Role of differentiation in glioblastoma invasion

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TGF-β as a therapeutic target in high grade gliomas - promises and challenges

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ABSTRACT

Transforming growth factor-β (TGF-β) is a cytokine with a key role in tissue homeostasis and cancer. TGF-β elicits both tumor suppressive and tumor promoting functions during cancer progression, in a wide range of cancers. Here, we review the tumor promoting function of TGF-β and its possible promise as a therapeutic target in high grade gliomas, including glioblastoma multiforme (GBM), a disease with very poor prognosis. TGF-β signalling is highly active in high grade gliomas and elevated TGF-β activity has been associated with poor clinical outcome in this deadly disease. Common features of GBMs include fast cell proliferation, invasion into normal brain parenchyma, hypoxia, high angiogenic - and immunosuppressive activity, characteristics that all have been linked to activation of the TGF-β pathway. TGF-β signaling has also been connected with the cancer stem cell (CSC) phenotype in GBM. CSCs represent a subset of GBM cells thought to be responsible for tumor initiation, progression and relapse of disease. Following the description of these different properties of TGF-β signaling and the underlying mechanisms identified thus far, the promise of TGF-β targeted therapy in malignant gliomas is discussed. Several drugs targeting TGF-β signaling have been developed that showed potent antitumor activity in preclinical models. A number of agents are currently evaluated in early clinical studies in glioma patients. Available results of these studies are highlighted and a perspective on the promise of TGF-β-targeted therapy is given.
1. TGF-β signaling in cancer

Transforming growth factor-β (TGF-β) represents a member of a large family of cytokines that include the bone morphogenic protein (BMPs), nodals and activins, which are involved in the regulation of embryonic development and tissue homeostasis (1). There are three isoforms of TGF-β in mammals, namely TGF-β1, -β2 and -β3, which are all synthesized as latent dimers. Latent TGF-βs need to be converted into their active forms by processing of precursors by proteases (2). At the cellular level TGF-β affects processes such as cell growth, cell survival, differentiation, migration and immune cell activation in a cell type-dependent fashion and depending on the cellular context. In order to execute these various effects TGF-β works through a complex network of different ligands and receptors leading to a range of downstream signals (1, 2).

In cancer the TGF-β pathway has both tumor suppressive and promoting functions. TGF-β is considered a tumor suppressor as it is a very potent inhibitor of proliferation in epithelial cells, astrocytes, and immune cells. Some tumors escape from the cytostatic responses of TGF-β, for example by acquiring mutations in elements of the TGF-β pathway. Certain malignant tumors, including gliomas, selectively lose the capacity of TGF-β to inhibit proliferation while maintaining other functions of the pathway intact (1). In such tumors TGF-β can promote proliferation, angiogenesis, invasion, metastasis and immune suppression. Thus TGF-β can have a dual role in tumorigenesis and depending on the stage and type of the tumor, it either acts as a tumor suppressor or a tumor promoter (3, 4). This switch from tumor suppressor to oncogenic activity is also known as the ‘TGF-β paradox’ (5, 6).

Within the TGF-β superfamily signaling via the TGF-β polypeptide is most extensively studied and is briefly described below (extensively reviewed by for example Santibanez et al 2011) (7). Though all the three different isoforms of TGF-βs are encoded by different genes, they all depend on the same receptor signaling system (3, 8). TGF-β binds and activates a membrane receptor serine/threonine kinase complex (TGF-β-RI/II/III), which phosphorylates SMAD2 and SMAD3, also known as receptor-regulated (R)-SMADS. The phosphorylated SMADs together with SMAD4 or common-mediator (co)-SMAD are translocated into the nucleus resulting in the formation of transcriptional regulatory complexes that modulate the expression of many target genes in a gene- and cell-specific way. Additional diversity in TGF-β signaling is achieved via activation of SMAD-independent effector molecules, also known as non-canonical TGF-β signaling (9). These include activation of mitogen-activated protein kinases (MAPK), cell survival enhancing PI3K/AKT, inflammatory regulators NF-κB and cyclooxygenase-2 (Cox-2), small GTP-binding proteins RAS and RhoA, and non-receptor protein tyrosine kinases SRC and Abl (9, 10). Interestingly, it is suggested that whereas SMAD-dependent signals result in growth inhibition and apoptosis, non-canonical
signals predominate in more advanced and metastatic tumor cells (11).

To prevent continuous TGF-β signaling several negative regulators have been identified. For example, BMP and activin membrane-bound inhibitor (BAMBI) can intercalate with TGF-β receptors preventing activation (12). Binding of nuclear SMAD7 to the U3 ubiquitin ligase SMURF2 results in cytoplasmic localization and binding to TGF-β -RI/II thus inhibiting SMAD2/3 activation. SMAD7 can be regulated by different stimuli, including TGF-β, IFN-γ, TNF-α as well as ultraviolet light, and mediates the crosstalk between TGF-β and other signaling pathways (13). Overall, TGF-β secreted by tumor cells (autocrine) and the microenvironment (paracrine) has an impact on tumor development and progression. The more precise determinants of TGF-β signaling and its opposing effects in cancer remain to be identified. However, it has become increasingly clear that this pathway provides an interesting and important target for the development of novel therapeutic strategies.

Here we will review the effects of TGF-β specifically on the formation and progression of brain tumors, in particular gliomas. Therapeutic approaches that have been explored in preclinical models will be described, and clinical studies with TGF-β signaling-targeted agents and perspectives are discussed.

2. Malignant Gliomas

Malignant gliomas account for eighty percent of malignant tumors that develop in the central nervous system and are essentially incurable, constituting a spectrum of clinicopathological entities, from low-to high-grade malignancies, and almost all low-grade tumors eventually progress to high-grade malignancy (15). These tumors progress through a series of developmental steps, which include the transformation from a cell of origin, activation of cellular proliferation signals and abrogation of cell cycle control, acquisition of invasive phenotype, enhanced angiogenesis, and further clonal evolution (15). Traditional pathological approaches divide adult gliomas into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. This classification is largely based on the predominant glial cell type (astrocytes or oligodendrocytes) present in tumor and the protein expression pattern as seen in immunohistochemistry. Oligodendroglial tumors tend to have better prognosis and are more chemosensitive than astrocytomas (15). Traditional pathological approaches also grade the malignant gliomas into three degrees of malignancy: World Health Organization (WHO) grade II, grade III (anaplastic) and grade IV (glioblastoma multiforme, GBM). GBM is the most aggressive brain tumor characterized by diffuse infiltration of the brain parenchyma, aberrant proliferation, resistance to chemotherapy and radiation, and recurrence after surgical resection (15, 16). In a clinical setting WHO grading is an important component among a combination of criteria used to predict response to therapy and outcome (16). Patients
with WHO grade II tumors typically survive for more than 5 years and those with grade III tumors survive 2-3 years (16). GBM has the worst prognosis, despite multimodality treatment consisting of open craniotomy with surgical resection of as much of the tumor as possible, followed by concurrent and sequential chemo/radiotherapy (17). Over the past two decades, extensive cytogenetic and molecular genetic studies have identified a number of recurrent genetic alterations and chromosomal abnormalities in malignant gliomas, particularly in GBM. Advances made in molecular technologies such as high-density microarray and genome sequencing, have made it possible to evaluate the genetic and epigenetic changes in these tumors at the genome-wide level (14). Recent gene expression profiling studies have revealed molecular subtypes of glioma based on the preferential expression of genes characteristic of neural progenitor cells (proneural, PN), neurons (neural), proliferating cells and receptor tyrosine kinase activation (classical), or mesenchymal tissues (mesenchymal, MES) (reviewed in 18). Malignant gliomas in the MES sub-class are aggressive, invariably coincide with disease recurrence, and are resistant to treatment modalities, whereas patients with a PN signature perform better in the clinic (19). This indicates that the aggressiveness of GBM may be governed by the genes that are involved in cell fate choice during neurogenesis or alternatively gene expression patterns that resemble mesenchymal cells. The poor prognosis of patients with malignant glioma calls for the development of novel therapeutic approaches to improve the survival of these patients.

3. TGF-β and glioblastoma

GBM is characterized by extensive heterogeneity at the cellular and molecular levels. There is compelling evidence suggesting that the different tumor cell populations can establish a complex network of interactions between each other and with the tumor microenvironment that promote tumor growth and providing an increased chance to escape therapy (20). The traditional idea that tumors are composed of a mass of malignant cells is drastically changing. It is now increasingly appreciated that tumors contain a proportion of host cells and tissues that infiltrate the tumor, attracted by tumor secreted molecules that include cytokines, chemokines and growth factors. This proportion of normal tissue and the variable amount of tissue immediately surrounding the tumor, make up the tumor microenvironment. The microenvironment of brain tumors is composed of a wide variety of cells that include the microglia, macrophages, astrocytes, oligodendrocytes, neurons, glial and neuronal progenitors, extracellular matrix, pericytes and endothelial cells. This complex mixture of neoplastic and non-neoplastic cells interact among themselves, hosting a deadly team work that favor tumor cell growth, invasiveness, immune escape and therapy resistance (20).

Among the multiple pathways associated with gliomas, the TGF-β pathway plays a very crucial role in regulating the behavior of these tumors (21). Elevated levels of TGF-β have
been reported in the blood serum of patients with malignant glioma and a striking correlation was observed between elevated TGF-β levels and high tumor grade, advanced tumor stage, and poor patient outcome (21-24). The study by Sasaki et al. showed that culture supernatants from malignant gliomas contained both active and latent forms of TGF-β1 and TGF-β2 (22). Thus far, no evidence has been obtained for possible antitumorigenic activity of TGF-β signaling in gliomas, such as the induction of senescence that has been observed in epithelial cancers (1). Instead, TGF-β expression in malignant brain tumors was found to render the tumor cells survival advantage by enhancing cell growth, migration, invasion, angiogenesis, immune suppression and stem cell properties (24) (see also Figure 1). TGF-β in gliomas is either secreted in an autocrine fashion by glioma cells or is derived from the microglial cells (22, 25). The autocrine secretion of TGF-β has been observed in established cell lines and in surgically resected malignant gliomas cells.

3.1. TGF-β and glioblastoma stem cells

A subpopulation of cells with stem cell-like properties has been identified in gliomas. This cell population named cancer stem cells or glioma stem cells (GSCs) is considered to be responsible for the initiation, propagation, maintenance and recurrence of these tumors, indicating that therapies that target the GSCs might significantly improve the poor prognosis.
associated with gliomas (26, 27). GSCs are characterized by the expression of neural stem cell (NSC) antigens and possess the capacity of self-renewal, multilineage differentiation and the ability to generate spherical cellular structures called neurospheres when cultured in serum-free medium in presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Thus GSCs share several characteristics with normal NSCs (28). The origin of GSCs is still an area of debate and some investigators argue that GSCs could originate from the malignant transformation of the normal NSCs or glial/neuronal progenitors, while others advocate for the idea that differentiated cells could revert back to the stem cell state (29, 30). It was reported that CD133+ GSCs in vitro were more resistant to radiation compared to the CD133- non-stem population due to activation of DNA repair pathways by CHK1/CHK2 (27). In addition, GSCs overexpress certain ATP-binding cassette transporters (ABCTs) that can cause resistance, such as ABCG2 that can excrete chemotherapeutic drugs like the DNA alkylating agent temozolomide (TMZ) out of GSCs (31). Furthermore, ABCG2 has also been mechanistically linked to the PTEN/P13K/AKT pathway governing survival signalling. Although elimination of GSCs has been regarded as a prerequisite for developing successful therapeutic strategies against gliomas, this has not been formally proven in patients. In genetically engineered mouse models, however, recently proof for this hypothesis was obtained (32). It was shown that the eradication of a subset of relative quiescent GSCs effectively blocked tumor growth, and remaining tumor cells were non-invasive and displayed low proliferative potential. However, the molecular mechanisms underlying GSC maintenance have not been fully elucidated as yet and optimal treatments remain to be developed.

The TGF-β superfamily plays a critical role in morphogenesis and cell lineage specification during brain development (33, 34). In an analogous way a study by Ikushima et al. showed that autocrine TGF-β signalling plays a crucial role in retention of stemness in GSCs (35). The inhibition of TGF-β signalling in GSCs by pre-treatment with inhibitors of the TGF-β pathway exhibited less lethal potency in intracranial transplantation assays in immune-compromised mice. TGF-β maintains the stemness of these cells by inducing the expression of the transcription factor Sox2, known to be an important regulator of stemness. Sox2 expression was found to be induced by Sox4, which is a direct target of TGF-β (34) (see also Figure 2). BMP4 another member of the TGF-β family on the other hand enhances differentiation and depletes the pool of GSCs (36).

The association of TGF-β with stemness was also demonstrated in the mammary epithelium, where it was observed that a brief exposure of mammary epithelial cells to TGF-β activated the epithelial-mesenchymal transition (EMT) program and enhanced the mammosphere formation capacity of these cells (37). Similarly, exposure to TGF-β enhances neurosphere
formation capacity in primary material-derived cultures of brain tumor cells in a dose dependent manner, showing that TGF-β enhances GSCs self-renewal capacity (38). This effect was compromised following the addition of TGF-β receptor 1 (TβR1) inhibitor concomitantly with TGF-β. Furthermore, a transcriptomic analysis of U373MG glioma cells treated with TGF-β and/or a TβR1 inhibitor revealed leukemia inhibitory factor (LIF) as one among the 63 TGF-β responding genes that were dependent on TβR1 activity (38). The role of LIF-LIFR/gp130-JAK-STAT signaling pathway has been implicated as a promoter of NSC self-renewal in the adult brain (39). Exposure of patient material derived neurospheres to any of the TGF-β family member TGF-β1, TGF-β2 or TGF-β3 could induce SMAD-dependent LIF transcription,
and this effect was mediated by TβR1 (38). It was found that TGF-β activates the JAK-STAT pathway via induction of LIF secretion that acts through an autocrine/paracrine loop and enhances the self-renewal potential of GSCs. TGF-β and LIF also prevent the differentiation of GSCs as the addition of TGF-β or LIF to differentiation medium containing serum delayed the differentiation process of neurospheres. The treated neurospheres maintained the expression of neuroprogenitor markers, such as Musashi, Sox2 and Nestin, and became less attached to the culture plate, maintaining the spherical morphology (38).

Inhibition of TβR1 with a selective chemical compound in glioblastoma primary material-derived cultures resulted in downregulation of the expression of the DNA-binding proteins and transcription regulators Id1 and Id3 in a SMAD4-dependent manner (40). This effect could also be seen with the in vivo intracranial GBM mouse models where Id1 downregulation was more pronounced over Id3 downregulation following treatment with TβR1 inhibitor. Furthermore, TβR1 inhibition reduced the neurosphere formation potential and decreased the mRNA levels of Id1 and Id3. In neurospheres high expression of Id1 correlated with high expression of CD44 and the CD44<sup>high</sup>/Id1<sup>high</sup> population showed properties of GSCs both in vitro and in vivo (40). In analogy, the increase of CD44<sup>high</sup> stem cell population in mammary epithelial cells following treatment with TGF-β has also been reported (37). The role of Ids in stem cell biology has been previously elucidated, where it was suggested that the GFAP<sup>+</sup> Id1<sup>high</sup> B1 type astrocytes are the bona fide stem cells of the adult murine subventricular neurogenic lineages. Id1 and Id3 also play a vital role in regulating the self-renewal capacity of stem cells and it was shown that the ablation Id1 and Id3, but not Id1 alone, was sufficient to reduce secondary neurosphere formation by ~50% (41). Treatment with TβR1 inhibitor in a phase 1-2 clinical trial including GBM showed a repressed expression of Id1 and CD44 in the tumor of the patient, confirming that inhibition of the TGF-β pathway targets the CD44<sup>high</sup>/Id1<sup>high</sup> cell population and by a proposed transdifferentiation of this GSC fraction into CD44<sup>low</sup>/Id1<sup>low</sup> cells (40). It has recently been reported that GSCs express higher levels of TGF-β2 than the more differentiated glioma cells, and the secretion of TGF-β2 also appears correlated with higher grades of gliomas (42). Together these evidences indicate a role of TGF-β in GSC biology, and that targeting TGF-β pathway might help eliminate the GCS fraction of gliomas.

### 3.2. TGF-β and angiogenesis

The formation of new microvasculature by capillary sprouting, or angiogenesis, is a prerequisite for solid tumor growth. Tumor vascularization is a complex process that is largely influenced by the interaction between the tumor cells and their microenvironment and also depends on specific molecular interactions between vascular cells and extracellular matrix components. Angiogenesis is the hallmark of malignant gliomas, in particular a property of high grade gliomas, and is positively correlated with the expression of vascular endothelial growth factors (VEGF).
factor (VEGF) (24). High vascular density, vascular abnormalization, hyper-proliferation and formation of glomeruloid vascular structures are characteristic features of GBM, hence anti-angiogenic therapy is a promising strategy against malignant gliomas (43, 44). This has led to the FDA approval of Bevacizumab (Avastin®), a VEGF neutralizing monoclonal antibody for the treatment of recurrent GBM (45). The connection between angiogenesis and TGF-β was first demonstrated in studies using transfected Chinese hamster ovary (CHO) cells overexpressing recombinant TGF-β1 (46). Following subcutaneous injection into nude mice, it was observed that stable ectopic expression of TGF-β1 in CHO cells enhanced proliferation and, moreover, showed extensive tumor-associated angiogenesis when compared to tumors derived from parental CHO cells. Enhanced growth and angiogenic pattern in TGF-β1 transfected cells could be inhibited by the use of a TGF-β1 neutralizing antibody, confirming the role of TGF-β1 in angiogenesis (46).

A recent study by Dieterich and coworkers identified 95 genes differentially expressed in glioblastoma vessels and interestingly a significant number of genes that are differentially expressed in GBM vessels included genes encoding extracellular matrix proteins, such as collagens, fibronectin, laminins and nidogens that are known to be regulated by TGF-β signaling (47). TGF-β signalling in the vasculature is mainly dependent on two type 1 receptors, activin receptor-like kinase (Alk)5 (TGFBR1), which is broadly expressed, and Alk1 (ACVRL1) which is restricted to the endothelium. An increase in SMAD2/SMAD4 and SMAD3/SMAD4 signalling complexes was observed in GBM vessels in situ. SMAD3/SMAD4 signalling complexes were mainly localized to the vasculature and the perivascular areas. These results suggest that TGF-β2 signalling contributes to the aberrant vascular gene expression pattern in GBM associated blood vessels (48).

Insulin-like growth factor binding protein 7 (IGFBP7) has been found to be a highly selective biomarker of tumor vessels in GBM (49). IGFBP7 was detected in GBM endothelial cells and extensively deposited into the vascular basal lamina. The selective upregulation of IGFBP7 in GBM vessels when compared to the nonmalignant brain vessels indicate a possible role of IGFBP7 in tumor vascular function. It was demonstrated that human brain endothelial cells (HBECs) could be induced to produce IGFBP7 by GBM cell-secreted mediators, including TGF-β1, through the TGF-β1/ALK5/SMAD2 signaling pathway (49). In this study HBECs exposed to GBM-U87 cell-conditioned media (CM) exhibited fourfold upregulation of IGFBP7 mRNA and protein when compared to the control cells. U87-CM contained a sufficient concentration of ~5pM TGF-β1 to stimulate IGFBP7 production by HBEC. The production of IGFBP7 by HBEC could be blocked both by pan-TGF-β-neutralizing antibody (ID11) and the TGF-β1 receptor (ALK5) antagonist, SB431542. Moreover, SB431542 also inhibited the increase in capillary-like tube (CLT) formation by HBECs in matrigel after exposure to
either U87-CM or IGFBP7 protein. Together this indicates that TGF-β1 is an important tumor-secreted effector capable of IGFBP7-dependent proangiogenic activity in brain endothelial cells (49). Thus, the TGF-β pathway could be a new target in addition to VEGF-A for vascular normalization therapy.

### 3.3 TGF-β and invasive behavior of glioblastoma

Malignant gliomas are characterized by a high rate of invasiveness. Around 90% of all GBMs relapse at the surgical margins in close proximity to the resection cavity and only a small fraction of GBM, 5-10%, recur at a greater distance from the main tumor mass (24). The invasive phenotype of malignant gliomas has been associated with activation of several cell surface receptors including receptor tyrosine kinases (RTKs), G protein–coupled receptors (GPCRs), TGF-β receptor, integrins, immunoglobulins, tumor necrosis factor (TNF) family, cytokine receptors and protein tyrosine phosphatase receptors (50). The physical interaction between heterodimeric αβ cell surface receptors, termed integrins, with extracellular matrix components, such as collagen, fibronectin, laminin and tenascin, is also been considered to mediate the migratory and invasive phenotype of glioma cells (24). This part of the review mainly focuses on the role of TGF-β pathway in regulating migration/invasion in malignant gliomas.

TGF-β is an important player in the invasion metastasis cascade in a wide range of cancers. For example, the majority of metastases to lung, liver and brain in breast cancer patients stain positive for phosphorylated-SMAD2, suggesting an active role of this pathway in metastasis activated by locally released TGF-β (2). High expression of TGF-β has been reported in glioma tumor specimens and cell lines (22, 23). The soluble factors secreted by glioma cells recruit inflammatory cells, including brain microglia and transform them into tumor supportive cells. The specific function of the microglia in malignant glioma biology remains largely unclear (51). However recent research suggests that activated microglia can secrete various cytokines and growth factors that may contribute to immune evasion, growth and invasion of brain neoplasms (51). Microglia are able to strongly enhance tumor invasion in cell co-cultures and brain organotypic slices models (25, 52). Primary rat microglial cultures were found to secrete a considerable amount of TGF-β1 (81 pg/ml) that was increased by 230% when co-cultured with glioma cells. Microglia-enhanced invasiveness of glioma cells was diminished after stable knockdown of TβRII using a specific short-hairpin (sh) RNA, indicating a critical role played by microglia-derived TGF-β in regulating tumor-host interactions (25). Proteases such as the matrix metalloproteinases (MMPs) and cathepsins, contribute to glioma invasion by degrading the extracellular matrix (24, 53). A role of TGF-β in enhancing the expression of MMPs and suppression of tissue inhibitors of metalloproteinase (TIMP) has been demonstrated in human gliomas cells in matrigel invasion assays (54, 55).
Radiation is an effective strategy in prolonging survival of GBM patients; however, the vast majority of GBM patients demonstrate progression at or near the site of original treatment (56). Several studies have reported that ionizing radiation (IR) enhances the invasion of tumor cells in malignant gliomas, but the mechanisms for this effect are not well understood. Irradiation elevates the secretion of TGF-β and β1-integrin and enhances the invasion capacity of U87 cells in a matrigel-based assay (57). Hence, the TGF-β signaling pathway is emerging as a promising therapeutic target to prevent invasion of glioma cells.

3.4. TGF-β-dependent immunosuppression

Anti-tumor immune responses may be greatly compromised during various stages of malignant glioma progression. The role of TGF-β as a potent immunosuppressant cytokine is now being appreciated not only in malignant gliomas but also in various other neoplasms (24). The lack of an effective anti-tumor response in glioma patients could be attributed to the absence of specific tumor antigens on glioma cells or to the lack of professional antigen-presenting cells in the brain (24). The secretion of immunosuppressive cytokines such as interleukin (IL)-10 and TGF-β2 and also prostaglandin E (PGE) has been shown to play a major role in inhibiting the antitumor immune response in malignant gliomas (58, 59). TGF-β exerts pleiotropic effects on all cells of the immune system. For example, several inhibitory effects on mature T cells have been observed, which include the inhibition, proliferation, abrogation of cytotoxic activity and induction of apoptosis (24, 58). TGF-β1 inhibits the progression of human T cells by downregulating proliferative signals mediated through IL-2R (60). TGF-β also enhances the generation of immunosuppressive regulatory T (T_{reg}) cells (61). T_{reg} cells play a vital role in immunosuppression regulated by TGF-β, but themselves do not enhance the secretion of TGF-β (62). T_{reg} cell-mediated immunosuppression occurs via multiple ways, which includes the inhibition of T cell activation and effector function as a consequence of downregulation of IL-2 production, inhibition of IFN-γ production, increase of Th2 cytokine production and suppression of pro-inflammatory cytokine production (TNF-α, IFN-γ, IL-6). Other TGF-β mediated immunosuppression mechanisms involve the downregulation of MHC class II antigen on glioma cells, the deactivation of mononuclear phagocytes, microglia and natural killer (NK) cells (63-65).

The use of a novel TGF-βR1 kinase inhibitor, SD-208 on intracranial glioma mouse models showed an increased tumor infiltration by natural killer cells, CD8 T cells and macrophages. In parallel the treated cells also showed an enhanced release of proinflammatory cytokines such as IFN-γ and TNF-α and a reduced release of IL-10. SD-208 treatment also significantly prolonged the median survival of glioma bearing mice (66). In a study by Ueda et al. a combinational strategy was developed using anti-TGF-β-neutralizing monoclonal antibody (ID11) with glioma-associated antigens (GAA) peptide-based vaccines for glioma
immunotherapy in mice bearing orthotopic GL261 gliomas (67). The neutralization of TGF-β with TGF-β mAb led to improvement of the therapeutic efficacy of GAA vaccine in two ways: firstly, by improving both systemic induction of GAA-specific CTL responses and secondly, by homing of GAA-reactive and IFN-γ producing CD8+ T cells into the tumor. ID11 treatment suppressed the phosphorylation of Smad2 and reduced the level of T\textsubscript{reg} cells in the glioma microenvironment. Neutralization of TGF-β also enhanced systemic production of type-1 cytokines/chemokines, including the upregulation of plasma levels of interleukin-12, macrophage inflammatory protein-1α and IFN-inducible protein-10. Together the systemic inhibition of TGF-β by ID11 could reverse the suppressive immunologic environment of orthotopic tumor–bearing mice systemically and locally, thereby enhancing the therapeutic efficacy of GAA vaccine, which significantly prolonged the survival of mice (67). Interestingly, the secretion of immunosuppressive cytokines like TGF-β2 and IL-10 was found to be higher in the GSC compartment when compared to more differentiated cells, and also showed a correlation with the pathological grade of gliomas (42). Overall, immunotherapeutic strategies have promise to contribute to better prognosis of patients with malignant gliomas, and the suppression of TGF-β signaling may be important in this respect (67, 68).

4. TGF-β affects chemo- and radio-resistance

In glioma cells treated with a large dose of radiation the secretion of TGF-β remained intact and, moreover, the level of TGF-β secretion per glioma cell was found to increase (69). Therefore, methods to inhibit TGF-β or downregulate its expression are thought to be of additional benefit in improving the efficacy of radiation therapy. Two recent studies provided evidence for this notion (70, 71). The first study by Zhang et al. provide evidence that TGF-β signalling blockade by the small molecule TGF-βR1 kinase inhibitor, LY2109761, significantly enhanced radiation-induced cytotoxicity and cooperated with radiation to suppress clonogenic survival of established glioblastoma cell lines. Their study revealed that LY2109761 may act in concert with radiation to enhance the radiation-induced DNA damage and apoptosis. LY2109761 reduced the self-renewal and proliferation capacity of primary material derived stem-like cells in GBM and also enhanced the radio sensitivity of these cells (70). As mentioned earlier in section 3.1., the role of stem-like cells in GBM radioresistance has been previously also linked with elevated activation of DNA repair pathways by CHK1/CHK2 (27). LY2109761 also enhanced radiation induced tumor growth delay in a subcutaneous and orthotopic brain tumor model; in both these models administration of this compound increased the radiation induced prolongation of the animal life span. The blockade of the TGF-β signalling using LY2109761 also inhibited both constitutive and radiation induced tumor cell invasion in the orthotopic model (70). The upregulation of genes like AKT, ALF5 and LMO2, which are activated to enhance tumor invasiveness, angiogenesis and radioresistance in GBM following irradiation, was also reversed by LY2109761.
A very recent study by Hardee et al. (71), demonstrated that the radiation sensitivity of cultured murine and human glioma cell lines could be increased by approximately 25% when treated with LY364947, a small-molecule inhibitor of TGF-β type1 receptor kinase, before irradiation. A significant decrease in tumor growth was also observed following irradiation on mice bearing flank tumors treated with ID11, a pan-isoform TGF-β neutralizing antibody. LY364947 treatment on GBM derived neurosphere culture before irradiation could decrease the primary neurosphere formation by 75% and secondary neurosphere formation by 68%, but radiation alone could decrease the primary neurosphere formation by 28% and did not have any effect on the secondary neurosphere formation. LY364947 treatment on neurosphere culture before irradiation also decreased the DNA damage responses and p53 phosphorylation along with reduction in induction of self-renewal signals like Notch1 and CXCR4 (71). These studies rationalize further translational studies of compounds targetting the TGF-β pathway alone and in combination with radiotherapy in the treatment of glioblastoma. Reminiscent to the situation in GBM, elevated plasma TGF-β1 has also been linked to poor patient outcome in hepatocellular carcinoma and breast, lung and prostate cancers (72). The role of TGF-β in radiotherapy is very crucial, as DNA damage response and subsequent cell fate decisions are severely compromised if TGF-β is inhibited prior to irradiation in mouse epithelial tissues, human mammary epithelial cells and lung cancer cells (72). TGF-β depletion or signal inhibition does not directly affect ATM protein abundance, but blocks the ATM kinase activity, which can promote apoptosis or cell cycle arrest following radiation-induced DNA damage (73).

In yet another study by Zhang et al. (74) a trimodal glioblastoma treatment regimen combining the TGF-βR1 kinase inhibitor LY2109761 with radiotherapy and the chemotherapeutic agent TMZ was tested. This preclinical study demonstrated that the addition of LY2109761 to fractionated radiation alone or in combination with TMZ drastically increased the antitumor effect of the standard treatment in human glioblastoma cells in vitro and in vivo. This observation is indicative for the potential of TGF-βR1 kinase inhibitor to improve the clinical outcome in human glioblastoma, when used in combination with radiation and TMZ (74). Though the direct effect of TGF-β on chemotherapy induced treatment resistance in GBM has not yet been investigated in greater detail, an indirect mechanism may also cause therapy resistance, as TGF-β enhances stem cell properties as described earlier in section 3.1, thus leading to cells with high resistance.

5. TGF-β-targeted therapy in high grade gliomas

From the preclinical data presented above it is clear that TGF-β-targeted therapy may be of great value for the treatment of gliomas. A list of therapeutics targeting of the TGF-β pathway that have been tested, or are currently being studied in glioma patients is summarized in Table 1.
Table 1. Summary of TGF-β pathway targeting agents clinically evaluated in patients with high grade glioma.

<table>
<thead>
<tr>
<th>TGF-β targeting drug</th>
<th>Treatment</th>
<th>Phase</th>
<th>Patients</th>
<th>Remarks</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabedersen (AP 12009)</td>
<td>Intratumor administration</td>
<td>1/2</td>
<td>n=24 recurrent, refractory GBM, AA</td>
<td>Completed. Good tolerability and safety; Indications for efficacy (7/24 responded, 2/24 complete responses)</td>
<td>Hau et al. 2007</td>
</tr>
<tr>
<td>Trabedersen - TGF-β2-specific</td>
<td>10 or 80 µM trabedersen vs.</td>
<td>2</td>
<td>n=141 recurrent GBM, AA</td>
<td>Completed. In AA significant beneficial effects at 10 µM trabedersen</td>
<td>Bogdahn et al. 2011</td>
</tr>
<tr>
<td>antisense oligodeoxynucleotide</td>
<td>standard chemotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabedersen - TGF-β2-specific</td>
<td>10 µM trabedersen vs. standard</td>
<td>3</td>
<td>N= 27 Recurrent, refractory GBM, AA</td>
<td>Terminated due to lack of patients</td>
<td><a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a> NCT00761280</td>
</tr>
<tr>
<td>antisense oligodeoxynucleotide</td>
<td>chemotherapy</td>
<td></td>
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<tr>
<td>GC 1008 TGF-β neutralizing antibody</td>
<td>Intravenous administration</td>
<td>2</td>
<td>n=32 recurrent, refractory GBM</td>
<td>Ongoing; safety and imaging study</td>
<td><a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a> NCT01472731</td>
</tr>
<tr>
<td>LY 2157299- Small molecule TGF-βR</td>
<td>Oral administration, alone or</td>
<td>2</td>
<td>n=180 Recurrent GBM</td>
<td>Ongoing</td>
<td><a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a> NCT01582269</td>
</tr>
<tr>
<td>kinase inhibitor</td>
<td>in combination with lomustine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY 2157299- Small molecule TGF-βR</td>
<td>Combined with radio-chemotherapy</td>
<td>1/2</td>
<td>n=62 Newly diagnosed GBM, AA</td>
<td>Ongoing</td>
<td><a href="http://www.clinicaltrials.gov">www.clinicaltrials.gov</a> NCT01220271</td>
</tr>
<tr>
<td>kinase inhibitor</td>
<td>with temozolomide</td>
<td></td>
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</tbody>
</table>

AP12009, an antisense oligonucleotide targeting TGF-β2 mRNA, was examined in three phase 1/2 open-label dose-escalation studies in recurrent or refractory WHO grade III and IV glioma patients (n=24) (75). AP12009, also known as trabedersen, was applied directly to the tumor by convection-enhanced delivery thus bypassing the blood-brain-barrier and achieving a homogenous drug concentration. Tumor growth was evaluated by MRI scans. The drug was well tolerated at a maximal dose of 80µM. Although not primarily designed as an efficacy study, a prolonged survival compared to literature data was observed; median overall survival after recurrence of trabedersen versus TMZ-treated patients, respectively 146 weeks versus 42 to 49 weeks in anaplastic astrocytoma (AA) patients and 44 versus 32 in GBM patients (for additional details see (75)). Furthermore, 7 out of 24 patients showed stable disease after 28 days as determined by MRI. Even more two patients with AA were reported with long-lasting complete tumor remissions. The authors postulate that the effects of TGF-β targeting are consistent with reversal of tumor-induced immunosuppression and
the restoration of an effective antitumor immune response. Based on these remarkable and promising results, a randomized phase 2b study in patients with recurrent or refractory high grade gliomas (n=141) was conducted (76). Patients were randomized to receive either 10 or 80µM trabedersen or standard chemotherapy and were evaluate on safety and efficacy. Six month tumor control rates, defined as the percentage of patients with complete response (CR) + partial response (PR) + stable disease (SD), were similar between patients, however, after 14 months the tumor control rate in AA patients treated with 10µM trabedersen showed a significant benefit compared to standard chemotherapy. Also indications were obtained for extended overall survival in AA patients treated with 10µM trabedersen over 80µM trabedersen and chemotherapy. To further examine the clinical efficacy of trabedersen (10µM) a phase 3 study was started that, however, was recently terminated due to lack of patients as a result of changes in brain tumor definitions and standard of care (NCT00761280 clinicaltrials.gov).

A number of clinical trials have been initiated with other TGF-β targeting agents. LY2157299, an oral drug, is a potent TβRI and TβRII kinase inhibitor and is currently examined in combination with radiochemotherapy and TMZ in phase 1/2 trials in patients (n=62) with newly diagnosed grade 3 and 4 gliomas (NCT01220271 clinicaltrials.gov). More recently, a phase II study with LY2157299 alone or combined with lomustine in recurrent glioblastoma patients (n=180) was initiated (NCT01582269 clinicaltrials.gov). The human anti-TGF-β monoclonal antibody, GC1008, is able to sequester and neutralize TGF-β. A phase 2 study has been started to examine if GC1008 may have efficacy in glioblastoma patients by first determining if the antibody reaches the tumor by making use intravenously applied 89Zr-labelled GC1008 in combination with PET imaging. In the second part GC1008 will be applied in patients with recurrent and refractory GBM (NCT01472731 clinicaltrails.gov).

6. Summary and perspectives

High grade gliomas are characterized by a high degree of therapy resistance followed by inevitable local and/or disseminated recurrence. Over the past two decades researchers have witnessed many new approaches to study and understand the molecular basis of malignant gliomas. Amongst them, the observation that TGF-β acts on multiple levels to promote the malignant phenotype of gliomas including angiogenesis, invasiveness, stemness and immunosuppression, has prompted the development of approaches to antagonize the biological effect of TGF-β as a promising experimental strategy to combat malignant gliomas. As discussed above results in preclinical models demonstrated potent antitumor activity of TGF-β inhibition alone or in combination with radio-chemotherapy. These findings have spurred the development and testing of TGF-β targeting agents in patients with high grade gliomas as listed in table 1. Initial studies show good tolerability and safety of TGF-β targeting agents. Which particular strategy, antisense oligonucleotides, neutralizing
antibodies, antagonistic antibodies or small molecule inhibitors, will be most potent remains to be demonstrated. In this respect the route of administration of these modalities, intratumoral, intravenous or oral may also be of importance anticipating reduced drug penetration caused by limitations in passing the blood-brain barrier. Currently running and future clinical studies will need to reveal the efficacy of TGF-β targeting therapy for the treatment of gliomas.

**FOOTNOTES**

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REFERENCES


