Chapter 7

Exploitation of Angiopoietin-2 pleiotropy by glioblastomas following VEGFA inhibition

S. Conroy
W.F.A. den Dunnen
Glioblastoma (GBM) is the most common primary brain tumor with no curative or efficacious therapies available. Due to its highly vascularized nature, therapies targeting this modality are extensively sought after. The initial reported responses were promising and even resulted in the approval of anti-vascular endothelial growth factor a (VEGFA) treatment for recurrent GBM, but the development of treatment resistance in the long term is limiting the benefit from this type of therapy. Although several evasive mechanisms have been suggested, the exploitation of Angiopoietin-2 (Ang-2) signaling in response to VEGFA inhibition in GBM is becoming evident. In this chapter, we discuss the physiological roles of VEGFA and Ang-2, the interaction between both signaling pathways and their role in GBM angiogenesis. We conclude with a discussion of more recent reports that shed light on potential resistance mechanisms that Ang-2 could mediate outside the context of GBM angiogenesis.
Introduction

Glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults with an average survival of 15 months despite optimal treatment [1-3]. It is among the most vascularized human cancers, and the fact that vascular proliferation is one of the pathological hallmarks of GBM reinforces the importance of vascularization in GBMs [4,5].

Early studies on vascularization of GBMs have mostly focused on neo-angiogenesis, a process through which new vessels are formed from pre-existing ones. The rate of neo-angiogenesis has been found to be inversely correlated with patient survival [6]. More recently, several alternate angiogenic mechanisms have also been described, including vascular co-option [7], vascular mimicry [8] and glioblastoma-endothelial cell or pericyte transdifferentiation [9-11]. Although the exact contributions of these mechanisms is not completely elucidated [12], it is becoming evident that the process of tumor-induced vascularization is not limited to neo-angiogenesis and much more complicated than was initially envisioned.

As it was generally assumed that vascularization is induced when a lack of oxygen in GBM cells develops, a state called hypoxia, the majority of studies have assessed the role of hypoxia signaling in the induction of angiogenesis. A second pathological hallmark of GBMs is the presence of necrosis, which are sites that result from massive cell death that are often surrounded by cell-dense and relatively hypoxic pseudopalisades [13,14]. Low oxygen pressure results in hypoxia-inducible factor (HIF-1) stabilization and through that route triggers the up-regulation multiple pro-angiogenic factors, including VEGF, which in turn stimulates endothelial cell proliferation and ultimately results in vessel growth [15,16]. The mechanisms regulating vascularization in more normoxic areas of GBM have been studied less extensively and are less well understood.

Since therapeutic inhibition of angiogenesis is being explored in GBM, with a primary focus on vascular endothelial growth factor A (VEGFA), a better understanding of angiogenic mechanisms has become of the utmost importance. The exploitation of vascular co-option and other non-VEGFA-instructed vascularization mechanisms are escape options available to GBMs [17-19], and recently the involvement of Angiopoietin-2 (Ang-2) has been implicated as a mediator of anti-VEGFA therapy refractoriness [20-22].

In order to provide a scientific background for the exploration of the role of Ang-2 in the context of VEGFA inhibition in GBM, we summarize literature reporting on the differential roles of both factors, describe their interrelation and their involvement in GBM angiogenesis. We conclude the chapter with potentially important Ang-2-mediated resistance effects outside of the angiogenesis context.
Initial discovery and physiological function

VEGFA

The growth factor VEGFA was originally identified for its mitogenic effect on endothelial cells [23], and in the light the vascular permeabilizing effects it was first named ‘vascular permeabilizing factor’ [24]. VEGFA is a member of the larger VEGF family that also includes the ligands VEGFB, VEGFC, VEGFD and placental growth factor (PIGF) [25], but due to the extensive cross-talk between VEGFA and Ang-2 we focus on VEGFA exclusively here. Post-translational processing (alternative splicing) of the VEGFA-transcript results in several distinct isoforms, each with different abilities to bind to heparin-containing proteins [26]. As a result thereof, both stimulatory and inhibitory effects have been reported with different isoforms, and the spatial patterning is highly regulated.

The receptors of the VEGF pathway are VEGFR1 (Flt-1), VEGFR2 (KDR) and VEGFR3 (Flt-4). VEGFA binds to VEGFR1 with relatively stronger affinity than to VEGFR2, but the phosphorylation (i.e. activation) of VEGFR1 is weaker [26]. It is therefore though to function as a decoy receptor, and VEGFR2 is considered the major receptor for VEGFA signaling in endothelial cells mediating angiogenesis [27]. Alternatively, VEGFA could also signal through neuropilin (NRP) receptors [28,29].

Embryonic lethality has been described with VEGFA knockout [30,31], thereby illustrating the importance of VEGFA for embryonic vasculogenesis. Additionally, the requirement of VEGFA for endothelial cell survival was illustrated by the induction of vascular regression following VEGFA inhibition [32,33]. Through binding to its receptors, VEGFA stimulates endothelial cell proliferation, migration and increases microvascular permeability.

Physiologically, VEGFA instructs increased vascular permeability through endothelial detachment from the parental vessel, which allows for sprouting angiogenesis to occur. Hypoxia is a direct mediator of VEGFA [34], and as a result of hypoxic stimulation, tumor cells secrete altered levels of several VEGFA isoforms [35]. This alters the tightly regulated VEGF-coordinated tip cell selection in angiogenesis and results in the deregulated vessel growth [36,37].

Ang-2

The second endothelial-specific growth factor system that was discovered concerned the Ang/Tie-2 system [38]. Within this signaling family Ang-1 and Ang-2 represent the most important ligands, and Tyr kinase with Ig and epidermal growth factor homology domains (Tie-2) is the best characterized receptor [39]. Signaling through the Tie-2 receptor is regulated through competitive binding of Ang-1 and Ang-2, and since Ang-1 was initially studied in much more detail, the first reported effect of Ang-2 was also related to the inhibitory effect it exerted on Ang-1/Tie-2 signaling [40]. Ang-1 and Tie-2 deletion has been shown to be
embryonically lethal in mice [41-43], but Ang-2 deletion instead limited the survival of mice to approximately 2 weeks postnatally [44]. Since the overexpression of Ang-2 on the other hand resulted in a phenotype similar to Ang-1 or Tie-2 knockout [40], this led to the postulation of the Tie-2 inhibition hypothesis. Additionally, Ang-2 was found to bind the Tie-2 receptor with similar affinity as Ang-1, but resulted in very weak activation of Tie-2, and when Ang-1 and Ang-2 were provided together, Tie-2 activation was decreased as compared to Ang-1 stimulation alone.

Ang-2 is stored in endothelial Weibel-Palade bodies and these bodies are released in response to various stimuli such as thrombin, histamine and sphingosine-1-phosphatase [45-47]. Functionally, Ang-1 has been shown to reduce vascular leakiness as a result of vascular stabilization and maturation [48,49]. In confluent cells this is mediated by PI3-kinase/AKT signaling that becomes activated in response to Ang-1/Tie-2 binding that results in cell-cell contract translocation of Tie-2/Tie-2 complexes, but contextual dependency is illustrated by the alternative employment of ERK signaling in sparse cells which results in cell-matrix localization of Tie-2 receptors [50].

The multimeric status of Ang proteins could also be of importance for eventual downstream signaling. Ang proteins form homomorphic variable-sized multimers, and for Ang-1 it has been shown that minimally a tetrameric size was required to phosphorylate the Tie-2 receptor [51]. Ang-2 displayed a multimeric state similar to Ang-1, but the context-dependent was not attributed to the multimeric state of the protein. Instead, the context-dependent antagonistic function of Ang-2 was found to originate from the fibrinogen-like domain. From a molecular biological perspective, besides antagonizing canonical Ang-1/Tie-2 signaling, Ang-2 has been shown to cause pericyte detachment [52].

**Role in GBM angiogenesis**

The expression of both VEGFA and Ang-2 has been reported to increase with glioma grade [53-55], and a relative increase of Ang-2 versus Ang-1 expression has been correlated with GBM patient survival [56]. These results signify the clinical importance of these factors, and the originally identified expression patterns first resulted in the implication of these factors in angiogenesis. Ang-2 expression was observed exclusively in tumor vasculature [54,55,57-59], and preferentially in the smaller vessels [40,55,57], while VEGFA was not restricted to a specific cell type [60,61].

Ang-2 and VEGFA are highly expressed after development of necrosis in a rat glioma model [7], and another report described that even in microtumors (<1 mm³) neo-angiogenesis was induced by C6 glioma cells [62]. During early tumor angiogenesis VEGFA, Ang-2, and VEGFR2 are simultaneously expressed, while Ang-2 expression in this study was limited further to activated endothelial cells [62]. The expression levels of Ang-2 and VEGFA increased in relation with the expansion of the tumor mass and the developing vascular tree [63]. Spatially, Ang-2 was expressed more robustly in the tumor center and the perimeter,
and in contrast, VEGFA was upregulated exclusively in the tumor center.

Ang-2 and VEGFA are considered important regulators of vascular regression and vascular growth [64], and the paradigm for the physiological cross-talk between these factors is that Ang-2 results in angiogenesis or vascular sprouting when VEGFA is present, but in the absence of VEGFA instructs vascular regression [7,65-68]. Studies that have explored the inhibition of VEGFA have provided insight into the cross-talk between these pathways in a tumor environment. Of interest was the suggestion that, in response to Bevacizumab treatment in GBM patients, Ang-2 upregulation could represent a mode of resistance [22].

In vivo ectopic expression of Ang-2 in GBM cells has been able to mimic these clinical effects by limiting the efficacy of the anti-VEGF therapy through the increase of vascular permeability [69]. Alternatively, recruitment of bone marrow-derived cells was also reported by Ang-2 which protected the vulnerable vasculature from degradation [70]. According to the paradigm, the inhibition of VEGFA should have left Ang-2 in the absence of VEGFA and result in vascular regression, but apparently the tumor is able to respond to this situation in a way that is different from healthy physiology.

**Non-angiogenic signaling effects of Ang-2**

Besides the well-recognized roles that Ang-2 and VEGFA serve in angiogenesis, recent insights are shedding light on alternative effects that these factors could exert. It has been shown that Ang-2 correlates with the expression of tissue proteases in glioma patient specimens [71], and functionally Ang-2 stimulation has been reported to mediate invasion through the induction of matrix metalloproteinase 2 (MMP2) [72,73]. Increasing numbers of reports also suggest that the immune compartment could mediate refractoriness against anti-angiogenic therapy, which is of great interest in the paradigm that we examine here [74].

The brain has long been considered an immune-privileged organ that was maintained by the blood-brain barrier (BBB), but recent insights have drastically changed this concept [75,76]. In gliomas, lymphocytic infiltration was reported in the 1970s [77], and BBB impairment in gliomas was first used to explain this observation. Assessment of the distribution of immune cells in brain tumors indicated that increased numbers of activated microglia and macrophages are found around necrosis, and activated microglia were increased in peripheral areas of diffuse infiltration [78]. Reduced microglial invasion in GBMs associated with a mutation in the chemokine receptor CX3CR1, and the presence of this mutation has been associated with improved patient survival [79]. Conversely, increased numbers of microglia/macrophages were observed in the GBM mesenchymal molecular subclass, and this molecular subclass is in turn associated with shorter patient survival [80-82]. Furthermore, correlations between microglia/macrophages counts and tumor grade have also been reported [80,83,84], all together suggestive of an association between tumoral immune infiltration and enhanced glioma malignancy.
Whether the microglia are able to exert physiological immune functions remains to be established [85], and the few reports available question this ability. The innate immune response (i.e. cytokine release) of tumor-invaded microglia has been reported to be substantially reduced [86], and additionally, opposite to healthy physiology, peripheral blood monocytes (PBMCs) from glioma patients were shown to exert immunosuppressive functions in vitro [87].

Recent reports suggest that Ang-2 could be a key mediator of this immunological tumor response [88]. The endothelial sensitizing effect of Ang-2 has been recognized as critical for physiological immune responses [89], but in glioma the chemo-attracting properties are better described. Ang-2 has been shown to recruit Tie-2-expressing monocytes (TEMs) from the circulation, a process that could be prevented by the administration of a soluble Tie-2 receptor [90]. With the reported Ang-2-mediated resistance against anti-VEGFA therapy we discussed above, the importance of immunologic mediation in combined VEGFA and Ang-2 inhibition is compellingly illustrated by two in vivo studies. Both these studies showed that the combined inhibition of Ang-2 and VEGFA had superior effects over single therapy, which was in both studies strongly associated with a phenotypic shift of the macrophages [91,92]. Interestingly, these studies did not report a reduction in macrophage recruitment, but rather a switch from a M2 tumor-promoting phenotype to a M1 anti-tumor phenotype. These findings highlight that not only the number of immune cells, but also the type of infiltrate and its phenotype are relevant for the ultimate functional effects. The substantial attenuation of survival when macrophage recruitment is inhibited [91], which has already been reported as a successful monotherapeutic approach in glioma [93], provides convincing indications that the immune compartment is crucially involved in the context of angiogenesis inhibition.

Conclusion

In this chapter we have summarized relevant literature describing the function of Ang-2 and VEGFA in physiology, and we have provided examples of how tumor physiology can deviate from that paradigm. We showed that the impact of Ang-2 and VEGFA cannot be considered in an exclusively angiogenic context, which has important implications for anti-angiogenic therapeutic approaches.

In the context of this thesis, several questions will remain unanswered regarding the approach that we employed. We examine the unconventional effect of Ang-2 and VEGFA stimulation on GBM tumor cells, for which we selected the VEGFA_{165} isoform, the most pro-angiogenic variant. Additionally, we modulate Ang-2 levels, but do not regard the relative influence in regards to Ang-1 levels. Furthermore, for the inhibition we use Bevacizumab and L1-10, but also here we are unaware of the effect on different splice variants or how the inhibition is affected by the multimeric status of proteins present in the xenografted mice.

Awareness of the current limits of our understanding, including the signaling effects of both systems and the relatively uncontrolled manipulation that we nowadays perform through
in vitro and in vivo experimentation, could inspire for a vast variety of studies. Studying these pathways in more detail could advance our understanding of the complexity of GBM and tumor angiogenesis.
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