Anti-angiogenic therapy in orthotopic human glioma models in nude mice.

Vessel normalization impairs radiological detection and chemotherapy delivery in high-grade, diffuse gliomas.

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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Chapter 1

General introduction: Diffuse glioma growth: a guerrilla war.

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Abstract

The brain is a complex, highly organized organ. It consists of a variety of neuronal and glial cells, embedded in a unique extracellular matrix (ECM). Furthermore, the brain contains a high number of blood capillaries which are normally characterized by the presence of a blood-brain barrier (BBB) that limits penetration of many substances into the brain and contributes to an immune-protected site. Diffuse gliomas, primary brain tumors derived from glial cells or precursors there of, are among the most common brain tumors. In contrast to e.g. metastatic tumors in the brain, diffuse gliomas infiltrate extensively into the neuropil, i.e. the dense network of interwoven neuronal and glial cell processes. This growth pattern hampers radiological visualization of the invasive front of diffuse gliomas and is a major factor in therapeutic failure. Although the knowledge about mechanisms of migration of (tumor) cells is rapidly increasing, the exact molecular underpinnings of diffuse infiltration of glioma cells in the surrounding brain parenchyma have not yet been elucidated. As the efficacy of conventional methods, comprising surgery, radiotherapy and chemotherapy, to fight diffuse infiltrative glioma is limited, a more targeted approach is needed that takes advantage of the typical molecular features of these tumors. Anti-angiogenic therapy, an example of such targeted therapy, has since 10 years been considered as a promising approach for high-grade malignant gliomas as these neoplasms often show a striking angiogenic response.
Clinical features of gliomas

Gliomas form a heterogeneous group of tumors of the central nervous system (CNS), encompassing many different histological types and malignancy grades. The most malignant diffuse infiltrative glioma, glioblastoma multiforme (GBM), is by far the most common. It accounts for more than 50% of all primary CNS gliomas and for 20% of all primary CNS tumors.\(^{37}\) Despite this high prevalence among primary brain tumors, GBM are relatively uncommon: they occur in only 2-5 per 100,000 people per year in Western Europe and North America,\(^ {155}\) this in contrast to lung, breast and colorectal cancer which have an annual incidence in Western Europe of approximately 63, 85 and 72 per 100,000 people per year respectively.\(^ {186}\) However, gliomas are associated with a disproportionately high morbidity and mortality.\(^ {32}\) Median survival is only 12 to 15 months for patients with GBM and 2 to 5 years for patients with anaplastic gliomas.\(^ {275}\) Astrocytic tumors are therefore the third leading cause of cancer-related death among middle aged men and the fourth leading cause of death for women between 15 and 34 years of age.\(^ {241}\)

Glioma symptoms vary depending on tumor size and location. Patients may suffer from headache caused by increased intracranial pressure, seizures, and neurological deficits, although low-grade tumors can remain asymptomatic for years. Increased intracranial pressure may also be associated with nausea and vomiting. The most prevalent symptom, however, is progressive failure in neurological or mental functioning due to involvement of the temporal and frontal lobe. Also venous thromboembolism and fatigue can occur.\(^ {155,275,276}\)

Radiology

Magnetic resonance imaging (MRI) is now the gold standard for diagnosing brain tumors.\(^ {208}\) Low-grade diffuse gliomas (World Health Organization (WHO) grade II) are typically hypointense lesions on T1-weighted MR images with limited edema and mass effect and lack of gadolinium diethylenetriaminepenta-acetic acid (Gd-DTPA) enhancement.\(^ {100}\) On T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences, low-grade diffuse gliomas are generally hyperintense. Distinguishing edema from infiltrating glioma is difficult using T1, T2, and FLAIR MR images. The lack of neovascularization and the apparently limited changes to the pre-existent, incorporated vessels explain the absence of contrast-enhancement in MRI examinations of these tumors.\(^ {8}\) As the main histopathological difference between WHO grade II and III diffuse astrocytic neoplasms is increased mitotic activity in the latter,\(^ {155}\) it is not surprising that part of the non-enhancing diffuse gliomas are histopathologically diagnosed as grade III at the time of biopsy.\(^ {8}\)
Compared to low-grade diffuse gliomas, high-grade (i.e. WHO grade III and IV) tumors are radiologically more heterogeneous and accompanied by more severe edema. The occurrence of contrast enhancement in diffuse gliomas generally signifies a more malignant biological behavior. The central area in “ring-enhancing” high-grade diffuse gliomas most often represents necrosis, while the enhancing rim contains vital glioma tissue with microvascular changes including increased vascular permeability (Figure 1). Some therapeutic interventions (e.g. surgical removal of glioma tissue, radiotherapy) contribute to contrast-enhancement. Increased enhancement after treatment with radiotherapy and temozolomide (TMZ) may reflect a transient increase in vessel permeability. This phenomenon is called pseudoprogression. Furthermore, it is important to note that contrast-enhancement in non-diffuse gliomas such as pilocytic astrocytomas does not imply high-grade malignancy.

Contrast enhanced (CE)-MRI is not only used for early diagnostic imaging, but also for evaluation of the response to therapy. Using standard Macdonald criteria or RECIST criteria, a decreased or stable Gd-DTPA contrast-enhanced area is regarded as evidence for tumor regression or stabilization respectively. However, conventional radiological investigations tend to significantly underestimate the extent of diffuse infiltrative glioma growth. Correlation of whole brain histological sections of high-grade gliomas with computerized tomography (CT) scans revealed that tumor cells were present even outside the peritumoral areas of low density. Similar observations have been made when correlating histology to hyperintensive regions in T2-weighted MR images. Importantly, to diminish edema-related symptoms, patients are often subjected to palliative treatment with corticosteroids. Such reduction of edema further complicates accurate therapy evaluation.

New MR modalities may contribute to better radiological classification and delineation of glial brain tumors as well as assist in identification of the best spot for a biopsy. With diffusion-weighted imaging (DWI) and a related approach called diffusion tensor imaging (DTI), differences in motility of water due to differences in cellularity, cell membrane permeability, intra- and extracellular diffusion, and tissue structure can be visualized. Theoretically, DWI can thus be used to indirectly image infiltration of glioma cells in normal brain tissue. Perfusion weighted imaging (PWI) is a technique which allows for quantitative assessment of the cerebral blood volume (CBV). With PWI, vascularization and perfusion of gliomas can be measured. The (relative) CBV correlates with both vascularization and malignancy grade as assessed by histology. As long as tumor infiltration is accompanied by changes in vascularization and perfusion, PWI may also indirectly visualize the presence of infiltrating glioma cells. Proton MR Spectroscopy (MRS) allows for obtaining metabolic spectra from tissues which may help to differentiate between tumor types or between tumor tissue and necrosis. For instance, in
the normal brain, high levels of the neuronal marker N-Acetyl-Aspartate (NAA) can be found, next to the proliferation marker choline, and other markers such as inositol and creatine. In high-grade gliomas, however, NAA is significantly decreased (reflecting a decrease in neuronal components) whereas choline is increased. Such spectra can be obtained in a single voxel or in multiple voxels in two or three dimensions.\textsuperscript{9,249} Several studies suggest that MRS may be helpful for better delineation of diffuse gliomas.\textsuperscript{46,78,164,197} Combining different MR modalities (e.g. DWI, PWI, MRS) is expected to further improve these results.\textsuperscript{54,203}

![Figure 1: Examples of MR images in two glioblastoma patients (kindly provided by Dr. Mathé Prick, Dept. of Neurology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands). The T1-weighted image of patient 1 (A) reveals bifrontal Gd enhancement of a tumor that crosses the corpus callosum (arrow), resulting in a so called “butterfly glioma”. The T1-weighted images of patient 2 with (B,C) and without Gd (D) suggest multiple, independent lesions. In the T2-weighted image (E), however, these bifrontal lesions appear to be interconnected via diffuse infiltrative growth in the corpus callosum (arrow). The lack of contrast enhancement in this latter area indicates that here disruption of the blood-brain barrier by infiltrating glioma cells is limited. A,B: coronal plane; C-E: axial plane.](image)
Up till now, a major drawback of most novel MR modalities is the limited spatial resolution: for conventional T1-weighted MRI at 3 Tesla this resolution is about 0.5 x 0.5 x 0.5 mm, while DWI and PWI reach a resolution of about 2 x 2 x 2 mm, and MRS of 10 x 10 x 10 mm. None of these new imaging techniques is therefore expected to replace conventional MRI soon. Obviously, visualization of dispersed infiltrative glioma cells will improve when the technical development of these MR imaging modalities advances.

Direct visualization of infiltrative glioma cells may also be performed by positron emission tomography (PET) and single photon emission computerized tomography (SPECT) imaging.15,105,159,196 Promising compounds for PET imaging of gliomas are O-(2-18F-fluoroethyl)-L-tyrosine (FET)202 and 18F-Galacto-RGD, an αvβ3 binding molecule.98 Furthermore, in the near future MR and PET imaging may be significantly improved by the application of nanoparticles 18,124,264 and labelled antibodies.98 Yet, it remains to be seen how these approaches will be affected by an intact BBB in diffuse gliomas.

Conventional therapies

The fact that diffuse infiltrative glioma cells tend to blend in extensively in the brain microenvironment makes it hard to plan an effective counterattack. Additional complicating factors are that the brain is very susceptible to therapy-induced damage and that neuronal tissue has, in contrast to many other organs, only very limited capacity to repair itself.

The optimal management of low-grade diffuse gliomas remains to be defined. Although such patients may survive for multiple years, these tumors lead to death of the patient sooner or later, often after progression to high-grade malignancy. In young patients with low-grade gliomas presenting with seizures only, treatment may be deferred until clinical or radiological progression occurs, in order to postpone the risk for radiotherapy-induced cognitive decline.260

It is generally accepted that patients with high-grade diffuse gliomas should be treated without delay. Surgery is the first step of treatment of GBM. However, whereas surgery of most other tumors aims at complete resection (with or without a margin of normal tissue), the diffuse growth of gliomas in the brain parenchyma precludes complete tumor removal. Already in the early days of neurosurgery, Dandy and Gardner noticed that even after performing a hemispherectomy glioma patients were not necessarily cured.49,80 Still, for patients with a high-grade glioma, radical resection of the contrast-enhancing part of the tumor without worsening neurological impairment is an independent prognostic factor for improved overall survival.101,207 Unfortunately, intraoperative assessment of the extent of resection by the neurosurgeon is notoriously inaccurate.2,242 Adequate removal
of the tumor might be increased when surgery is guided by the fluorescent dye 5-aminolevulinic acid. This is an orally-available, non-fluorescent prodrug that leads to intracellular accumulation of fluorescent porphyrins in glioma cells. Maximal resection of contrast-enhancing tumor cells leads to an improved progression-free survival and overall survival in glioma patients.\textsuperscript{242,243}

Radiotherapy after surgery can further inhibit tumor growth. However, although radiotherapy was proven to be beneficial for high-grade glioma patients, eradication of diffuse infiltrative glioma cells without significantly damaging the infiltrated brain parenchyma has been difficult to achieve.\textsuperscript{119,138,139} Up till now, limited field irradiation (generally with an arbitrary 2 cm beyond the contrast enhancing mass) rather than whole brain irradiation is the standard treatment,\textsuperscript{139} with an optimal total radiation dose of 60-65Gy.

The success of chemotherapy is hampered by the marked intratumoral heterogeneity of gliomas.\textsuperscript{207} Especially in areas where the original tissue architecture is relatively preserved, the BBB may form an obstacle for optimal delivery of chemotherapeutics to diffuse infiltrative tumor cells. Recently, temozolomide (Temodar/ Temodal\textsuperscript{®}, TMZ) treatment (concomitant and adjuvant with radiotherapy) was shown to result in modest improvement of median overall survival and increased 2 years survival in GBM patients up to 70 years of age with minimal additional toxicity.\textsuperscript{244} TMZ is an orally-available DNA-alkylating agent. In the circulation, it undergoes rapid conversion to its active derivative MTIC (3-methyl-(triazen-1-yl)imidazole-4-carboxamide) which then exhibits anti-neoplastic activity by adding alkyl groups to the DNA, thereby interfering with DNA replication. Promotor methylation of the DNA repair enzyme O\textsuperscript{6}-methylguanine methyltransferase (MGMT) gene is an important predictive factor for response to therapy.\textsuperscript{99} Such methylation causes silencing of the MGMT gene and lack of repair of the TMZ-induced DNA-damage in the tumor cells. In addition to TMZ, the topoisomerase I inhibitor irinotecan is currently often used for the treatment of glioma patients. It has demonstrated its efficacy in patients with recurrent glioma, mainly when used in combination with other chemotherapeutic compounds like TMZ.\textsuperscript{267}

Current standard of care for GBM is thus surgical resection to maximal feasible extent, followed by radiotherapy and systemic TMZ chemotherapy. In addition, patients often receive supportive treatment to relieve symptoms: e.g. corticosteroids to reduce intracranial edema. Even with such aggressive treatment, median survival after diagnosis is still only one to two years. Death is usually due to cerebral edema and, consequently, increased intracranial pressure. A small percentage of treated patients survives for more than three years. Such long-term survival has been associated with young age at diagnosis, good initial performance score, MGMT promotor methylation, and surgical removal of
tumor to maximal feasible extent.\textsuperscript{128} However, much remains unknown about why a small minority of GBM patients survive longer. A recent study revealed that mutations in the active site of isocitrate dehydrogenase 1 (IDH1) are relatively frequent in young patients and in patients with secondary GBM and are associated with an increase in overall survival.\textsuperscript{187,283} Such knowledge may be helpful to design therapeutic approaches that more specifically target the molecules involved in the oncogenesis of tumors of individual patients.

**Histopathology of diffuse gliomas**

Diffuse gliomas can be histologically classified in three main subtypes: astrocytic, oligodendroglial, and mixed/oligoastrocytic tumors. The nomenclature is based on the resemblance of the tumor cells with non-neoplastic astrocytes and oligodendrocytes. However, it is still unclear whether tumor cells are derived from derailed mature glial cells or from neural stem cells (NSCs) or glial progenitor cells.\textsuperscript{78,235,241,287} The genetic aberrations underlying astrocytic and oligodendroglial tumors are significantly different. Sixty-70\% of oligodendroglial tumors carry loss of chromosomal arms 1p and 19q,\textsuperscript{209,260} whereas astrocytic tumors often show amplification of chromosome 7 (including the Epidermal Growth Factor Receptor (EGFR) gene located on 7p12) and loss of chromosome 10.\textsuperscript{155} The discrimination between different subtypes has major implications for prognosis of patients: those with malignant oligodendroglial tumors (especially those with 1p/19q loss) often show better survival and better response to chemotherapy using alkylating agents.\textsuperscript{33,258}

The WHO-2007 classification\textsuperscript{155} contains four malignancy grades for glial brain tumors, with the non-diffuse pilocytic astrocytoma as the least aggressive and GBM (WHO grade IV) as the most malignant one. Cellularity, cytological atypia, and mitotic activity generally increase with grade. In addition, prominent (often glomeruloid) microvascular proliferation and necrosis emerge in high-grade gliomas\textsuperscript{277,278} (Figure 2). In fact, these latter phenomena are used for histopathological grading of glial tumors.\textsuperscript{155} Often, around areas of necrosis in GBM, accumulation of pseudopalisading tumor cells occurs. These cells show signs of hypoxia and expression of hypoxia-response genes such as glucose transporter-1 (Glut-1) and Vascular Endothelial Growth Factor (VEGF). The latter factor plays a crucial role in the induction of angiogenesis.\textsuperscript{26,27,67} While the diffuse infiltrative growth pattern is characteristic for both low- and high-grade diffuse gliomas, especially high-grade gliomas frequently show marked phenotypical heterogeneity with spatial differences in cellular phenotype and malignancy grade.\textsuperscript{155}
Diffuse infiltrative growth of tumor cells in the neuropil is almost unique for gliomas. Only very few non-glial tumors (esp. small cell lung carcinoma, lymphoma) occasionally display “pseudo-gliomatous” growth in the neuropil.\textsuperscript{12,212,270} In diffuse gliomas, the cells preferentially invade along myelinated fibers in white matter tracts (intrafascicular growth). Additionally, subpial, perivascular (in the Virchow-Robin space), and perineuronal accumulation of tumor cells is frequently encountered\textsuperscript{42,86} (Figure 3). These collections of tumor cells induced by pre-existent structures are called secondary structures of Scherer.\textsuperscript{191,227}
Gliomas can arise in both white and gray matter. From the originating area, tumor cells often extensively infiltrate into the surrounding brain. Many diffuse gliomas occupy more than one lobe and not infrequently, glioma cells cross the corpus callosum, sometimes resulting in the radiological presentation as a butterfly-shaped tumor (Figure 1A). The most extreme example of diffuse infiltrative glioma growth is represented by gliomatosis cerebri. According to the WHO-2007 classification, this neoplasm involves at least three cerebral lobes, usually bilaterally, and even the entire neuraxis may be involved.47,155,162 Glioma cells can also spread to distant sites via cerebrospinal fluid (CSF). Interestingly, even though GBM may show an extreme angiogenic phenotype, extraneural metastases of these tumors are extremely rare.

Diffuse infiltration of gliomas
The typical diffuse infiltrative growth of glioma cells in the neuropil warrants specific, tightly regulated and converging interactions between these cells and their microenvironment. Up till now, it is not known what exactly initiates this behavior of glioma cells. As the group of diffuse gliomas is genotypically heterogeneous, it is unlikely that one particular genetic aberration accounts for this growth pattern in all diffuse gliomas. Several studies suggest that gliomas are derived from neural stem cells (NSCs) or...
glial progenitor cells rather than from derailed mature glial cells. CD133 (Prominin-1) is frequently used as a marker for identification of NSC features in glioma cells, but other markers such as nestin, CD90, CD44, CXCR4, musashi homolog 1 (Msi1), and maternal embryonic leucine zipper kinase (MELK) are also used for this purpose. Interestingly, in vivo and in vitro experiments CD133-positive glioma cells displayed a higher tumorigenic potential than CD133-negative cells, showed increased radio- and chemo resistance, and contributed in a major way to angiogenesis via VEGF-A production.

During normal development of the CNS, extensive proliferation and migration of stem cells and progenitor cells is essential. In contrast, in the normal adult brain only in some locations (e.g. subventricular zone, dentate gyrus of the hippocampus and sub-cortical white matter, rostral migratory system) some of these phenomena can be observed. Clues for elucidation of the molecular mechanisms enabling diffuse infiltrative glioma growth may thus be provided by the rapidly expanding research focussing on such stem cells and progenitor cells. Although the molecular biology underlying NSC migration is far from clear, molecules like nuclear factor kappa B (NF-κB), macrophage chemoattractant protein-1 (MCP-1), stem cell factor (SCF), stromal cell-derived factor-1 (SDF-1), and platelet derived growth factor (PDGF) were demonstrated to play an important role in the regulation of this process (reviewed in Widera). For most of these factors, however, the role in glioma cell migration is not yet known.

For a more systematic discussion of the mechanisms and factors that are relevant for diffuse infiltration of glioma cells in the neuropil, a comparison with guerilla warriors may be helpful. One would like to know not only what exactly initiates the migratory behavior of such “warriors”, but also which qualities and environmental factors enable them to successfully perform this behavior. With regard to these latter aspects, one could recognize a) an internal system that coordinates input and output of signals, b) a locomotor apparatus, c) trails to travel on, d) parts that directly interact with these trails, e) tools to remove obstacles, f) microenvironmental signals that guide the way, and g) other stimulatory or permissive microenvironmental factors (Figure 4). Some factors will be discussed in more detail. It is important to realize that (the interactions of) these underlying mechanisms are complex and that the list of factors associated with glioma cell invasion/migration given below is far from complete. A more extended overview of molecular factors involved in glioma cell infiltration is discussed in our published review.
Figure 4: Schematic overview of factors and mechanisms important for diffuse infiltration of glioma cells in the neuropil. The following aspects relevant for the diffuse growth pattern can be recognized: (a) an intracellular system that coordinates all incoming and outgoing signals via a complex set of pathways, (b) a locomotor apparatus in which the actin cytoskeleton plays a crucial role, (c) a scaffold (ECM, surface of cells/cell processes) on which the glioma cells can travel, (d) cell–ECM and/or cell–cell receptors that allow direct interaction with the ECM and cellular microenvironment, (e) tools to remove obstacles like ECM degrading proteases, (f) growth factors that guide the way, and (g) other stimulatory or permissive microenvironmental factors (e.g., chemokines derived from inflammatory cells). In this scheme, the protrusion on the right side of the cell represents the lamellipodium at the front.

a. Intracellular integration of signals
Interactions of glioma cells with their microenvironment via membrane receptors (integrins, growth factor receptors) induces intracellular signals which are transmitted through effectors like the focal adhesion kinase (FAK) family of cytoplasmic, non-receptor tyrosine kinases. The FAK family consists of two proteins, FAK and pyruvate kinase (Pyk2), which both play an important role in intracellular events such as proliferation, migration, survival, and apoptosis.168 Glioma cells have been reported to show increased expression of FAK, especially at the invasive front.289 FAK is activated by phosphorylation on critical tyrosine residues234 and subsequently it phosphorylates cytoskeleton-associated substrates (e.g. Src, paxillin).188 While some studies suggest a role for FAK activation mainly in glioma cell proliferation,150 other studies show involvement in activation of Rac, which in turn leads to actin polymerization and formation of cell protrusions, focal adhesion and subsequent motility.28,210 Pyk2 has a similar sequence and structure as FAK and, upon activation by phosphorylation, interacts with many of the same intracellular proteins as FAK.150 A recent study shows that knockdown of FAK and Pyk2 in an
intraprenal xenograft model of glioma significantly increased survival of mice compared to control mice. This substantiates the relevance of both signalling proteins in glioma and suggests a potential therapeutic approach.\textsuperscript{151}

b. Locomotion
Cell migration requires dynamic remodelling of the actin cytoskeleton through assembly, disassembly and organization of actin filaments into functional networks, which direct protrusion at the front of the cell and retraction at the rear. One of the first steps in cell migration is the formation of actin-rich structures, termed lamellipodia, at the leading edge of the motile cell.\textsuperscript{73,210} These lamellipodia are broad, sheet-like protrusions containing short-branched actin filaments.\textsuperscript{198} In addition to lamellipodia, more slender cytoplasmic protrusions containing bundles of cross-linked actin filaments (filopodia) can be formed.\textsuperscript{210} Members of the Rho family of small GTP binding proteins, especially Rac and cell division cycle protein (Cdc42), are pivotal regulators of these processes. When bound to GTP, these proteins can interact with downstream target proteins, including protein kinases, phosphatases, and WASP/WAVE proteins (Wiskott-Aldrich Syndrome protein/Wiskott-Aldrich Syndrome protein family members). These latter proteins are activators of the Actin-related protein (Arp2/3) complex, a nucleator of new actin filaments at the leading edge of the cell and thereby instrumental for protrusion of lamellipodia and filopodia.\textsuperscript{65,181,210} Several studies showed that inhibition of Rac1, one of the three Rac isoforms, inhibits glioma cell migration and invasion \textit{in vitro}.\textsuperscript{38,40} Depletion of the phosphoinositide phosphatase synaptojanin-2 (another effector of Rac1) using small interfering RNA was reported to inhibit glioma cell invasion through Matrigel and rat brain slices \textit{in vitro}.\textsuperscript{40} Interestingly, Rac is one of the downstream targets of phosphatidylinositol 3-kinase (PI3K), and the effect of PI3K (i.e. phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2)) is counteracted by the tumor suppressor protein phosphatase and tensin homolog (PTEN).\textsuperscript{35} By dephosphorylating PIP3, PTEN may inhibit glioma cell invasion in two ways: by modulation of glioma cell motility via inactivation of Rac and Cdc42 as well as by suppression of extracellular matrix (ECM) degradation via MMPs.\textsuperscript{75} As loss of chromosome 10q, which contains the PTEN gene (locus: 10q23.3), is a frequent event in especially GBM,\textsuperscript{55,94} such loss may theoretically thus result in increased migration.

c. Scaffold for migration
In normal brain, common ECM components such as collagens, laminin, and fibronectin are essentially restricted to the vessel walls and the perivascular and subpial glial limiting membrane.\textsuperscript{87} The exact composition of the ECM in the neuropil is not yet fully elucidated, but hyaluronan, glycosaminoglycans, and proteoglycans are considered to be major ECM components in this compartment.\textsuperscript{14,87,90} Due to the dense network of cell processes the volume of the extracellular space in the normal neuropil is limited. In diffuse gliomas, this
space increases in volume, becomes more irregular, and abnormal ECM components accumulate.291 The fact that, in contrast to almost all other tumors, glioma cells have the capacity to diffusely infiltrate in the neuropil suggests that unique cell-ECM or cell-cell interactions are involved.254 Glioma cells may create their own microenvironment by synthesizing and depositing ECM molecules such as vitronectin, tenasin-C and laminin.24,104,127,163,190,224,256,290,291 Increased expression of tenasin-C was described to correlate with higher malignancy grade104 as well as to promote endothelial cell adhesion, spreading and migration, which are critical steps in the process of angiogenesis.290 Apart from ECM components, glioma cells may also use the surface of neighbouring neuronal and glial cells (including myelin sheaths) as a scaffold for diffuse infiltration in the neuropil. Interestingly, myelin was reported to be one of the most permissive substrates for attachment and migration of glioma cells.85 This phenomenon may at least partly explain the histopathological finding that glioma cells preferentially migrate in white matter tracts.

d. Cell-ECM and cell-cell interactions
Glioma cell migration requires dynamic expression of adhesion molecules, adequate positioning of these molecules, attachment to a relevant substrate, and detachment when the cell moves on. Integrins are considered to play a major role in glioma cell-ECM adhesion. Integrins are a family of calcium-dependent, transmembrane molecules that mediate cell-ECM and cell-cell adhesion and consist of a non-covalently linked α and β subunit. ECM binding integrins bind especially to the arginine-glycine-aspartic acid (RGD) sequence in the ECM components. Through the cytoplasmic domain of the β subunit, integrin activation can lead to activation of FAK, and of its intracellular signal transduction pathway.108,210 Subsequently, cytoskeletal rearrangements may occur and lead to cell movement.74 Integrins that were described to be upregulated on glioma cells are α3β1, αvβ1, αvβ3, αvβ5, the two latter integrins being receptors for vitronectin. In addition, αvβ3 can also bind to laminin, fibronectin, and tenascin-C.142

e. Proteases
In analogy with invasion of other cancer cells it is often hypothesized that glioma cells remodel their microenvironment by degrading the surrounding ECM to render it permissive for migration. Based on in vitro studies, several glioma derived proteolytic enzymes involved in cell migration were identified, such as MMP-2 (synonym: Gelatinase A), MMP-9 (Gelatinase B), and urokinase-type plasminogen activator (uPA).91,205,224,280,285 These proteases are synthesized and secreted as inactive pro-enzymes and activated by proteolytic cleavage outside the cell. For some of these proteases a role in glioma invasion has been confirmed in in vivo studies,91,135,226 their expression being correlated with glioma grade and infiltrative capacity. The expression of these proteases is tightly regulated and can, for example, be activated by interaction of the glioma cell with the
surrounding ECM. Several studies showed that the activation of ERK and Akt pathways stimulates secretion of MMP-2 and -9. Overexpression of secreted protein acidic and rich in cystein (SPARC) by glioma cells was described to cause increased expression of uPA, uPAR, MMP-2 and -9, which then leads to upregulation of PI3K and RhoA.

f. Growth factors and related signalling molecules
While in vitro studies revealed that Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (bFGF), and Transforming Growth Factor β (TGF-β) significantly affect invasion of glioma cells (see review Chicoine), many questions remain about the origin (tumor cells? inflammatory cells? pre-existent brain cells?) and exact role of such growth factors in vivo. Especially GBM cells often show amplification and mutation of the EGF receptor gene and overexpression of this receptor on the cell surface. Other studies indicate that Scatter Factor/ Hepatocyte Growth Factor (SF/HGF) is important for glioma cell migration. HGF binds to the tyrosine kinase receptor c-Met, and both HGF and its receptor are frequently overexpressed in gliomas. HGF-binding to c-Met results in autophosphorylation of the receptor, subsequent activation of several signalling pathways (e.g. MAPK-, Jak/Stat-, PI3K-pathways), and various cellular responses including migration. Recently, it was shown that hypoxia-induced HIF-1α causes up-regulation of c-Met and thereby enhances the effect of HGF on glioma migration.

g. Inflammatory cells and other factors
While high-grade malignant gliomas were described to contain large numbers of microglial cells and macrophages, lower numbers of microglial cells were found in low-grade diffuse gliomas. These cells are able to produce cytokines and growth factors and may contribute to evasion of immune attack as well as stimulate tumor growth, but the exact effect of such inflammatory cells in gliomas is not known. The findings that glioma patients show an increased number of immune-suppressive regulatory T-cells (not only the tumor tissue, but also in peripheral blood) and that expression of MHC class I and II molecules is downregulated on invading glioma cells may explain that diffuse infiltrative glioma cells can evade an immuneresponse (a phenomenon that has been called “stealth invasion of the brain”).

Angiogenesis
Angiogenesis is a normal feature in growth, development and wound healing involving proliferation of endothelial cells, degradation of the ECM, formation of sprouts and tubes and recruitment of pericytes. Under normal physiological conditions, it is a tightly regulated process which depends on an equilibrium between a variety of pro- and anti-angiogenic signalling factors. VEGF-A (formerly known as vascular permeability factor (VPF)) is the most important pro-angiogenic factor and is a member of a larger family of
structurally related proteins. Within this family, VEGF-A is unique in its capacity to induce migration and proliferation of endothelial cells, stimulate outgrowth of new blood vessels from the pre-existent vasculature and the formation of the blood vessel lumen. From now on, VEGF-A will be referred to as VEGF in this thesis. In addition, VEGF increases microvascular permeability by induction of fenestrae in endothelial cells and transendothelial cell transport involving vesiculovacuolar organelles, which eventually form transendothelial cell pores. To date, six isoforms of VEGF are known, produced by alternative splicing. VEGF-121 and -165 are the most prevalent and potent isoforms. VEGF-121 is the soluble form and does not bind to ECM molecules, whereas 50-70% of VEGF-165 remains bound to the cell surface and ECM. VEGF-165 induces endothelial cell proliferation and migration, whereas VEGF-121 appears to be a less potent inducer of migration. VEGF mainly exhibits its cellular response by binding to the transmembrane tyrosine kinase receptor VEGFR-2 (also named kinase-insert domain containing receptor (KDR)). VEGF-binding leads via dimerization of the receptor to a cascade of different signalling pathways with various cell regulating effects. For instance, VEGF induced, intracellular activation of the MAPK pathway leads to initiation of DNA synthesis and cell growth. The activation of the PI3K-Akt pathway on the other hand leads to increased endothelial cell survival, whereas activation of src can lead to actin cytoskeleton changes and induction of endothelial cell migration. In addition, co‐receptors like neuropilins (NRP-1, NRP-2) enhance the ability of VEGF to bind and activate its receptors. Neuropilins are multifunctional non-tyrosine kinase receptors that bind to class 3 semaphorins and are normally involved in regulation of axon guidance in the developing nervous system. Neuropilins are also expressed on several types of tumor cells, however, their role here is not yet fully understood. Studies suggest that NRP-1 expression protects tumor cells from apoptosis via binding of VEGF-165. Additionally, NRP-1 may mediate cancer cell migration and metastasis (shown in breast cancer, colon cancer and melanoma) also via binding of VEGF. VEGF-165 can bind to both NRP-1 and VEGFR-2, thus forming a ternary complex. The presence of NRP-1 enhances the binding of VEGF-165 to VEGFR-2 and potentiates VEGFR-2 signalling and pro-angiogenic activities. VEGF-121 can also bind NRP-1, but only VEGF-165 can bridge the NRP-1 – VEGFR-2 complex.

Most types of human tumor cells express VEGF, often at elevated levels. This is mostly due to various environmental stimuli (e.g. hypoxia, cytokines, sex hormones, growth factors, chemokines) and (epi-) genetic changes in the tumor cells (mutant p53, VHL, PTEN-suppressor genes, activated oncogenes (e.g. ras, src, EGFR, and erbB-2/HER2)). Hypoxia is an important inducer of VEGF. In hypoxic conditions, the transcription factor HIF-1α is stabilized due to a block of ubiquitin-dependent degradation. HIF proteins in turn stimulate the production and release of VEGF. Other important factors involved in the regulation of angiogenesis include basic fibroblast growth factor (bFGF/ FGF2),
hepatocyte growth factor/ scatter factor (HGF/SF), PDGF and deltoid ligand 4 (DII-4). bFGF promotes endothelial cell proliferation and organization of endothelial cells into tube-like structures.\textsuperscript{23} While bFGF may promote angiogenesis by a direct effect on endothelial cells, it mainly works indirectly by the up-regulation of VEGF in endothelial cells.\textsuperscript{173,240} Also HGF can induce diverse angiogenesis. Via activation of downstream signalling pathways, HGF mediates various processes involved in angiogenesis: cell proliferation (via Ras/MAPK), cell migration and invasion (via Ras/MAPK), cell survival and resistance to apoptosis (via PI3K/Akt), tubule formation (via both Ras/MAPK and PI3K/Akt) and epithelial tubule morphogenesis and endothelial cell proliferation (via STAT). HGF secreted by tumor cells, vascular smooth muscle cells, and pericytes has been found to regulate all of these mechanisms through activation of c-Met receptors on endothelial cells.\textsuperscript{1} As mentioned before, HGF and its receptor c-Met are also frequently overexpressed in gliomas where they are thought to also play a role in glioma cell migration.\textsuperscript{93,126} PDGF functions through binding to its tyrosine kinase receptors. It is involved in pericyte recruitment during vascular development and maintenance of vasculature via association with endothelial cells.\textsuperscript{17} Recent studies have revealed a pivotal new angiogenesis signalling pathway: notch-DII-4. DII-4, a transmembrane ligand that is exclusively expressed on endothelial cells, and binds notch receptors 1 and 4 on adjacent endothelial cells. The induction of DII-4-notch signalling is thought to prevent excessive angiogenesis and to promote the orderly development of new blood vessels.\textsuperscript{121,153,216}

Folkman hypothesized in 1971 that for growth, solid tumors need to induce angiogenesis for sustained supply of nutrients and oxygen and removal of waste products.\textsuperscript{68} Before that time, it was widely believed that tumors grew along pre-existent blood vessels. Recent studies with experimental subcutaneous tumor models have strengthened the concept of strict angiogenesis-dependency. However, it is more likely that in human tumors both angiogenesis-dependent and -independent tumor areas are present. Angiogenesis is indeed an absolute requirement for rapidly proliferating tumors growing in avascular spaces. For tumors growing in vessel-dense organs like brain and lung, this strict dependency is far less clear. For example, in 16\% of patients with non-small-cell lung cancer, tumors were growing exclusively along pre-existent blood vessels without any sign of angiogenesis.\textsuperscript{194} This is also obvious in high-grade gliomas. While such gliomas may focally show an extreme angiogenic phenotype, quantitative studies revealed that the vascular density in many regions of both low- and high-grade diffuse gliomas and of gliomatosis cerebri is in the range of that for normal cerebral grey or white matter, indicating that in these areas angiogenesis is lacking.\textsuperscript{19,278,279} In such areas, the diffuse infiltrative glioma cells seem to behave like guerilla warriors that do not construct their own “supply lines” but incorporate and abuse pre-existent ones, a process which is known as vessel co-option.\textsuperscript{132,146}
Therapeutic options: anti-angiogenic agents

Since the discovery of angiogenic growth factors and their receptors, strategies to inhibit angiogenesis have become within the realm of possibilities. Various approaches to inhibit angiogenesis have been developed including neutralization of VEGF by monoclonal antibodies and inhibition of growth factor signalling by tyrosine kinase inhibitors (TKIs) with specificity for the angiogenic receptors. Preclinical testing of inhibitors of VEGF signalling in a variety of, predominantly subcutaneous, animal models (e.g. lung, prostate, breast, ovarian, colon, glioma or vulval human tumor xenografts) resulted in tumor regression or at least stabilization. These promising results led to a rapid introduction of anti-angiogenic therapies in the clinic.

Bevacizumab (Avastin®, Genentech/Roche) is the first Food and Drug Administration (FDA)-approved anti-angiogenic substance. It is a humanized monoclonal neutralizing antibody directed against VEGF. It has been approved as part of first line treatment for advanced colorectal cancer, non-small-cell lung cancer and breast cancer, in combination with chemotherapeutics. Also TKIs, which inhibit signalling from e.g. VEGF and/or PDGF receptors are rapidly entering the clinic like Sunitinib (Sutent®, Pfizer) which is directed against VEGFR, PDGFR, RET, KIT and flt-3; vandetanib (ZD6474, Zactima®, AstraZeneca) directed against VEGFR2, EGFR and RET; sorafenib (Nexavar®, Bayer) directed against VEGFR, PDGFR and Raf; cediranib (AZD2171, Recentin®, AstraZeneca) directed against VEGFR, PDGFR and c-Kit; and vatalanib (PTK787/ZK222584, Novartis) directed against VEGFR.

The promising results achieved in preclinical experiments with mono-therapies of these compounds could so far for most tumor types not be reproduced in the clinical setting. Only for renal cell carcinoma, mono-therapy of sunitinib and sorafenib prolonged progression-free survival compared with other therapies. These compounds are now approved for treatment of advanced renal cell carcinoma, imatinib-resistant gastrointestinal stromal tumor (sunitinib), advanced renal cell carcinoma and advanced hepatocellular carcinoma (sorafenib). Yet, combinations with chemotherapy have been shown to improve progression-free and overall survival in certain tumor types. Bevacizumab combined with chemotherapy for advanced colorectal cancer was reported to improve overall survival by approximately 4.5 months compared to chemotherapy alone. The biological mechanisms underlying this observation are still not completely understood. One hypothesis to explain this phenomenon is that of normalization of tumor vasculature. Unbalanced VEGF expression in tumors results in an irregularly dilated, tortuous vasculature with numerous sprouts and anastomoses (Figure 5). The irregular shape of blood vessels has consequences for blood flow: at numerous locations in tumor vasculature the blood flow is turbulent or even temporarily stopped. Furthermore, VEGF induces hyperpermeability of tumor vessels, resulting in extravasation of plasma proteins,
edema and a high interstitial pressure, thereby potentially impairing uptake of chemotherapeutic compounds from the vascular compartment. The normalization hypothesis states that inactivation of the surplus VEGF in tumors would result in a more regular vascular morphology, normalization of the permeability and reduction of the interstitial pressure. Combined, this would result in better perfusion of the tumor and better delivery of chemotherapeutic compounds to the tumor cells (Figure 5).^{107,110}

Solid proof for this hypothesis was recently presented by Eichhorn et al.^{59} who analyzed blood vessel functionality in subcutaneous melanoma tumors after anti-VEGF treatment with the tyrosine kinase inhibitor SU5416. Despite a significant decrease in vessel density in treated tumors, the uptake of the MRI-contrast agent was significantly higher in SU5416-treated tumors as compared to controls. Concomitantly, pO2 was increased upon treatment and red blood cell velocities increased. Analysis of vessel morphology in these tumors revealed that SU5416 resulted in a lower density of vessels that were however significantly larger in diameter. Because the vasculature in treated tumors lacked the extensive anastomoses which are seen in untreated tumors, blood flow was more efficient and conductive upon treatment.^{59} The clinical relevance of these findings should be put in perspective of the tumor-microenvironment. Indeed, angiogenesis is a prerequisite for rapidly proliferating tumors growing in avascular spaces, which encompasses most of the subcutaneous murine tumor (including subcutaneous glioma) models. Such subcutaneous tumors were shown to be very responsive to anti-angiogenic therapy. However, an experimental mouse tumor may grow to several grams in weight (up to 20% of body weight) within weeks, implying that blood vessel maturation is more or less synchronized and that these vessels are therefore relatively homogeneously sensitive to VEGF inhibition. Human tumors, on the other hand, grow more slowly and angiogenesis will be less synchronized than in rapidly growing tumors in animal models leaving only a relatively small subpopulation susceptible to VEGF inhibition. This is yet another example of how important it is to choose an appropriate model system for preclinical drug-testing. Interestingly, up till now, the most commonly used experimental glioma models are cell lines and subcutaneous animal models that do not recapitulate the typical diffuse infiltrative growth pattern of most human gliomas.
Figure 5: Schematic overview of possible effects of anti-angiogenic therapies on tumors.

A. Pre-treatment: tumor vasculature is characterized by turbulent blood flow, hyperpermeability and presence of various anastomoses and sprouts. Antibodies and contrast agents can easily access the interstitial space. A counteracting interstitial pressure may be present.

B. Anti-angiogenesis: newly formed blood vessels are attacked by the treatment, anastomoses disappear, the laminar flow in the vessels is restored and tumor cells beyond a minimal diffusion distance from vessels become hypoxic and apoptotic.

C. Normalization: the more regular vasculature with normalized permeability due to anti-angiogenic treatment results in a more conductive blood flow and a better delivery of chemotherapeutic compounds to the tumor. However, extravasation of large molecules like antibodies may be blocked by the reduction of permeability.
Outline of the thesis

Diffuse gliomas, ranging from low-grade to high-grade tumors (astrocytic, oligodendrogial and mixed oligo-astrocytic neoplasms), are primary brain tumors that are characterized by diffuse infiltration of tumor cells in the brain parenchyma. Partly due to this diffuse growth pattern, these tumors are notorious for their poor response to current therapies. In Chapter 1, we discussed several characteristics of diffuse gliomas: clinical features, radiological consequences of diffuse infiltrative growth, histopathology, molecular mechanisms of diffuse infiltration, angiogenesis and anti-glioma treatments using anti-angiogenic therapies.

In order to improve the prognosis of glioma patients, there is an urgent need for new therapeutic agents. Ideally, such agents are tested in representative preclinical model systems. However, many animal models for human gliomas do not display typical glioma characteristics like the diffuse infiltrative growth pattern or do not adequately represent the different genetic backgrounds of the subsets of human gliomas (e.g. oligodendrogial vs astrocytic tumors). The U87 glioma cell line is the most widely used glioma model despite its inability to grow diffusely into the brain. In Chapter 2, we discuss several glioma animal models, varying from existing glioma cell lines to xenograft models we recently established. Intracerebral inoculation of existing glioma cell lines in nude mice often gives rise to compact growing lesions. However, the E98 human glioma xenograft line (propagated through subcutaneous growth) consistently produces intracerebral tumors displaying diffuse infiltrative growth in the brain parenchyma when injected in the mouse brain. In addition, we established four other intracerebral glioma models (E434, E468, E473, E478) by direct inoculation of Surgically removed human glioma cells in the brain of nude mice. These models consistently show extensive diffuse infiltration throughout the brain and the tumor cells carry typical chromosomal aberrations (-1p/-19q in anaplastic oligodendroglioma, +7/-10 in glioblastoma (GBM)). Especially these latter four models and the E98 line thus represent adequate geno- and phenocopies of human gliomas and form an attractive platform to investigate different therapeutic approaches in a preclinical setting.

As discussed in Chapter 1, proper delineation of gliomas using contrast-enhanced magnetic resonance imaging (CE-MRI) poses a problem. The intact blood brain barrier (BBB) in diffuse infiltrative areas precludes extravasation of contrast agents like gadolinium diethylene triamine penta-acetic acid (Gd-DTPA) and therefore hampers MR-based detection of the contrast agent. Treatment with anti-angiogenic compounds may further complicate tumor detection as such compounds can restore the BBB in angiogenic regions. Chapter 3 describes a study in which MRI is performed on the intracerebral glioma U87 model with and without the anti-angiogenic compound vandetanib, a tyrosine
kinase inhibitor directed against Vascular Endothelial Growth Factor Receptor type 2 (VEGFR2), Epidermal Growth Factor Receptor (EGFR) and Rearranged during Transfection (RET). Vandetanib indeed reduced the visibility of tumors when using Gd-enhanced MRI. However, CE-MRI using ultrasmall particles of iron oxide (USPIO, Sinerem®) as blood pool contrast agent has additional value for detection of glioma in the brain of nude mice since tumors remain visible due to the decrease in microvessel density caused by anti-angiogenic therapy.

The hypothesis formulated in Chapter 3 that anti-angiogenic therapy can restore the BBB is further tested in Chapter 4. Here we studied the effect of three different anti-angiogenic regimens (the monoclonal antibody against VEGF bevacizumab, the tyrosine kinase inhibitor sunitinib (directed against VEGF, Platelet Derived Growth Factor Receptor (PDGFR), RET, KIT and flt-3) and a combination of sunitinib and vandetanib) on intracerebral E98 tumors. More specifically, we tested whether inhibition of vessel maturation via targeting of PDGFR increases therapeutic benefit in the orthotopic E98 glioma animal model. We found that such additional inhibition of PDGFR did not improve the therapeutic efficacy. While angiogenesis in gliomas was effectively inhibited, the diffuse infiltrative growth was not notably affected. Furthermore, all regimens induced restoration of the BBB, resulting in reduced visibility in Gd-DTPA enhanced MRI-scans. We thus provided evidence that the BBB is closed upon anti-angiogenic treatment and showed that this hampers leakage of contrast agent in the tumor, thereby complicating radiological visualization of tumors.

To test whether restoration of the BBB can hamper delivery of not only contrast agents such as Gd, but also of chemotherapeutic compounds to the tumor, we treated animals carrying orthotopic E98 and U87 lesions with a combination of the angiogenesis inhibitor vandetanib and the DNA-alkylating agent temozolomide (TMZ). Anti-angiogenic compounds have been shown to synergize with chemotherapeutic compounds in other tumor types, possibly due to vessel normalization. Driven by the failure of conventional therapeutic approaches for diffuse gliomas, such combinations are also introduced in the clinic for the treatment of glioma patients. However, in Chapter 5 we show that, in our models, vandetanib antagonizes the effects of TMZ, presumably by restoration of the BBB. Vessel normalization in brain thus obstructs chemo-distribution of TMZ to the tumor cells. Combination of anti-angiogenic compounds with chemotherapeutics for brain tumors should thus be applied with caution in the clinic.

Overall, although vessel normalization is generally considered to result in a better delivery of chemotherapeutic agents in tumor tissue, we show in this thesis that it strongly depends on the tumor growth pattern and microenvironment whether anti-angiogenic therapy will be effective. Especially in vessel-dense organs like the brain, infiltrative tumor
cells can use the pre-existent blood vessels for supply of nutrition and oxygen and removal of waste products. As such tumors can theoretically grow and disperse without induction of angiogenesis, and anti-angiogenic treatments of such tumors may have limited impact.

The unique presence of the BBB in the brain causes an additional problem: vessel normalization implicates restoration of the BBB, thereby inhibiting substances like contrast agents and chemotherapeutics to reach the tumor cells. We conclude that diffuse infiltrative glioma cells are difficult to attack by both conventional therapeutic modalities and by anti-angiogenic therapy. Therefore, there is an urgent need for new therapeutic approaches that specifically target the diffusely infiltrating glioma cells and, equally important, that are able to reach those cells \textit{in vivo}. 
Chapter 2

Phenotypic and genotypic characterization of orthotopic human glioma models and its relevance for the study of anti-glioma therapy

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Abstract

Most human gliomas are characterized by diffuse infiltrative growth in the brain parenchyma. Partly due to this characteristic growth pattern, gliomas are notorious for their poor response to current therapies. Many animal models for human gliomas, however, do not display this diffuse infiltrative growth pattern. Furthermore, there is need for glioma models that represent adequate genocopies of different subsets of human gliomas (e.g. oligodendrogliomas). Here we assessed intracerebral growth patterns and copy number changes (using MLPA / CGH) of fifteen human glioma lines in nude mice. Most xenografts present with compact growing lesions intracerebrally. Only the E98 and, to a lesser degree, E106 xenograft lines (propagated through subcutaneous growth) consistently produce intracerebral tumors displaying diffuse infiltrative growth in the brain parenchyma. In contrast, four xenograft lines (E434, E468, E473, E478) established by direct intracerebral inoculation of human glioma cells, and serially propagated intracerebrally, consistently show extensive diffuse infiltration throughout the brain. After several passages, the neoplastic cells still carry typical chromosomal aberrations (-1p/-19q in oligodendroglioma, +7/-10 in glioblastoma multiforme). Especially these latter four models and the E98 line thus represent adequate geno- and phenocopies of human gliomas and form an attractive platform to investigate different therapeutic approaches in a preclinical setting.
Introduction

Gliomas are the most common primary human brain tumors. While non-glial brain tumors generally form relatively circumscribed masses, most gliomas in adult patients are characterized by extensive, diffuse infiltration in the brain parenchyma. This diffuse growth pattern suggests the existence of specific interactions between glioma cells and their microenvironment, but the exact nature of such interactions is still largely unknown. Histopathologically, glioma cells seem to preferentially invade along myelinated fibers in white matter tracts. In addition, migration and accumulation of tumor cells in the perivascular (Virchow-Robin) spaces and areas with a more compact phenotype may be present in human gliomas. Although gliomas rarely metastasize to distant sites, curative treatment using surgery, radiotherapy or chemotherapy is generally impossible, partly due to the diffuse infiltrative growth of these tumors in the brain. Most diffuse infiltrative glial tumors can be classified as astrocytic, oligodendrogial, or mixed oligo-astrocytic neoplasms. Typical oligodendrogial tumors are characterized by loss of chromosome arms 1p and/or 19q. The 1p/19q loss is associated with chemosensitivity and prolonged survival, even when chemotherapy is not provided. Typical genetic aberrations of high-grade astrocytomas are loss of chromosome 10 and gain of chromosome 7. In these latter tumors, EGFR (located at 7p12) amplification and overexpression are frequently detected. Also epigenetic changes like methylguanine-DNA-methyltransferase (MGMT) promoter hypermethylation can occur in gliomas. The resulting reduced expression of MGMT predicts better response to alkylating agents like Temozolomide.

A variety of experimental models has been used to study gliomas. Most of these are models for astrocytic tumors, and currently only few oligodendroglioma models are available, among which cell lines like TC620, subcutaneous xenografts, and transgenic mouse models. The more commonly used models for (astro)glial tumors encompass human glioblastoma multiforme (GBM) cell lines, e.g. U87, or the rat glioma cell line C6; subcutaneous xenograft models using human glioma cells; orthotopic models like transgenic animal models, and intracerebral xenograft models using human glioma cells. Although all these different models can be valuable in specific experiments, it is important to realize the limitations of each system. While in vitro systems can provide a lot of information on e.g. the biochemical and biological properties and on the intrinsic chemoresistance of glioma cells, in these systems, the brain microenvironment with e.g. the blood brain barrier is not recapitulated. Moreover, cultured glioma cells can lose their typical genetic alterations like EGFR amplification. Subcutaneous xenograft models can be used to test chemosensitivity of glioma cells for different chemotherapeutic compounds. These heterotopic tumors are characterized by synchronicity and reproducibility of tumor formation and have the advantage of being
easy to monitor and genetically stable.\textsuperscript{71,83} However, diffuse infiltrative growth in the brain parenchyma is also lacking, while such growth can be essential when studying tumor behavior. It is therefore now generally accepted that in many respects orthotopic models are clinically most relevant.\textsuperscript{217,247,248,252} However, even orthotopic glioma models not necessarily mimic the growth pattern (and thus are not necessarily adequate phenocopies) of human gliomas.\textsuperscript{195,218}

In the present study, we assessed the intracranial growth pattern and copy number changes of seven human glioma cell lines (U87, U373, Hs683, U251, U343-31L, U343-C12:6, U410) and four human subcutaneous GBM xenograft lines (E34, E49, E98, E106). In addition, we established four orthotopic xenograft lines by direct inoculation of surgically obtained fresh human anaplastic oligodendroglioma cells (E434, E478) and GBM cells (E468, E473) in the brain of nude mice. Especially these latter models appear to resemble both phenotypically and genotypically the original human tumors.
Material and methods

Animals
Female nude Balb/c mice (6-8 weeks old), weighing 18-25 g, were obtained from the central animal facility of the Radboud University Nijmegen Medical Center. The animals were kept under specific pathogen free conditions in plastic cages with air filters and received food and water ad libitum. The local ethics committee for animal use approved the experimental procedures.

Glioma cell lines
Seven human glioma cell lines were used: U87, U373 and Hs683, kindly provided by Dr Robert Kiss (Free University of Brussels, Belgium) and U251, U343-31L, U343-C12:6 and U410, kindly provided by Prof Dr Joop van Zoelen (Radboud University Nijmegen, The Netherlands). Another stock of U87 cells was provided by the Department of Radiotherapy (Radboud University Nijmegen Medical Centre, The Netherlands). U87 and U373 cells were cultured in Eagle Minimum Essential Medium (EMEM, with L-Glutamine) supplemented with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Hs683 cells were grown in RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. U251, U343-31L, U343-C12:6 and U410 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide. All culture reagents were obtained from Cambrex Bio Science (Verviers, Belgium).

Subcutaneous and intracerebral xenograft lines
The subcutaneous GBM xenograft lines (sc-xenograft lines: E34, E49, E98, E106) were established in the early 1990s by implantation of surgically obtained original and fresh tumor tissue in the flanks of nude Balb/c mice. These lines are maintained by regular passaging to new mice via subcutaneous transplantation of tumor pieces (± 10 mm³).²⁰

The intracerebral xenograft lines (ic-xenograft lines: E434, E468, E473, E478) were established by direct intracerebral inoculation of human tumor material as described below. Fresh surgical specimens were obtained from patients with anaplastic oligodendroglioma (n=2), GBM (n=2) or anaplastic oligoastrocytoma (n=2).

In vivo orthotopic grafting of glioma cells
The glioma cells of the glioma cell lines were trypsinized at 80% confluence, using 0.125% trypsin (0.1% EDTA, 0.1% glucose, in phosphate buffered saline (PBS)) and collected in medium. The cells were pelleted by centrifugation for 5 minutes at 900 rpm, washed once
in PBS and resuspended to a concentration of $5 \times 10^6$/ml. Subcutaneous E34, E49, E98 and E106 xenografts were excised, gently minced with a sterile scalpel and filtered through a 70 μm mesh filter to make a cell suspension. In the same way, a tumor cell suspension was prepared from the human surgical specimens. The resulting tumor cell suspension (20 μl or a suspension of about 100,000 cells in the case of cell lines) was inoculated in the brain of anaesthetized (mixture of 1.3% isoflurane in N$_2$O/O$_2$) mice (at least five animals per cell line) using a standardized procedure with guided injection through the skull 2 mm from the midline in the right parieto-occipital region and at a depth of 3 mm from the skin. The animals were monitored closely and upon showing discomfort or weight loss, they were sacrificed and their brains harvested. For this study, the brains were formalin-fixed, cut into five or six coronal slices and embedded in paraffin. Histological sections were prepared according to standard procedures.

**Maintaining intracerebral xenograft lines**

After intracerebral inoculation of a cell suspension derived from a human surgical specimen, the animals were monitored closely and when they started to display discomfort, they were sacrificed, their brains were harvested and examined macroscopically. Parts of the brain suspicious for tumor growth were excised and used to prepare a cell suspension. Using this procedure, xenograft lines were generated that are maintained by serial intracerebral inoculation.

**Immunohistochemistry**

Immunohistochemistry was performed according to standard procedures using antibodies against vimentin to highlight human tumor cells (Clone Vim 3B4, DakoCytomation, Glostrup, Denmark), CD34 for mouse endothelial cells (Clone MEC14.7, Hycult Biotechnology bv, Uden, The Netherlands), Glut-1 for hypoxic cells and for endothelial cells of the brain vasculature (Glut-1 is considered to be a BBB marker$^{95}$) (DakoCytomation), and Ki-67 for proliferating cells (Clone SP6, Lab Vision Corporation, Fremont, CA, USA). After incubation with a biotin-labeled secondary antibody, this latter antibody was detected using the ABC-method (Vector Laboratories, Burlingame, CA, USA) and specific signal was demonstrated by staining with 3-amino-9-ethyl-carbazole solution (Scytek Laboratories, Logan, UT, USA). All sections were mounted in Immol Mount medium (Klinipath BV, Duiven, The Netherlands).

**In situ hybridization for VEGF mRNA**

VEGF mRNA in situ hybridization was performed as previously described.$^{147}$ In brief, 4μm sections were dewaxed, rehydrated and digested with 5μg/ml proteinase (Invitrogen, Carlsbad, CA, USA) after which the tissue was cross-linked in 4% formaldehyde / phosphate-buffered saline (PBS). Non-specific binding sites were blocked by incubation with acetic anhydride. Tissue was hybridized with dioxigenin-labeled VEGF-A sense or
antisense probe in a humidified chamber at 63°C overnight. Sections were incubated with alkaline phosphatase-conjugated anti-dioxigenin (Roche, Neuilly, France) and developed by incubating the slides in 4-nitroblue-tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate solution (Roche), briefly counterstained with nuclear fast red solution, dehydrated in absolute ethanol and xylene and finally mounted with Permount (Klinipath BV).

Transmission Electron Microscopy
To study the diffuse infiltrative growth of glioma cells in more detail, small fragments of the diffuse infiltrative E98 tumor in corpus callosum were fixed by immersion in 2.5% glutaraldehyde, dissolved in 0.1M sodium cacodylate buffer overnight at 4°C and washed in 0.1M sodium cacodylate buffer, pH 7.4. The tissue fragments were postfixed in Palade-buffered 2% OsO4 for 1 hour, dehydrated, and embedded in Epon 812 (Merck, Darmstadt, Germany). Ultrathin serial sections were contrasted with 4% uranyl acetate for 45 min and subsequently with lead citrate for 5 min at room temperature. Sections were examined in a JEOL 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Genetic and epigenetic analysis of human tumors and xenografts
DNA was isolated from the cell lines, sc- and ic-xenograft lines and from the human tumor tissue samples using the DNeasy Tissue Kit (Qiagen, Venlo, The Netherlands). For the ic-xenograft lines, DNA was isolated from various generations: P1, P15 and P16 for the E434 line, P1, P4 and P8 for E468, P1, P2, P3, P9 and P10 for E473, and P1, P4 and P6 for E478. Genetic aberrations in these tumors were studied using Comparative Genomic Hybridization (CGH) and Multiplex Ligation-dependent Probe Amplification (MLPA) (for the ic-xenograft lines: on P1 with CGH and on all generations just mentioned with MLPA). MLPA kit P105 (MRC Holland, Amsterdam, The Netherlands) was used to detect EGFR, TP53 and CDKN2A aberrations. These two techniques were performed as previously described.111,113 MGMT promotor hypermethylation analysis was performed using methylation specific (MS) - MLPA. The MS-MLPA kit with probe mix was prepared by MRC-Holland (Amsterdam, The Netherlands). It contains 10 control probes and eight specific probes to detect hypermethylation including four MGMT probes. These eight methylation specific probes all contain an HhaI restriction site. All MLPA probe pairs were designed and prepared as described by Schouten et al (http://www.mlpa.com)228 and MS-MLPA was performed as previously described by the manufacturer.112,182 Methylation-ratios, indicative for the percentage of methylated sequences, were calculated for all samples. Based on this ratio, semiquantitative assessment of the methylation status of the MGMT promotor of the glioma cell and xenograft lines was possible (absent (R < 0.25), mild (0.25 < R < 0.50), moderate (0.50 < R < 0.75) and extensive (R > 0.75)).
Results

Orthotopic inoculation of human glioma cell lines, sc-xenograft lines and fresh human tumor material
Intracerebral injection of tumor cell suspensions reproducibly resulted in tumor development in nine out of eleven cell and subcutaneous xenograft lines. Direct intracerebral injection of human tumor homogenates reproducibly led to tumor take in four out of six human tumors. Intracerebral inoculation of two cell lines (U343-31L and U410) and two fresh tumor suspensions (E474 and E560, both oligoastrocytomas) did not result in detectable tumor growth even after six months. The intracerebral tumors were characterized by reproducible, line-specific growth patterns and growth rates (Table 1). Dependent on the specific tumor line, mice started to display general discomfort and weight loss between 2 and 15 weeks after injection. Interestingly, subcutaneous inoculation or transplantation of fresh human tumor cells or tissue from the original human E434 and E468 tumors did not result in tumor growth (six months follow-up). The four ic-xenograft lines have been passaged intracerebrally in mice for at least six and up to sixteen times (in three years).

Orthotopic growth pattern of human glioma cell and xenograft lines
With the exception of E98 and the ic-xenograft lines E434, E468, E473 and E478 which will be discussed below, all lines showed a predominantly compact growth pattern. Additionally, E49, U87, U373, U343-cl2:6 and Hs683 tumors showed variable perivascular infiltration in the brain parenchyma. Diffuse growth along white matter tracts was never observed in these five xenografts (Figure 1A,B,C). Orthotopic E34 and U251 lesions presented as compact growing tumors with limited diffuse infiltrative growth in the neuropil. E106 xenografts grew to expansive tumors with dispersed necrotic foci and a mild (in four of six mice) to extensive (in two of six mice) diffuse infiltrative component.

Orthotopic growth pattern of E98 sc-xenograft line
The intracerebral E98 xenograft lesions consistently showed both extensive diffuse infiltrative and compact growth (Figure 1D-I) and dispersed perivascular accumulation of tumor cells, resulting in severe neurological symptoms and death 24-28 days after injection. The diffuse infiltrative component was preferentially present in white matter tracts, especially in and adjacent to the corpus callosum (Figure 1F). Ultrastructurally, the tumor cells were located in between the myelinated fibers, which showed variable swelling and disintegration (Figure 1G). The compact tumor component was localized within the ventricles, occasionally with limited central necrosis. In addition, a compact or sheet-like tumor component was present in the leptomeninges in 80% of the mice, with some extensions in the Virchow-Robin space in the underlying brain. Focal central
hypoxia, as demonstrated by Glut-1 immunostaining, was observed in the compact E98 tumor areas, whereas such staining was never seen in the diffuse infiltrative components (Figure 1H). In line with hypoxia-driven VEGF-A production, VEGF mRNA expression was absent in the diffuse infiltrative components but consistently co-localized with Glut-1 staining in compact E98 areas (Figure 1I). Moreover, glomeruloid microvascular proliferations (MVPs) were present in 80% of the tumors and these MVPs were only found in close proximity to the VEGF-A positive regions in the compact areas (Figure 1E). A proliferation index of approximately 80% was observed by Ki-67 staining in both the compact and diffuse infiltrative tumor components (not shown).

Figure 1. Orthotopic growth pattern of glioma cell lines and sc-xenograft lines. H&E staining of U87 (A), E49 (B,C) and E98 (D–F), Transmission Electronmicroscopy of E98 (G), Glut-1 immunohistochemical staining (H) and VEGF mRNA in situ hybridisation of E98 (I). The U87 line (A) gives rise to compact growing tumors without diffuse infiltrative growth in the surrounding brain. The E49 line (B) gives rise to tumors showing expansive growth with perivascular extensions in the surrounding brain (C). The E98 line shows a combination of diffuse infiltrative growth (D, arrow), especially in white matter tracts, and intraventricular compact growth (D, arrow head). Only in the compact areas of the E98 tumors, focal florid microvascular proliferation was found (E). E98 tumor cells invade between the myelinated nerve fibers (arrows) of the corpus callosum (F), which show swelling and disintegration (G). In the compact E98 tumor areas, focal central hypoxia as is shown by the Glut-1 staining (H), co-localizes with (hypoxia-driven) VEGF-A expression (I). Original magnifications: A,B,D: x12; C,E,F: x400; G: x2000; H,I: x100.
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<td>U410</td>
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Glioma cell lines

Chapter 2
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Table 1: Overview of the different glioma cell and xenograft lines: phenotypical and genotypical characteristics.

For every cell or xenograft line, tumor take, time till symptoms, intracerebral growth pattern, chromosomal aberrations as detected by CGH and (epi)genetic changes detected by MLPA are given. Genetic gains or losses are indicated by light grey and dark grey, respectively. For the ic-xenograft lines, the genetic aberrations shown are from the last tested generations. MGMT promotor methylation status is indicated by - (absent), + (mild), ++ (moderate) or +++ (extensive) methylation. For the ic-xenograft lines, the genetic aberrations which are present in both the originating human tumor and in the xenograft are marked in bold. Abbreviations used: GBM: glioblastoma multiforme, Anap Oligo: anaplastic oligodendroglioma, (*): n>30 mice, (§): n=5, (Φ): high copy number gain of EGFR, (§): MLPA analysis of one stock of the U87 cell line showed (also after repeated analysis) a low-level gain of EGFR, while in another stock this aberration was not found.
Orthotopic growth pattern of GBM ic-xenograft lines E468 and E473

The GBM ic-xenograft lines E468 and E473 consistently showed extremely diffuse infiltration of tumor cells throughout the entire brain (Figure 2A). Tumor cells were disseminated in both white and grey matter (Figure 2B). In the cortex, secondary structures of Scherer like accumulation of tumor cells around neurons (perineuronal satellitosis) (Figure 2C) and blood vessels was present. Pre-existent brain structures like white matter tracts were present in between the tumor cells. No hypoxic tumor cells were detected by Glut-1 immunostaining. The tumor-invaded brain showed the normal (i.e. high) vessel density, with vessels staining strongly positive for Glut-1 (Figure 2D). Florid MVPs were absent. Around P3, the E473 line showed a minor shift in phenotype and tumor cells were found predominantly in white matter tracts instead of both white and grey matter.

Figure 2. Orthotopic growth pattern of GBM ic-xenograft lines. H&E staining of E468 (A,B) and E473 (C) and Glut-1 immunohistochemical staining of E473 (D). Both xenograft lines give rise to extensive diffuse infiltrative growth in the mouse brain in both white (B, arrow indicates corpus callosum) and grey matter (B, arrowhead indicates deep nuclei). Secondary structures like perineuronal satellitosis are present (C, neurons indicated by arrowheads; tumor cells by arrows). The tumors show a high vessel density with vessels that are strongly positive for the BBB marker Glut-1 (D), consistent with incorporation of pre-existent brain microvasculature. Original magnifications: A: x12; B,D: x100; C: x400.
Orthotopic growth pattern of anaplastic oligodendroglioma ic-xenograft lines E434 and E478

The anaplastic oligodendroglioma ic-xenograft line E434 is the oldest, currently in the sixteenth passage (in three years). The first passages showed an extensive diffuse growth in both white and grey matter (Figure 3A) whereas in later generations tumor cells were diffusely invading mainly in white matter and dispersed fibrin lakes occurred in between the tumor cells. Additionally, perivascular accumulation of tumor cells was more prominent in these later generations. The other anaplastic oligodendroglioma ic-xenograft line E478 grew to diffusely infiltrative, highly cellular tumors (Figure 3B) with some isolated multinucleated cells (Figure 3E) and remnants of pre-existent brain tissue in between the tumor cells. The tumor cells of esp. the E434 line displayed the typical “fried egg” morphology (Figure 3C,D). No hypoxic tumor cells could be detected by Glut-1 immunostaining in neither E434 nor E478 lesions. Both xenografts presented with a high vessel density, with vessels positive for Glut-1 and CD34. Florid MVPs were absent in the E434 line and focially present in the E478 lesions (Figure 3F). A high proliferation index (± 90% and 70% in E434 and E478 lesions, resp.) was observed in the Ki-67 staining (not shown).

Genetic and epigenetic aberrations in glioma cell lines, sc- and ic-xenograft lines

In Table 1, an overview is given of the (epi-)genetic aberrations of the glioma cell and xenograft lines. For the ic-xenograft lines, the aberrations detected in the original human gliomas were comparable with those in the derived xenografts (Table 1, matching aberrations are marked in bold). Also after repeated passaging of the ic-xenograft lines, overall the same genetic aberrations were detected: the generations tested of the E434, E468 and E478 show the same copy number changes for EGFR, CDKN2A and TP53. Only the E473 ic-xenograft line changed pheno- and genotypically around P3 and in comparison with P1 lost its high copy number increase of EGFR. However, from P3 onwards, also this line is genetically stable. The GBM ic-xenograft line E468 shows typical GBM aberrations: loss of chromosome 10 and gain of chromosome 7 (CGH) as well as loss of CDKN2A and copy number gain of EGFR (MLPA). Both anaplastic oligodendroglioma ic-xenograft lines show loss of chromosome arms 1p and 19q, copy number gain of EGFR and loss of CDKN2A. The majority of the studied cell and xenograft lines show copy number gain of EGFR (10 out of 15 lines), mostly low-level gains (9 of 10), possibly due to chromosome 7 trisomy. Only the E468 line carries a high copy number gain of EGFR, probably due to actual genetic amplification. Interestingly, two different stocks of U87 cells showed different EGFR copy numbers, illustrating the importance to check cell and xenograft lines at regular intervals for both genotypic and phenotypic characteristics. The most abundant aberration is loss of CDKN2A (15/15), fourteen lines show a homozygous deletion of CDKN2A and one line (E478) a hemizygous loss of this gene.
Figure 3. Orthotopic growth pattern of anaplastic oligodendroglioma ic-xenograft lines. H&E staining of E434 (A,C) and E478 (B,D-F). E434 lesions are extremely diffuse infiltrative (A), whereas the E478 line grows to diffusely infiltrative, highly cellular tumors (B). The tumor cells of esp. the E434 (C) display the typical “fried egg” morphology. Frequent mitoses (D, arrows), dispersed multinucleated giant cells (E, white arrowheads) and occasional florid microvascular proliferations (F, black arrowhead) are present in the E478 xenograft. Original magnifications: A: x12; B: x50; C-F: x400.

Epigenetic analysis using MS-MLPA detected absent, mild, moderate and extensive MGMT promoter hypermethylation respectively in 2 (E468, U343-31L), 1 (E434), 3 (E34, E48, U251) and 9 (E49, E98, E106, E473, Hs683, U343C12:6, U373, U410, U87) lines. The majority thus shows hypermethylation of the MGMT promoter, predicting response to chemotherapy using alkylating agents like Temozolomide. Indeed, preliminary results show that both (orthotopic) E98 and U87 tumors show complete or partial response to temozolomide.
Discussion

While many tumors in the human brain show a compact, expanding growth pattern (e.g. meningioma), perivascular growth (e.g. metastatic carcinomas) or a combination of these, gliomas are unique in displaying diffuse infiltrative growth in the brain parenchyma. Many models are used in glioma research and the ideal preclinical test model is one in which a relevant genotype is combined with a relevant phenotype. In in vitro tumor models, tumor spheroids, and heterotopic glioma models in animals, the important glioma feature of diffuse infiltrative growth in the brain parenchyma is lacking. It is therefore now widely accepted that orthotopic tumors most closely resemble the situation of the original human gliomas. However, as we show here, orthotopic inoculation of human glioma cells does not necessarily lead to an adequate phenocopy of human gliomas. For example, upon transcranial injection U87 cells grow to expansive tumors with perivascular infiltration, but without the characteristic diffuse infiltrative growth in the neuropil. Also, U373, U343-C12:6, U251 and Hs683 cells and our stable GBM sc-xenograft lines E34 and E49 show no or only very limited diffuse infiltrative growth in the mouse brain. Hence, we consider these glioma models as suboptimal phenocopies of diffuse infiltrative human gliomas.

In contrast to these lines, the E98 and E106 xenografts show a combination of diffuse infiltrative and expansive growth. However, whereas the intracerebral E98 lesions consistently mimics the diffuse infiltrative growth pattern that is characteristic for human gliomas, this component is less pronounced in the E106 xenografts. The E98 xenograft line generates intracerebral lesions that not only show key histological features of human GBM (including nuclear atypia, mitotic activity, focal glomeruloid microvascular proliferation, and necrosis) but are also genetically similar to human GBM and stable in time for over 15 years. Genetic analysis using CGH and MLPA revealed that the E98 xenograft line carries genetic aberrations characteristic for GBM (gain of chromosome 7, copy number gain of EGFR, loss of chromosome 10 and loss of CDKN2A). In addition, this xenograft line shows MGMT promotor hypermethylation. According to previously reported guidelines for the use of glioma animal models, the facts that i. the E98 xenograft model represents both a genocopy and a phenocopy of a human GBM, ii. tumor take is 100%, and iii. orthotopic tumors grow to relevant sizes in a relatively short time (time from inoculation to neurological damage is 3-4 weeks) make the E98 line a unique and clinically relevant xenograft model for the study of human glioma.

Although the E98 xenograft is a good in vivo model for GBM, there is still a need for other models that represent the spectrum of human gliomas, including suitable oligodendroglioma models. Here we describe the establishment of orthotopic glioma animal models that feature both genotypical and phenotypical characteristics of GBM or...
oligodendrogial tumors. Interestingly, direct intracerebral inoculation of human tumor cells gave rise to neoplasms in four out of six tumors. In 2 cases (2/4), simultaneous subcutaneous grafting of the same human tumor tissue did not result in growth. In the past, to establish xenograft lines, human glioma cells were generally inoculated subcutaneously, or cultured first and then inoculated subcutaneously or intracerebrally. However, confronting glioma cells with an artificial (in vitro or subcutaneous) environment may result in selection of tumor cells that have lost the ability to diffusely infiltrate the brain. Also, it is important to realize that cell cultures or xenograft models can only be established from a subset of malignant gliomas and optimal human xenograft models for low-grade astrocytomas, oligodendroglialomas and oligoastrocytomas are up till now not available, possibly due to the low mitotic rate in these tumors. The E434 line is our longest standing ic-xenograft line, currently in the sixteenth passage. It has been described that intracerebral xenografts in rat adopt a more circumscribed phenotype in later generations (≥ 10th). Although we noticed a minor shift in phenotype in the E434 line, from dispersed growth in both white and grey matter to a preference for white matter tracts, extensive diffuse infiltrative growth remains the prototypic growth pattern of the E434 lesions. As the typical chromosomal losses of 1p and 19q and the histopathological honeycomb morphology are clearly present, we conclude that this E434 line is a stable, pheno- and genotypically relevant anaplastic oligodendroglioma animal model. Based on the pheno- and genotypical features, the E478 xenograft is a highly malignant oligodendroglioma model since it shows, in addition to oligodendrogial features, also microvascular proliferations, high mitotic activity and giant multinucleated tumor cells. This xenograft line displays, next to -1p and -19q, gain of chromosome 7, a hallmark of malignant progression of high-grade oligodendrogial tumors.

Remarkably, all studied glioma cell and xenograft lines show loss of CDKN2A. Malignant progression of oligodendrogial and astrocytic tumors is associated with accumulation of genetic abnormalities and one of these is homozygous CDKN2A deletion, which is present in up to one third of the anaplastic oligodendroglialomas and in the majority of the high-grade astrocytic tumors. CDKN2A and B encode for p16 and p14, important regulators of G1/S phase cell cycle transition. These proteins inhibit phosphorylation of the retinoblastoma protein (pRb) by the cyclin-dependent kinases Cdk4 and Cdk6. Loss of this pRb-dependent G1/S-phase cell cycle checkpoint is a frequent event in high-grade malignant gliomas and gives these tumor cells a growth advantage which appears to be of major importance when culturing or inoculating human glioma cells in nude mice. It has been described that homozygous deletions of CDKN2A occur more frequently in GBM cell lines than in primary tumors. However, loss of CDKN2A alone is not a guarantee for success when inoculating tumor cells in nude mice: two glioma cell lines (U343-31L and U410) did not give rise to tumor growth in vivo although they also show loss of CDKN2A. In
contrast to TP53 mutations, copy number changes of the TP53 gene are not very common in gliomas,
\textsuperscript{111,155} which is in line with our results detecting TP53 loss in only two of the cell and xenograft lines (2/15).

Orthotopic grafting of human glioma cells has the advantage of bringing the tumor cells in their natural environment thus enabling an extremely diffuse growth pattern, as is also shown by others.\textsuperscript{83,217,225} However, passaging glioma cells intracerebrally from one mouse to the next also has some practical disadvantages. At present, non-invasive imaging of invading glioma cells \textit{in vivo} and measurement of the degree of intracerebral tumor burden is difficult. Although the passaging itself causes only minor trauma for the animal, it is time-consuming and requires skilled personnel. Furthermore, in human glioma xenografts in immunodeficient animals, the role of immune response to the tumor can obviously not adequately be studied and should rather be investigated in other (e.g. genetically engineered transgenic) models in immunocompetent animals.\textsuperscript{13,71}

In conclusion, we show that the intracerebral E98 xenograft model both phenotypically and genotypically represents diffuse infiltrative human GBM. We also show that direct intracerebral inoculation of fresh human glioma material in nude mice gives rise to glioma models with extremely diffuse tumor growth and the typical glioma-related copy number changes. We have now a panel of GBM and anaplastic oligodendroglioma models, all displaying characteristic aspects of glioma biology. These models are characterized by high tumor take and short time to tumor formation, essential features of animal models. Adequate anaplastic oligodendroglioma models are reported here for the first time. Such genotypically and phenotypically relevant models are crucial for e.g. further elucidation of the mechanisms underlying diffuse infiltrative growth and assessment of response to various (novel) therapeutic modalities for diffuse infiltrative gliomas.
Chapter 3

Magnetic Resonance Imaging (MRI)-based detection of glial brain tumors in mice after anti-angiogenic treatment

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Bob Hamans,
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Cathy Maass,
Arend Heerschap,
William Leenders
Abstract

Proper delineation of gliomas using contrast-enhanced magnetic resonance imaging (CE-MRI) poses a problem in neuro-oncology. The blood brain barrier (BBB) in areas of diffuse infiltrative growth may be intact, precluding extravasation and subsequent MR-based detection of the contrast agent gadolinium diethylenetriaminepenta-acetic acid (Gd-DTPA). Treatment with anti-angiogenic compounds may further complicate tumor detection as such compounds can restore the BBB in angiogenic regions. The increasing number of clinical trials with anti-angiogenic compounds for treatment of gliomas calls for the development of alternative imaging modalities. Here we investigated whether CE-MRI using ultrasmall particles of iron oxide (USPIO, Sinerem®) as blood pool contrast agent has additional value for detection of glioma in the brain of nude mice.

We compared conventional T1-weighted Gd-DTPA-enhanced MRI with T2*-weighted USPIO-enhanced MRI in mice carrying orthotopic U87 glioma which were either or not treated with the anti-angiogenic compound vandetanib (ZD6474, ZACTIMA™).

In untreated animals, vessel leakage within the tumor and a relatively high tumor blood volume resulted in good MRI visibility with Gd-DTPA- and USPIO-enhanced MRI, respectively. Consistent with previous findings, vandetanib treatment restored the BBB in the tumor vasculature, resulting in loss of tumor detectability in Gd-DTPA MRI. However, due to decreased blood volume, treated tumors could be readily detected in USPIO-enhanced MRI scans.

Our findings suggest that Gd-DTPA MRI results in overestimation of the effect of anti-angiogenic therapy of glioma and that USPIO-MRI provides an important complementary diagnostic tool to evaluate response to anti-angiogenic therapy of these tumors.
Introduction

Glioblastoma multiforme (GBM) is the most frequent and most malignant glial brain tumor with dismal prognosis and median survival of less than two years, despite development of novel chemotherapeutic compounds.\(^{244}\) GBM are characterized by extensive diffuse infiltrative growth in the brain parenchyma making curative treatment by surgery or radiotherapy impossible.\(^{84,125}\) One of the hallmarks of GBM is the regional occurrence of prominent angiogenesis. The disrupted blood brain barrier (BBB) in such regions allows magnetic resonance (MR)-based detection of these tumors by extravasation and accumulation in the interstitial spaces of contrast agents like gadolinium diethylenetriaminepenta-acetic acid (Gd-DTPA).\(^{82,157,278}\) Importantly, however, the blood vessels in especially diffuse-infiltrative tumor regions may not be leaky, causing difficulties in radiological delineation of these tumors.\(^{200}\) This is of critical importance since neuro-oncologists depend on MRI for staging of tumors, monitoring response to therapy, distinguishing recurrent tumor from radiation necrosis, and planning stereotactic biopsies and surgical resections.\(^{82}\)

Due to the occurrence of angiogenesis in GBM, the proposition is that this tumor type may be candidate for anti-angiogenic therapy.\(^{76}\) Several agents have been developed that target angiogenic signaling pathways. An example is bevacizumab, a monoclonal antibody which neutralizes Vascular Endothelial Growth Factor-A (VEGF-A), the main inducer of angiogenesis.\(^{179}\) Tyrosine kinase inhibitors with specificity towards angiogenic receptors have also been developed and are entering clinical trials now.\(^{72,232,272}\)

Preclinical testing of inhibitors of VEGF signalling resulted in potent antitumor effects in a variety of animal models of cancer.\(^{89,272}\) However, in the clinical setting, results of monotherapy with angiogenesis inhibitors have not met the initial expectations. Yet, combination of bevacizumab with chemotherapy in patients with advanced colorectal cancer has resulted in prolongation of median survival of four months as compared to chemotherapy alone.\(^{107}\) It has been hypothesized that the beneficial effect of combination therapy is based on bevacizumab-induced normalization of tumor vasculature, resulting in reduced interstitial pressure and, consequently, improved distribution of cytotoxic compounds to tumor cells.\(^{110,286}\) Such vessel normalization also occurred in a mouse model of cerebral Mel57 melanoma metastases in response to vandetanib (ZD6474, ZACTIMA™),\(^{147}\) a tyrosine kinase inhibitor with specificity towards VEGFR2 and Epidermal Growth Factor Receptor (EGFR).\(^{272}\) Although vandetanib treatment effectively inhibited angiogenesis, it did not induce tumor regression or even dormancy: tumors progressed via co-option of pre-existent vasculature.\(^{130,147}\) Importantly, vessel normalization resulted in restoration of the BBB and, consequently, invisibility of progressing tumors in Gd-DTPA-enhanced MRI scans.\(^{147}\)
We also previously reported that Mel57 melanoma xenografts may grow in brain parenchyma in an angiogenesis-independent fashion by vessel co-option.\textsuperscript{133} Although these infiltrative lesions could not be detected in Gd-DTPA contrast enhanced MRI (CE-MRI), the relatively low vascular volume in the tumors, as compared to the surrounding tissue, could be exploited to detect these lesions using blood pool contrast agents such as ultrasmall particles of iron oxide (USPIO).\textsuperscript{144} Because anti-angiogenic treatment may result in conversion of an angiogenic tumor phenotype into a co-opting one, USPIO imaging may therefore be an attractive complementary tool to detect glioma and evaluate response to anti-angiogenic therapy. USPIO (Sinerem\textsuperscript{®}, Guerbet, France) consists of iron oxide crystals, coated by a dextrane layer.\textsuperscript{116} Because of its physicochemical characteristics (mean particle size 29.5 nm +/- 23.1 (volume weighted)), it remains in the blood circulation for a long time (plasma half-life: 25-30 hours) and can therefore be considered to be a blood pool contrast agent early after intravenous injection.\textsuperscript{45,263} USPIOs have a low toxicity profile and have been tested in clinical trials for detection of lymph node metastases in patients with bladder and prostate carcinoma.\textsuperscript{45,96}

Here we utilized the blood pool contrast agent property of Sinerem to investigate whether use of this contrast agent allows better detection of gliomas after treatment with vandetanib.
Materials and Methods

Animal tumor models
All experiments were approved by the Animal Experimental Committee of the institution. Balb/c nude mice (6–8 weeks old, weighing 18–25 g) were used in all experiments. U87 glioma cells were cultured in DMEM supplemented with 10% fetal calf serum until 80% confluency. After trypsinization and washing, 100,000 cells in 2 µl phosphate buffered saline were injected through the skull of anaesthetized mice (1.3% isoflurane in N₂O/O₂) at a depth of 3 mm. This procedure reproducibly results in tumor growth and obvious tumor-related symptoms three weeks after intracerebral injection.

Anti-angiogenic treatment
Mice carrying intracerebral U87 xenografts received vandetanib [50 mg/kg (n = 8) or 100 mg/kg (n = 9)] as a suspension in 1% polysorbate-80, ZD6474, Zactima™, kindly provided by Andy Ryan, AstraZeneca, Macclesfield, UK) once daily by oral gavage in a volume of 100µl. Mice were treated from day 7 till day 21 after tumor injection. A control group (n=8) received 1% polysorbate-80 vehicle only. Throughout the experiment, mice were monitored closely and after 16 to 20 days, when tumor-related symptoms became apparent (weight loss and neurological defects), CE-MRI was performed according to the protocol described below. Afterwards, mice were sacrificed and their brains removed and formalin-fixed for immunohistochemical analyses.

Magnetic resonance imaging
Gd-DTPA CE-MRI was performed on tumor-bearing mice as described previously. Animals were anaesthetised using 1.3% isoflurane in an N₂O/O₂ gas mixture. The tail vein was catheterized for injection of the two contrast agents. Animals were positioned on a warm water bed (37°C) to maintain body temperature. A 12 mm diameter transmit/receive coil was placed over the skull. MR imaging was performed on a 7T/200 mm horizontal-bore MR spectrometer (MR Research Systems Ltd, Guilford, UK). After recording navigational scout images, sixteen T1-weighted coronal images (Tₑ = 8 ms; Tₑ = 100 ms; flip angle = 90°; number of averages = 1; field of view = 25x25 mm; matrix size = 256x256; slice thickness = 1 mm) were acquired. A bolus of 0.2 ml of Gd-DTPA (20 mMol/l, Magnevist®, Schering, Germany) was injected intravenously and additional sets of T1-weighted images were acquired immediately after and at 2 and 10 minutes after administration. Upon completion of the final set of images the animal was removed from the setup and allowed to recover for a period of two hours. During this time, Gd-DTPA is allowed to clear from the bloodstream. Subsequently the animal was repositioned in the setup as described above. A reference T2*-weighted multi slice gradient echo (Tₑ = 7 msec; Tₑ = 1500 msec; number of averages = 1; field of view = 35 x 35 mm; matrix size =
256 x 256; slice thickness = 1 mm) was acquired before injection of USPIO (Sinerem®, Guerbet, France) at a dose of 12.5mg/kg. Two minutes after injection another set of T2*-weighted images was acquired. Additional T2-weighted spin echo images were also routinely acquired before and after Sinerem injection (T₁ = 50 ms; T₂ = 3000 ms).

For analysis of the data sets, MR spectrometer bundled image analysis software was used (MR Research Systems Ltd, Guildford, UK). In two mice of each group, regions of interest, retrospectively defined from histology and the appearance of the images, were selected that encompassed a complete lesion and intensity within such regions was measured pre-contrast and at different time points post contrast agent injection.

**Immunohistochemistry**
Formalin-fixed mouse brains were cut in six coronal slices of approximately 2 mm and paraffin embedded. Sections of 4 µm were subjected to H&E staining or immunohistochemical stainings using antibodies against alpha-smooth muscle actin (aSMA, Sigma, Zwijndrecht, The Netherlands) to highlight activated pericytes and vascular smooth muscle cells, Ki-67 (Clone SP6, Lab Vision Corporation, Fremont, CA, USA) for proliferating cells, mouse immunoglobulins (Vector Laboratories, Burlingame, CA, USA) to detect extravasated IgG (i.e. vessel leakage), CD34 (MEC14.7, Hycult Biotechnology bv, Uden, The Netherlands) to detect mouse brain capillary endothelial cells and Glut-1 (DakoCytomation) to highlight brain vasculature with an intact BBB, as well as hypoxic tumor cells.

**Quantitative analysis of hypoxia and microvessel density**
A computer program (KS 400 3.0 Zeiss) was used to measure the percentage of hypoxia (Glut-1 staining area / total tumor area), as well as the microvessel density (MVD; number of microvessels / tumor area) in 4 random, non-overlapping microscopic fields (magnification x200) in the tumors of vehicle-treated and vandetanib-treated mice (n ≥ 6). For the MVD measurement, a positive single vessel (identified by CD34 or Glut-1 staining of endothelial cells) was counted when (clusters of) positive endothelial cells were present with or without a recognizable lumen.

**Statistical analysis of hypoxia and microvessel density**
MVD data (number of vessels per mm²), hypoxia data (percentages) and proliferation indices (percentages) were subjected to a one-way ANOVA with treatment as factor. A post-hoc T-test (two-sided) was performed where appropriate and a p-value <0.05 was considered significant.
Results

Vasculature in intracerebral U87 tumors

Intracranial injection of U87 tumor cells reproducibly resulted in relatively large tumors three weeks after inoculation. Tumors grew to large, mostly sharply demarcated lesions with dilated vessels at the tumor periphery (Figure 1E,F). The tumors presented as highly proliferative lesions (proliferation index of 80-90%, Ki-67 immunohistochemistry, Figure 1G).

![Figure 1. CE-MRI of intracerebral U87 glioma lesions of vehicle-treated mouse. Representative images of intracerebral lesions before (A,B) and after (C,D) injection of contrast agent. Tumor lesions are not visible pre-contrast, but become visible as hyper-intense lesions in Gd-DTPA-enhanced MR images (C) due to leakage of contrast agent in and immediately around the tumor, and as hypo-intense lesions in USPIO-enhanced images (D) due to a higher vessel density in the tumor. H&E (E), Glut-1 (F) and Ki-67 (G) stainings of the corresponding brain slice show large viable tumors with regular vascular patterns and dilated vessels in the peritumoral rim (F, arrow). These latter vessels cause a a dark rim around the tumor lesion in the USPIO-enhanced MR image (D, arrow). Original magnifications: x25 (E) and x100 (F,G).](image)

Vessel densities in U87 tumors were relatively high and vessels were leaky, as established by T1-weighted Gd-DTPA-enhanced MRI (Figure 1A,C and Figure 5). When the same animals were subjected to T2*-weighted USPIO-enhanced MRI, a decrease in signal intensity was observed throughout the brain, but in the tumor this decrease was more
prominent (140%, compared to signal loss in normal brain, Figure 1B,D and Figure 4A). This was consistent with the high blood volumes within the tumors, as determined by vascular surface area (VSA) measurements using the KS400 software package (VSA in tumor: 4.1% vs 2.5% in contralateral brain). The dilation of peritumoral vasculature was reflected in a dark rim around the tumor (arrow in Figure 1D).

Effects of vandetanib treatment on tumor vasculature
In a previous study in mice carrying intracerebral VEGF-A expressing Mel57 melanoma lesions, we observed that vandetanib at a dose of 100 mg/kg restored the BBB in tumor-associated vessels, whereas a 50 mg/kg dose resulted in effective inhibition of angiogenesis but without full restoration of the BBB. Based on these results, we decided to treat mice carrying intracerebral U87 tumors with vandetanib at doses of 50 mg/kg (n=8) or 100 mg/kg (n=9). Treatment was well tolerated, but did not confer a prolonged survival of animals in neither the 50 mg/kg nor the 100 mg/kg group.

There was a clear dose effect on the tumors: vandetanib caused a significant decrease in microvessel density and a significant increase of hypoxia (one-way ANOVA: treatment effect: p<0.001 for MVD; p<0.001 for hypoxia). Tumors of mice treated with 50 mg/kg vandetanib showed a significant decrease in microvessel density (185 vessel/mm² in 50 mg/kg treated mice vs 309 vessels/mm² in vehicle-treated mice, p<0.05) and a significant increase of hypoxic tumor area as determined by immunostaining with the hypoxia
Chapter 3

marker Glut-1 (13.6% in 50 mg/kg treated mice vs 3.6% in vehicle-treated mice, p<0.05) vehicle (Figure 2C and Figure 5). In spite of these features, the tumors still presented locally with a high proliferation index although in the hypoxic tumor areas only occasionally Ki-67 positivity was observed (Figure 2C,F). The low proliferation index in hypoxic tumor regions has been described before and is consistent with an unmet energy demand in these areas. Consistent with previous data, tumor vessels were still leaky after treatment with 50 mg/kg vandetanib, indicated by Gd-DTPA MRI (Figure 2A,D).

Figure 3. CE-MRI of cerebral U87 lesions in mice treated with 100 mg/kg vandetanib. Representative images of intracerebral lesion before (A,B) and after (C,D) injection of contrast agent. Tumor lesions are not visible pre-contrast and stay invisible after injection of Gd-DTPA (C). Note that contrast agent did reach the brain, as indicated by contrast enhancement of large meningeal vessels (arrow). The corresponding H&E staining (E) shows a large, intraparenchymal tumor. Changes in tumor vessel density caused by anti-angiogenic therapy still allow visualization in USPIO-enhanced images. Tumors are visible as hyper-intense lesions due to a lower vascular volume compared to the surrounding normal brain parenchyma (D). The lesions present with only limited vascularization (F, Glut-1) whereas there was still a remarkably high positivity for Ki-67 in non-hypoxic regions (G). The arrows in F and G point at a regions of hypoxia. Original magnifications: x25 (E) and x100 (F,G).

Figure 4 shows representative USPIO-enhanced MR images of tumors of vehicle- and vandetanib-treated mice. Analysis of the images obtained from treated mice revealed that the signal intensity decrease in the tumor after USPIO administration was reproducibly
less than in the contralateral, unaffected hemisphere. This is consistent with the lower vascular volume in the tumor as compared to normal brain parenchyma (Figure 4B and C).

![Image of Figure 4](image)

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Figure 4. Relative MR signal intensities of regions of interest in representative examples of U87 tumors before (pre) and after (post) USPIO administration in vehicle (A), 50 mg/kg (B) and 100 mg/kg (C) vandetanib-treated mice. Signal intensities in regions of interest (ROIs, circles), covering the tumor core and in contralateral brain tissue, were measured using SMIS analysis software before and after USPIO injection. Differences in absolute signal intensities are indicated (Δ). Percentage signal decrease in the tumor, as compared to that in the contralateral normal hemisphere, are indicated in bold.

The decrease in microvessel density and occurrence of hypoxia in the tumor was even more prominent in 100 mg/kg vandetanib-treated mice (78 vessel/mm² vs 309 vessels/mm² in vehicle-treated mice, p<0.05 and 38.8% hypoxia in 100 mg/kg treated mice vs 3.6% in vehicle-treated mice, p<0.05 (Figure 5)). The proliferation index in vital tumor parts was not significantly different from that in control tumors (one-way ANOVA: treatment effect: p=0.068; proliferation index of 77% in vital tumor regions of vandetanib-treated animals vs 86% in vehicle-treated mice, see Ki-67 immunostaining in Figure 3G) although again the number of Ki-67 positive cells was much lower in hypoxic tumor areas (region effect: p=0.001 (T-test, two-sided); 14% in hypoxic tumor regions vs 77% in vital tumor regions of vandetanib-treated animals). Consistent with our previous finding, treatment with the high dose of vandetanib resulted in a complete lack of Gd-DTPA
extravasation, i.e. blood vessels which were incorporated in the tumors were characterized by an intact BBB, precluding conventional Gd-DTPA MRI-based detection (Figure 3A,C, note that in these mice Gd-DTPA did reach the brain, as evidenced by the signal increase in the large leptomeningeal vessels directly after injection, arrow). Importantly, the low vessel density and, consequently, low blood volume in the tumors did allow imaging of these tumors using USPIO. Signal decrease in the tumors was 15±10% of that in normal brain parenchyma, resulting in relatively hyperintense lesions (Figure 3D and Figure 4C).

**Figure 5. Effects of vandetanib treatment (50 or 100 mg/kg) on microvessel vessel density (MVD) and hypoxia development in U87 xenografts.** There is a significant decrease in MVD (*: p=0.01; **: p=0.00005; ***: p=0.002) and a significant increase in amount of hypoxia (*: p=0.02; ^^: p=0.0009; ^^^: p=0.02).

Discussion

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Treatment of high-grade gliomas is still cumbersome. Astrocytic tumors in adults are often characterized by the presence of highly motile tumor cells which grow in a diffuse infiltrative manner.\textsuperscript{84,125} This growth pattern complicates reliable diagnosis since radiological imaging using the conventional contrast agent Gd-DTPA mostly fails to properly delineate tumor margins. This is caused by the presence of an often intact BBB in many infiltrative parts, precluding extravasation of contrast agent.\textsuperscript{144,200} The diffuse growth characteristics also make curative treatment by conventional therapies impossible since it is unachievable to destroy all tumor cells without causing unacceptable side effects.\textsuperscript{84,125} Thus, for treatment of astrocytic tumors, there is an urgent need for new therapeutic approaches. One of these is anti-angiogenic therapy\textsuperscript{76} since angiogenesis, especially occurring at the rim of a necrotic centre, is one of the hallmarks of GBM.\textsuperscript{377} The angiogenic response is accompanied by increased leakiness of the vasculature and results in the characteristic ring-like enhancement in Gd-DTPA-enhanced MR images. It must be stressed that this phenotype always occurs only regionally in high-grade astrocytomas, next to large, diffuse-infiltrative, non-enhancing tumor areas, and presumably anti-angiogenic therapies will have no or only little effect on these growth aspects. Apart from inhibiting the angiogenic response, anti-angiogenic treatment of patients with high grade gliomas may also be useful as palliative therapy: the hyperpermeability of angiogenic blood vessels may result in potentially fatal brain edema. Anti-angiogenic therapies may effectively restore the BBB and diminish edema.\textsuperscript{147}

It is currently not known how anti-angiogenic therapies exactly affect disease progression in patients with high-grade astrocytic tumors. One complication of evaluating the effects of therapy is that anti-angiogenic compounds may close the BBB and preclude extravasation of contrast agents. We already described that this phenomenon renders tumors invisible in CE-MRI,\textsuperscript{147} up till now one of the few diagnostic modalities that are available in a routine diagnostic setting to detect brain tumors.

In a previous study we showed that tumors that expand in brain without induction of angiogenesis, have a relatively low blood volume which can be visualized in MRI by using a blood pool contrast agent such as USPIO.\textsuperscript{144} Here we show that intracerebral U87 tumors in mice treated with the VEGFR2/EGFR inhibitor vandetanib at a dose of 100 mg/kg can be very effectively visualized by employing T2*-weighted USPIO-enhanced gradient echo MRI. In some mice, both vehicle and vandetanib-treated, tumors were also visualized as hyperintense lesions in T2-weighted spin echo images. However, T2w-spin echo imaging did not reproducibly visualize these tumors in pre-contrast imaging and was always less optimal than USPIO-enhanced T2*-weighted imaging. It has been described before that in T2-weighted spin echo edema in brain tumors can be readily visualized.\textsuperscript{183} Vandetanib-induced closure of the BBB would result in reduction of edema, and thus reduction of
visibility in T2-weighted images. Although we were not able to show this in this study (data not shown), we conclude that USPIO-enhanced T2* imaging is a sensitive, proper and safe method to detect tumors on the basis of altered blood volume.

The relative signal intensities in the tumors, compared to unaffected brain tissue, correlated with relative vessel densities. Importantly, we found that when tumor bearing animals were treated with lower doses of vandetanib, vessel density decreased, as established by both immunohistochemical stainings and USPIO MRI, but vessels remained leaky as established by Gd-DTPA MRI. Thus, the actual dose of anti-angiogenic compounds is an important determinant of whether a tumor can be visualized via conventional Gd-DTPA imaging.

There are several options how to translate these results to the clinic. In other orthotopic glioma models we found that treatment with lower doses of vandetanib may also result in restoration of the BBB (unpublished results). Probably the actual levels of VEGF-A produced by the tumor, determine the dose of vandetanib required to annihilate VEGF effects. High-grade astrocytic tumors display remarkable intra- and intertumoral heterogeneity, also with regard to VEGF-A production. Thus, it must be anticipated that in a tumor, regions will always be present where anti-angiogenic therapy induces closure of the BBB, thus potentially leading to misinterpretation of therapeutic efficacy based on Gd-DTPA MRI.

Obviously, it will be crucial to know whether the effect of BBB closure is a vandetanib-specific effect. The selectivity of many tyrosine kinase inhibitors is rather broad. Vandetanib blocks tyrosine phosphorylation of VEGFR2, EGFR and RET, although the compound is less potent towards the latter two receptors. U87 tumor cells show only minor expression of EGFR and since tumor cell proliferation in non-hypoxic tumor areas is not notably inhibited by vandetanib, it is likely that the effects of vandetanib are on the level of VEGFR2 inhibition. In a previous study, using an intracerebral melanoma metastasis model in which tumor cells either or not express recombinant VEGF-A, we showed that vandetanib converted the phenotype of VEGF-A expressing melanoma lesions into that of non-VEGF-A expressing lesions, suggesting that vandetanib indeed neutralizes VEGF-A effects without affecting tumor cells. We therefore expect that similar effects may be observed with other anti-angiogenic compounds which are currently FDA approved and in various trials, such as Bevacizumab and Sutent. Indeed, a recent clinical study using Bevacizumab revealed that contrast-enhancement shrank in half of the patients with recurrent GBM. Whether in this study this reduction is the result of actual tumor regression or whether therapeutic efficacy is overestimated due to closure of the BBB cannot be answered without further examination. Evidently, further investigations in animal models are necessary.
Altogether, our results suggest that blood volume imaging using USPIOs will be a valuable addition to conventional Gd-DTPA enhanced MRI when evaluating the effect of anti-angiogenic therapy. Although not demonstrated in the present study, our results also suggest that T2*-weighted USPIO imaging may enable a better visualization and delineation of the diffuse infiltrative components in astrocytic tumors, which have blood volumes that are distinct from that in normal brain. Alternative methods for radiological measurement of vascular volume, such as dynamic susceptibility contrast (DSC) MRI and vascular space occupancy (VASO) functional MRI have been described. Advantage of these imaging protocols is that no contrast agents are required, but it remains to be established in comparative studies whether these methods are as sensitive as T2*w-USPIO-imaging.

The USPIO Sinerem has been previously used in clinical trials and was shown to have a safe toxicity profile. In these trials, Sinerem was used for its property to be phagocytosed by macrophages within one to two days, thereby enabling macrophage tracking. This principle has been used to detect lymph node metastases of prostate carcinoma. When using Sinerem as a blood pool contrast agent, patients should be imaged directly after intravenous injection, when macrophage uptake has not occurred yet.

In conclusion, our results show that tumors that are treated with anti-angiogenic therapy, and (regions of) which may be missed by conventional Gd-DTPA-enhanced MR imaging due to restoration of the BBB, are readily visualized via blood pool contrast-enhanced imaging. Therefore, complementary imaging of brain tumors using USPIOs will reduce the chance on false conclusions about the efficacy of anti-angiogenic therapy which are based on Gd-DTPA MR scans.
Chapter 4

Lack of additive effect of targeting multiple angiogenic pathways in glioma

Submitted for publication

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Abstract

Currently available compounds that interfere with VEGF-A signaling effectively inhibit angiogenesis in gliomas but affect diffuse infiltrative growth to a much lesser extent. Here we tested whether additional inhibition of vessel maturation via targeting of PDGF receptors increases therapeutic benefit in an orthotopic animal xenograft model of glioma. We used bevacizumab and vandetanib as VEGF-inhibitors and sunitinib as additional PDGF-inhibitor. We showed that combination therapy of sunitinib and vandetanib does not improve therapeutic efficacy compared to treatment with sunitinib, vandetanib or bevacizumab alone. Furthermore, all compounds induced restoration of the blood-brain barrier, resulting in invisibility in Gd-DTPA enhanced MRI-scans.
Introduction

Glioblastoma multiforme (GBM) is a primary brain tumor which is characterized by angiogenic tumor areas, but also extensive diffuse infiltrative dispersion in the brain parenchyma.\textsuperscript{155,277} Treatment consists of surgery when possible, followed by combined radiotherapy and chemotherapy.\textsuperscript{139,244} However, curative treatment is rare since complete removal of tumor cells is very difficult to achieve\textsuperscript{84} and the majority of patients die within 15 months after diagnosis. In the angiogenic areas of GBM, the vasculature is leaky, allowing contrast enhanced magnetic resonance imaging (CE-MRI)-based detection. In contrast, the extensive diffuse infiltrative tumor regions may have incorporated pre-existing blood vessels with an intact blood-brain barrier (BBB), preventing extravasation of contrast agents and thereby hampering radiological delineation when using T1-weighted Gd-DTPA MRI.\textsuperscript{8,19}

Because of the presence of prominent angiogenesis in GBMs, anti-angiogenic therapies are considered for these tumors.\textsuperscript{76} Many compounds have been developed that effectively inhibit the action of Vascular Endothelial Growth Factor-A (VEGF-A), the main inducer of angiogenesis (Figure 1). The neutralizing anti-VEGF-A antibody bevacizumab (Avastin, Genentech, San Fransisco, US) has been FDA-approved for treatment of advanced colorectal, breast and non-small cell lung cancer (in combination with chemotherapy)\textsuperscript{102,107,167}, the VEGF- and Platelet Derived Growth Factor (PDGF) receptor tyrosine kinase inhibitor sunitinib (Sutent, Pfizer, New York, US) is now widely used for treatment of renal cell carcinoma.\textsuperscript{72,107,122,169,272} Previous studies from our laboratory have revealed that vandetanib (ZD6474, Zactima™, AstraZeneca, Macclesfield, UK), a tyrosine kinase inhibitor with specificity against VEGFR2, Epidermal Growth Factor Receptor (EGFR) and Rearranged during Transfection (RET) affects angiogenesis-dependent tumor growth in brain tumor models of metastatic melanoma and different orthotopic glioma xenograft models.\textsuperscript{41,44,147,261} However, vandetanib treatment did not improve survival in the different orthotopic mouse models, and this could be attributed to a lack of inhibition of the diffuse infiltrative growth component. Furthermore, vandetanib caused a significant reduction of vessel leakage, resulting in difficulties with CE-MRI-based detection.\textsuperscript{41,44} The diminished penetration of contrast agent due to the restored BBB may lead to an overestimation of drug efficacy in clinical studies.

In the diffuse infiltrative areas, pre-existing blood vessels with a mature phenotype are incorporated, and these vessels may not be susceptible to anti-VEGF treatment.\textsuperscript{17} This may indeed explain the lack of effect of VEGFR2 inhibition on these tumor areas. It has been described, that additional targeting of mature tumor vessels via specific inhibition of PDGFR activity significantly enhances therapeutic effects.\textsuperscript{17} A recent phase II clinical study supports this proposition as cediranib (Recentin, AZD2171, AstraZeneca, Macclesfield, UK),
an inhibitor of VEGFR, c-KIT and PDGFR, improves progression-free survival in patients with recurrent GBM. However, it is important to notice that this is a small, uncontrolled phase II study and therefore the beneficial effect on survival is yet to be proven.

As anti-angiogenic compounds have now entered phase II clinical trials for GBM, it is very important to investigate whether such compounds have similar effects on tumor biology as vandetanib and whether additional targeting of the PDGFR by sunitinib increases anti-tumor activity. Here we performed in depth analysis of the effects of bevacizumab and sunitinib, either alone or in combination with vandetanib, in the well-characterized E98 model of intracerebral glioma. We show that normalization of the BBB is a common result of different anti-angiogenic treatments and that additional targeting of the PDGFR does not improve outcome.

Figure 1. Schematic overview of growth factor signaling pathways and some of their inhibitors. The tyrosine kinase activity of growth factor receptors is stimulated upon ligand binding and receptor dimerization. This results in activation of multiple signaling cascades which result in proliferation, migration and cell survival, cellular activities which are central to both tumor progression and angiogenesis. Targets of bevacizumab, sunitinib and vandetanib are indicated. Abbreviations: VEGF(R): vascular endothelial growth factor (receptor), EGF(R): epidermal growth factor (receptor), PDGF(R): platelet-derived growth factor (receptor), MEK-1/2: mitogen-activated protein kinase and extracellular signal regulated protein kinase-1/2 kinase, MAPK/ERK-1/2: mitogen-activated protein kinase/extracellular signal-regulated protein kinase-1/2, PLC: phospholipase C, PKC: protein kinase C, PI3K: phosphatidylinositol-3-kinase, mTOR: mammalian target of rapamycin, Akt: protein kinase B.
Materials and Methods

Animal tumor model
All experiments were approved by the Animal Experimental Committee of the institution. Balb/c nude mice (6- to 8-week-old, weighing 18–25 g) were used in all experiments. Orthotopic gliomas were xenografted as previously described.\(^{42}\) In short, a mouse carrying a subcutaneous E98 xenograft was sacrificed and its tumor surgically removed. A tumor cell suspension was prepared by mincing the tumor in small pieces and gently filtering it through a 70 μm filter. The tumor cells were suspended in phosphate buffered saline. Twenty μl of this cell suspension was injected through the skull of anesthetized mice (1.3% isoflurane in N2O/O2) at a depth of 3 mm. The E98 xenograft line consistently produces intracerebral tumors displaying diffuse infiltrative and angiogenesis-dependent growth in the brain parenchyma.\(^{43}\) Mice show tumor-related symptoms three weeks after inoculation.

Anti-angiogenic treatment
Mice were randomly divided into groups of five, except for the control group (P) which consisted of seven animals. Three different anti-angiogenic agents were used in four treatment groups: bevacizumab (group B), sunitinib (S), vandetanib (V) and vandetanib plus sunitinib (VS). Treatments started on day 8 after intracerebral injection. On that day, two mice from the control group were sacrificed to check tumor burden. Bevacizumab was administered four times (day 8, 10, 12 and 14 after intracerebral injection) by intraperitoneal injection in a volume of 100 μl (5 mg/kg in phosphate buffered saline). Mice received vandetanib (50 mg/kg as a suspension in 1% polysorbate-80) or sunitinib (30 mg/kg as a suspension in 12.5% cremaphor/12.5% ethanol in water) once daily by oral gavage in a volume of 100 μl. Mice were treated from day 8 until day 21 after tumor injection. A fourth group (VS) was treated with a combination of vandetanib and sunitinib using the dosage scheme as described for the individual compounds. The control group (P, n=5 remaining animals) received 1% polysorbate-80 vehicle only.

CE-MRI
Throughout the experiment, mice were monitored closely and after 16 to 20 days, when tumor-related symptoms became apparent (weight loss and neurological defects), CE-MRI was performed as previously described.\(^{41}\) In short, 16 T1-weighted coronal images (TE=8 ms; TR = 100 ms; flip angle = 90°; number of averages = 1; field of view = 25 × 25 mm2; matrix size = 256 × 256; slice thickness = 1 mm) were acquired. A bolus of 0.2 ml of Gd-DTPA (20 mMol/l, Magnevist®, Schering, Germany) was injected intravenously via a pre-inserted tail vein catheter and additional sets of T1-weighted images were acquired immediately after and at 2 and 10 min after administration. CE-MRI was carried out on at
least two mice per treatment group. For mice receiving daily doses of sunitinib and/or vandetanib, CE-MRI was performed on the same day as the last dose. Mice in the bevacizumab group received the last dose on day 14 and CE-MRI was performed five to seven days later.

**Immunohistochemistry**

After MRI, all mice were sacrificed and their brains removed and formalin-fixed for immunohistochemical analyses. Formalin-fixed mouse brains were cut in six coronal slices of approximately 2 mm and paraffin embedded. Sections of 4 µm were subjected to H&E staining or immunohistochemical stainings using antibodies against α-smooth muscle actin (αSMA, Sigma, Zwijndrecht, The Netherlands) to highlight activated pericytes and vascular smooth muscle cells, Ki-67 (Clone SP6, Lab Vision Corporation, Fremont, CA, USA) for proliferating cells, mouse immunoglobulins (Vector Laboratories, Burlingame, CA, USA) to detect extravasated IgG (i.e. vessel leakage), CD34 (MEC14.7, Hycult Biotechnology bv, Uden, The Netherlands) to detect mouse brain capillary endothelial cells and Glut-1 (DakoCytomation, Glostrup, Denmark) to highlight brain vasculature with an intact BBB, as well as hypoxic tumor cells. The quantitative analysis of hypoxia was performed as previously described41 with a computer program (KS 400 3.0 Zeiss). The complete angiogenesis-dependent, compact growth area and diffuse infiltrative growth area was measured. Hypoxia was expressed as percentage hypoxic tumor area (Glut-1 staining area)/ total tumor area x100. Proliferation indices were measured in 4 random, non-overlapping microscopic fields (magnification x200), in both compact and diffuse infiltrative growth areas. A proliferation index of 50% or more was considered high, 20% or less was considered low. All measurements were performed in tumors of all placebo-treated and anti-angiogenesis-treated mice (n=5 per group). Hypoxia data (percentages) were subjected to a one-way ANOVA with treatment as factor. A post-hoc T-test (two-sided) was performed where appropriate and a p-value <0.05 was considered significant.
Results

Anti-angiogenic treatment

Mice were treated with three different anti-angiogenic agents: bevacizumab, sunitinib, vandetanib, or a combination of sunitinib and vandetanib. All treatments were well tolerated. Mice from all groups were sacrificed when they showed obvious tumor-related symptoms (P: day 22 +/- 9; B: day 22 +/- 3; S: day 18 +/- 2; V: day 19 +/- 3; VS: day 19 +/- 3). Although none of the treatments induced prolonged survival, treatments did cause anti-tumor effects in the expansive, compactly growing tumor areas which, in this E98 xenograft model, are reproducibly found in ventricles and leptomeninges.

![Figure 2. Percentage of hypoxia in different treatment groups.](image)

Anti-angiogenic treatment causes an increase in hypoxia in the compact tumor compartments. Hypoxia in compact tumors in mice treated with vandetanib (V) or combination vandetanib-sunitinib (VS) was significantly higher than in compact tumors in placebo-treated mice (P) (*: p<0.01). A similar increase, though not significant, was present in compact tumors of mice treated with bevacizumab (B) and sunitinib (S).

Mice that received vandetanib and the combination vandetanib and sunitinib showed a significantly higher percentage of hypoxia per tumor area in the compact tumors compared with the vehicle-treated mice (Figure 2, one-way ANOVA: treatment effect: p<0.05; 13.4% in P vs 32.7% in V and 29.9% in VS groups, p<0.01). In mice treated with bevacizumab or sunitinib alone, there was a similar trend although significance was not reached (Figure 2, 13.4% in placebo vs 22.4% in A (p=0.1) and 22.9% in S groups (p=0.2)). One of the most prominent effects of all therapies was the near absence of microvascular proliferations, which were almost always found in vehicle-treated animals, but were only occasionally present in anti-angiogenesis treated mice (Figure 3C,D).
Figure 3. Effect of anti-angiogenic treatment on mice carrying intracerebral E98 tumors. H&E stainings of vehicle-treated (A,C) and vandetanib+sunitinib-treated (B) mice, representative Glut-1 (E) and Ki-67 (F) stainings of intracerebral E98 lesions of a bevacizumab-treated mouse. In 80% of vehicle-treated mice, microvascular proliferations were detected in the tumor (C,D), whereas this phenotype was only occasionally observed in 5% of treated mice (D). The diffuse infiltrative areas (E,F, arrowheads) were not notably affected by the treatment. The compact tumor areas show a striking increase in hypoxia (E, asterisk). The proliferation index of tumor cells remains high in both diffuse infiltrative (F, arrowhead) and compact (F, arrow) tumor areas despite anti-angiogenic treatment. Only in hypoxic tumor regions in the compact tumor areas (E, asterisk), there is a strong decrease in proliferation index (F, asterisk). There is no hypoxia detectable in the diffuse infiltrative areas (E, arrowhead). Original magnifications: A,B: x12; C: x400; E,F: 100x.
Importantly, although inhibition of VEGF signaling resulted in an increase of hypoxia in the compact tumors (Figure 2 and 3E, asterisk), tumor growth was still possible here to some extent, presumably due to diffusion of nutrients from the surroundings. This hypothesis is supported by a high proliferation index in the tumor rim whereas the proliferation index was strongly decreased in hypoxic areas (60-70% in vital tumor parts vs 10% in hypoxic areas, Ki-67, Figure 3E,F). In sharp contrast, no treatment protocol notably affected diffuse infiltrative tumor areas (Figure 3A,B,E,F), similar to previous observations with vandetanib treatment alone.44 No hypoxia was present in the diffuse infiltrative tumor areas (Figure 3E, arrowhead) and the proliferation index remained high in these areas (± 70%, Figure 3F, arrowhead).

Effect of treatment on the blood-brain barrier
Tumors in vehicle-treated mice could be readily detected using Gd-enhanced MRI, as described in previous reports.44 In diffuse infiltrative areas, leakage is lower than in the compact tumor parts, possibly due to a reduced vessel activation state and/or co-option of pre-existent blood vessels with an intact BBB. Bevacizumab, sunitinib, vandetanib and the combination of sunitinib and vandetanib all resulted in restoration of the BBB and, consequently, invisibility in CE-MRI, despite the presence of large tumors. Representative examples of MR images and corresponding histology of tumor bearing brains after the different treatment modalities are presented in Figure 4.
Figure 4. Correlation between CE-MRI and histopathology of intracerebral E98 glioma lesions. For each treatment group, a representative image is shown of the intracerebral lesion before (pre) and after (post) injection of Gd-DTPA contrast agent. H&E stainings of corresponding slices are shown on the right. Tumors in vehicle-treated mice (P) were readily detectable using Gd-enhanced MRI. No tumor was detected in MRI of mice treated with anti-angiogenic compounds (bevacizumab (B), sunitinib (S), vandetanib (V) and combination vandetanib-sunitinib (VS)). H&E staining of the corresponding brain slices, however, shows large tumors. Original magnification of H&E stainings: x12.
Discussion

Glioblastoma multiforme (GBM) is a highly malignant brain tumor characterized by the presence of areas with a compact, angiogenesis-dependent growth pattern as well as large diffuse infiltrative areas which make these tumors essentially untreatable. Characteristically, in the compact tumor components angiogenesis is present suggesting that gliomas may be good candidates for anti-angiogenic therapies. However, emerging data suggest that the angiogenesis-dependency of glioma is not as strict as in other tumor types. This is mainly due to the very high density of pre-existent blood vessels in the brain which may be co-opted by tumor cells. Still, the improved progression free survival seen with anti-angiogenic therapies in other tumor types, together with a disturbing lack of other treatment options has resulted in implementation of bevacizumab and sunitinib, in combination with chemotherapy, in phase II clinical trials for glioma. Interim analyses of these trials suggest beneficial effects based on decreased tumor volume on CE-MRI. However, as we describe here and showed previously, anti-angiogenic treatment (bevacizumab, sunitinib and/or vandetanib) resulted in restoration of the disrupted BBB and therefore in reduced vessel leakage, a phenomenon that leads to overestimation of response to treatment on Gd-DTPA-enhanced MRI. It is therefore important to take this knowledge into account when evaluating treatment response based on standard CE-MRI. In previous work, we showed that closure of the BBB may have an antagonizing effect on the distribution of the alkylating agent temozolomide to the tumor cells. Since the current data show that also other VEGFR/PDGFR-targeting compounds close the BBB, antagonism of chemotherapies could also be anticipated if the present preclinical observations translate to the clinic. Yet, it has to be realized that restoration of the BBB in response to anti-VEGF therapies also gives a clear clinical benefit since edema, a very aggravating and possibly life-threatening complication of leaky vasculature in brain tumors, is alleviated. Clinically, this may be misinterpreted as tumor regression and this effect may also account for a seemingly improved progression-free survival.

Anti-angiogenic therapies in brain tumors do not result in tumor regression but rather induce a shift from angiogenic to infiltrative, but progressive growth. We show here that also monotherapy with bevacizumab and sunitinib potently inhibits angiogenesis in the compact growing components in gliomas which is reflected in an increase in hypoxia in angiogenic tumor areas and complete inhibition of microvascular proliferations. The therapies however do not affect progression of the diffuse tumor areas. This progression may be entirely attributed to co-option of pre-existing brain vessels as a mechanism of escape from anti-angiogenic therapy. Furthermore, there was no additional benefit of targeting both VEGF and PDGF receptors, even with combinations of tyrosine kinase inhibitors in this study. Whereas PDGFR inhibition is effective in preventing the maturation process during later stages of angiogenesis, it does not affect vasculature in diffuse
growth aspects in our tumor model. This provides further evidence that these co-opted vessels are pre-existing brain vessels and not newly formed.

Being an antibody, bevacizumab is unlikely to be transferred over the BBB. We show here that bevacizumab can expedite its anti-angiogenic effect on the angiogenesis-dependent areas in the orthotopic E98 GBM model and that it reduces vessel leakage. Probably, bevacizumab can leak into the tumor via hyperpermeable blood vessels and neutralize tumor-derived VEGF-A before it can act on the endothelium.

Two recent clinical studies using bevacizumab or cediranib (AZD2171, AstraZeneca) revealed that contrast-enhancement was reduced in half of the patients with recurrent GBM.\textsuperscript{11,201} However, in the cediranib study, the observed radiographic responses did not translate into prolonged survival of the patients. The difficulties in obtaining tumor material from such patients to perform histopathology make it very hard to determine whether radiographic reduction is the result of true tumor regression or whether therapeutic efficacy is overestimated due to restoration of the BBB.

In conclusion, our data show that the incorporation of pre-existing brain vasculature in the diffuse infiltrative growth phenotype in high-grade brain tumors prohibits effective curative therapy using approaches targeting VEGF, but suggest that anti-angiogenic therapies may have palliative effects.
Chapter 5

Anti-angiogenic compounds interfere with chemotherapy of brain tumors due to vessel normalization

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Abstract

Glioblastomas are highly aggressive primary brain tumors. Curative treatment by surgery and radiotherapy is generally impossible, due to the presence of diffusely infiltrating tumor cells. Furthermore, the blood brain barrier in infiltrative tumor areas is largely intact, and this hampers chemotherapy too. The occurrence of angiogenesis in these tumors makes these tumors attractive candidates for anti-angiogenic therapies. Because anti-angiogenic compounds have been shown to synergize with chemotherapeutic compounds in other tumor types, based on vessel normalization, there is a tendency towards such combination therapies for primary brain tumors also. However, vessel normalization in brain may result in restoration of the blood brain barrier with consequences for the efficacy of chemotherapeutic agents. In this study we investigated this hypothesis.

Balb/c nude mice with intracerebral xenografts of the human glioblastoma lines E98 or U87 were subjected to therapy with different dosages of vandetanib (an angiogenesis inhibitor), temozolomide (TMZ, a DNA-alkylating agent) or a combination (n>8 in each group).

Vandetanib selectively inhibited angiogenic growth aspects of glioma and restored the blood brain barrier. It did not notably affect diffuse infiltrative growth and survival. Furthermore, vandetanib antagonized the effects of TMZ, presumably by restoration of the blood brain barrier and obstruction of chemo-distribution to tumor cells.

The tumor micro-environment is an extremely important determinant for the response to anti-angiogenic therapy. Particularly in brain, anti-angiogenic compounds may have adverse effects when combined with chemotherapy. Thus, use of such compounds in neuro-oncology should be reconsidered.
Introduction

Growth of solid tumors depends on angiogenesis for sufficient supply of oxygen and nutrients and disposal of waste products. The realization that inhibition of angiogenesis may represent an effective anti-tumor therapy has elicited an enormous amount of research which resulted in an unravelling of the molecular determinants of angiogenesis. The most important angiogenic factor, Vascular Endothelial Growth Factor-A (VEGF-A), and its mode of action have since then become center of attention for the development of anti-angiogenic compounds. An example is bevacizumab (Avastin, Genentech), a neutralizing anti-VEGF-A antibody and the first FDA-approved anti-angiogenic compound. Additionally, a number of small compound tyrosine kinase inhibitors with specificity towards angiogenic receptors has been developed and some of these are entering clinical trials now.

VEGF-inhibition indeed results in potent anti-tumor effects in a variety of tumor xenograft models in nude mice. The results of monotherapy with angiogenesis inhibitors in clinical trials, however, have been disappointing so far. Treatment of patients with metastatic renal cancer did not result in prolonged survival, although a delay in time to progression was observed. Yet, anti-angiogenic compounds do have beneficial effects when combined with chemotherapy, possibly due to normalization of the tumor vasculature.

We previously reported on the effects of vandetanib (ZD6474, ZACTIMA™), a tyrosine kinase inhibitor with specificity towards VEGFR2, Epidermal Growth Factor Receptor (EGFR) and Rearranged during Transfection (RET), on tumor growth in brain. Vandetanib treatment of mice carrying intracerebral angiogenic melanoma metastases resulted in efficient inhibition of angiogenesis, but not in tumor regression. Instead, tumors progressed via growth along pre-existent brain vessels (vessel co-option), a phenomenon which has also been observed by others. This change of phenotype was accompanied by vessel normalization and restoration of the blood brain barrier (BBB), resulting in the inability to detect these tumors via contrast-enhanced Magnetic Resonance Imaging (MRI). Lowering of the vandetanib dose resulted in inhibition of angiogenesis, while the BBB remained disrupted by the action of tumor-derived VEGF-A.

Glioblastoma multiforme (GBM) is the most frequent and most malignant primary brain tumor. One of the hallmarks of GBM is the occurrence of regions of sprouting angiogenesis and florid microvascular proliferations, but large areas generally exist in which tumor cells grow in a diffuse infiltrative manner. These regions are often missed in conventional Gd-DTPA-enhanced MRI, indicating that the incorporated tumor blood vessels have an intact BBB. The diffuse infiltrative growth aspects make curative surgery...
and/or radiotherapy impossible. Presumably due to the BBB, susceptibility to chemotherapy is also generally poor, despite the introduction of the alkylating agent temozolomide (TMZ).\textsuperscript{97,207,259} The occurrence of regions of angiogenesis in GBM\textsuperscript{277} has led to the idea that these tumors may be candidate for anti-angiogenic therapy. Based on results from clinical trials with other tumor types, there is now a tendency to combine anti-angiogenic and chemotherapeutic compounds also for treatment of GBM patients.

We hypothesized that vessel normalization in brain tumors in response to anti-angiogenic therapy might adversely affect biodistribution of chemotherapeutic agents across the brain capillaries to the tumor cells. We tested this in two orthotopic glioma xenograft models. Our data indeed show that anti-VEGF therapy should be used with caution in patients with glial tumors as normalization of blood vessels in these neoplasms may have an adverse effect on chemotherapeutic efficacy.
Materials and Methods

Animal tumor models
All experiments were approved by the Animal Experimental Committee of the Radboud University Nijmegen Medical Centre. Balb/c nude mice (6-8 weeks old) were kept under specific pathogen free conditions and received food and water ad libitum. E98 is an in house developed xenograft model. E98 tumors were maintained in BALB/c nude mice by grafting 8 mm³ tumor fragments subcutaneously in the flank. A nude mouse carrying a subcutaneous E98 xenograft was sacrificed, the tumor was removed under sterile conditions, minced to small pieces using a sterile scalpel and a tumor cell suspension was prepared by gently filtering the homogenate through a 70 µm mesh filter. Twenty µl of this cell suspension was transcranially injected to a depth of 3 mm measured from the skin in 6-8 week old anaesthetized BALB/c nude mice (1.3% isoflurane in N₂O/O₂). This procedure reproducibly results in extensive tumor growth 24-30 days after injection. U87 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s modified medium (DMEM), supplemented with 10% fetal calf serum. Cells were trypsinized, washed in serum-containing medium, and resuspended in phosphate buffered saline at 5x10⁵ cells/ml. Twenty µl of this cell suspension was injected transcranially as described for E98. Subcutaneous U87 tumors were grown by injection of 2x10⁶ cells in 200 µl PBS.

Treatment protocols
To test the susceptibility of E98 and U87 xenografts to temozolomide (TMZ) under conditions without BBB, we first treated mice carrying subcutaneous tumors (n=3 for each tumor line). Treatment started when tumors reached a volume of approximately 100 mm³ at day 23 post tumor implantation. TMZ (32 mg/kg, kindly provided by Robert Bishop, Schering-Plough, Kenilworth, NJ, USA) in 10% DMSO/0.9% NaCl was administered via intraperitoneal injection on day 23, 27, 30, 32 and 34 post tumor implantation. A control group (n=3) received 10% DMSO/0.9% NaCl only. Tumor volumes were measured twice weekly with callipers, calculated as hwxwxd and expressed relative to the tumor volume at the start of therapy (set at 100%). After completion of the therapy, mice were sacrificed and analysed histologically for presence of tumor.

For treatment of mice carrying intracerebral E98 and U87 xenografts, groups were formed that were subjected to different treatment schemes. Groups V (n=14 for U87, n=8 for E98) received vandetanib only (25, 50 or 100 mg/kg, orally once daily in 100µl 0.5% Tween-80, ZD6474, Zactima™, kindly provided by Andy Ryan, AstraZeneca, Macclesfield, UK) starting on day 9 after tumor inoculation. Groups T (n=3 for U87, n=12 for E98) received one cycle of TMZ (32 mg/kg in 10% DMSO/0.9% NaCl via i.p. injection at day 12, 14, 16, 19 and 21), groups P (n=7 for U87, n=11 for E98) received placebo (i.p. injection of 10% DMSO/0.9%
NaCl). Groups VT (n=8 for U87, n=13 for E98) received the combination therapy (25, 50 or 100 mg/kg vandetanib starting at day 9 after tumor inoculation, followed by i.p. injection of 32 mg/kg TMZ in 10% DMSO/0.9% NaCl at days 12, 14, 16, 19 and 21). Throughout the experiment, mice were monitored closely for development of tumor-related symptoms. Symptomatic mice were sacrificed and brains removed. In some vandetanib-treated mice, contrast-enhanced MRI of the brain was performed before sacrifice. Statistical analyses were performed with a two-sided Fisher’s test.

Magnetic resonance imaging
To test the status of the BBB in intracerebral U87 and E98 xenografts, we performed Gd-DTPA MRI on tumor bearing mice as described previously. In short, animals were anaesthetized using 1.3% isoflurane in N2O/O2. A 12 mm diameter transmit/receive coil was placed over the skull and imaging was performed in a 7T/200 mm horizontal-bore MR spectrometer interfaced to a SMIS console and equipped with a gradient insert (gradient strength = 150 mT/m). After recording scout images, sixteen T1 weighted coronal images (T1s = 8 ms; TR = 100 ms; flip angle = 90°; number of averages = 1; field of view = 25x25 mm; matrix size = 256x256; slice thickness = 1 mm) were acquired before and 2 minutes after intravenous injection of Gd-DTPA (Magnevist®, Schering, Germany) and analysed using SMIS software.

Immunohistochemistry
Formalin-fixed mouse brains were cut in six coronal slices of approximately 2 mm and paraffin embedded. Sections of 4 µm were subjected to H&E staining or immunohistochemical stainings using antibodies against human vimentin (Vim 3B4, DakoCytomation, Glostrup, Denmark) to highlight tumor cells, CD34 (MEC14.7, Hycult Biotechnology bv, Uden, The Netherlands) to detect mouse endothelial cells and alpha-smooth muscle actin (αSMA, Sigma, Zwijndrecht, The Netherlands) to highlight pericytes. Immunohistochemistry for Ki-67 was performed to calculate the proliferation index, Glut-1 (DakoCytomation) immunohistochemistry was performed to highlight brain vasculature with an intact BBB, as well as hypoxic tumor cells. KS 400 software (3.0 Zeiss) was used to calculate relative hypoxia in tumor sections. In cases where no tumor was detected on H&E, and vimentin-stainings yielded inconclusive results, sections were subjected to in situ hybridisation with a probe specific for human centromer 1. Deeper sections of the paraffin blocks were prepared and stained to reduce the chance of false negative results.

TUNEL assay
To determine the amount of apoptosis in the tumor sections, TUNEL analysis (Terminal deoxynucleotidyl Transf erase Biotin-dUTP Nick End Labeling) was performed using the ApopTag Plus Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) according to the manufacturer’s instructions. KS 400 software (3.0 Zeiss) was used to
quantify the amount of apoptosis. The apoptotic rate was defined as percentage of apoptotic tumor cells. Student T-tests were performed for statistical analyses.
Susceptibility of subcutaneous E98 and U87 xenografts to temozolomide

Susceptibility of glioma cells to TMZ is largely determined by the methylation status of MGMT promoter. We previously assessed that the MGMT promoter in both U87 and E98 cells is hypermethylated (unpublished results). To test whether this indeed translates into susceptibility to TMZ, we first treated mice with established subcutaneous E98 and U87 tumors with one cycle of TMZ therapy, consisting of five intraperitoneal injections with 32 mg/kg TMZ on days 23, 27, 30, 32 and 34 post tumor implantation. Already 4 days after the first TMZ administration, a dramatic reduction in tumor volume was observed in both U87 and E98 xenografts whereas tumors in placebo treated mice grew to large volumes (Figure 1A and B). After five TMZ injections, E98 tumors had completely regressed (no remnants of tumor were detected both macroscopically and via histological analysis of the site of tumor injection, not shown) whereas U87 tumors stabilized. Thus subcutaneous E98 xenografts showed a complete and U87 xenografts a partial response to TMZ using this treatment protocol.
Figure 1. Growth of subcutaneous E98 (A) and U87 (B) xenografts and response to TMZ. Upon implantation of small tumor fragments, xenografts were allowed to grow for 23 days prior to start of TMZ therapy or placebo. Note the rapid response to TMZ. The X-axes represents days after tumor injection, the Y-axes displays relative tumor volumes, where the volume on the first day of treatment is set at 100%.

Morphology of intracerebral tumor xenografts

Upon intracerebral injection, U87 grew to large, circumscribed tumors with high vessel densities, as previously described. This phenotype is not representative of human GBM which typically grows with very heterogeneous phenotypes, i.e. areas of angiogenesis and large areas of diffuse infiltrative growth. This heterogeneity is also observed in E98 xenografts: these reproducibly present with extensive diffuse infiltrative growth along white matter tracts, especially in the corpus callosum, perivascular growth and compact growth (Figure 2A). The compact tumor component, which is most prominent in the ventricles (arrowhead in Figure 2A) characteristically contains hotspots of activated vessels resembling glomeruloid-like microvascular proliferations, as observed with anti-CD34 immunohistochemistry (Figure 2B,C).
Figure 2. Intracerebral growth pattern of E98 xenografts. H&E staining (A) and CD34 staining (B,C) of an intracerebral E98 tumor. Tumors present with extensive diffuse infiltrative growth along white matter tracts (A, arrow) and compact growth in the ventricles (A, arrowhead). In this latter component, activated vessels with strong CD34-positivity resembling the glomeruloid-like microvascular proliferations are present (B,C). Original magnifications: A: x25, B: x100, C: x400.

Blood brain barrier in orthotopic glioma xenografts
In intracerebral E98 xenografts, the BBB is disrupted as can be concluded from the fact that these tumors are readily detected in Gd-DTPA-enhanced MRI (Figure 3A-B). Interestingly, leakage in the diffuse infiltrative parts of E98 tumors was always lower than in the compact tumor parts (Figure 3B, arrow and arrowhead respectively). This difference may be explained by variations in VEGF-A expression, as was revealed by mRNA in situ hybridisation: VEGF-A expression co-localized with hypoxia in the compact tumor areas, whereas it was undetectable in the diffuse infiltrative parts (data not shown). We described before that vandetanib treatment results in closure of the BBB in cerebral Mel57-VEGF-A165 lesions. To examine whether this is also true in the E98 model, we performed MRI on animals with intracerebral E98 tumors that were treated with vandetanib only. The results are depicted in figure 3D and 3E and clearly show that, also in this model, vandetanib restores the functionality of the BBB. In intracerebral U87 tumors similar results were obtained. Gd-DTPA MRI showed extensive leakage of the tumor vessels, which was completely annihilated by treatment with 100 mg/kg vandetanib.45 Thus, high concentrations of vandetanib restore a previously disrupted BBB in tumor vessels.
Response of intracerebral glioma xenografts to vandetanib

The high density of activated vasculature in U87 and areas in E98 xenografts would predict a good response to vandetanib. Indeed, vessel densities decreased dramatically in U87 xenografts, but this affected tumor size only moderately, presumably because in the brain parenchyma tumor cells could progress via co-option of pre-existing vasculature, as described before.\textsuperscript{147} The lower vessel density in treated U87 xenografts resulted in a higher ratio of apoptotic cells (67 +/-20 apoptotic cells/mm\textsuperscript{2} vs 38 +/-13 /mm\textsuperscript{2} in placebo controls, p=0.03, Figure 4).

In E98 xenografts, only the compact regions responded in a dose-dependent manner to vandetanib treatment, as reflected in a smaller size and a higher percentage of hypoxic tumor cells (Figure 5D, and representative stainings for the hypoxia marker Glut-1 in Figure 5A-C). Importantly, the diffuse-infiltrative tumor areas were not notably affected by different dosages of vandetanib treatment (see arrow in Figure 5C).

Survival of both U87 and E98 carrying mice was not significantly prolonged by treatment with vandetanib.

Figure 3. Representative T1-weighted MR images of placebo-treated (A-C) or 100 mg/kg vandetanib-treated (D-F) intracerebral E98 xenografts. A and D represent pre-contrast images, B and E are images, recorded 2 minutes after intravenous injection of Gd-DTPA. Tumor vessels are leaky in placebo-treated mice as is clear from the Gd-DTPA-enhanced image. Leakage in the diffuse infiltrative parts (B, arrow) of E98 tumors was always lower than in the compact tumor parts (B, arrowhead). Vandetanib-induced restoration of the BBB precludes extravasation of Gd-DTPA from tumor vessels in vandetanib-treated mice (E). Panels C and F show H&E stainings of corresponding brain slices. Original magnifications: x12 (C,F)
Figure 4: Number of apoptotic cells in tumor. Effects of vandetanib and TMZ on apoptotic index of U87 xenografts, measured by TUNEL staining (*: p = 0.03; **: p=0.004; ***: p=0.0007). P: placebo, V: vandetanib-treated, VT: combination of vandetanib and TMZ, T: TMZ.

Figure 5. Vandetanib treatment of intracerebral E98 xenografts. Glut-1 staining of placebo-treated (A), low dose vandetanib-treated (25 mg/kg, B) and high dose vandetanib-treated (100 mg/kg, C) E98 carrying mice. Glut-1 staining shows a dose-dependent increase in hypoxia in the compact ventricular tumor areas (arrowheads), whereas no such staining could be detected in the diffuse infiltrative component (arrows). This increase is also shown in the graph (D) where the amount of hypoxia is given for the diffuse infiltrative and compact ventricular areas. Original magnifications: x12.
Response of intracerebral glioma xenografts to TMZ

The leakiness of vessels in E98 and U87 xenografts predicts good penetration and accessibility to tumor cells of chemotherapeutic drugs, irrespective of its ability to be transported across an intact BBB. In line with the results of the subcutaneous xenografts, mice carrying intracerebral E98 tumors responded very well to 32 mg/kg TMZ treatment. Whereas untreated mice had to be sacrificed on day 24 (range 21-30) after tumor inoculation due to severe tumor-related symptoms (cachexia, weight loss), mice in the TMZ-group appeared healthy. Brains of six mice were subjected to (immuno)histological analysis at day 65. No tumor cells could be detected in these brains, even after in situ hybridisation using a human chromosome 1 centromer probe, and after repeating histological analyses at three deeper levels of the paraffin blocks. Brains of the six remaining mice were analysed at day 83, and small tumors were observed in four mice, suggesting that one cycle of 32 mg/kg TMZ therapy still allowed escape of tumor cells in these mice (Figure 6). We did not examine here whether this escape could be prevented with additional cycles of TMZ therapy.

Because U87 was already identified as a partial responder, the almost absolute chemoresponse to TMZ of E98 was not seen. Still, there was a tendency towards smaller tumors upon TMZ treatment. Because TMZ-induced DNA damage forces cells into apoptosis, we performed TUNEL analysis on brains of treated and non-treated mice. A significant induction of apoptosis was seen as a result of TMZ treatment (137 +/-22 apoptotic cells/mm² in TMZ-treated mice vs 38 +/-13 in placebo-treated mice (p=0.004), Figure 4). Consistent with this finding, the proliferation index, determined by Ki-67 staining, was significantly lower in treated mice (40% as compared to 90% in control, p<0.0001). In line with these findings, at the time of sacrifice mice appeared to be more healthy than the controls. Because we decided to perform time-matched experiments with the U87 xenografts, we did not generate survival curves.
Combination therapies of TMZ and vandetanib

_E98_ - Survival in the VT groups was significantly prolonged as compared to the placebo groups (more than 2 months). Although animals in the VT groups were treated with three different concentrations of vandetanib, no significant dose-dependent differences were observed. Therefore, we considered all VT mice as one group. Although animals appeared healthy, microscopic evaluation of the brains revealed presence of tumor in 80% of all mice (Figure 6).

_U87_ – Mice that received the VT combination appeared healthy on day 20 after tumor inoculation whereas at that time placebo mice had to be sacrificed due to tumor-related symptoms. Yet, histological evaluation revealed presence of small tumors in all VT mice (Figure 6) with low proliferation indices (40% as compared to 90% in placebo controls, p<0.001). In the VT group, the apoptotic index was significantly lower than in the T group (Figure 4, p=0.0007), suggesting an inhibitory effect of vandetanib on TMZ-induced apoptosis. As time-matched experiments were performed, we cannot comment on survival of treated mice.

Figure 6. Percentage of tumor-bearing mice in different treatment groups in both E98 and U87 (*: p=0.015). P: placebo, V: vandetanib-treated, VT: combination of vandetanib and TMZ, T: TMZ.
Discussion

Treatment of high-grade gliomas is still troublesome. The extensive diffusely infiltrative growth makes curative surgery and/or radiotherapy virtually impossible. An additional complication is that radiological imaging mostly fails to properly delineate tumor margins because blood vessels in the infiltrative parts generally display an intact BBB, precluding extravasation of MR contrast agents.\textsuperscript{144} TMZ is currently the chemotherapy of choice for high-grade astrocytomas and oligodendroglomas, adjuvant to surgery and/or radiotherapy.\textsuperscript{51,259} However, curative treatment is still exceptional. One of the hallmarks of high-grade astrocytomas is angiogenesis.\textsuperscript{277} Adjuvant anti-angiogenic therapies are therefore considered to be potentially beneficial for the treatment of these tumors, although it is clear that anti-angiogenesis alone will probably not suffice to eradicate diffusely growing areas. Our present results confirm this: although angiogenesis is very effectively inhibited by vandetanib, tumor cells in both xenograft models are still able to progress in the brain parenchyma in an angiogenesis-independent fashion. Yet, tumor vessel densities in U87 tumors and in the remaining compact areas of E98 were significantly lower than in control treated animals, hypoxia was significantly increased and vessel leakage was annihilated.

Based on the results of clinical trials with other tumor types,\textsuperscript{107} it is postulated that this reduction of vessel leakage and concomitant reduction of interstitial fluid pressure (referred to as vessel normalization) will improve biodistribution of cytotoxic compounds to tumor cells.\textsuperscript{110} We show here that in brain, vessel normalization has an antagonizing, rather than a synergistic or additive effect. E98 glioma xenografts in mice brain are very sensitive to TMZ and are essentially eliminated by this therapy whereas this sensitivity is markedly reduced by co-treatment with vandetanib.

Because U87 xenografts are only partially susceptible to TMZ (apparent from treatment of subcutaneous tumors), antagonism of vandetanib and TMZ did not translate in an absolute increase in tumor burden. However, TMZ-induced apoptosis was significantly reduced in tumors which were co-treated with vandetanib. Obviously, solid proof for our hypothesis would require that TMZ concentrations are measured in tumor tissues of the different treatment groups, but our studies did not allow such analyses.

In TMZ-treated patients with malignant gliomas, the agent is found in the cerebrospinal fluid (CSF), suggesting that TMZ passes the BBB.\textsuperscript{184} However, in these patients it cannot be excluded that TMZ leaks to the CSF via leaky tumor vasculature. As our results show that TMZ is more active in the absence of a functional BBB, we hypothesize that transport of this compound over an intact BBB is less efficient than transport through leaky tumor vessels. Interestingly, a recent pharmacokinetic study revealed that co-treatment with the
anti-angiogenic compound TNP-470 led to reduced uptake of TMZ in intracerebral glioma xenografts.\textsuperscript{158}

Combination trials of anti-angiogenic compounds with TMZ and/or radiotherapy are now being performed in human glioma patients. The combination of thalidomide and TMZ is currently in phase I/II, and similar trials are ongoing in which TMZ is combined with PTK787, a tyrosine kinase inhibitor with activity against VEGFR2 and Platelet Derived Growth Factor Receptor (PDGFR, EORTC phase I/II study 26041). To our knowledge, it is not known whether thalidomide and/or PTK787 also have BBB restoring capabilities. Since increased vessel permeability is a VEGFR2 effect,\textsuperscript{145} it is reasonable to assume that PTK787 will have similar effects as vandetanib. Indeed, it has recently been shown that AZD2171, also an inhibitor of VEGF receptors and PDGF receptors, normalizes tumor vessels in glioblastoma patients.\textsuperscript{31} It will therefore be extremely important when recruiting patients for such trials, to take into account specific information on the tumor vessel permeability.

In a previous study we reported on the effects of vandetanib on Mel57 melanoma cells that constitutively secreted high amounts of VEGF-A\textsuperscript{147} and showed that lower doses of vandetanib effectively inhibited angiogenesis while leaving the BBB disrupted.\textsuperscript{147} Thus, it might be argued that low doses may be more effective in combination therapies than high doses. In the E98 model we found, however, that both low and high dose vandetanib were equipotent in inhibiting TMZ activity. Since VEGF-A expression in E98 xenografts is only limited (restricted to centrally located hypoxic tumor cells in the expansive tumor regions), it is likely that lower concentrations of vandetanib are required to fully silence VEGF-A effects than in the Mel57-VEGF-A tumor model.

In conclusion, our results suggest that normalization of tumor blood vessels, which occurs in response to anti-angiogenic therapy and which results in beneficial effects when combined with chemotherapy in patients with advanced colorectal or renal tumors, may have an adverse effect on the efficacy of chemotherapeutic compounds in brain. It will therefore be crucial to rationally design tailor-made treatment protocols based on the levels of VEGF-A produced by tumor cells. The amount of tumor vessel leakiness, which may be deduced from radiological imaging, may provide a clue to the dosing of anti-angiogenic compounds.
Discussion: Concerns about anti-angiogenic therapies in high-grade gliomas
Diffuse gliomas are the most frequent primary tumors of the Central Nervous System (CNS) and are characterized by diffuse infiltrative dispersion throughout the brain parenchyma. This unique phenotype has important diagnostic, prognostic, and therapeutic implications. Partly because of this diffuse infiltration, gliomas cannot be eradicated by current treatment modalities and are notorious for their short survival in many patients.

Unraveling the mechanisms that allow glioma cells to diffusely infiltrate in the neuropil may provide novel therapeutic targets for recognizing, attacking, and killing these cells. Investigations using glioma models have already provided a wealth of information on the biological mechanisms responsible for glioma cell migration. However, many of these experiments were performed in in vitro and in vivo models, e.g. subcutaneous xenograft models, that poorly recapitulate the interactions of human glioma cells with the brain microenvironment. For example, the blood-brain barrier (BBB) is not present in subcutaneous tumor models and also the typical diffuse infiltrative growth of gliomas is lacking. Invasion studies in vitro are mostly performed in Matrigel. However, Matrigel mainly consists of collagen and laminin, two extracellular matrix (ECM) proteins that are largely restricted to the vessel walls in the brain. Selection of proper experimental models for preclinical studies is of utmost importance for successful translation of novel therapeutic modalities to the clinic. We tested several mouse models of glioma, varying from existing glioma cell lines to newly established xenograft models (Chapter 2). Intracerebral inoculation of tumor cells predominantly produces tumors with a compact phenotype. Only the E98 model, and to a lesser degree the E106 model, reproducibly gave rise to tumors with both diffuse infiltrative and compact areas. By direct inoculation of fresh, surgically removed human glioma cells in the brain of nude mice we established four models which consistently show extensive diffuse infiltration throughout the brain (E434, E468, E473 and E478). In addition, the tumor cells in these models carry typical chromosomal aberrations (-1p/-19q in anaplastic oligodendroglioma, +7/-10 in glioblastoma (GBM)). Interestingly, so far only few oligodendrogial tumor models are available. Together with the E98 model, we now have a panel of glioma models representing adequate geno- and phenocopies of human high-grade gliomas. These models can be used to investigate different therapeutic approaches in a preclinical setting.

The difference in growth pattern of glioma xenografts in brain versus the subcutaneous space underlines that interaction with the tumor microenvironment is a very important determinant for tumor phenotype. Tumor cells inoculated in the brain encounter highly vascularised tissue where they can grow by use of the pre-existent blood vessels without the need of angiogenesis. However, tumor cells inoculated in the subcutaneous space are obliged to induce angiogenesis in order to survive and grow beyond a certain volume. In subcutaneous tumors that grow fast in an avascular space, blood vessel growth is more or
Discussion

less synchronized, this in contrast to clinical brain tumors that grow more slowly even if they are highly malignant. Consequently, vessels in human brain tumors are more heterogeneous. Subcutaneous tumors from various origins (lung, prostate, breast, ovarian, colon, glioma or vulvar tumors) were shown to be very responsive to anti-angiogenic therapy and this initially led to high expectations for anti-angiogenic treatment of high-grade gliomas, also because microvascular proliferations are a hallmark of these latter neoplasms. However, in high-grade gliomas, angiogenic areas are present next to large diffuse infiltrative areas where intratumoral vessels may be incorporated rather than newly formed (= vessel co-option). The actual effect of anti-angiogenic therapy in the clinic remains to be established. Indeed, as we described for animal models of orthotopic glioma and metastatic melanoma in brain, vessel co-option may allow the tumor to escape from anti-angiogenic therapy (Chapter 4,5). We treated mice with intracerebral E98 lesions with the anti-angiogenic compound vandetanib (a tyrosine kinase inhibitor of VEGFR, EGFR and RET) and found that this compound increases the amount of hypoxia in the compact growing tumor areas, whereas the diffuse infiltrative areas are not notably affected (Chapter 3-5). Thus, the co-opted vessels in diffuse infiltrative tumor areas are refractory to anti-angiogenic treatment. Moreover, also bevacizumab (a monoclonal antibody against VEGF-A), sunitinib (a tyrosine kinase inhibitor directed against VEGFR and PDGF), and a combination of sunitinib and vandetanib increase hypoxia in compact tumor areas in orthotopic E98 lesions but do not have a clear effect on the diffuse infiltrative tumor areas (Chapter 4). Thus, also the additional inhibition of vessel maturation via targeting of PDGF receptors does not improve the efficacy of anti-angiogenic therapy in our E98 model.

The intact BBB in diffuse infiltrative glioma areas precludes extravasation of contrast agents like gadolinium diethylenetriaminepenta-acetic acid (Gd-DTPA), making proper delineation of gliomas using contrast-enhanced magnetic resonance imaging (CE-MRI) problematic. Anti-angiogenic treatments may complicate tumor detection as these compounds can restore the BBB in angiogenic regions. Indeed, vandetanib treatment of mice carrying U87 glioma lesions, Mel57 intracerebral metastatic melanoma lesions or E98 glioma lesions resulted in effective inhibition of angiogenesis, restoration of the BBB and reduced visibility of lesions in Gd-DTPA enhanced MRI-scans (Chapter 3,5). Bevacizumab, sunitinib and a combination of sunitinib and vandetanib caused the same reduced visibility on CE-MRI in angiogenic tumor areas in orthotopic E98 lesions (Chapter 4). These findings corroborate the hypothesis that anti-angiogenic compounds lead to restoration of the BBB. However, CE-MRI using ultrasmall particles of iron oxide (USPIO, Sinerem®) as blood pool contrast agent did allow for detection of these lesions in the brain of nude mice after anti-angiogenic therapy (Chapter 3). Using USPIO-enhanced scans, highly vascularized tumors are visible as hypointense lesions compared to the surrounding brain parenchyma, whereas after anti-angiogenic treatment, tumors are
visible as hyperintense lesions compared to the surrounding brain parenchyma because of the decreased microvessel density.

Also in GBM patients, anti-angiogenic treatment results in a reduced CE-MRI signal when using Gd-DTPA as contrast agent. However, it is unclear whether this reduction in contrast-enhancement represents a reduction in tumor volume. A positive effect of this restoration of the BBB is that reduction in vessel hyperpermeability leads to a decrease in interstitial pressure and edema. In the past, corticosteroids were used to alleviate the symptoms caused by edema. However, there is currently little doubt amongst clinicians that angiogenesis inhibitors are more effective than corticosteroids in reducing these symptoms and that these inhibitors reduce the need for corticosteroid treatment in recurrent GBM patients. Typically, several patients return to normal activities of daily living shortly after bevacizumab therapy, while being severely impaired before, even under corticosteroid therapy. This rapid “anti-edema” effect of anti-VEGF therapy thus provides a strong palliative benefit.

Restoration of the BBB may also have a negative effect, despite the initial benefit for the patients well-being, as it may hamper the delivery of chemotherapeutic drugs to the tumor cells. Temozolomide (TMZ) is currently the chemotherapy of choice for the treatment of high-grade gliomas, causing a modest increase in overall survival of two months when given in combination with radiotherapy. As combinations of anti-angiogenic agents with chemotherapeutics seem successful in other tumor types similar regimens are currently being tested in clinical trials in patients with a recurrent GBM. However, the BBB is normalized upon anti-angiogenic treatment. We found that in mice bearing orthotopic E98 or U87 tumors, treatment with TMZ alone was more effective than when TMZ was combined with anti-angiogenic treatment (Chapter 5). Chemotherapeutic drugs are generally not very effective against GBM, partly due to intrinsic resistance but probably also because the BBB is intact in the diffuse infiltrative parts of the tumor. Even TMZ, which is described to be able to cross the BBB to some extent, probably acts more effectively against tumor cells in more accessible angiogenic parts of the tumor. Whereas the strategy of combining anti-angiogenic and chemotherapeutic agents may work well in tumors outside the brain, in case of GBM, normalization of the tumor vascular bed and reduction of interstitial pressure comes at the expense of restored functionality of the BBB as evidenced by the reduced permeability for Gd-DTPA. The accompanying reduced accessibility of chemotherapeutic drugs holds the risk of antagonizing the efficacy of such agents. Thus, whether vessel normalization will result in better delivery of chemotherapeutic agents depends on specific features of the tumor-microenvironment. Our results strongly indicate that, especially for patients with brain tumors, anti-angiogenic therapy should be applied with caution.
Clinical anti-angiogenic trials for glioma patients

Despite potential caveats just discussed, anti-angiogenic therapy was recently introduced in the clinic for GBM. Several clinical trials are now ongoing in which patients with recurrent GBM are treated with a combination of chemotherapeutics and anti-angiogenic agents (mostly bevacizumab). Preliminary results of phase II trials at first seemed promising, with impressive improvements of median progression free survival (PFS) and 6 months progression free survival (PFS6). First non-randomized phase II trials conducted in recurrent GBM patients indicated high response rates and improvement of PFS6 from 20% with single agent TMZ, to 30% with single agent AZD2171 (Cediranib) or 35.1% with single agent bevacizumab. PFS6 further improved to 50.2% when bevacizumab was combined with chemotherapy. It is important to realize that these data are based on PFS, rather than overall survival, and that they have been obtained by taking overall survival of historical controls as reference group. The results of such non-randomized phase II studies should be interpreted with caution as they are notorious for overestimation of response rates by inevitable selection bias of patients. Consequently, to unequivocally assess efficacy of such treatment approaches, the outcome is needed of ongoing randomized controlled trials where angiogenesis inhibitors and appropriate control treatments are compared. Furthermore, PFS may not be a good endpoint for this type of study because absence of clinical symptoms and diminished radiological detection, brought about by bevacizumab, clearly is not synonymous with absence of tumor cell proliferation. A recent study, performed in collaboration with the Academic Medical Centre Amsterdam, in which patients with recurrent GBM are treated with bevacizumab in combination with TMZ, showed temporary decrease of hyperintense area on T2-weighed images, strong temporary decrease of contrast enhancement on Gd-enhanced MRI (Figure 1), temporary decrease of tumor perfusion and of permeability of tumor vessels. However, resection material of these tumors (taken at least 6 weeks after last infusion of bevacizumab), shows high numbers of tumor cells co-opting pre-existent blood vessels (Figure 2). During anti-angiogenic therapy, tumor cells thus indeed seem to be able to migrate furtively into the surrounding brain parenchyma using pre-existent vasculature (personal communication J.J. Verhoeff, W.R. van Furth).
Another fundamental problem with the assessment of the effect of angiogenesis inhibition in GBM is that next to PFS, response to therapy is evaluated by MRI using standard Macdonald Criteria or RECIST criteria. A decreased or stable Gd-DTPA contrast-enhanced area is regarded as evidence for tumor regression or stabilization. As discussed above, also in patients with GBM, the impressive decrease of contrast enhancement in these tumors upon bevacizumab treatment is not necessarily synonymous with anti-tumor effects, but may well be due to restoration of the BBB.
Figure 2. Resection material of recurrent GBM, after bevacizumab treatment. H&E staining (A,C), Glut-1 (B,D).

A,B: Resection material of recurrent GBM, 6 weeks after last infusion of bevacizumab. Tumor cells co-opt pre-existent vessels with relatively intact BBB (arrows). C,D: Recurrent GBM, autopsy 10 weeks after last infusion of bevacizumab. Tumor cells invade the brain parenchyma along white matter tracts. Original magnifications: x200 (A,B,C), x100 (D).
Future perspectives
Anti-angiogenic treatment of glioma patients should be implemented in the clinic with caution, since problems may arise because of the specific tumor-microenvironment. Restoration of the BBB by anti-angiogenic compounds may lead to overestimation of treatment effect and antagonism with chemotherapy. Despite the wide spread belief, not all tumors depend on angiogenesis for survival. Tumors arising in vessel-dense organs like the brain may grow via pre-existent blood vessels without inducing angiogenesis. The phenomenon of vessel co-option may apply not only to primary brain tumors, but also to metastatic brain tumors. GBM can theoretically be separated into a more compact growing, angiogenesis-dependent component and a less cellular, diffuse infiltrative component. This latter component is likely to be largely inert to anti-angiogenic therapy and to be of key importance for disease progression and survival. While symptoms caused by glioma are tempered by anti-angiogenic treatment, the tumor may progress furtively by invasion, at first unrecognized by standard imaging modalities. It will therefore be of huge importance

i. to identify the tumor types that are most suitable for anti-angiogenic therapy,
ii. to carefully select patients who will benefit from it based on amount of (functional) angiogenesis in the tumor,
iii. to investigate the optimal dosage and timing of anti-angiogenic therapy,
iv. to test the effect of anti-angiogenic compounds in combination with other, conventional therapeutic modalities (e.g. radiotherapy, chemotherapy) and,
v. to develop better tools for monitoring response to treatment.

Although angiogenesis inhibition has been shown to be of value for symptom reduction in GBM patients (reduction of edema/intracranial pressure), the possible lack of a true anti-tumor effect raises concerns about the place of this type of therapy in the treatment of GBM. Also other therapies currently used, e.g. surgery and chemoradiation, have only a modest effect on survival of patients with these tumors. New therapeutic approaches are urgently needed to improve therapy of glioma patients. As anti-angiogenic therapies do not have an effect on diffuse infiltrative tumor regions because the blood vessels are incorporated pre-existent blood vessels rather than newly formed, anti-vascular therapies, targeting all tumor associated blood vessels, may be an alternative option for the treatment of glioma patients. Such an anti-vascular approach is based on the hypothesis that tumor endothelial cells express a specific set of proteins. This could theoretically result in secondary tumor cell death caused by disruption of the blood supply to all tumor cells. An interesting candidate to target in this respect is Plexin D1 (PLXND1), a member of the plexin membrane protein family which bind to semaphorins. PLXND1 is present on some but not all tumor associated vessels. Interestingly, tumor cells also frequently express PLXND1, making this protein a potential candidate for simultaneous targeting of tumor vessels and tumor cells. However, as diffuse glioma cells blend in extensively in the normal brain parenchyma, also normal brain cells will be attacked by anti-vascular
therapy. It is therefore still unclear how much damage this approach will cause to the infiltrated brain parenchyma.

Since diffuse infiltration is a characteristic shared by both low- and high-grade gliomas, interference with glioma cell motility and invasion may be exploited as a novel therapeutic approach for all diffuse gliomas. Experimental, mostly preclinical, studies are ongoing which target different aspects of glioma cell migration. It is important to realize that the unique tumor-microenvironment of gliomas is not recapitulated in most of the pre-clinical model systems and therefore, it is not yet clear whether these results can be extrapolated to the human situation. However, a small number of compounds has already reached the clinic and are currently being tested for the treatment of glioma patients. A selection of these potentially efficient anti-invasive compounds is discussed below.

Glioma cells can create their own microenvironment by synthesizing and depositing ECM molecules such as tenascin-C. Increased expression of tenascin-c has been shown to be correlate with higher glioma malignancy grade. In a phase II clinical trial, injection of the anti-tenascin antibody 131I-m81C6 (44 Gy) in the surgically created resection cavity of patients with recurrent malignant glioma followed by standardized chemotherapy resulted in prolonged median survival compared to that of historical controls treated with surgery plus 125I brachytherapy. The integrin family of calcium-dependent, transmembrane molecules is considered to play a major role in glioma cell-ECM adhesion. Integrins consist of a non-covalently linked α and β subunit and several of these αv integrins were described to be overexpressed in glioma where their activation may lead to cytoskeletal rearrangements and cell movement. In preclinical trials using orthotopic U87 glioma lesions in nude mice, αv integrin inhibitor EMD 121974 (cilengitide, cyclo Arg-Gly-Asp-D-Phe-(N-methyl)-Val, a cyclic RGD pentapeptide) was described to induce anoikis (apoptosis supposedly induced by detachment from the ECM) in angiogenic blood vessels and tumor cells. In a phase I trial, including 51 malignant glioma patients that were treated with this inhibitor, complete response was seen in two patients and partial response in three patients. Recently, a phase III trial was started to investigate clinical activity, safety, and tolerability of cilengitide in the treatment of first recurrence of GBM. Epidermal Growth Factor Receptor (EGFR), which is frequently overexpressed in GBM, can be targeted with EGFR kinase inhibitors like gefitinib and erlotinib or monoclonal antibodies directed against EGFR like cetuximab. In the first studies conducted, only a small number of the GBM patients treated with such inhibitors showed response (especially those in which the glioma cells co-expressed variant III of EGFR (EGFRvIII, with deletion of exons 2-7 resulting in auto-activation of the signaling pathway) and tumor suppressor gene PTEN. However, a recent phase I/II trial in which GBM patients were treated with erlotinib in combination with TMZ and radiotherapy showed no benefit of erlotinib treatment compared with TMZ controls. In
these patients, presence of EGFRvIII, p53, PTEN, combination of EGFR and PTEN, and EGFR amplification status was not predictive of survival.²⁹

Other novel treatment options for diffuse gliomas include EGFRvIII vaccinations, immunotherapy with dendritic cells, and specific delivery systems like convection-enhanced delivery (CED). Glioma patients can be vaccinated with dendritic cells that are loaded with tumor-associated peptides. Ideally such dendritic cells then stimulate a cytotoxic T-cell response against the tumor. In gliomas, this approach is often hampered by the large inter- and intratumoral antigenic heterogeneity and the lack of a universally expressed tumor antigen. EGFRvIII, a relatively common tumor-specific gene mutation in glioma cells may be a good candidate for immunotherapy. EGFRvIII vaccination was shown to be an efficacious immunotherapy in syngeneic murine models.²²⁰ In Phase II trials vaccines targeting EGFRvIII induced potent T- and B-cell immunity in newly diagnosed patients with high-grade gliomas (grade III or IV), and led to a prolonged survival. However, in many patients, EGFRvIII-negative tumors recurred, which highlights the need for targeting a broader repertoire of tumor-specific antigens.²²⁰ Alternatively, dendritic cells can be loaded with a cell lysate derived from the patient’s own glioma. Using this latter method, an overall prolonged median survival was found.³² As an increased number of immune-suppressive, regulatory T cells was detected in GBM,⁶⁰ interference with such cells also represents a potential way of immunotherapy of malignant gliomas.⁹² Reaching the diffusely infiltrating tumor cells behind the intact BBB remains a problem for the treatment of gliomas. By using convection-enhanced delivery (CED) different therapeutics (e.g. chemotherapeutics, endotoxins, radioisotopes, chimeric products) may reach diffuse infiltrative glioma cells in the brain parenchyma.¹⁷¹,¹⁷² With CED, one or more small-caliber catheters are placed through a burr hole into the target tissue under image guidance, and an infusate is actively pumped into the brain parenchyma. This infusate will then disperse through the interstitial space.²⁰⁶,²¹⁹,²⁶² Although the pre-clinical results are promising,¹³¹,¹⁸⁸,²⁷¹ it is clear that the positioning of the catheter is crucial for the success of this approach, and that the distribution of the infusate should be closely monitored during treatment.¹²¹

While these new approaches are attractive from a theoretical point of view, many more studies are needed to determine which are the most promising (combinations) of these. So far, glioma patients are unfortunately still far from being cured. In line with the statement (in 1987) of Peter Burger, one of the experts in the neuropathology of (glial) brain tumors, “Diffuse gliomas are unlikely to be cured by techniques that cannot selectively destroy the neoplastic cells”³⁰, future research should in our opinion focus on unraveling the mechanisms underlying diffuse glioma growth in order to find more specific targets for anti-glioma therapy.
Chapter 8

Summary

Nederlandse samenvatting
Summary

Diffuse gliomas are the most frequent primary CNS tumors encompassing different histological sub-types that are characterized by diffuse infiltrative dispersion throughout the brain parenchyma. This unique phenotype of diffuse gliomas has important diagnostic, prognostic, and therapeutic implications.

In Chapter 1, we discuss several characteristics of diffuse gliomas: clinical features, radiological consequences of diffuse infiltrative growth, histopathology, molecular mechanisms of diffuse infiltration, angiogenesis and anti-glioma treatments using anti-angiogenic therapies. The diffuse growth pattern is not just the result of malignant progression as both low- and high-grade diffuse gliomas display this phenomenon. Especially the high-grade lesions frequently show marked phenotypical heterogeneity with spatial differences in cellular phenotype and malignancy grade. The presence of necrosis, frequent mitoses and florid microvascular proliferation points to high-grade malignancy. Partly because of this diffuse infiltration, gliomas are notorious for their poor response to therapies and short survival. Current standard of care for glioblastoma multiforme (GBM), the most aggressive diffuse glioma, is surgical resection to maximal feasible extent, followed by radiotherapy and systemic temozolomide (TMZ) chemotherapy. However, even with such aggressive treatment, median survival after diagnosis is still only one to two years since the current treatments fail to eradicate tumors completely. In addition, the visualization of tumors using Magnetic Resonance Imaging (MRI) techniques is difficult due to the lack of neovascularization and the apparently limited changes to the pre-existent, incorporated vessels in the diffuse infiltrative tumor areas.

Chapter 2 describes several glioma animal models, varying from existing glioma cell lines to xenograft models we recently established. Unraveling the mechanisms that allow glioma cells to diffusely infiltrate in the neuropil may provide novel therapeutic targets for recognizing, attacking, and killing these cells. Investigations using glioma models have already provided a wealth of information on the biological mechanisms responsible for glioma cell migration. However, many of these experiments were performed in in vitro and in vivo models that poorly recapitulate the glioma cell-micro-environment interactions of human glioma cells in the brain micro-environment or that do not adequately represent the different genetic backgrounds of the subsets of human gliomas (e.g. oligodendrogial vs astrocytic tumors). The exact culture conditions may also have a major influence on the results obtained. We tested several glioma animal models, varying from existing glioma cell lines (U87, U373, Hs683, U251, U343-31L, U343-C126; U410) and human subcutaneous GBM xenograft lines (E34, E49, E98, E106) to newly established xenograft models (E434, E468, E473, E478). Inoculation of tumor cells intracerebrally in nude mice mostly gave rise to compact growing lesions. Only the E98 and, to a lesser
degree, E106 xenograft lines (propagated through subcutaneous growth) consistently produced intracerebral tumors displaying diffuse infiltrative growth in the brain parenchyma. By direct inoculation of human glioma cells in the brain of nude mice we established four models which consistently show extensive diffuse infiltration throughout the brain. In addition, the tumor cells in these models carry typical chromosomal aberrations (-1p/-19q in oligodendrogliaoma, +7/-10 in glioblastoma (GBM)). Together with the E98 model, we now have an attractive panel of glioma models representing adequate geno- and phenocopies of human gliomas. These models can be used to investigate different therapeutic approaches in a preclinical setting. Critical evaluation of the geno- and phenotype of glioma models before using them in glioma research is of utmost importance to extrapolate the results to the human situation.

**Chapter 3** deals with the problem of proper delineation of diffuse gliomas using contrast-enhanced (CE-)MRI. The blood-brain barrier (BBB) is intact in diffuse infiltrative areas and thereby precludes extravasation of contrast agents like gadolinium diethylenetriaminepenta-acetic acid (Gd-DTPA). As the efficacy of conventional methods to fight diffuse infiltrative glioma is limited, a more targeted approach is needed that takes advantage of the typical molecular features of these tumors. Anti-angiogenic therapy, an example of such targeted therapy, has since 10 years been considered as a promising approach for high-grade gliomas as these neoplasms often show a striking angiogenic phenotype. However, anti-angiogenic compounds may further complicate tumor detection as such compounds can restore the BBB in angiogenic regions. We treated mice with intracerebral glioma U87 lesions with the anti-angiogenic compound vandetanib, a tyrosine kinase inhibitor of vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and rearranged during transfection (RET). We found that this compound has a clear effect on the amount of hypoxia in the tumors, however, this is not immediately reflected by changes in tumor volume or proliferation index. Additionally, treatment restored the BBB and as a result tumors could not be visualized any longer using Gd-DTPA as contrast agent. We thus provided evidence that the BBB is closed upon anti-angiogenic treatment and showed that this hampers leakage of contrast agent in the tumor, thereby complicating radiological visualization of tumors. However, CE-MRI using ultrasmall particles of iron oxide (USPIO, Sinerem®) as blood pool contrast agent has additional value for detection of glioma in the brain of nude mice. With this contrast agent the decrease in microvessel density caused by anti-angiogenic therapy can be visualized.

In **Chapter 4**, the hypothesis that anti-angiogenic therapy can restore the BBB (formulated in Chapter 3) is studied further. Vandetanib treatment of mice carrying intracerebral E98 glioma lesions resulted in effective inhibition of angiogenesis, restoration of the BBB and reduced visibility of lesions in Gd-DTPA enhanced MRI-scans. Moreover, also the
monoclonal antibody against VEGF-A, bevacizumab, the tyrosine kinase inhibitor sunitinib directed against VEGFR and platelet-derived growth factor receptor (PDGFR), and a combination of sunitinib and vandetanib caused the same reduced visibility on CE-MRI and increased hypoxia in angiogenic tumor areas in orthotopic E98 lesions. Restoration of the BBB is thus a common result when applying various anti-angiogenic regimens in various animal models. Furthermore, additional inhibition of vessel maturation via targeting of PDGF receptors did not improve the therapeutic efficacy in our model.

In Chapter 5, we tested whether restoration of the BBB can hamper delivery of not only contrast agents such as Gd, but also of chemotherapeutic compounds like TMZ to the tumor. The DNA-alkylating agent TMZ is currently the chemotherapy of choice for the treatment of gliomas. Unfortunately, implementation of this compound in the treatment regimen of patients has resulted in only a modest increase in overall survival. Combinations of anti-angiogenic agents with chemotherapeutics seem successful in other tumor types like advanced colorectal cancer. It is postulated that in these tumors, angiogenic agents cause a reduction of vessel leakage and concomitant reduction of interstitial fluid pressure which improves biodistribution of cytotoxic compounds to tumor cells. Driven by the failure of conventional therapeutic approaches for diffuse gliomas, similar regimens are currently being tested in clinical trials in patients with recurrent GBM. However, as we showed before, the BBB is closed upon anti-angiogenic treatment. This not only complicates visualization of tumors, but more importantly, it potentially also hampers delivery of chemotherapeutic compounds to the tumor cells. Combining vandetanib with TMZ in the intracerebral E98 glioma model antagonized the effects of TMZ, presumably by restoration of the BBB. Also in the orthotopic U87 model, vandetanib antagonized the effects of TMZ, but as U87 xenografts are only partially susceptible to TMZ, antagonism of vandetanib and TMZ did not translate in an absolute increase in tumor burden. However, TMZ-induced apoptosis was significantly reduced in tumors which were co-treated with vandetanib. Vessel normalization in brain thus results in obstruction of chemo-distribution to the tumor cells. The tumor micro-environment is an extremely important determinant for the response to anti-angiogenic therapy. Particularly in brain, anti-angiogenic compounds may have adverse effects when combined with chemotherapy. However, as restoration of the BBB causes reduction in vessel hyperpermeability and thereby leads to a decrease in interstitial pressure and cerebral edema, anti-angiogenic therapy may at the same time provide a strong palliative benefit for GBM patients.

In this thesis we described several studies related to the treatment of diffuse gliomas. Chapter 6 contains concluding remarks and a critical evaluation of our findings compared to current ideas. GBMs can grossly be separated into a more compact, angiogenic and a diffuse infiltrative, non-angiogenic component. Whereas this latter component seems
inert to anti-angiogenic therapy, it is of key importance for disease progression and survival. While symptoms are tempered by anti-angiogenic treatment, disease may progress furtively by invasion, at first unrecognized by standard imaging modalities. This possible lack of a true anti-tumor effect raises concerns about the place of this type of therapeutics in the treatment of GBM. Moreover, inhibition of angiogenesis in GBM may antagonize the efficacy of chemotherapeutic drugs by normalizing the BBB function. Combination of anti-angiogenic compounds with chemotherapeutics for brain tumors should thus be applied with caution in the clinic. We show in this thesis that it strongly depends on the tumor growth pattern and microenvironment whether anti-angiogenic therapy will be effective. Especially in vessel-dense organs like the brain, tumors (including metastatic lesions) may grow via pre-existent blood vessels without inducing angiogenesis. It will therefore be of huge importance to identify the tumor types that are most suitable for anti-angiogenic therapy and to carefully select patients who will benefit from it based on amount of angiogenesis in the tumor. Additionally, there is an urgent need for better tools to monitor the effect of such therapy.

We conclude that diffuse infiltrative glioma cells are difficult to attack by both conventional therapeutic modalities and by anti-angiogenic therapy. Therefore, a more targeted therapy specifically directed against the diffusely infiltrating tumor cells is urgently needed. Future research focussing on unraveling the molecular mechanisms underlying the diffuse phenotype will hopefully provide new therapeutic approaches that specifically target the diffusely infiltrating glioma cells and that are able to reach these cells behind an intact BBB.
Nederlandse samenvatting

Diffuse gliomen zijn de meest frequente tumoren die ontstaan uit het hersenweefsel zelf. Deze groep tumoren kan verder onderscheid worden gemaakt in verschillende histologische subtypes die allen gekarakteriseerd zijn door vaak heel uitgebreide, diffuus infiltratieve groei in de hersenen. Dit unieke groeipatroon heeft belangrijke consequenties voor diagnose, prognose en therapie van patiënten met deze tumoren.

In Hoofdstuk 1 worden verschillende karakteristieken van diffuse gliomen besproken: klinisch beeld, consequenties voor radiologische detectie, histopathologie, moleculaire factoren belangrijk voor diffuus groei en mogelijke therapieën. Daarnaast wordt ook dieper ingegaan op angiogenese (vorming van nieuwe bloedvaten), een belangrijk kenmerk van hooggradige gliomen, en op mogelijk gebruik van anti-angiogene therapie bij gliomen. Diffuus groei is niet slechts het resultaat van maligne progressie aangezien zowel laaggradige als hooggradige tumoren dit groeipatroon tonen. De hooggradige gliomen kunnen daarnaast binnen een tumor ook grote fenotypische verschillen tonen met diffuus infiltratieve gebieden naast delen met uitgebreide necrose en floride microvasculaire proliferatie (MVP). Dit laatste is een bijzondere vorm van angiogenese, waarbij clusters van prolifererende capillaire bloedvaten dicht op elkaar gelegen zijn. Door de vaak heel uitgebreide diffuus infiltratieve groei in de hersenen kunnen gliomen bijna nooit geheel door de neurochirurg verwijderd worden. Ook met behulp van andere behandelingen zoals radiotherapie en chemotherapie zijn de tumorcellen meestal niet definitief uit te roeien. Momenteel worden patiënten met glioblastoma multiforme (GBM), het meest agressieve diffuse gloom, behandeld met een operatie om zo veel mogelijk tumorweefsel te verwijderen, gevolgd door radiotherapie en systemische temozolomide (TMZ) chemotherapie. Echter, ondanks deze agressieve behandeling is de gemiddelde overleving van deze patiënten na diagnose nog steeds slechts één tot twee jaar. Ook het precies in beeld brengen van deze tumoren met beeldvormende technieken als contrast-enhanced Magnetic Resonance Imaging (CE-MRI) is lastig. Met behulp van het contrastmiddel gadolinium diethyleentriaminepenta-acetaat (Gd-DTPA) kunnen tumoren aangetoond worden door de lekkage ervan uit kleke bloedvaten. Echter, in de infiltratieve gebieden van gliomen is de bloed-hersen barrière (blood-brain barrier, BBB) nog intact waardoor Gd-DTPA niet uit de vaten lekt en deze delen onzichtbaar blijven op standaard CE-MRI.

Hoofdstuk 2 beschrijft verschillende diermodellen die gebruikt worden bij het bestuderen van gliomen. Om nieuwe therapeutica te ontwikkelen en om meer informatie te verkrijgen over de diffuse gliomen zijn modelstudies die pre-klinisch onderzoek mogelijk maken essentieel. Echter, de typische diffuus infiltratieve groei wordt meestal niet goed nagebootst in proefdiermodellen. De meest gebruikte modelstudies zijn in vitro-systemen en proefdiermodellen (vaak subcutane modellen). Echter, in dit soort modellen
ontbreekt de typische diffuse groei. Ook tonen de gebruikte modellen vaak niet de goede chromosomale en genetische samenstelling van de verschillende glioomsubtypes (zoals astrocytaire en oligodendrogiale tumoren). We hebben verschillende diermodellen met intracerebraal groeiende humane gliomen geno- en fenotypisch gekarakteriseerd, variërend van bestaande glioma cellijnen (U87, U373, Hs683, U251, U343-31L, U343-C12:6, U410) en humane subcutane GBM xenograaf lijnen (E34, E49, E98, E106) tot nieuw opgestarte intracerebrale xenograaf modellen (E434, E468, E473, E478). We zagen dat de meeste tumorcellijnen aanleiding gaven tot compacte tumoren wanneer ze worden ingespoten in de hersenen van naakte muizen. Dergelijke modellen zijn dan ook niet geschikt voor onderzoek specifiek gericht op het ontrafelen van kenmerkende eigenschappen van diffuus infiltratieve groei van gliomen. Enkel het E98 GBM model en in mindere mate het E106 GBM model tonen steeds een combinatie van de typische diffuse groei en meer compacte groei. Daarnaast hebben we vier nieuwe diermodellen gecreeerd door tumorcellen van patiënten met diffuse hooggradige gliomen direct na de operatie in te spuiten in de hersenen van naakte muizen. Deze glioommodellen tonen zeer uitgebreide diffuse groei in de hersenen en ook typische chromosomale en genetische afwijkingen zoals verlies van chromosoomarmen 1p en 19q in de oligodendroglioomb-modellen E434 en E478 en verlies van chromosoom 10 en amplificatie van chromosoom 7 in de GBM modellen E98, E468 en E473. Hierdoor hebben we nu de beschikking over vijf unieke diermodellen die de situatie bij de mens goed nabootsen. Dit onderzoek onderstreept dat het voor het uitvoeren van experimenten heel belangrijk is om bij een bepaalde vraag een geschikt diermodel te selecteren. Immers, hoe passender het model, hoe makkelijker behaalde resultaten zijn te extrapoleren naar de humane situatie.

In Hoofdstuk 3 hebben we ons gericht op het probleem van de moeilijke detectie en vooral afbakening van diffuse gliomen via CE-MRI. Het contrastmiddel Gd-DTPA lekt niet uit de vaten met een intacte BBB in de diffuus infiltratieve gebieden van gliomen waardoor deze tumorgebieden onzichtbaar blijven op standaard CE-MRI. Zoals eerder vermeld is het effect van de huidige behandelingen voor patiënten met een diffuus glioom beperkt. Nieuwe, meer effectieve therapieën zijn daarom dringend nodig. Anti-angiogene therapie is een voorbeeld van zulk een meer gerichte aanpak. Aangezien angiogenese een belangrijk kenmerk is van veel hoog-maligne gliomen, worden deze al enige tijd beschouwd als goede kandidaten voor anti-angiogene therapie. Echter, anti-angiogene stoffen kunnen de BBB herstellen waardoor tumoren minder goed aantoonbaar worden via Gd-DTPA-MRI. Dit fenomeen hebben we aangetoond in het intracerebrale U87 glioommodel. Muizen met intracerebrale U87 tumoren werden door ons behandeld met vandetanib, een tyrosine kinase remmer gericht tegen Vascular Endothelial Growth Factor Receptor type 2 (VEGFR2), Epidermal Growth Factor Receptor (EGFR) en Rearranged during Transfection (RET). Vandetanib remde de angiogenese in deze tumoren zoals bleek uit de stijging van hypoxie en de afwezigheid van MVP's in de tumoren van behandelde
dieren. Dit vertaalde zich echter niet in een afname van tumorgrootte of proliferatie index. Bovendien konden de tumoren na vandetanib behandeling niet meer goed zichtbaar gemaakt worden met standaard Gd-DTPA-MRI, hoewel tumoren wel nog aanwezig waren. De BBB was dus door de anti-angiogene behandeling minder doorgankelijk geworden met als gevolg dat het lekken van contrastmiddelen naar de tumor werd gehinderd en de radiologische detectie van deze tumoren werd belemmerd. Dit betekent ook dat hierdoor het effect van anti-angiogene therapie bij gliomen overschat. Een interessant bevinding was dat door gebruik te maken van USPIOs (ultrasmall particles of iron oxide) als contraststof de tumoren na anti-angiogene behandeling nog wel zichtbaar gemaakt konden worden. Dit is te verklaren doordat USPIOs lange tijd in de circulatie aanwezig blijven en zo beschouwd kunnen worden als blood-pool agents. Door de daling in vaatdichtheid, geïnduceerd door de anti-angiogene behandeling, zijn tumoren behandeld met een hoge dosis vandetanib zichtbaar als hyperintense lesies, terwijl onbehandelde, vaatdense tumoren zichtbaar zijn als hypointense lesies ten opzichte van het normale hersenweefsel.

In Hoofdstuk 4 wordt verslag gedaan van een studie die het fenomeen van herstel van de BBB onder invloed van anti-angiogene therapie verder bestudeert. We hebben vier verschillende therapiën getest, namelijk 1) het monoclonale antilichaam gericht tegen VEGF-A, bevacizumab, 2) de tyrosine kinase inhibitor sunitinib gericht tegen VEGFR en platelet-derived growth factor receptor (PDGFR), 3) vandetanib en 4) de combinatie van sunitinib en vandetanib. Sunitinib werd gebruikt om naast angioenese via VEGFR ook mature vaten te remmen via PDGFR. Alle therapieën resulteerden in verminderde zichtbaarheid op Gd-DTPA-MRI en stijging van hypoxie in de compacte tumorgebieden in het E98 glioom model. De diffuus infiltratieve tumorgroei werd niet aangepakt door deze therapie. Additionele remming van vaatmaturatie via remming van de PDGF receptoren resulteerde ook niet in een betere effectiviteit van therapie.

In Hoofdstuk 5 tonen we aan dat herstel van de BBB niet alleen lekkage van contrastmiddelen zoals Gd-DTPA hindert maar dat het ook transport van chemotherapeutische middelen zoals TMZ naar de tumor belemmerd wordt. In sommige klinische studies met andere typen tumoren zoals colorectale tumoren) werd gevonden dat combinatie van anti-angiogene middelen met chemotherapie de effectiviteit van de chemotherapie kan vergroten en daarmee een gunstig effect kan hebben op de overleving van patiënten met dergelijke tumoren. Dit zou verklaard kunnen worden door aan te nemen dat anti-angiogene therapie het vaatbed normaliseert, waardoor de interstitiële druk lager wordt en de chemotherapeutica de tumorcellen makkelijker kunnen bereiken. Dit succes in andere tumoren heeft geleid tot het testen van combinaties van anti-angiogene middelen en chemotherapie bij GBM patiënten. Echter, omdat in het hersenmilieu anti-angiogene middelen de BBB herstellen zou daardoor ook de distributie
van chemotherapeutica naar de tumorcellen kunnen worden gehinderd. We hebben muizen met intracerebrale E98 en U87 tumoren behandeld met een combinatie van vandetanib en TMZ en vonden, vooral in het E98 model, inderdaad aanwijzingen dat deze combinatie het effect van TMZ antagoneert. Belangrijk is daarbij te bedenken dat U87 minder gevoelig is voor TMZ dan E98 waardoor in het U87 model dergelijk antagonisme minder makkelijk aantoonbaar is. De micro-omgeving van tumorcellen is dus erg belangrijk voor de respons op anti-angiogene therapie: in de hersenen dreigt vaatnormalisatie te leiden tot een verlaagd transport van chemotherapeutica naar de tumorcellen. Herstel van de BBB heeft echter ook een positief effect: verlaging van de vaathyperpermeabiliteit en interstitiële druk leidt tot vermindering van intra- en peritumoraal oedeem, hetgeen (tijdelijk) een positieve bijdrage kan leveren aan het welbevinden van de patiënt.

**Hoofdstuk 6** bevat conclusies over het onderzoek beschreven in dit proefschrift en plaatst onze resultaten naast bevindingen beschreven in de literatuur. We hebben laten zien dat de behandeling van gliomen nog steeds moeizaam is. Nieuwe benaderingen, zoals anti-angiogene therapiën moeten met grote voorzichtigheid toegepast worden bij glioompatiënten. De effectiviteit van deze therapie is sterk afhankelijk van het soort weefsel waarin de tumor zich bevindt. In vaatrijke weefsels zoals de hersenen kunnen infiltratieve tumorcellen doorgroeien zonder angiogenese door (meer) gebruik te maken van de bestaande bloedvaten. Ook metastases zijn vaak aanwezig in vaatrijke weefsels en hoeven derhalve niet angiogenese-afhankelijk te zijn. Anti-angiogene therapie is dus zeker niet altijd effectief in het belemmeren van de groei van tumoren. Daarnaast kunnen in het geval van anti-angiogene behandeling van hersentumoren contrastmiddelen en chemotherapeutica de tumor niet of minder goed bereiken, hetgeen dreigt te leiden tot moeizamer radiologische detectie en verminderde effectiviteit van chemotherapie. Wel kan anti-angiogene therapie tijdelijk een sterke verbetering van levenskwaliteit geven voor de patiënten door het verminderen van hersenooedeem en daardoor het verlagen van de intracraniële druk.

We concluderen dat aangezien infiltratieve gliomcellen erg moeilijk te elimineren zijn via conventionele therapiën, nieuwe stoffen moeten ontwikkeld worden die specifiek deze cellen opspreen en uitschakelen. Toekomstig onderzoek zou zich dan ook moeten toespitsen op het verder in kaart brengen van factoren bepalend voor het infiltratieve karakter van gliomcellen om zo nieuwe aangrijpingspunten te vinden voor therapie. Mogelijk interessante factoren waar in de toekomst extra onderzoek naar gedaan kan worden zijn o.a. tenascines, integrines, en immunotherapie. Ook is het heel belangrijk om methoden te ontwikkelen die het beter mogelijk maken om (lieftz op voorhand en bij individuele patiënten) de tumoren te identificeren die geschikt zijn voor anti-angiogene behandeling en zo de patiënten te selecteren die baat zullen hebben bij deze therapie. Er is dus nog veel onderzoek nodig om de prognose voor glioompatiënten te verbeteren.
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References


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## Abbreviations

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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>Cdc</td>
<td>cell division cycle</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CT</td>
<td>computerized tomography</td>
</tr>
<tr>
<td>CXCR4</td>
<td>chemokine (C-X-C motif) receptor 4</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF(R)</td>
<td>epidermal growth factor (receptor)</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>gadolinium diethylenetriaminepenta-acetic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NSC</td>
<td>neural stem cell</td>
</tr>
<tr>
<td>PDGF(R)</td>
<td>platelet-derived growth factor (receptor)</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>protein phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during tranfection</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>SF</td>
<td>scatter factor</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein acidic and rich in cystein</td>
</tr>
<tr>
<td>TMZ</td>
<td>temozolomide</td>
</tr>
<tr>
<td>uPA(R)</td>
<td>urokinase-type plasminogen activator (receptor)</td>
</tr>
<tr>
<td>USPIO</td>
<td>ultrasmall particles of iron oxide</td>
</tr>
<tr>
<td>VEGF(R)</td>
<td>vascular endothelial growth factor (receptor)</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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List of publications


Curriculum vitae

Dankwoord

Voilà, dit is het! Na vier jaar onderzoek is mijn proefschrift een feit! Hoog tijd dus voor een dankwoord want een proefschrift bij elkaar pipetteren en schrijven heb ik natuurlijk niet alleen gedaan. Ik wil dan ook graag verschillende mensen bedanken die hieraan hebben bijgedragen.

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Dankwoord

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Figure 2: Schematic overview of different diffuse astrocytoma grades. A: normal brain, B: low-grade astrocytoma (WHO grade II), C: anaplastic astrocytoma (WHO grade III) and D: GBM (WHO grade IV). Diffuse infiltrative growth is characteristic for all grades, and is often accompanied by perivascular accumulation of tumor cells (vessels in red, tumor cells in blue), perineuronal satellitosis (neurons in green), subpial growth of tumor cells, and/or intrafascicular growth in the corpus callosum. Cellularity, cytological atypia, and mitotic activity generally increase with grade. Mitotic tumor cells are depicted in black. In addition, prominent (often glomeruloid) microvascular proliferation and necrosis (dark grey area) emerge in the most malignant tumors (D).
Figure 3: Typical secondary structures of Scherer present in GBM. In A (H&E), asterisk indicates subpial growth, arrows indicate perineuronal satellitosis, and arrow head indicates perivascular accumulation of tumor cells. Image B (combined Luxol Fast Blue and H&E staining) shows increased cellularity with diffuse infiltration of tumor cells in the relatively well preserved myelinated tracts of the corpus callosum. In image C (H&E) asterisk indicates area of necrosis, arrow indicates perinecrotic pseudopalisading tumor cells, and arrow heads indicate glomeruloid microvascular proliferation. Original magnification: A,C: x100, B: x200.
Figure 4: Schematic overview of factors and mechanisms important for diffuse infiltration of glioma cells in the neuropil. The following aspects relevant for the diffuse growth pattern can be recognized: (a) an intracellular system that coordinates all incoming and outgoing signals via a complex set of pathways, (b) a locomotor apparatus in which the actin cytoskeleton plays a crucial role, (c) a scaffold (ECM, surface of cells/cell processes) on which the glioma cells can travel, (d) cell–ECM and/or cell–cell receptors that allow direct interaction with the ECM and cellular microenvironment, (e) tools to remove obstacles like ECM degrading proteases, (f) growth factors that guide the way, and (g) other stimulatory or permissive microenvironmental factors (e.g., chemokines derived from inflammatory cells). In this scheme, the protrusion on the right side of the cell represents the lamellipodium at the front.
A. Pre-treatment: tumor vasculature is characterized by turbulent blood flow, hyperpermeability and presence of various anastomoses and sprouts. Antibodies and contrast agents can easily access the interstitial space. A counteracting interstitial pressure may be present.

B. Anti-angiogenesis: newly formed blood vessels are attacked by the treatment, anastomoses disappear, the laminar flow in the vessels is restored and tumor cells beyond a minimal diffusion distance from vessels become hypoxic and apoptotic.

C. Normalization: the more regular vasculature with normalized permeability due to anti-angiogenic treatment results in a more conductive blood flow and a better delivery of chemotherapeutic compounds to the tumor. However, extravasation of large molecules like antibodies may be blocked by the reduction of permeability.

Figure 5: Schematic overview of possible effects of anti-angiogenic therapies on tumors.
Figure 1. Orthotopic growth pattern of glioma cell lines and sc-xenograft lines. H&E staining of U87 (A), E49 (B,C) and E98 (D-F), Transmission Electronmicroscopy of E98 (G), Glut-1 immunohistochemical staining (H) and VEGF mRNA in situ hybridisation of E98 (I). The U87 line (A) gives rise to compact growing tumors without diffuse infiltrative growth in the surrounding brain. The E49 line (B) gives rise to tumors showing expansive growth with perivascular extensions in the surrounding brain (C). The E98 line shows a combination of diffuse infiltrative growth (D, arrow), especially in white matter tracts, and intraventricular compact growth (D, arrow head). Only in the compact areas of the E98 tumors, focal florid microvascular proliferation was found (E). E98 tumor cells invade between the myelinated nerve fibers (arrows) of the corpus callosum (F), which show swelling and disintegration (G). In the compact E98 tumor areas, focal central hypoxia as is shown by the Glut-1 staining (H), co-localizes with (hypoxia-driven) VEGF-A expression (I). Original magnifications: A,B,D: x12; C,E,F: x400; G: x2000; H,I: x100.
Figure 2. Orthotopic growth pattern of GBM ic-xenograft lines. H&E staining of E468 (A,B) and E473 (C) and Glut-1 immunohistochemical staining of E473 (D). Both xenograft lines give rise to extensive diffuse infiltrative growth in the mouse brain in both white (B, arrow indicates corpus callosum) and grey matter (B, arrowhead indicates deep nuclei). Secondary structures like perineuronal satellitosis are present (C, neurons indicated by arrowheads; tumor cells by arrows). The tumors show a high vessel density with vessels that are strongly positive for the BBB marker Glut-1 (D), consistent with incorporation of pre-existent brain microvasculature. Original magnifications: A: x12; B,D: x100; C: x400.
Figure 3. Orthotopic growth pattern of anaplastic oligodendroglioma ic-xenograft lines. H&E staining of E434 (A,C) and E478 (B,D-F). E434 lesions are extremely diffuse infiltrative (A), whereas the E478 line grows to diffusely infiltrative, highly cellular tumors (B). The tumor cells of esp. the E434 (C) display the typical “fried egg” morphology. Frequent mitoses (D, arrows), dispersed multinucleated giant cells (E, white arrowheads) and occasional florid microvascular proliferations (F, black arrowhead) are present in the E478 xenograft. Original magnifications: A: x12; B: x50; C-F: x400.
Figure 1. CE-MRI of intracerebral U87 glioma lesions of vehicle-treated mouse. Representative images of intracerebral lesions before (A,B) and after (C,D) injection of contrast agent. Tumor lesions are not visible pre-contrast, but become visible as hyper-intense lesions in Gd-DTPA-enhanced MR images (C) due to leakage of contrast agent in and immediately around the tumor, and as hypo-intense lesions in USPIO-enhanced images (D) due to a higher vessel density in the tumor. H&E (E), Glut-1 (F) and Ki-67 (G) stainings of the corresponding brain slice show large viable tumors with regular vascular patterns and dilated vessels in the peritumoral rim (F, arrow). These latter vessels cause a a dark rim around the tumor lesion in the USPIO-enhanced MR image (D, arrow). Original magnifications: x25 (E) and x100 (F,G).
Figure 2. CE-MRI of cerebral U87 lesions mouse treated with 50 mg/kg vandetanib. Representative images of intracerebral lesions before (A,B) and after (D,E) injection of contrast agent. Tumor vessels are still leaky and are thus readily detectable after injection of Gd-DTPA (D). Tumors are visible as hyper-intense lesions compared to the normal brain parenchyma in USPIO-enhanced images (E). The histology and vascular profile of the corresponding tumor is displayed in C (Glut-1) and F (Ki-67). Note the relatively low vessel density and the increase in hypoxia (C, arrow). Original magnification: x100 (C,F).
Figure 3. CE-MRI of cerebral U87 lesions in mice treated with 100 mg/kg vandetanib. Representative images of intracerebral lesion before (A,B) and after (C,D) injection of contrast agent. Tumor lesions are not visible pre-contrast and stay invisible after injection of Gd-DTPA (C). Note that contrast agent did reach the brain, as indicated by contrast enhancement of large meningeal vessels (arrow). The corresponding H&E staining (E) shows a large, intraparenchymal tumor. Changes in tumor vessel density caused by anti-angiogenic therapy still allow visualization in USPIO-enhanced images. Tumors are visible as hyper-intense lesions due to a lower vascular volume compared to the surrounding normal brain parenchyma (D). The lesions present with only limited vascularization (F, Glut-1) whereas there was still a remarkably high positivity for Ki-67 in non-hypoxic regions (G). The arrows in F and G point at a regions of hypoxia. Original magnifications: x25 (E) and x100 (F,G).
Figure 3. Effect of anti-angiogenic treatment on mice carrying intracerebral E98 tumors. H&E stainings of vehicle-treated (A,C) and vandetanib+sunitinib-treated (B) mice, representative Glut-1 (E) and Ki-67 (F) stainings of intracerebral E98 lesions of a bevacizumab-treated mouse. In 80% of vehicle-treated mice, microvascular proliferations were detected in the tumor (C,D), whereas this phenotype was only occasionally observed in 5% of treated mice (D). The diffuse infiltrative areas (E,F, arrowheads) were not notably affected by the treatment. The compact tumor areas show a striking increase in hypoxia (E, asterisk). The proliferation index of tumor cells remains high in both diffuse infiltrative (F, arrowhead) and compact (F, arrow) tumor areas despite anti-angiogenic treatment. Only in hypoxic tumor regions in the compact tumor areas (E, asterisk), there is a strong decrease in proliferation index (F, asterisk). There is no hypoxia detectable in the diffuse infiltrative areas (E, arrowhead). Original magnifications: A,B: x12; C: x400; E,F: 100x.
Figure 4. Correlation between CE-MRI and histopathology of intracerebral E98 glioma lesions. For each treatment group, a representative image is shown of the intracerebral lesion before (pre) and after (post) injection of Gd-DTPA contrast agent. H&E stainings of corresponding slices are shown on the right. Tumors in vehicle-treated mice (P) were readily detectable using Gd-enhanced MRI. No tumor was detected in MRI of mice treated with anti-angiogenic compounds (bevacizumab (B), sunitinib (S), vantданib (V) and combination vantdanib-sunitinib (VS)). H&E staining of the corresponding brain slices, however, shows large tumors. Original magnification of H&E stainings: x12.

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Figure 2. Intracerebral growth pattern of E98 xenografts. H&E staining (A) and CD34 staining (B,C) of an intracerebral E98 tumor. Tumors present with extensive diffuse infiltrative growth along white matter tracts (A, arrow) and compact growth in the ventricles (A, arrowhead). In this latter component, activated vessels with strong CD34-positivity resembling the glomeruloid-like microvascular proliferations are present (B,C). Original magnifications: A: x25, B: x100, C: x400.
Figure 3. Representative T1-weighted MR images of placebo-treated (A-C) or 100 mg/kg vandetanib-treated (D-F) intracerebral E98 xenografts. A and D represent pre-contrast images, B and E are images, recorded 2 minutes after intravenous injection of Gd-DTPA. Tumor vessels are leaky in placebo-treated mice as is clear from the Gd-DTPA-enhanced image. Leakage in the diffuse infiltrative parts (B, arrow) of E98 tumors was always lower than in the compact tumor parts (B, arrowhead). Vandetanib-induced restoration of the BBB precludes extravasation of Gd-DTPA from tumor vessels in vandetanib-treated mice (E). Panels C and F show H&E stainings of corresponding brain slices. Original magnifications: x12 (C,F)
Figure 5. Vandetanib treatment of intracerebral E98 xenografts. Glut-1 staining of placebo-treated (A), low dose vandetanib-treated (25 mg/kg, B) and high dose vandetanib-treated (100 mg/kg, C) E98 carrying mice. Glut-1 staining shows a dose-dependent increase in hypoxia in the compact ventricular tumor areas (arrowheads), whereas no such staining could be detected in the diffuse infiltrative component (arrows). This increase is also shown in the graph (D) where the amount of hypoxia is given for the diffuse infiltrative and compact ventricular areas. Original magnifications: x12.
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Figure 2. Resection material of recurrent GBM, after bevacizumab treatment. H&E staining (A,C), Glut-1 (B,D). A,B: Resection material of recurrent GBM, 6 weeks after last infusion of bevacizumab. Tumor cells co-opt pre-existent vessels with relatively intact BBB (arrows). C,D: Recurrent GBM, autopsy 10 weeks after last infusion of bevacizumab. Tumor cells invade the brain parenchyma along white matter tracts. Original magnifications: x200 (A,B,C), x100 (D).