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Vascular Normalization in Cerebral Angiogenesis: Friend or Foe?

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Abstract

Current antiangiogenic therapies have led to the observation that such agents can lead to improved tumor vessel structure and function termed “vascular normalization” which reduces tumor burden. However, vessel normalization is a transient process, and patients often develop resistance/poor response to anti-vascular strategies that remains an important clinical challenge. Therefore, increasing effort has been made to better understand the cellular and molecular mechanisms of vascular normalization and its contribution to immunomodulation. Herein, we summarize the recent effort to better understand the cellular and molecular mechanisms of vascular normalization with a focus on preclinical genetic models. These studies remain important directions for a mechanistic understanding of the complexities of the maintenance of BBB integrity and the impact of its breakdown on tumor dissemination and pharmaco-distribution of therapeutics.

Keywords

Vascular normalization; Angiogenesis; Blood–brain barrier; Glioma; Antiangiogenic therapy; Bevacizumab

1 Introduction

Cerebral vascular normalization refers to the process in which the central nervous system (CNS) microvasculature, when damaged in disease and/or by aging, is no longer compromised by surrounding metabolic, pharmacological, or immunological challenges. Under normal circumstances, an intact blood–brain barrier (BBB) protects the brain by limiting access of blood-borne molecules and circulating immune cells [1, 2]. While angiogenesis and remodeling of the BBB occur in normal development and retain many of the essential cellular and molecular parameters of an adult BBB [3], breakdown of the BBB is generally associated with CNS injury, ischemia, and brain cancers. The tight regulation and organization of brain endothelia, pericytes, astrocytes, and neurons, is termed the neurovascular unit (NVU) and functions within the extracellular matrix (ECM) microenvironment [4]. Because dysregulation of the NVU occurs in response to various CNS injury pathologies particularly in CNS tumors, understanding the mechanism of cerebral angiogenesis and BBB dysfunction is essential for the delivery of therapeutics and restoration of BBB homeostasis. Furthermore, the mobilization of local (i.e., microglia) vs. circulating immune cells (i.e., blood monocytes/macrophages and lymphocytes) to CNS tumors can influence the vascular endothelium and BBB integrity to regulate tumor progression.

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2 Antiangiogenesis Therapy and Vascular Normalization in CNS Tumors

While many growth factors and cytokines are known to mediate various responses to CNS injury, vascular endothelial growth factor (VEGF/VPF) is unique among these factors as both a potent growth factor and inducer of vascular permeability (VP) of blood vessels. With the discovery of its central role in vascular biology and the development of VEGF antagonists to starve tumors of their blood supply (i.e., antiangiogenesis), the efficacy of VEGF blockade on reducing angiogenic blood vessel density, VP, and vessel diameter has proven therapeutically valuable. Tumor-associated blood vessels have elevated VP, vessel tortuosity, and decreased patency which lead to a hypoxic tumor microenvironment with high interstitial fluid pressure (IFP) [5], that there is also a disruption of efficient drug delivery and reduced immunosurveillance by circulating leukocytes. Targeting tumor vessels with antiangiogenic therapies (i.e., bevacizumab) has led to the paradoxical observation that such therapies can have the effect of restoring blood vessel integrity (i.e., vascular normalization) [6–9]. Vascular normalization has been identified as a novel mechanism for balancing several molecular and physiological properties of angiogenesis-related vascular remodeling. For example, Goel et al. detail the specific effects of the mouse anti-human VEGF mono-clonal antibody (mAb, A.4.6.1), the humanized variant of A.4.6.1 (i.e., bevacizumab), an anti-mouse VEGF mAb, and a VEGF trap (i.e., aflibercept) as well as various anti-VEGFR agents (DC101 and tyrosine kinase inhibitors) [10]. Dosing of these anti-VEGF agents, primarily in tumor-bearing animal models, typically results in a reduction in vessel diameter, tortuosity, and permeability thus underscoring the link between VEGF and the vascular normalization phenotype. While the vascular normalization phenotype is also observed in colorectal, melanoma, breast, and prostate models, glioma-associated tumor vessels have been the focus of the vascular normalization phenotype based in part on the availability of preclinical models with intravital imaging and the number and scope of clinical reports of anti-VEGF therapy in brain tumors. In fact, with the FDA approval of bevacizumab for glioma and the increasing numbers of breast and melanoma cancer patients with brain metastases secondary to managed primary neoplasms, there has been an increased effort to better understand the mechanisms of action of vascular normalization and develop strategies to exploit vascular normalization for reducing tumor burden.

For example, in the brain, preclinical models of orthotopic glioma treated with agents that induce the vascular normalization phenotype are associated with a recruitment of perivascular pericytes, changes in endothelial proteins regulating endothelial stability/permeability (VEGF, bFGF, Ang-1/Ang-2, TSP-1, iNOS, and Src/FAK), and changes in the ECM microenvironment of blood vessels [6]. The effect of antiangiogenic therapies of increasing tumor oxygenation further complicates approaches designed to starve a tumor of a blood supply where there may be only a narrow time frame in which therapy-induced vascular normalization occurs. The transient nature of pharmacologically induced vascular normalization contrasts with genetic models and presents important opportunities for a better understanding of experimental models and a translational path.

Interestingly, metronomic dosing of sustained yet lower doses of cytotoxic chemotherapy agents leads to reduced tumor burden [11, 12]. Such dosing strategies are generally associated with an increase in better tumor vascular perfusion and tumor oxygenation and

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reductions in IFP. While the mechanisms of action of such approaches are poorly understood, the efficacy of combined antiangiogenic/chemotherapeutic strategies may involve vascular normalization and be even more relevant to emerging drug delivery technologies. For example, even with adequate tumor perfusion, a significant limitation of targeted nanoparticle delivery across the BBB remains due to the complexity of multicellular NVU, ECM, and elevated IFP, all three being barriers that prevent efficient drug delivery.

3 Cell Biology of Vascular Normalization

Tumor angiogenesis is a multicellular process that is coordinated by factors that are produced from tumor and stromal cells that influence the proliferation and maturation of endothelial cells. Accordingly, the vascular normalization process also involves endothelial cells and stromal cells that are responsible for regulating the steps of vascular remodeling.

3.1 Endothelial Cells

Prolonged exposure to angiogenic factors (e.g., VEGF) secreted from tumor and stroma induces structurally and functionally abnormal vessels in the tumor. Tumor vessels are tortuous and have irregular diameter and length and discontinuous lining with sometimes multiple layers of endothelial cells. Functionally, they are poorly perfused and leaky, allowing tumor cells to intravasate and metastasize and also inhibiting trafficking of leukocytes. In contrast, normalized vessels appear to have reduced vessel diameter, less tortuous with regular branching, and the endothelial cell layer is continuous with smooth lining, and there is improved perfusion and oxygenation. Genetic modification of oxygen sensor protein prolyl hydroxylase domain-containing protein 2 (PHD2) [13] or Sox17 transcription factor [14] in endothelial cells is associated with a vascular normalization phenotype, indicating the importance of these particular pathways in endothelial cells. In the brain, endothelial-specific deletion of focal adhesion kinase (FAK) demonstrated vascular normalization in a model of glioma [6].

3.2 Pericytes and Basement Membrane

Maturation of the new vessels involves pericyte recruitment and deposition of extracellular matrix. In normal vessels, pericyte-deficient mice display BBB breakdown and neuronal defects [15, 16]. Angiogenic tumor vessels often lack sufficient coverage of pericytes or the endothelial–pericyte interaction is lost, leaving “empty sleeves”—pericytes and basement membrane that are devoid of endothelial cells. The thickness of the basement membrane is also irregular, inhibiting the appropriate matrix binding of growth factors that are important for vascular maturation. This leads to irregular flow and perfusion, causing uneven distribution of oxygen and nutrients, and hence, hypoxia in the tumor. Normalized tumor vessels demonstrate improved pericyte coverage and perfusion, which can also help with the delivery of chemotherapeutic agents to the tumor core. For example, Hamzah et al. demonstrated that loss of regulator of G-protein signaling 5 (*Rgs5*) protein induces maturation of pericytes that cover endothelial cells and induce vascular normalization [17]. Another study demonstrated that Ras [18] is important for pericyte association and maturation of tumor vessels, and upregulation of Ras signaling induced vascular

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normalization, indicating pericytes as alternative targets for inducing normalization of tumor vasculature.

3.3 Astrocytes

In the brain, the recruitment of pericytes and astrocytes is an essential process for the formation of tight junctions that are unique to the BBB. The tight junctions play an essential role in controlling the permeability of the BBB. Astrocytes form perivascular astrocytic end feet to maintain the astrocyte–endothelial interaction, whereas in tumors, this contact is lost, which correlates with increased permeability. Restoration of the astrocyte contact is found in a vascular normalization model in glioma as shown by the upregulation of aquaporin-4 surrounding the tumor vasculature [6].

4 Genetic Models of Vascular Normalization

Knockout mouse models have been used to establish the functional relevance of specific molecules in vascular normalization where in some models deletions are cell-type restricted. For example, a role for the Ang/Tie2 pathway in endothelium is supported by knockout animal models and pharmacological approaches showing a vascular normalization phenotype, while Ang/Tie2 also functions in tumor-infiltrating monocytes [19, 20]. Notably, antagonists of placental growth factor (PlGF), angiopoietins, and integrins have also been shown to exhibit a vascular normalization phenotype in various models [21].

Using a glioma model, our lab has shown that a conditional and endothelial-restricted deletion of FAK in the endothelium leads to a vascular normalization phenotype in tumor-associated brain–blood vessels [6]. The conditional nature of this model enables testing of the possible temporal restriction of the vascular normalization phenotype at different developmental stages, in a specific cell type. The fundamental importance of the FAK pathway in regulating endothelial barrier integrity is further demonstrated by knock-in mutation of the kinase domain in response to recombinant VEGF [22]. The importance of tumor hypoxia in the vascular normalization phenotype is demonstrated functionally in knockout mice lacking the Siah family of ubiquitin ligases [23]. In this model, the loss of Siah led to vascular normalization and reduced breast tumor burden, but with tumors having increased perfusion and pericyte coverage of blood vessels. While Siah2 was shown to attenuate the hypoxia-responsive transcription factor HIF1α, consistent with a link between vascular normalization and hypoxia, the $Siah2^{-/-}$ mice also exhibited an increased sensitivity to chemotherapy. Recently, Yang et al. demonstrated that mice lacking the Sox17 transcription factor in endothelial cells have reduced tumor burden associated with a vascular normalization phenotype associated with increased number of infiltrating myeloid cells [14]. Thus, while these genetic examples (summarized in Table 1) define the functional relevance of specific signaling/transcriptional pathways in vascular normalization, these models also demonstrate that the vascular normalization phenotype observed in preclinical and clinical paradigms following antiangiogenic therapy can also be mediated by the inflammatory response.

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5 Immunomodulation of Vascular Normalization

Tumor-associated macrophages (TAMs) are an abundant myeloid-derived cell type of the innate immune system that exist in both quiescent and activated states to regulate vascular remodeling and tumor progression. Extensive infiltration of myeloid-derived cells (i.e., $CD45^+$, $CD11b^+$) contributes to a significant percentage of the overall tumor burden (i.e., 10–20 %) of tumor progression. Cytokines secreted by infiltrating myeloid-derived cells (i.e., VEGF, PDGF, PlGF) influence endothelial cell proliferation and angiogenesis. For example, deletion of myeloid-cell-derived VEGF-A induced vascular normalization in lung tumors [24]. Therefore, regulation of TAM activation to affect tumor vessel physiology is emerging as an important intersection of tumor immunology and vascular biology. Strategies to switch these tumor macrophages into becoming tumor-inhibiting phenotypes (i.e., M1) with host-produced factors such as histidine-rich glycoprotein [25] establish experimental paradigms for regulating tumor vessel permeability, patency, and therefore perfusion of chemotherapeutics. Establishing a link between switching TAM phenotypes and vascular patency for the purpose of enhancing drug delivery is an important translational research area in tumor biology. For example, in a screen of antifibrotic drugs, losartan was identified as a potent enhancer of nanoparticle penetration into solid tumors of the breast, pancreas, and skin [26]. Regulating the balance of growth factors and cytokines holds promise in obtaining a better understanding of the mobilization/switching of macrophages and other myeloid and stem cell-like cell populations to the tumor microenvironment. Across various tumor models, there is a strong correlation of TAMs with angiogenesis and tumor progression, and brain tumors, with gliomas in particular, are no exception [27]. In the CNS, microglia, the resident macrophage of the brain, provide a dynamic and abundant innate immune response to tumors, infection, and trauma; however, the recruitment of inflammatory myeloid-derived monocytes augments the initial immune response. With time, this local and mobilized immune response converts to an overall immunosuppressive tumor microenvironment. The immunosuppressive, proangiogenic state of myeloid cells in tumors can be altered in response to the secreted factors in the microenvironment. Therefore, a better understanding of the mechanisms that regulate the switching to the immunosuppressive phenotype and identification of therapeutic strategies to reverse the phenotype will provide novel therapeutic approaches to treat CNS cancers.

6 Drug Delivery to Normalized Tumor Vasculature

Drug delivery to the brain remains to be a challenge as the BBB prevents endocytosis or transport of various molecules to the brain parenchyma. Recent progress has been made on strategies to overcome this barrier by utilizing vehicle-mediated transport of drugs, for example, nanoparticles [28–30]. In brain pathologies, much of the current CNS drug delivery dogma is based either on their hydrophobicity or the capacity of larger molecules (i.e., antibodies) to gain access to CNS injury sites through overtly compromised BBB. As such, it has been well established that IFP and tissue fibrosis impede drug delivery to brain tumors that are considered to have a “leaky” barrier. Furthermore, as oppose to the dysregulated tumor vessels in the hypoxic, necrotic tumor core, endothelial cells at the tumor–stroma interface display characteristics similar to those of normal BBB. This type of heterogeneity of tumor vessels causes the delivery of chemotherapeutic agents to be uneven

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throughout the tumor. Yet vascular normalization has been associated with a decrease in vascular tortuosity, increased patency, and increased perfusion and drug delivery. Increasing evidence in pre-clinical models of vascular normalization demonstrates that these changes in the tumor vasculature accompany improvement in the delivery of chemotherapeutic drugs [23, 31, 32].

7 Resistance and Escape from Antiangiogenesis Therapy

Patients subjected to antiangiogenic therapy have shown vascular normalization to be only a transient process, therefore only providing a short window of time when combined therapy gives the best outcome [33]. Another serious problem in the clinical management of gliomas is the diffuse and infiltrative nature of the CNS tumor burden that is generally refractory to complete surgical resection. Moreover, glioma patients develop resistance/poor response to anti-vascular strategies, which is accompanied with enhanced invasion of tumor cells [34]. Therefore, there is a critical need to determine optimal dosing of vascular normalization agents to promote more sustained/prolonged vessel normalization. Developing alternative vessel normalization targets and approaches can also overcome resistance to current antiangiogenesis therapies to control invasiveness of glioma cells. For example, targeting of the PI3K and mTOR pathways with the inhibitor NVP-BEZ235 [35] or EGFR inhibitors [36] to induce prolonged vascular normalization could be used as an alternative to VEGF targeting.

8 Conclusions

Although antiangiogenic strategies reduce tumor burdens by starving tumors of their blood supply in preclinical models, clinical studies indicate that several compensatory mechanisms exist in humans that generally result in only a modest improvement in disease-free survival [37, 38]. Therefore, a better mechanistic understanding of the “adaptive” vascular mechanisms of antiangio-genesis therapies is essential. The concept of enhancing perfusion of tumors to yield “normalized” vessels is paradoxical in the sense that one would assume that a “better” vascular supply would enhance, rather than diminish, tumor progression. So while an enhanced vascular supply improves access to nutrients and more efficient removal of toxins, efflux pumps may be more efficient in the removal of chemotherapeutic agents. Thus, mechanistic studies of the cellular and molecular basis of neovascularization and regulation of VP remain important directions for further research to understand the complexities of BBB integrity and their role on tumor dissemination and pharmaco-distribution of therapeutics.

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Table 1

Host-restricted loss-of-function models for vascular normalization

Target gene	Cell type	Model	Reference
Rgs5	General	RIP1-Tag5 pancreatic tumor	Hamzah et al. [17]
Ang2	General	s.c. lung tumor and melanoma	Nasarre et al. [20]
PlGF	General	Skin tumor, liver tumor, RIP1-Tag2 pancreatic tumor, s.c. mammary tumor, fibrosarcoma, Panc02 pancreatic tumor	Van de Veiri et al. [21], Rolny et al. [25]
R-Ras	General	s.c. lung tumor, melanoma, prostate tumor	Sawada et al. [18]
Siah2	General	PyMT mammary tumor	Wong et el. [23]
PHD2	Endothelial	Pancreatic tumor, s.c. lung tumor, and melanoma	Mazzone et al. [13]
FAK	Endothelial	Glioma	Lee et al. [6]
Sox17	Endothelial	s.c. lung tumor and melanoma	Yang et al. [14]
VEGF-A	Myeloid cell	MMTV-PyVT, s.c. lung tumor	Stockmann et al. [24]