The pro-migratory and pro-invasive role of the procoagulant tissue factor in malignant gliomas

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During the infiltration process, glioma cells are known to migrate along preexisting anatomical structures such as blood vessels, axonal fiber tracts and the subependymal space, thereby widely invading surrounding CNS tissue. This phenomenon represents a major obstacle for the clinical treatment of these tumors. Several extracellular key factors and intracellular signaling pathways have been previously linked to the highly aggressive, invasive phenotype observed in malignant gliomas. The glioblastoma (GBM), which is the most malignant form of these tumors, is histologically characterized by areas of tumor necroses and pseudopalisading cells, the latter likely representing tumor cells actively migrating away from the hypoxic-ischemic core of the tumor. It is believed that intravascular thromboses play a major role in the emergence of hypoxia and intratumoral necroses in GBMs. One of the most highly upregulated prothrombotic factor in malignant gliomas is tissue factor (TF), a 47 kDa type I transmembrane protein belonging to the cytokine receptor superfamily. In a recent study, we provided evidence that TF/FVIIa signaling via the protease-activated receptor 2 (PAR-2) promotes cell growth, migration and invasion of glioma cells. In this Commentary & View, we outline the key molecular players involved in migration and invasion of gliomas, highlight the potential role of TF for the pro-migratory and pro-invasive phenotype of these tumors and discuss the underlying mechanisms on the cellular level and in the tumor microenvironment.

Key words: brain tumor, blood coagulation, hypoxia, MAP kinase, cancer stem cells, tumor invasion

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Introduction

Gliomas are a group of glial-derived brain tumors, displaying morphological and immunohistochemical features of astrocytic, oligodendroglial or ependymal lineages.1,2 Subgrouping of glial tumors according to their infiltrative behavior allows discrimination into less malignant, more circumscribed tumors like the pilocytic astrocytomas, and the large group of highly infiltrative diffuse gliomas.1,2 Glioblastoma (GBM), assigned to WHO grade IV, is the most malignant and frequent primary brain tumor.1,2

During the infiltration process, tumor cells are known to migrate along preexisting anatomical structures such as blood vessels, axonal fiber tracts and the subependymal space,3–6 thereby widely invading surrounding CNS tissue. This phenomenon represents a major obstacle for the clinical treatment of GBM.7 Therapeutic strategies aimed at eradication of the tumor core, i.e., surgical resection and radiotherapy exhibit only limited efficiency because they do not target the migratory cells in the infiltration zone. Therefore, a high rate of tumor recurrence associated with poor outcome in patients suffering from GBM appears to be an inevitable consequence of current clinical therapies.8

Signals Promoting Glioma Cell Invasion and Migration: Interplay Between Tumor Cells and the Microenvironment

As briefly outlined below, there are several extracellular key factors and intracellular
signaling pathways that have been previously linked to the highly aggressive, invasive phenotype observed in malignant gliomas. Diffuse invasion is a multi-step process involving extracellular matrix (ECM) adhesion, ECM degradation and ensuing cell migration. Adhesion of tumor cells to specific components of the ECM confers the traction required for directed migration/invasion. Cell adhesion molecules such as integrins and members of the cadherin family are known to play a pivotal role in this context.\(^9\)-\(^{12}\) Subsequent ECM remodeling and degradation of ECM proteins is necessary to remove the physical barriers against tumor cell invasion. This is mainly achieved through secretion of proteolytic enzymes, i.e., serine proteases, cathepsins and matrix metalloproteases. 13 Moreover cytoskeletal rearrangements in the tumor cells and formation of lamellipodia and filopodia are necessary for cell locomotion. 14 Small, monomeric GTPases of the Rho family are important cellular regulators of cell motility as Rho stimulates formation of stress fibers and focal adhesions. Rac, another member of this family, is required for the formation of lamellipodia whereas Cdc42 mainly promotes cell polarity and filopodia formation. 15-18 Another pivotal player in glioma invasion is galectin-1 which exerts pro-migratory and pro-invasive effects via multiple mechanisms, including modulation of cytoskeleton organization, Rho expression and integrin recycling. 19-21 Constitutive overactivation of oncogenic signaling pathways implicated in propagation of cell migration and invasion, such as the STAT3 pathway 22-25 and the PI3K/Akt pathway, 26 is a common feature of malignant glioma cells. Downregulation of Stat3 can suppress the expression of proinvasive mediators 27 and the transcription factor C/EBP\(\beta\) can synergistically act with STAT3 to promote the expression of mesenchymal genes, thereby driving migration and invasion. 28 Inhibition of the PI3K/Akt pathway by RNA interference or by small molecule inhibitors exerted limiting effects on glioma cell migration and invasion. 29,30 Together with a complex interplay of tumor cell-derived autocrine factors, such as TGF\(\alpha\), 31 CXCR4 32 and glial cell line-derived neurotrophic factor (GDNF), 33 this suggests that a highly pro-invasive and promigratory phenotype to a significant degree is an intrinsic feature of malignant glioma cells. Furthermore, interaction between host brain cells such as microglia 34,35 and the brain vasculature 36 also contribute to tumor cell invasion and migration.

Invasion of surrounding brain tissue by glioma cells can be further enhanced by tumor hypoxia, a hallmark of malignant gliomas. 34,35 The Best documented signaling molecules mediating these processes are the transcription factors hypoxia-inducible factors 1 and 2 (HIF1, HIF2), which are stabilized and activated under hypoxic conditions, 36 thereby increasing the expression of invasion-related genes such as matrix metalloproteases, 37 transforming growth factor-alpha (TGF\(\alpha\)) and the metastasis factor c-Met. 38,39 Interestingly, the basal expression of HIF2 was recently found to be elevated under normoxic conditions in glioma stem cells in comparison to matched non-stem cancer cells. 40 The pro-invasive capacity of HIFs may be of critical importance for the response of malignant gliomas to anti-angiogenic therapy leading to enhanced tumor hypoxia. Indeed, the VEGF-specific antibody bevacizumab (avastin) was recently shown to trigger a phenotypic shift of gliomas to a predominantly infiltrative pattern. 41 Still, there is evidence for a positive impact of bevacizumab on progression-free survival 42 and bevacizumab was recently approved for second-line therapy in the US.

**Intravascular Thrombosis, Hypoxia and Tumor Necrosis: Hallmarks of Malignant Glioma**

Hypoxic GBMs are histologically characterized by areas of tumor necroses and pseudopalisading cells, the latter likely representing tumor cells actively migrating away from the hypoxic-ischemic core of the tumor. 34 It is believed that vascular factors play a major role in the emergence of hypoxia and intratumoral necroses in GBMs. 35,36 From a histological point of view, one of the most frequent vascular features of malignant astrocytomas is an intravascular thrombosis, which can initiate, propagate and accentuate tumor hypoxia and necrosis. A strong relationship between human malignant astrocytomas and abnormal blood clotting is well-established and when present in anaplastic astrocytomas, it appears to indicate a more aggressive clinical behavior. 34,35,44 Intravascular thromboses are a frequent intraoperative finding by the neurosurgeon and they can often be identified under the surgical microscope. Multiple factors likely contribute to intravascular thrombosis, including abnormal blood flow within the highly aberrant tumor vasculature and breakdown of the blood-brain barrier (BBB) with subsequent access of plasma clotting factors to tumoral tissue. 34 One of the most highly upregulated prothrombotic factors in malignant astrocytomas is tissue factor (TF), a 47 kDa type I transmembrane protein belonging to the cytokine receptor superfamily. 34,35,45

**Tissue Factor: A Procoagulant Whose Expression is Positively Correlated with the Grade of Malignancy in Glial Tumors**

TF is the main initiator of blood coagulation. It serves as a cell-surface receptor and cofactor for Factor VIIa (FVIIa). The complex of TF and FVIIa catalyzes the conversion of Factor X to Xa, which then forms the prothrombinase complex with factor Va. 45,46 Expression of TF by tumor cells correlates with the histological grade of malignancy, tumor invasiveness and prognosis in other types of cancer. 47,48 The expression of TF also positively correlates with the histological grade of gliomas and with the extent of necrosis. 50 Conversely, expression of the tissue factor pathway inhibitor-2 (TFPI-2), a structural homolog of TFPI-1 and broad spectrum protease inhibitor, is negatively correlated with glioma grade. 51 TF is known to exert multiple effects on cancer cell autonomic functions and the microenvironment of tumors. 49,52-54 Given its role in the blood clotting and the fact that TF is normally expressed only in subendothelial mesenchymal cells, i.e., is excluded from the vascular compartment, increased expression of TF in tumor cells and in tumor vascular endothelial cells (VECs) is correlated with a...
Tissue Factor and its Role for the Migratory and Invasive Phenotype of Glioma

In a recent study, we presented evidence that TF/FVIIa signaling plays an important role in cell growth, migration and invasion glioma cells.69 Our data also suggest that the effects of TF/FVIIa are mediated through downstream activation of PAR-2 and the p44/42 MAP kinase/extracellular regulated kinase pathway (Fig. 1).

Molecular Mechanisms of the Pro-migratory and Pro-invasive Effects of TF in Glioma Cells

In our study, we chose three cell lines with high expression levels of TF and co-expression of FVII, as well as PAR1 and PAR2 (MZ-18, MZ-304 and HS 683). TF-dependent signaling was inhibited by (1) stable lentiviral knockdown of TF expression and by (2) a neutralizing monoclonal antibody (mAB TF9-10H10) specifically interfering with TF/FVIIa-mediated signaling without affecting the pro-coagulatory function of TF/FVIIa.65 As an additional control, we also employed cell line U373, expressing only low amounts of TF and FVII (Fig. 2A) and lacking detectable expression of PAR-2 (Fig. 1B). To further analyze the effects of TF in a PAR-2-deficient system, we also established U373 cells stably overexpressing TF (Fig. 2A).

Inhibition of TF/FVIIa-mediated signaling by mAB TF9-10H10 evoked significant antiproliferative effects in TF- and PAR-2-expressing cells,65 while it had no discernible effect on the proliferation of U373 cells overexpressing TF (Fig. 3). This suggested that the reduced proliferation in TF- and PAR-2-expressing cells was indeed attributable to interference with TF/FVIIa-mediated PAR-2 signaling. Knockdown of TF and mAB TF9-10H10 also limited tumor cell migration and invasion under normoxic and hypoxic conditions,65 whereas mAB TF9-10H10 had no effect on migration and invasion in U373 cells overexpressing TF, even when they were exogenously stimulated TF with its natural agonist FVIIa (Fig. 3). Future experiments with PAR-1 and PAR-2 peptide agonists will shed further light on the role of PARs in mediating the protumorigenic effects of TF.

Since mAB TF9-10H10 specifically inhibits TF/FVIIa-mediated signaling without interfering with the blood coagulation cascade, TF-dependent activation of cell proliferation, migration and invasion obviously did not require Factor X and thrombin known to be expressed by malignant glioma cells.66,67 Since other studies have proposed a thrombin-mediated proliferative effect in glioma cells and other cell lines,66,68,69 the role of thrombin in this context remains controversial.

It is well established that hypoxia triggers activation of the ERK pathway,70 The knockdown of TF inhibited basal and hypoxia-triggered phosphorylation of ERK1/ERK2 in PAR-2-expressing cells,65 possibly limiting ERK-dependent activation of downstream targets involved in modulation of cell migration, such as myosin light chain kinase (MLCK), calpain and focal adhesion kinase (FAK).71 In addition, the ERK pathway can also promote MMP-9 expression in glioma cells and has previously been implicated in the shift to a more invasive phenotype of glioma,72,73 Collectively, the results of our study point at an important role of the TF/FVIIa/PAR2/ERK axis in tumor growth and invasion of gliomas and suggest that TF may be a suitable target for the development of novel therapies against gliomas displaying high expression levels of TF. To further strengthen this hypothesis, the impact of TF inhibition on tumor growth and survival needs to be tested in vivo glioma models.

The Vicious Cycle of Vascular Thrombosis, Tumor Hypoxia and Invasion in Malignant Glioma: TF as a Potential Driving Force?

There is evidence that the expression of vascular endothelial growth factor (VEGF) and TF is positively correlated in malignant astrocytomas.74 Interestingly, our histopathological analyses indicated that in human gliomas, TF expression is enhanced in perivascular tumor cells located in close vicinity to the endothelia,75 the structures that tumor cells use as guidance to migrate in the brain...
As stated above, pro-inflammatory cytokines also can increase TF expression. Thus, inflammatory cytokines (e.g., TNFα) released to the tumor environment by monocytic and lymphocytic infiltrates could increase TF levels on the surface of subendothelial tumor cells or tumor endothelial cells, leading to an ensuing increase in VEGF expression. Since VEGF is believed to play a pivotal role in blood-brain barrier breakdown, one now might hypothesize a key role of TF in astrocytoma progression. After leakage of the blood-brain barrier has occurred through VEGF synthesis, plasma clotting factors like FVII gain access to perivascular located tumor cells.
overexpressing TF (Fig. 5). TF then could theoretically get access to the intravascular space, either as a splice variant containing the TF ectodomain or as a cargo of membrane microvesicles.77 This would initiate the blood clotting cascade causing thromboses of the small blood vessels, thereby diminishing the oxygen supply in the microenvironment, which would further drive TF expression in the tumor cells. In addition, through generation of thrombin, TF might also increase VEGF expression, fueling a vicious cycle of thrombosis, hypoxia and overexpression of TF and VEGF (Fig. 5). Activation of the PAR-2 receptor by TF and FVIIa might then propagate the migratory and invasive behavior of the tumor and support migration away from the hypoxic focus and along the preexisting vascular structures (Fig. 5). TF expressed on tumor endothelial cells might play only a minor role in this context since (1) it is physiologically not expressed in these cells and (2) tumor formation and metastasis of several different cancer cell lines were not impaired in mice expressing extremely low levels of TF (low-TF mice), indicating that in general, tumor-derived TF may play a dominant role.78

Enhanced TF Expression in Malignant Glioma: Potential Link to the Mesenchymal Phenotype and to Cancer-Initiating Stem Cells

Recently the perivascular niche has emerged as a place necessary to maintain cancer stem cells in a stem cell-like state.79-81 Dissection of high-grade astrocytomas into prognostic subclasses recently demonstrated that patients with tumors displaying stem-like expression signatures had a shorter overall survival. In addition, recurrent tumors were found to shift towards a mesenchymal phenotype, a hallmark of tumor aggressiveness in malignant glioma.82 Of note, it has been proposed that TF expression is significantly increased in CD133 positive cancer stem cells.83 In light of the fact that epithelial-to-mesenchymal transition (EMT)-like changes in glioma cells are associated with enhanced expression of TF, one might speculate about the possible role of tumor cell-derived and host related TF in influencing the properties of cancer stem cells.78,84 Interestingly, cancer initiating stem cells can be located in close proximity to tumor vessels (vascular niche).79 Due to this vascular proximity, but also through extravascular leakage of coagulation factors from the hyperpermeable tumor microcirculation, the contact between TF derived from perivascular tumor cells and coagulation factors from the blood-stream (FVIIa) is highly facilitated. Supporting evidence for an important role of TF in cancer stem cells is provided by the fact that inhibition of TF interfered with tumor initiation of A431 squamous cell carcinoma cells.78 Future studies are necessary to clarify the
Figure 4. TF expression in human glioblastomas. (A and B) Perivascular tumor cells (arrowheads) expressing TF mainly in the perivascular space. (C) Perinecrotic tumor areas showing tumor cells strongly expressing TF (arrowheads). (D) In the infiltration zones of glioblastomas, TF expression is accented around vessels. Here, TF is not only restricted to neoplastic astrocytes but also seen in reactive astrocytes showing elongated processes directed to the vessel wall (arrowheads). (All vessel lumina are indicated with an asterisk).

Figure 5. Hypothetical mechanism of TF-triggered migration/invasion of perivascular tumor cells. After leakage of the blood-brain barrier (BBB) has occurred through activation of VEGF, plasma clotting factors like FVIIa gain access to tumor cells localized in the perivascular space. In reciprocal fashion, TF gets access to the intravascular space at high concentrations and initiates the blood clotting cascade causing thromboses of small blood vessels. Ensuing hypoxia in the microenvironment further enhances TF expression in the tumor cells. TF might also increase VEGF expression, fueling a vicious cycle of thrombosis, hypoxia and overexpression of TF and VEGF. Activation of the PAR-2 receptor by TF and FVIIa propagates the promigratory and proinvasive behavior of the tumor cells, leading to migration away from the hypoxic focus and along the preexisting vascular structures.
exact mechanisms and functional consequences of the heterogeneous expression of TF in cancer stem cells vs non-stem cells. If indeed TF plays a role in the tumor-initiating properties of cancer stem cells, it remains to be shown in which environments and settings this may be of importance.

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