harboring the tumor bulk are shown.

(D) Detection of tumor cells in brain sections using luminescence.

DBTRG-luc cells injected into mice were detected with administration of luciferin; 1 mm brain sections were collected and bioluminescent signal was imaged with a Lumina CCD imaging system. Brain sections with luciferase activity were cryosectioned for further immunohistochemical analysis. Units = photons/sec/cm²/steradian

Fig 3. Glioma-induced remodeling of the basal lamina

Immunohistochemical staining of tumor-bearing brain sections with ECM markers revealed changes in the tumor bed.

(A) Immunostaining of blood vessels at the tumor margin. Areas with primary tumor (1°, left panel) and secondary tumor (2°, right panel) were defined by dense nuclei staining (dotted line). Tumor-bearing vessels labeled with an anti-CD31 antibody (panel A) were evaluated by confocal microscopy and merged with images with staining for different markers of the ECM including: (B) laminin, (C) agrin, and (D) tenascin. Note the strong, multilayered staining of laminin and agrin at the edge of secondary tumors that is not associated with endothelial cells (B and C). Size bars = 100µm

Fig 4. Glioma-induced remodeling of the astrocytes

Immunohistochemical staining of tumor-bearing brain sections with different markers of astrocytes.

(A) Immunostaining of blood vessels at the tumor margin show areas with primary tumor (1°, left panel) and secondary tumor (2°, right panel) that are defined by dense nuclei staining (dotted line). Tumor-bearing vessels were identified by anti-CD31 immunostaining, analyzed by confocal microscopy by merging images with immunohistochemical markers for different classes of astrocytes: (B) GFAP for astrocytes, (C) AQ4 for astrocytic endfeet and (D) Aldh1L1 for prototypic astrocytes. Note the decreased immunoreactivity of anti-GFAP and anti-AQ4

antibodies in the primary tumor and the loss of contact between AQ4 and CD31 staining in the primary and secondary tumors. Size bars = 100µm