7. Summary, Discussion and Future Perspectives
Summary

A lot of the characteristic features of glioblastomas, be it radiological, immunohistochemical, or intraoperative, can be related to tumor angiogenesis. The angiogenic activity in glioblastomas is relatively high, although the relevance of this for human glioma growth and prognosis is not entirely clear. The process of angiogenesis is a complex sequence of events that involves multiple cell types and growth factors systems. The angiopoietin-Tie growth factor system is one of these and it is intimately involved in this process. The studies presented in this thesis analyze the role of this system in glioblastoma growth and development. The general status, expression pattern and activity of this system in human glioblastomas is studied and its relevance to prognosis is tested. Also, the possibility of interfering with the process of angiogenesis and the coincidental changes in the angiopoietin-Tie system are studied in an animal glioma model.

In chapter 2 the immunohistochemical assessment of the glioma vasculature is reviewed. The methods used to study tumor vasculature quality and quantity, as well as angiogenic activity are described. In doing so, the various aspects of the tumor vasculature that may be relevant in the process of tumor angiogenesis are reviewed: tumor vessel density, vascular pattern, the vessel constituents (endothelial cells, pericytes, basement membrane), the blood brain barrier, and growth factor activity (VEGF, HIF, angiopoietin-Tie). The possibilities for the clinical application of the immunohistochemical assessment of the tumor vasculature are critically discussed, also in the context of antiangiogenesis therapy. It is concluded that, possibly owing to a limited relevance of the angiogenic response to glioma growth, immunohistochemical assessment of the tumor vasculature in human glioblastomas lacks clinical relevance at this time.

Chapter 3 describes the study of the glioblastoma vasculature and its comparison with an entirely different glioma: the pediatric ependymoma. This chapter illustrates the relatively high intensity of the angiogenic response in glioblastomas as well as the high degree of heterogeneity that can be observed within these tumors. The vasculature of glioblastomas is found to be more immature and have a higher endothelial cell turn-over than the other tumor type. Despite these differences and in line with the findings of chapter 2, the MVD of both tumor types is similar. Regarding the angiopoietin-Tie system, remarkably, the main difference between the two tumor types is a lower expression of Ang-1 in glioblastomas. It is concluded that, as a group, glioblastomas show a higher intensity of the angiogenic response. However, most parameters show noticeable overlap between the tumor types.

The various quantitative aspects of the vasculature of human glioblastomas and their relation to prognosis are the main subject of chapter 4. In this chapter, MVD, endothelial cell turn-over, VEGF expression, and the angiopoietin balance are related to patient survival. The expression pattern of VEGF is described as well as the topographical variations of endothelial cell en tumor cell turn-over. The findings of VEGF expression pattern lead to contemplations on the degree to which the expression of VEGF is hypoxia driven. Most importantly, however, the angiopoietin balance is found to be positively correlated with survival. It is concluded that although this does not constitute a causal relation between angiopoietin balance and survival, it does illustrate the close
association of the angiopoietin-Tie system with glioma growth and development. The findings, therefore, warrant further exploration of the role of the angiopoietin-Tie system in glioblastomas and its potential as a target for therapy.

Whereas the analysis of the angiopoietin expression in glioblastomas described in chapter 4 was performed on the level of mRNA, chapter 5 describes the analysis of the angiopoietin-Tie system on a protein level. The expression patterns of angiopoietin-1 and -2 confirm findings in the literature. Angiopoietin-1 is predominantly seen in the tumor cell compartment. Angiopoietin-2 expression is largely limited to the vasculature. However, Tie-2, which was initially thought to be endothelial cell specific, is present in the non-vessel compartment. Double staining procedures identify the non-vascular Tie-2-positive cells as macrophages (so-called Tie2 expressing monocytes (TEM’s)). It is concluded that Tie-2 expression in glioblastomas is not limited to the endothelial cells and that this extravascular expression can largely be ascribed to the presence of TEM’s. The role of TEM’s in glioma growth and the role of angiopoietin-2 in the recruitment of TEM’s is speculated on.

The feasibility of interfering with the process of tumor angiogenesis is illustrated by the experiments described in chapter 6. It is shown that treating glioma harboring mice with a combination of radiation and a COX-2 inhibitor enhanced treatment response compared with radiation only. The treatment effect coincides with an increase in tumor vessel maturation status and a non-significant reversal of the angiopoietin-1/-2 ratio. It is concluded that the treatment effect, combined with the finding that in vitro the COX-2 inhibitor had no effect on the growth of glioma cells, suggests a possible involvement of the process of angiogenesis in the observed effects. In the context of the findings of chapter 5, it is interesting to note that the addition of a COX-2 inhibitor to the treatment coincides with an increase of an apoptotic cell population in vivo that colocalizes with a cell population as identified by CD45 immunohistochemical staining.
Discussion and Future Perspectives

Glioblastomas are highly aggressive tumors with a dismal prognosis despite advances in the various treatment entities. They are characterized by a prominent angiogenic response to local hypoxia. Because this angiogenic response may provide us with possible targets for therapy, different in vitro and in vivo methods of analyzing this response have been developed. For instance, in vitro, endothelial cell proliferation and migration can be studied, as well as tube formation by these endothelial cells. In vivo, gene knock-out models allow for the analysis of the effect of various growth factors on the process of angiogenesis and sophisticated techniques make it possible to view the formation of new blood vessels in brain tumors “live” through a cranial window. For the study of the angiogenesis in human brain tumors there are less methods available. The techniques that can be used are largely limited to culture and analysis of endothelial cells isolated from tissue samples obtained during surgery and radiological or nuclear imaging. The radiological techniques can be used to study parameters such as perfusion and vascular volume. Perfusion MRI may even be used in a clinical setting for distinguishing tumor progression from pseudoprogression after combined radio- and chemotherapy. However, most of the techniques used for the study of angiogenesis in human gliomas, including immunohistochemical assessment (reviewed in chapter 2), are used in scientific research and at this time lack a clear clinical benefit in the context of the treatment of glioblastoma patients. In this thesis, the experiments that are described mainly include immunohistochemical detection of proteins and RT-PCR analysis of gene expression levels in human glioma tissue samples (with the exception of the experiments described in chapter 6) focusing on tumor angiogenesis and, more specifically, on the behaviour of the Ang-Tie-2 system.

The process of tumor angiogenesis is a complex mechanism involving multiple cell types, and one in which multiple delimited steps can be recognized. Considering this complexity, and considering the wide range of molecules involved in the process of angiogenesis, it can be concluded that there is no one parameter that characterizes angiogenesis or that can be used to quantify it as a whole. In the studies described in chapter 3 and 4 the tumor neovascularization is analyzed using immunohistochemistry and RT-PCR, to monitor a set of genes involved in angiogenesis thereby covering many aspects of the process. In chapter 3, two types of brain tumors are compared of which one is considered to be angiogenically highly active (glioblastoma), while the other does show neovascularization but to a much lesser extent (pediatric ependymoma). By comparing these tumors the aspects that vary from one tumor to the other are highlighted. The microvascular density (MVD), once the center of attention in clinical angiogenesis research, was shown to be similar between the two tumor types. Despite this similarity, endothelial cell proliferation and apoptosis, basement membrane coverage, and pericyte coverage were significantly different. These results indicate a higher vascular turnover and lower vascular maturity in glioblastomas. In line with this relative immaturity of the glioblastoma vasculature, the expression of Ang-1 was lower in glioblastoma, whereas Ang-2 expression did not differ between the two tumor types. These results confirm the general idea of glioblastoma as an angiogenically active tumor, and the initial interpretation of the MVD as a measure of angiogenic activity turns out to be a misconception. Besides the fact that other tumors may have a comparable MVD, as shown in
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Chapter 3, it was earlier shown that the MVD of the surrounding grey and white matter can even supersede that of glioblastoma. That glioblastoma is an angiogenically active tumor nonetheless can be explained by the fact that MVD is not only determined by the angiogenic activity of a tumor but also by the resistance to hypoxia of the tumor cells (Hlatky). A clear limitation of the study described in chapter 3 is the lack of a parameter that can be used as a measure of vessel functionality, such as vessel perfusion or tissue oxygenation/hypoxia. Such a parameter may be of interest in the context of the concept of 'normalized' vessels. Jain et al have shown that the immature, aberrant tumor vasculature that results from the process of tumor angiogenesis is relatively dysfunctional and when targeted by an antiangiogenic therapy may gain maturity and functionality. In tumors with these so-called 'normalized' vessels there is less hypoxia due to an increased perfusion. It is postulated that these tumors may be more amenable to conventional therapies, such as chemo- and radiotherapy. In this context, it would be interesting to know whether or not the differences identified between the two tumor types described in chapter 3 are paralleled by differences in tumor vessel functionality.

Although the comparison of vascular parameters of two different types of tumors does not provide us with directly clinically relevant knowledge, it does, in a translational sense, corroborate in human tumors some of the concepts and ideas that come from preclinical studies. In chapter 4, the clinical relevance of these vascular parameters is tested. Using the tissue samples of a cohort of glioblastoma patients, the vascular parameters are analyzed for a possible correlation with survival. It is discussed that the pattern of gene expression of VEGF-A and –D suggests an influence of hypoxia. More importantly, the balance between Ang-1 and -2 was shown to be correlated to survival. Ang-2 dominance was associated with a worse prognosis. As stated in the introduction, the Ang balance is associated with the state of maturity of the vasculature. That is, dominance of Ang-2 over Ang-1 is associated with detachment of pericytes and breakdown of the basement membrane, as well as loosening of intercellular connections between the endothelial cells. In such a state the endothelial cells are vulnerable. Under these conditions, if VEGF is present, however, the endothelial cells are free to proliferate and angiogenesis can ensue. Therefore, the findings may indicate that a worse prognosis in glioblastoma is associated with a vasculature that is more immature, unstable, and more prone to angiogenesis. Of course, these findings do not constitute a relation of cause and effect. Moreover, the expression level of the Angiopoietins was measured at the level of mRNA. These may not be representative for the actual protein levels which may be influenced by post-transcriptional regulation.

In chapter 5, the expression level of Ang-1 and-2 is assessed by immunohistochemistry to analyse this expression at the protein level. Both Ang-1 and Ang-2 ligate the Tie-2 receptor. Therefore, the availability and distribution of the Tie-2 receptor may be of importance for the actual biological effect of the Ang balance within the tumor. For this reason, in chapter 5, the expression of Tie-2 in glioma samples is also studied. In this cohort of glioblastoma patients a semiquantative score of Tie-2 expression correlated with survival. A higher Tie-2 expression score was associated with a worse prognosis. The Angiopoietins, in contrast, did not show a correlation with survival, possibly due to the fact that a reliable balance between the Angiopoietins, such as the one calculated in
chapter 4, could not be given by the semiquantitive assessment of their protein levels. Others have presented similar findings of an increased expression of Tie-2 in human glioma and associated the increased expression with the grade of malignancy and so, indirectly with prognosis. In preclinical studies of glioma cell lines the Ang-Tie-2 activity in glioma was linked to invasion and angiogenesis, and drug resistance. Together these findings argue for an exploration of the Ang-Tie-2 system as a possible target for therapy. Several agents targeting the Ang-Tie-2 pathway have been developed and are tested in clinical trials. Preclinically, such agents have been successful in multiple tumor models. One of the agents that is tested clinically is Trebananib, a peptide Fc fusion protein that prevents binding of Ang-1 and Ang-2 to Tie-2. This drug is currently in 2 trials with or without Bevacizumab in recurrent adult glioma (www.clinicaltrials.gov). In ovarian carcinoma Trebananib has shown promising results in a phase 2 trial.

In order to further analyze the cellular mechanism by which the Ang-Tie-2 system influences glioma angiogenesis, chapter 5 describes the results of double staining procedures aimed at elucidating the cellular distribution of Tie-2 expression. This analysis shows that a large part of the expression of Tie-2 outside the vascular compartment is accounted for by tumor associated macrophages (TAM), or rather a subtype of these monocytic cells that express Tie-2 (TEM). The occurrence of TAM is well known and they are assigned a controversial role in tumor growth and development. There is evidence for both supporting as well as inhibiting effects of TAM on tumor growth. TEM have been identified in a brain tumor model and are thought to play a slightly different role in tumor growth, albeit equally controversial. TEM have been implicated to support angiogenesis and possibly play a role in resistance to anti-angiogenesis drugs. Targeting the Ang-Tie-2 pathway to reduce TEM activity or depleting TEM more directly has been successful in reducing angiogenesis and tumor growth in animal models. Because the results described in chapter 5 represent the first identification of TEM in human gliomas, future studies will have to investigate whether or not drugs like Trebananib are able to reduce TEM activity.

The feasibility of interfering with the process of tumor angiogenesis is illustrated by the experiments described in chapter 6. It is shown that treating glioma harboring mice with a combination of radiation and a COX-2 inhibitor enhanced the response compared with radiation only. The treatment effect coincided with an increase in tumor vessel maturation status, however, without major changes in the Angiopoietin-1/-2 ratio. The change in maturation status nonetheless led us to conclude that the treatment effect suggests a possible interference with the process of angiogenesis in the observed effects. A conclusion that was supported by the finding that in vitro the COX-2 inhibitor had no effect on the growth of glioma cells. In the context of the findings of chapter 5, it is interesting to note that in vivo the addition of a COX-2 inhibitor to the treatment coincides with an increase in the apoptotic cell population that is identified by CD45 expression. A further characterization of these cells has not been performed. It would be interesting to know whether this cell population contains TAM or TEM, particularly because the monocytic tumor associated cells may be a major source of COX-2 dependent prostaglandin production. Selective COX-2 inhibitors were developed as an alternative to NSAID, with the aim to reduce the side effects of these drugs, especially in the gastro-intestinal tract. They are associated with...
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an antiproliferative and antiangiogenic effect in tumors. COX-2 inhibitors were initially met with great enthusiasm because of their possible widespread application as analgesics. This enthusiasm suffered a blow when these drugs were found to carry an increased risk of adverse cardiovascular events. Research into the possible clinical application of COX-2 inhibitors decreased, primarily because the increased risk of cardiovascular events was considered unacceptable in the context of its use as a painkiller or anti-inflammatory drug. In oncological research, however, these side effects are less of an obstacle. In fact, within oncology, these drugs are considered to be of low toxicity. Nevertheless and despite encouraging results from preclinical research, trials studying the use of COX-2 inhibitors in human high grade glioma are scarce. This may, in part, be due to its bad publicity in the context of analgesia. Of note is, however, also the observation that not all results of the trials performed so far are equally promising (table 1).

Table 1: Overview of the results of clinical trials using COX-2 inhibitors for human high grade gliomas in terms of survival. n: number of patients studied; GBM/AA: Glioblastoma/Anaplastic astrocytoma; PFS: progression free survival; OS: overall survival. *: The series of Grossman et al studied newly diagnosed glioblastomas instead of recurrent high grade gliomas as was reported in the other 3 studies. This has, in all likelihood, major impact on the reported overall survival as compared to the other series.

<table>
<thead>
<tr>
<th>Citation</th>
<th>n</th>
<th>GBM/AA</th>
<th>Treatment</th>
<th>Phase</th>
<th>PFS</th>
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<tr>
<td>37</td>
<td>40</td>
<td>37/3</td>
<td>Irinotecan with celecoxib</td>
<td>II</td>
<td>11 wks</td>
<td>31.5 wks</td>
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<tr>
<td>25</td>
<td>25</td>
<td>25</td>
<td>13-cis-retinoic acid with celecoxib</td>
<td>II</td>
<td>8 wks</td>
<td>-</td>
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<tr>
<td>15</td>
<td>25*</td>
<td>25</td>
<td>Radiation and celecoxib</td>
<td>other</td>
<td></td>
<td>52 wks</td>
</tr>
<tr>
<td>16</td>
<td>14</td>
<td>10/4</td>
<td>Capecitabine or Temozolomide with pioglitazone and rofecoxib</td>
<td>II</td>
<td>7 wks</td>
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Interestingly, a celecoxib analog (2,5-dimethyl-celecoxib, DMC) has been developed that is devoid of COX-2 inhibitory function, but seems to sustain its antitumor effects. This is in line with earlier findings that suggest that part of the effects of COX-2 inhibitors are independent of the presence of COX-2, as was also mentioned in chapter 6. This drug (DMC) could possibly take away some of the reluctance that is felt regarding the use of COX-2 inhibitors and may be worthwhile to explore as an adjuvant in glioma therapy.

The studies presented here, analyzing the Ang-Tie-2 pathway, highlight part of the process of angiogenesis in glioma. While awaiting the results of ongoing trials in which this pathway is targeted, it is tempting to speculate on what could be the mechanisms of a possible antitumor effect. It seems that the Ang-Tie-2 pathway is active at least at three levels in the process of angiogenesis or tumor growth (Figure 1). First, as was outlined in the introduction, early research on this pathway, initiated by Holash et al, focused on the endothelial cells. The binding to Tie-2 on the endothelial cells by Ang-2 leads to vascular disintegration, marking the beginning of the angiogenic switch (see also the introduction). Second, the ongoing angiogenesis is supported...
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by TEM, which are either recruited or activated by Ang-2. This division of signalling via the endothelial cell vs TEM may suggest a concomitant temporal division of these signalling pathways (the endothelial cells in the initial tumor outgrowth vs TEM in the established tumor). However, although the disintegration of the vasculature is mostly associated with the initial outgrowth of tumor cells, it is likely that these events occur over and over again, for instance at the tumor border. Moreover, TEM activity may not be limited to advanced and established tumors, but has been associated with the angiogenic switch in its own right. Nevertheless, there are reports of tumor phase dependent effects of Ang-Tie2 modulation. Third, Ang-1 Tie-2 signalling is involved in the formation or upholding of the tumor stem cell niche. The evidence for the latter is much less extensive than for the first two (Tie-2 signalling in endothelial cells and TEM), yet it could account for the Tie-2 expression on the glioma cells.

Figure 1: Possible mechanisms via which the Ang-Tie-2 system can support angiogenesis or growth in human brain tumors and which may serve as targets for therapy. A: The angiogenic switch was first described in experimental gliomas. The initial outgrowth of tumor cells triggers the expression of Ang-2 in the local endothelium leading to vascular desintegration and deterioration. The subsequent hypoxia induces the production of VEGF, which, together with Ang-2, starts the process of sprouting angiogenesis. B: The mononuclear infiltrate of tumors has been shown to be involved in various processes supporting tumor growth. TEM are thought to specifically support tumor angiogenesis and are recruited or activated by Ang-2. C: The interaction of glioma stem cells with the tumor endothelium is mediated by Tie-2 creating a so-called niche for the stem cells alongside the vasculature.
Although all three levels of Ang-Tie-2 activity in tumors seem attractive targets for therapy, actually targeting the pathway may prove to be difficult or even hazardous. There is great uncertainty as to the biological effect of Ang-Tie-2 modulation for two reasons: There are multiple targets to choose from (Ang-1, Ang-2, Tie-2), and the biological effect of Ang-Tie-2 signalling is known to be context dependent on more than one level\(^4\). As to the targets, multiple agents have been developed that target either Ang-1 and Ang-2, Ang-2 alone, or Tie-2\(^10\). The context dependency of the effects of Tie-2 ligation concerns both Ang-1 and Ang-2 (figure 2). For Ang-1, downstream signaling of Tie-2 phosphorylation has been shown to depend on the cellular context. In confluent cells, where Tie-2 is located at the cell-cell junctions, Ang-1 expression leads to predominant AKT signalling. In isolated cells, where Tie-2 is located at the membrane bordering the extracellular matrix, stronger ERK signalling is produced\(^12, 38\). For Ang-2, which is generally considered to be an Ang-1 antagonist, some data suggest an agonistic role\(^6, 40\). These variable effects of Tie-2 ligation by Ang-2 may be related to the oligo- or multimeric state of Ang-2 or the composition of the extracellular matrix\(^34\).

Figure 2: Context dependency of Ang-Tie-2 signalling. A: In confluent cells Tie-2 is expressed in the cell membrane opposite the neighboring cells. Multimeric Ang-1 ligation results in so-called trans-association of Tie-2, leading to Akt signalling. In isolated cells, Tie-2 is translocated to the membrane opposing the substratum and binds Ang-1 that is bound to the extracellular matrix, leading to Erk signalling. B: Ang-2-Tie-2 signalling depends on the oligomerization state of Ang-2. Lower order oligomers (shown here as dimers) lead to redistribution of Tie-2 to focal adhesions containing α2β1 integrin with a weak agonistic effect. In the multimeric state of Ang-2, distribution of Tie-2 is similar to that in Ang-1 signalling in confluent cells. In that case Ang-2 may function as an antagonist. In angiogenic sprouts the tip cells are devoid of Tie-2 expression, but do release Ang-2, which may bind Tie-2 that is
expressed on the stalk cells of the sprout 9.

In addition, there should be concern about the effect that a drug that targets the Ang-Tie-2 system may have on the vascular beds of normal healthy tissue, or on physiological angiogenesis such as occurs during wound healing. The possible involvement of the Ang-Tie-2 in inflammation, haematopoiesis, and neuroprotection adds to these concerns.

Besides targeting the Ang-Tie-2 system as a means of therapy, an alternative application of these molecules may be the use of serum/blood Ang-2 or TEM levels as biomarkers. The dramatic radiological changes that may be seen in glioblastomas in response to antiangiogenic agents is not necessarily associated with an improvement in patient outcome. This is one of the reasons why, within the antiangiogenic treatment of glioma, the traditional radiological response criteria are questioned. It follows that there is a need for new markers to monitor the response to therapy. It has been shown that the serum level of Ang-2 can be used as such in lung carcinoma\(^2\), colon carcinoma\(^{13}\), melanoma\(^{17}\) and hepatocellular carcinoma\(^{28}\). For hepatocellular carcinoma the concentration of TEM in peripheral blood may even be superior in this regard\(^{28}\). Because of the abundant presence of TEM in glioblastomas, it seems worthwhile to analyse the possibility of their use as a biomarker in glioblastoma patients.

In recent years, much attention has been paid to the genetic profiling of glioblastomas. This led to the identification of three molecular subclasses of high grade glioma that are distinct in their gene expression patterns. Of these three subclasses, tumors of the proneural subclass, as opposed to those of either the proliferative or the mesenchymal subclasses, are associated with a longer survival\(^{33}\), and a reduced advantage from aggressive treatment protocols\(^{41}\). Although such genetic profiling has not become common practice in the clinic for several reasons\(^{19}\), these subclasses do have pathophysiologic significance besides their prognostic implications. For instance, the different subtypes have been shown to relate to CD133+ cell content and to the extent of contrast enhancement\(^{19}\). Among these subclasses, the mesenchymal type tumors are especially associated with angiogenesis and overexpression of VEGF and VEGFR\(^{33}\). It would be interesting to know whether the status of the Ang-Tie-2 system within GBM and their TEM content can be related to these molecular subtypes.

**Concluding remarks**

The results presented in this thesis emphasize the importance of the Ang-Tie-2 pathway in glioma tumor biology. Also, the results contribute to the filling of a pronounced gap between the clinical trials that are ongoing and the limited understanding of the exact role and relevance of this pathway in human gliomas. It is important that this gap is filled in order to adequately interpret the future results from currently ongoing trials. Although the data presented here certainly support such clinical trials, if the exploration of the possibility and usefulness of targeting the Ang-Tie-2 system is to be a success, it is crucial that we have thorough understanding of the role and significance of this system in the growth and development of human gliomas.
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