Chapter 3

Tie-2 in human glioblastomas is also expressed outside the vascular compartment

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submitted

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Abstract

In this study the authors analyzed the extent and distribution of expression of the Ang/Tie2 system in the tumors of 50 glioblastoma patients and related this expression to clinical outcome.

Tumor tissue was obtained from surgical specimens. Immunohistochemistry was used to study Ang-1, Ang-2 and Tie-2 expression and scored semiquantitatively. To analyze the contribution of the tumor cells and the tumor associated macrophages (TAM) to tumoral expression of Tie-2, immunofluorescent double staining was performed with GFAP, HLA-DR, CD68 and CD163, as well as qRT-PCR on glioma cell lines.

Tie-2 expression was shown to negatively correlate with survival in this group of glioblastoma patients. The scoring system used did not show a correlation of Ang-1 or Ang-2 with survival or vascular morphometric data. Tie2 expression was shown to occur mostly outside the vascular compartment, in the tumor cells and tumor associated macrophages (TAM), of which the latter made up 4-30% of the cells in the non vessel compartment.

Tie-2 expressing monocytes (TEM) are a subpopulation of TAM that account for a large part of the Tie-2 expression in human glioblastomas. This expression can be related to patient survival. These results support ongoing studies into the feasibility of targeting the Ang/Tie-2 system in the treatment of glioblastoma patients, but in a broader sense than just anti-angiogenesis.
Chapter 3    The angiopoietin/Tie-2 system in human glioblastomas

Introduction
Angiopoietin-1 and -2 (Ang-1, -2) are strongly associated with the angiogenic switch in tumors [1]. In surgical samples of human glioblastomas (GBM), we have previously shown that the balance between these angiopoietins, as measured by mRNA content, has prognostic value [2]. Tie-2, the cognate receptor of Ang-1 and -2, was initially thought to be expressed only by endothelial cells. However, other cell types that express Tie-2 have been identified. In tumors, neoplastic cells as well as the mononuclear infiltrate were found to express Tie-2. The latter are also known as “Tie-2 expressing monocytes” or TEM and may be considered a subtype of tumor associated macrophages (TAM). These TEM are thought to be either recruited or activated by the Ang-Tie-2 system [3-5]. Here, we further analyzed Ang-1 and Ang-2 on a protein level in human gliomas and study the distribution of Tie-2 expression within these tumors. A special emphasis is placed on the identification of TEM, which have not been studied before in human gliomas. These subjects are relevant in the context of ongoing trials that use agents that target the Ang-Tie-2 system [6]. For instance, AMG386, a peptide –Fc fusion protein that prevents binding of the angiopoietins to Tie-2, is currently being tested in recurrent glioma in two clinical trials (www.clinicaltrials.gov; NCTO 1609790 and NCTO 1290263). Such alternatives to the available anti-angiogenic agents are needed because current anti-angiogenic treatment of glioblastomas is characterized by low response rates [7,8] and the development of therapy resistance [9]. Amongst other mechanisms [9,10], the Ang-Tie-2 pathway is implicated in this resistance [11,12].

Materials and Methods

Patient Population and Baseline Characteristics
A total of 50 patients, meeting the following criteria, were included: 1. patients were diagnosed with a primary GBM, 2. patients had undergone
neurosurgical debulking, 3. patient follow-up was available, and 4. sufficient representative frozen tumor tissue was available for the study. Baseline patient characteristics were collected including: age, sex, postoperative adjuvant treatment, and survival. Age and survival were determined using the date of surgery.

Table 1. List of antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
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<td>Ang-1</td>
<td>N-18 / sc-6319</td>
<td>Goat Polyclonal</td>
<td>Santa Cruz</td>
<td>1:100</td>
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<tr>
<td>Ang-2</td>
<td>F-18 / sc-7017</td>
<td>Goat Polyclonal</td>
<td>Santa Cruz</td>
<td>1:50</td>
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<td>Tie-2</td>
<td>C-20 / sc-324</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz</td>
<td>1:50</td>
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<td>GFAP</td>
<td>--</td>
<td>Rabbit Polyclonal</td>
<td>Dako</td>
<td>1:800</td>
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<td>HLA-DR</td>
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<td>Mouse Monoclonal</td>
<td>Ortho</td>
<td>1:100</td>
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<td>CD68</td>
<td>PGM1</td>
<td>Mouse Monoclonal</td>
<td>Dako</td>
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<td>Novocastra</td>
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<td>Qbend10</td>
<td>Mouse Monoclonal</td>
<td>Immunotech</td>
<td>1:100</td>
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<td>ICN Biochemicals</td>
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<td>M0851</td>
<td>Mouse Monoclonal</td>
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<td>1:100</td>
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<td>Ki67</td>
<td>MIB1</td>
<td>Mouse Monoclonal</td>
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<tr>
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<td>Asp175</td>
<td>Mouse Monoclonal</td>
<td>Cell Signalling</td>
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<td>VEGF-A</td>
<td>Sc-152</td>
<td>Rabbit Polyclonal</td>
<td>Santa Cruz</td>
<td>1:50</td>
</tr>
</tbody>
</table>

**Immunohistochemical Staining**

For immunohistochemical staining of Ang-1, Ang-2 and Tie-2, 5 µm sections of frozen tissue from all 50 tumor samples were processed using the protocol previously described by Zeng et al. [13]. Immunohistochemical staining
procedures for VEGF-A, -B, -C, and -D as well as Ki67/CD34, and cleaved caspase/CD34 were previously described [2]. Staining procedures for CD68 and CD163 were performed on paraffin sections and for HLA-DR on frozen sections. For a complete list of antibodies and dilutions see table 1. Aspecific binding was evaluated by omission of the primary antibody.

**Histological Evaluation**
Analysis of all tissue slides for Ang-1, Ang-2 and Tie-2 protein expression was performed using a semiquantitative staining intensity score: no staining was scored as 0, low staining as 1, moderate as 2, and high intensity staining as 3. Normal brain parenchyma was used as a control for the staining procedure. Additionally, the fraction of Tie-2 expressing cells was scored using a dichotomous scale: either more (scored as 2) or less (scored as 1) than 50% of the cells staining positive for Tie-2. All slides were evaluated by a neuropathologist who was blinded to patient data. Analysis of the samples for microvascular density (MVD), proliferation fraction, apoptotic fraction, and VEGF-A, -B, -C, and -D protein expression were previously described [2].

**Immunofluorescent Double Staining**
After drying for 20 minutes, sections were postfixivated in aceton for 10 minutes. Sections were then treated with 0.05% sudan black solution (Merck) for 10 min and 1% sodium borohydrate (Sigma) for 10 min to quench autofluorescence. The slides were then blocked with 5% normal goat serum (Vector) in PBS containing 0.03% TritonX (Sigma) for 45 min. Subsequently, sections were incubated at 4°C overnight with the various primary antibodies (Table 1). The next day, sections were washed in PBS and incubated for 3h at room temperature with goat anti-mouse Alexa488 (Invitrogen) and goat anti-rabbit Cy3 (Jackson Immunoresearch) secondary antibodies. Sections were then washed in PBS and coverslipped with Mowiol (Sigma). Primary antibody omission served as negative controls.
Glioma cell lines

The human GBM cell line U87 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The newly generated glioma cell lines GG1, GG6, GG7, GG9, GG12, GG13, GG14 have been described before [14]. U87 cells were cultured in DMEM-Hg supplemented with 10% FCS and 1% pen/strep (Gibco Life Technologies). The GG cells were maintained as neurospheres in Neurobasal A-Medium (Gibco Life Technologies) supplemented with 2% B27 supplement (Gibco Life Technologies), 20 ng/mL EGF (R&D systems, Abingdon, UK), 20 ng/mL basic fibroblast growth factor (bFGF; Merck Millipore, Billerica, MA, USA), 1% pen/strep and 1%L-glutamine (Gibco Life Technologies).

Quantitative RT-PCR

Total RNA was obtained from U87 and GG1, GG6, GG7, GG9, GG12, GG13, GG14 cells. RNA was extracted from the cell lines using the RNeasy mini kit according to manufacturers guidelines (Qiagen) and from patient samples using the TRIzol® reagent (Ambion) method by adding 1mL of TRizol® per 4 slices of 10μm snap frozen tissue sections. TRizol® treated samples were incubated for 10 minutes, mixed with 200μL Chloroform per 1mL TRizol® and centrifuged at 4°C for 5 minutes at 14,000RPM. The aqueous phase was transferred into a new tube, samples were incubated with 500μL Isopropyl alcohol per 1mL TRizol® for 10 minutes and centrifuged at 4°C for 5 minutes at 14,000RPM. RNA pellet was washed with 75% Ethanol, centrifuged at 4°C for 5 minutes at 14,000RPM, air dried and quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). To decontaminate total RNA from DNA inclusions, TURBO DNA-Free Kit was used according to the manufacturer’s instructions (Ambion). RNA quality and integrity was checked on a 1.2% agarose gel (Invitrogen) in 50mL Tris Bore EDTA (TBE)/5μL Ethidium Bromide (Invitrogen). Samples were loaded in equal amounts in 10x loading buffer (10mM EDTA/50% glycerol/0.1% Bromophenol Blue) at 100V for 45
minutes. Synthesis of cDNA was performed following iSCRIPT guidelines (Bio-
Rad laboratories) and cDNA quality was checked with a ladder PCR.
Real-time PCR was performed in quadruplicates by loading 5ng (cell lines)
of cDNA in a 20μL reaction mix including the TaqMan Universal Master
Mix (Applied Biostystems) and Tie-2 primer (Hs00945153_m1) or GAPDH
(Hs02758991_g1, Gibco Life Technologies). Samples were measured with an
ABI PRISM 7900 HT real time sequence detection system (Applied Biosystems,
Forster city, CA) in a 384-well reaction plate. Raw data was extracted with
SDS software 2.3 (Applied Biosystems) and averages of the threshold cycle
(CT) were used for calculation of expression relative to GAPDH with the $2^{-ΔCT}$
method.

Results

Angiopoietin-1 and -2 show different expression patterns
Immunohistochemical staining of all GBM patient samples showed relatively
diffuse Ang-1 and Ang-2 expression without an obvious zonal distribution in,
for instance, the tumor margin or the perinecrotic zone. Out of 50 tumors,
Ang-1 staining was weak in 13, moderate in 19, and strong in 18 tumors.
Expression was found in the tumor cells, sometimes in a band-like pattern
across the tumor tissue. The vascular compartment (endothelial cells and
pericytes) was only weakly positive. The semiquantitative Ang-1 score did not
correlate with baseline patient characteristics or morphometric angiogenic
data [2]. All but 1 GBM showed Ang-2 expression. Staining was weak in 24
and moderate in 25 out of 50 tumors. The strongest expression was observed
in blood vessels (mainly endothelial cells). There was also staining in the
tumor compartment, but to a lesser extent. A zonal distribution for Ang-
2 was not found and Ang-2 scores did not correlate with baseline patient
characteristics nor with morphometric angiogenic data [2].
Tie-2 is more strongly expressed in the non-vessel compartment and shows a negative correlation with survival

In general in the tumors a relatively weak Tie-2 staining could be observed
in the endothelium and the pericytes / smooth muscle cells in blood vessels (Fig. 1D and F and Fig. 2A). In the non-vessel compartment of the tumour the Tie-2 expression varied considerably: 1 tumour was completely negative (Fig. 1A), 33% showed a weak staining intensity of 1+, 48% a moderate intensity, and 17% a strong staining (Fig. 1B). The expression was patchy (<50% of the surface area) in 40% of the tumours, whereas a more diffuse staining pattern could be observed in the remaining tumours (>50% of the surface area).

The resulting Tie-2 score (intensity * fraction of Tie-2 positive cells) showed a negative correlation with overall survival of the patients (R = -283, p = 0.05). The Tie-2 score did not correlate significantly with microvessel density or proliferation and apoptosis frequency of either endothelium or tumour cells. Interestingly, two specific staining patterns could be found in several of the tumours. One showed predominant Tie-2 staining of cells surrounding blood vessels (Fig. 1C and D) and the other had increased intensity and number of cells positive for Tie-2 in areas surrounding tumour necrosis (Fig. 1E and F).

**Glioma stem cell lines express Tie-2 mRNA**

In order to evaluate the expression of Tie-2 by tumor cells we performed quantitative RT-PCR analysis on mRNA of the glioma cell lines. Six out of 7 GG cell lines exhibited Tie-2 mRNA expression. Values of \(2^{-\Delta CT}\) ranged from \(4.1 \times 10^{-6}\) (GG14) to \(6.8 \times 10^{-4}\) (GG6). U87 cells also expressed Tie-2 mRNA \(2^{-\Delta CT} : 7.1 \times 10^{-5}\).

**GBMs contain large percentages of TAM and TEM**

All GBMs contained TAM. In the non-vessel compartment percentages ranged from 4-30%. CD68 (median 11, range 4-23), HLA-DR (median 10, range 5-30) and CD163 staining (M2 marker, median 12, range 5-25) highly overlapped. Outside the perinecrotic and necrotic zones these TAM had a ramified to activated phenotype, whereas in the perinecrotic TAM had a more amoeboid appearance. In the necrotic zones these cells increased in
size due to phagocytosis of debris. Interestingly, Tie-2 showed overlapping staining patterns with HLA-DR, CD163, and CD68 (Fig. 2 panel B). Double

**Figure 2.** Photomicrograph of double immunofluorescence of Tie-2 (middle column in red) and CD34 (panel A), CD68 (panel B), CD163 (panel C) and GFAP (panel D) in green in the first column respectively. The column at the right hand side shows the merged pictures. Staining is on frozen GBM tissues. Original magnification is 400x. Arrows indicate cells that are double positive for Tie-2 and either CD34, CD68, CD163, or GFAP. In panel A it can be observed that the endothelial lining of the blood vessel shows co-expression of CD34 and Tie-2 (both cell membrane markers). Moreover in the merged figure Tie-2 expression can also be observed on the outer border or the vessel wall. Panel B shows cells co-expressing CD68 in the cytoplasm (lysosomal marker) and Tie-2 on the cell membrane. Panel C shows M2-macrophage marker CD163 co-expressed with Tie-2 (both cell membrane markers). Panel D shows the cytoskeletal astrocytic marker GFAP in green, varying in staining intensity. These cell only show weak cytoplasmic (background) expression of Tie-2. In between the astrocytes, GFAP negative cells can be observed with strong membranous Tie-2 expression (indicated by the circle).
immunolabelling showed coexpression of Tie-2 and CD163 (Fig. 2 panel C), whereas many GFAP positive tumor cells were negative or only weakly positive for Tie-2 (Fig. 2 panel D). Double immunolabeling of Tie-2 with either HLA-DR or CD68 showed co-expression similar to that of Tie-2 and CD163 double staining (data not shown), indicating that a large part of the Tie-2 expression can be accounted for by the monocytic infiltrate.

**Discussion**

The regulation of tumor angiogenesis by the Ang-Tie-2 system through the activation of the Tie-2 receptor on the tumor endothelial cells has been well established. Here, we studied the expression and distribution of Tie-2 in GBM and show that Tie-2 is also strongly expressed outside the vascular compartment. This expression can mainly be ascribed to a subpopulation of TAM. In human glioblastomas, on an mRNA level, the balance between Ang-1 and Ang-2 is associated with survival [2]. Here, we show that Tie-2 expression also correlates with survival.

Initial studies on Tie-2 expression in human tumors localized Tie-2 specifically on the tumor endothelial cells [15]. We also observed Tie-2 expression in endothelial cells, but the intensity is much weaker when compared with the non-vessel compartment. Others have demonstrated Tie-2 expression on hematopoietic and neural stem cells [16,17]. Lee et al. reported that Tie-2 immunohistochemical staining in human GBM exceeded, by far, the expression that could be accounted for by the CD34+ and CD31+ cells [18]. Using double staining procedures they found colocalization of Tie-2 with GFAP expression, indicating that the neoplastic glial cells were largely responsible for the Tie-2 staining observed in the glioblastoma tissue samples. Our outcomes are, in part, in line with these findings. We found expression of Tie-2 in tumor cells (double staining of GFAP and Tie-2) and in glioma stem cell lines (qRT-PCR). However, we also found that the predominant part of extra-endothelial Tie-2 expression can be ascribed to the TEM within the tumor.
De Palma et al. first identified this subpopulation of TAM that express Tie-2 [19] and measured the presence of TEM in a range of human tumors [5]. They illustrated the contribution of these cells to the tumor angiogenesis process in a brain tumor model. Moreover, they showed that Ang-2 is able to induce monocyte migration, suggesting that Ang-2 expression may be instrumental in the recruitment of TEM. Others have found that Ang-2 does not actually recruit TEM, but induces Tie-2 expression in tumor associated monocytic cells [4] and increases the tumor growth promoting properties of these cells [3]. Du et al. also identified a tumor associated Tie-2 expressing monocyte subpopulation and similarly show the influence of these cells on tumor growth in a brain tumor model [20]. More specifically, they suggest that these cells determine the angiogenic phenotype of the tumor.

The subpopulations of TAM described by De Palma et al. and Du et al. may not be entirely comparable, nonetheless, both research groups presented data that point in the same general direction, namely the existence of a Tie-2 expressing subpopulation of tumor associated monocytic cells that is involved in the stimulation of angiogenesis and tumor growth. In our study immunohistochemistry was used to identify these TEM in human glioma samples. CD68, HLA-DR, and CD163, as a marker for M2/tumor-supportive-macrophages [21,22], were co-stained with Tie-2 and co-localization was observed.

Analysis of TEM in human GBM in the literature is scarce. Venneri et al. mention a measurement of TEM in a single GBM in their supplementary material [5]. In this tumor no TEM could be identified. The reason for this is not entirely clear as we found TEM in almost all the GBM tested. Since there is some zonal distribution in Tie-2 expressing cells in GBM, differences in the outcomes of both studies could be the result of sampling error.

Our findings are in line with the preclinical evidence which suggests that the Ang-Tie-2 pathway and TEM are intimately involved in glioma growth and angiogenesis, as presented by De Palma et al. and Du et al.. Preclinical
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Evidence supports the idea that this involvement exists at different levels. First, in the initial tumor outgrowth, Ang-2 upregulation leads to desintegration of the vasculature, which serves as a prerequisite for angiogenesis [23]. Second, the recruitment and/or activation of TEM supports tumor growth and angiogenesis [19,20]. Third, the Tie-2 activation by Ang-1 is associated with the upholding of the vascular stem cell niche [24]. The importance of the Ang-Tie-2 system and TEM for other tumors has been extensively reported [25]. Moreover, serum Angiopoietin and TEM levels have been presented as tumor markers in lung carcinoma [26], colon carcinoma [27], hepatocellular carcinoma [28], and melanoma [29]. Also, targeting the Ang-Tie-2 pathway has shown promising results in ovarian cancer [30].

Conclusion

In conclusion, this paper shows that Tie-2 protein expression in human glioblastoma correlates with patient survival. The tumor cells and TEM largely account for this expression and TEM are abundant in most high grade gliomas. Although no direct causal relations can be revealed using our study set-up, our results, together with the arguments provided above, support the validity of ongoing attempts to target the Ang-Tie-2 pathway in the treatment of glioblastoma.
References


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