Review

Normalizing Tumor Vasculature to Reduce Hypoxia, Enhance Perfusion, and Optimize Therapy Uptake

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Simple Summary: In order for solid tumors to grow, they need to develop new blood vessels in order to support their increasing metabolic requirements. To facilitate the novel vessel formation, the tumor initiates an aggressive pro-angiogenic program. As a result of the aggressive angiogenesis, blood vessels form very rapidly and are often malformed and dysfunctional. There is a reduction in perfusion to the tumor, and often the tumors exhibit significant areas of tumor hypoxia. This review paper discusses the pro-tumorigenic environment induced by tumor hypoxia and how this can be targeted through normalization of the tumor vasculature. Here, we review tumor angiogenesis, the development of a hypoxic phenotype, and how this contributes to sustained tumorigenesis and resistance to therapy. We further discuss the potential of vascular normalization to reduce tumor hypoxia and facilitate uptake and efficacy of a variety of therapies.

Abstract: A basic requirement of tumorigenesis is the development of a vascular network to support the metabolic requirements of tumor growth and metastasis. Tumor vascular formation is regulated by a balance between promoters and inhibitors of angiogenesis. Typically, the pro-angiogenic environment created by the tumor is extremely aggressive, resulting in the rapid vessel formation with abnormal, dysfunctional morphology. The altered morphology and function of tumor blood and lymphatic vessels has numerous implications including poor perfusion, tissue hypoxia, and reduced therapy uptake. Targeting tumor angiogenesis as a therapeutic approach has been pursued in a host of different cancers. Although some preclinical success was seen, there has been a general lack of clinical success with traditional anti-angiogenic therapeutics as single agents. Typically, following anti-angiogenic therapy, there is remodeling of the tumor microenvironment and widespread tumor hypoxia, which is associated with development of therapy resistance. A more comprehensive understanding of the biology of tumor angiogenesis and insights into new clinical approaches, including combinations with immunotherapy, are needed to advance vascular targeting as a therapeutic area.

Keywords: hypoxia; angiogenesis; vascular normalization; drug delivery; therapy resistance

1. Introduction

1.1. Sprouting Angiogenesis in Normal Physiology

Angiogenesis is the complex and highly regulated formation and maturation of vasculature from pre-existing vessels throughout the body. Typically, the process is kept quiescent through a balance of growth factors and inhibitors. Normal human processes that necessitate angiogenesis in the adult include placentation in the pregnant uterus, formation of the endometrium in the menstrual cycle, growth of the mammary gland in preparation for lactation, and supply of granulation tissue for wound healing [1–3]. In any of these situations, angiogenesis consists of a series of events including removal of structural pericytes in the area of the developing sprout, degradation of the capillary...
basement membrane, migration and proliferation of the endothelial cells comprising the new sprout, nascent tube formation, and vascular stabilization [4].

The presence of angiogenic stimuli such as hypoxia, mechanical stress, or inflammation leads to the release of growth factors, as summarized in Figure 1. These signaling events ultimately lead to the activation of cellular effectors, which aim to form the nascent vessel [4]. Upon effector stimulation, smooth muscle cells called pericytes located at intervals along the capillary wall are first removed from the sprouting area of a mother vessel. VEGF stimulation triggers intricate calcium oscillations within endothelial cells allowing for the selection of an endothelial cell distinguished by specialized filopodia, called a tip cell [5]. The tip cell guides the developing sprout through chemotaxis, following angiogenic stimuli secreted by the target tissue requiring increased perfusion [5]. As tip cells are highly influenced by even minute fluctuations in growth factor signaling, a loss of growth factor balance in this system may lead to disorganized vasculature. The tip cell releases matrix metalloproteases (MMP), which degrade basement membrane components in its path [6]. A second group of specialized endothelial cells, called stalk cells, are highly proliferative and interact with tip cells through delta-notch signaling to elongate the nascent sprout [7]. At a point of anastomoses between the tip cell of another nascent vessel or stabilized vessel, junctional adhesion proteins are deposited at the contact site of the two tip cells. A lumen is formed through cell membrane invagination or cord hollowing, forming a functional vascular network [8]. Circulating endothelial progenitor cells also contribute to the nascent vessels, which are haphazardly branched and in need of organization. Local differences in blood flow and pressure lead to the elimination of poorly perfused branches (pruning) or recycling of their component endothelial cells to areas of significant flow [9,10]. Conversely, highly perfused sprouts are stabilized through deposition of basement membrane, reduced endothelial cell activity, tightening of cell junctions, and recruitment of pericytes [10].

**Figure 1.** Hypoxia induced by the growing tumor mass triggers an “angiogenic switch” within the tumor microenvironment, resulting in a crude version of angiogenesis.

1. In the earliest stage of tumor growth, oxygen and nutrients is supplied by diffusion.
2. As tumors grow beyond 2mm³, hypoxia triggers stabilization of HIF-1α in tumor cells, leading to expression of genes and resultant proteins related to the angiogenic process.
3. An HIF-induced factor gradient forms a chemotactic route for growth of the nascent vessel. Interactions between proteins and their receptors results in degradation of basement membrane and pericycle destabilization (MMPs), endothelial cell growth and tube formation (VEGF, bFGF), sprouting and branching (Ang-1). Other cell types within the TME (Macrophages, neutrophils) exacerbate abnormal vasculature by releasing more angiogenic factors.
1.2. Tumor Control of Angiogenesis

In many ways, tumors can be considered functional organs as opposed to a group of aberrant cells. The tumor stroma includes mesenchymal-derived cells, inflammatory cells, and vascular cells, albeit in an irregular fashion that has been modified by the tumor to tailor to its survival needs [11,12]. Tumors are therefore capable of inducing angiogenesis by co-opting the same pro-angiogenic program. Small tumors devoid of vasculature are often observed in solid tumor types—their oxygen and nutrient demands being supplied by passive diffusion from nearby vessels [13]. However, as tumors grow beyond 2 mm², the tumor core becomes increasingly hypoxic and the process of angiogenesis begins to fuel oxygen and nutrient demands [14]. This moment has been termed “the angiogenic switch” in which tumor cells respond to low oxygen perfusion by releasing many of the angiogenic factors represented in Figure 1 [15]. Cellular responses to low oxygen are primarily regulated by DNA-binding transcription factors known as hypoxia inducible factors (HIF). HIFs are heterodimeric proteins that consist of a constitutively expressed HIF-1ß subunit and an oxygen-regulating subunit (HIF-1α or HIF-2α) [16,17]. These alpha subunits are composed of an amino terminal basic Helix-Loop-Helix (bHLH) necessary for DNA binding to hypoxia response elements (HRE), transactivation domains (N-TAD and C-TAD) that are vital for activation of HIF target genes, PAS-A and PAS-B domains for protein-protein dimerization, and an oxygen-dependent degradation domain (ODDD). Redundancy in HIF-1α stabilization is evident as a secondary lysine residue within the ODDD can be acetylated by an acetyl transferase enzyme called arrest-defective-1 (ARD-1) to favour degradation of HIF-1α [18]. The expression of ARD-1 is decreased in hypoxia, resulting in stabilized HIF-1α under this condition [18].

Under normoxic conditions, the prolyl hydroxylase domain (PHD) uses oxygen as a rate-limiting substrate and iron as a cofactor to hydroxylate two proline residues within the ODDD [18]. Hydroxylated HIF-1α becomes associated with Von Hippel Lindau factor (pVHL) and elongins B and C, cullin-2 (cul-2) and rbx1 co-factors, forming a complex with E3 ubiquitin ligase activity (HIF-1α-VBC complex) [19]. However, under hypoxic conditions, HIF-1α is stabilized through limited PHD activity. This allows generation and accumulation of non-hydroxylated HIF-1α. Further, HIF-1α stability is controlled by ubiquitin ligases that are PHD enzymes themselves, as well as pVHL-interacting deubiquitinating enzyme (VDU2), which acts to destabilize ubiquitin ligases on HIF-1α [18]. Given the significantly short half-life of HIF-1α (<1 min in a perfused lung), it is constantly being degraded at physiological oxygen levels in normal cells and is subject to tight regulation should oxygen levels decline [20]. In contrast, the median oxygenation of an untreated tumor falls between approximately 0.3% and 4.2%, with most untreated tumors exhibiting median oxygen levels <2% [21]. This level of hypoxia triggers the release and stabilization of HIF-1α while also inducing oncogenic mechanisms that further derail the HIF pathway and make tumors less dependent on oxygen [21]. Tumor-induced mutations in the binding pocket of pVHL have been shown to disrupt HIF-1α interactions and thereby disassemble the E3 ubiquitin ligase (VEC) complex [22]. More directly, in lung cancer, TP53 mutants have been shown to exert a gain of function on HIF-1, leading to heightened expression of hypoxia-response genes [23]. HIF-1α is capable of binding directly to the tumor suppressor, favouring mouse double minute 2 homolog (Mdm2) ubiquitination and proteosomal degradation of HIF-1α, which is not possible in TP53 mutants or knockouts [24]. Several studies have shown that HIF expression is abrogated upon phosphoinositide 3-kinase (PI3K) pathway inhibition regardless of oxygen levels [25,26]. Similarly, HIF-1 is upregulated by AKT in human gastric cancer, breast cancer, and non-small cell lung cancer [27,28].

1.3. Factors Contributing to Tumor Vascular Dysfunction

Tumors initiate the angiogenic process through activation of multiple factors including the most prominent angiogenic ligand, vascular endothelial growth factors (VEGF), and its receptors including VEGFR2 [29–31]. The VEGF family of proteins includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF) [31]. VEGF-C
and VEGF-D are studied as regulators of lymphangiogenesis, while VEGF-A is commonly referred to simply as VEGF due to its dominant role in angiogenesis. VEGF undergoes alternative splicing, leading to several isoforms that differ based on heparin binding affinity, localization to the extracellular matrix, or diffusive potential. The VEGF gene is transcriptionally regulated in response to HIF, and its levels must be tightly controlled to prevent aberrant angiogenesis [31]. Due to their control over HIF, tumor cells release exaggerated levels of VEGF to the extracellular space in response to hypoxia [32]. High concentrations of VEGF surrounding endothelial cells select for excess tip cells, which then contribute to irregular branching and tortuous vascular networks. The basement membrane of tumor vessels, which serves as a physical barrier for cancer cell metastasis to surrounding tissues, is often absent or thin due to chemical degradation by tumor-derived proteases [33,34]. The monolayer of endothelial cells is often disorganized and cells are plagued with abnormal gene expression profiles, karyotypic abnormalities, and chromosomal instability [35–37]. Compared with normal endothelial cells, tumor endothelial cells contain four times the amount of total RNA, indicating enhanced gene expression. Indeed, tumor endothelial cells have enhanced expression of VEGFR-1 and -2 and are therefore more responsive to VEGF stimulation [38]. Recently, tumor endothelial cells have been shown to have enhanced expression of markers of angiogenesis and stemness such as CD61, CD105, Sca-1, CD34, CD90, and ALDH [39,40]. These expression profiles contribute to the escalated angiogenic potential of tumor endothelial cells compared with normal endothelial cells, which facilitates the aberrant vascular arrangement seen in tumors [41]. Extracellular factors such as VEGF, PMA, TGF-β, and cytochalasin B, which are overexpressed in the tumor microenvironment, have been shown to impact fenestration formation in endothelial cells [42,43]. Given that these plasma membrane microdomains are vital for the exchange of solutes and water at the interface of tissue and vasculature, tumor endothelial cells are often more porous compared with normal counterparts [43]. Abnormal VEGF signaling in tumor endothelial cells also leads to downregulation of connexin expression, causing gap junction dysfunction, increasing vascular fenestrations, and increasing vascular permeability [44–46]. In fact, VEGF was initially identified based on its ability to increase vascular permeability and extravasation of plasma proteins, such as fibrinogen [47].

Pericytes are specialized smooth muscle cells that are recruited to mature and stabilized vessels through release of PDGF-β by ECs [48]. Mice deficient in PDGF-β signaling lack pericytes and succumb to micro hemorrhaging, demonstrating the importance of these cells for proper vascular function [48,49]. Signals secreted by pericytes maintain EC survival by leading to enhanced expression of BCL-w antiapoptotic protein [49]. Pericytes therefore also shelter normal vessels from anti-angiogenic therapies, allowing for tumor-targeted action of these agents. Hypoxia and downstream angiogenic factors released by tumor cells disengage pericytes from endothelial cells as the initial step to the formation of the nascent vascular sprout. Therefore, tumor-associated vessels are largely devoid of pericytes or demonstrate weak connections between pericytes and endothelial cells, contributing to an immature vascular phenotype and facilitating continued angiogenesis [50].

1.4. Abnormal Vasculature Results in Limited Treatment Delivery

The abnormalities of the tumor vasculature result in poor tissue perfusion, which poses a physical barrier to therapy delivery to tumors. Of the number of delivery and uptake impediments, elevated interstitial pressure (IFP) is considered to be the most significant barrier to therapy access to the tumor [51–53]. The etiology of IFP elevation is multifactorial and involves high vascular permeability and mechanical compression of lymphatic blood vessels [54,55]. Disrupted vascular morphology with reduced pericyte coverage is associated with a loss of endothelial cell junction integrity and an activated endothelium, resulting in vessels that are leaky and extravasate fluid into the tumor environment, thereby increasing pressure within the tumor [56,57]. Combined with solid stress, in which accumulation of cancer cells, stromal cells, cancer-associated fibroblasts
pericyte coverage. The immature tumor vessels are characterized by blind end shunts, torturous pathway, sacculations, and decreased luminal size, and increased fenestrations. Excessively fenestrated vessels allow for fluid extravasation and their associated extracellular matrix create high mechanical pressure within the tumor, IFP leads to a significant elevation in intratumoral pressure [58].

This high IFP causes a stasis in flow throughout the tumor, which results in tumor hypoxia and acidosis [59]. Elevated hypoxia as a consequence of high IFP is associated with poor outcome in cancer patients and is considered an early response marker for cancer therapeutics such as chemotherapy and radiation [60,61]. As another consequence of reduced perfusion and flow within the tumor, there is impediment of drug uptake and delivery within the tumor tissue [62]. With the elevated IFP, there is an attenuated transvascular osmotic pressure difference, resulting in impaired delivery of drugs throughout the tumor [63]. Even in tumors in which there is vascular heterogeneity, drugs will become concentrated in regions that have sufficient blood supply but will have limited migration to areas in which IFP is higher and vessel density is decreased [64]. Although IFP is often discussed in relation to the primary tumor, it is important to note that larger metastatic tumors also demonstrate elevated IFP and decreased drug uptake, potentially contributing to the development of drug-resistant metastatic disease.

While intra-tumoral treatment delivery decreases off-target toxicities, it fails to account for metastatic disease and has not led to significant survival benefit compared to systemic administration [65]. Clinical use of intra-tumoral drugs is also impractical for some tumor subtypes such as ovarian and pancreatic cancers, which are inaccessible through transdermal injection. In order to prove effective, systemic agents must not only navigate from the injection site to the tumor vasculature but must also gain access and disperse throughout a tumor, which is often plagued with impediments to this process, posing a therapeutic challenge [66]. The properties of the tumor microenvironment that pose issues for treatment delivery are depicted in Figure 2.

Figure 2. Tumor hypoxia activates several tumorigenic processes. Tumor vasculature has altered morphology, with reduced pericyte coverage. The immature tumor vessels are characterized by blind end shunts, tortuous pathway, sacculations, decreased luminal size, and increased fenestrations. Excessively fenestrated vessels allow for fluid extravasation and increased interstitial fluid pressure (IFP) and facilitate intravasation and migration of metastatic tumor cells. Elevated IFP and disrupted tissue perfusion contribute to areas of acute and chronic hypoxia, which can activate numerous pro-tumorigenic processes.
1.5. Hypoxia and Tumor Metabolism

As tumors grow beyond their vascular supply, skewed supply and demand of oxygen and heterogenous blood supply contribute to the development of an O2 gradient within the tumor. In this setting, rapidly proliferating peripheral cells consume available oxygen supplied by vessels, thus limiting oxygen diffusion to the core. This heterogenous distribution of oxygen and subsequent hypoxic regions select for an aggressive phenotype and fuel metastasis as well as treatment resistance, which has been extensively reviewed [67,68]. Hypoxia can alter metabolic pathways within the tumor cells including the impairment of oxygen-dependent processes of mitochondrial oxidative phosphorylation, hindering ATP production. As a result, the tumor cells rewire their metabolism from oxidative phosphorylation to aerobic glycolysis through a process known as the Warburg Effect [69]. Here, a significant increase in glucose consumption results in excess production of lactate [67]. Tumor cells are able to produce ATP at a much more rapid rate through glycolysis and become addicted to glucose consumption to aid in their ability to proliferate at a higher rate compared with normal cells.

With the tumor cells consuming copious amounts of glucose in order to survive and proliferate, an accumulation of lactate occurs. While hypoxic tumor cells favour glucose consumption, normoxic tumor cells, which are located closest to the vasculature, have the option to consume glucose or lactate to fuel their metabolics [70]. Interestingly, these normoxic cells choose to use lactate over glucose, and the accumulated lactate waste produced by hypoxic tumor cells is recycled and reused by normoxic tumor cells, fueling metabolic symbiosis between the tumor cells and the tumor microenvironment [71].

When glucose levels are low, excess lactate signals oxidative-tumor cells to use glutaminolysis, another metabolic avenue that tumor cells rely heavily on to fuel their energy source, as glutamine is imperative to assist rapidly proliferating tumor cells [72]. Cancer cells also require a strong demand for NADPH and many other biosynthetic precursors making glutaminolysis a perfect avenue to maintain the tricarboxylic acid (TCA) cycle and replenish these depleted energy ATP levels. In addition to ATP production, lactate is also produced further contributing to the metabolic symbiosis in the tumor microenvironment [73].

Extracellular lactate accumulation as a result of tumor aerobic glycolysis and glutaminolysis leads to the development of an acidic tumor microenvironment, thereby influencing the potentiality of more aggressive and invasive tumor cells [74]. Extracellular acidic environments have been shown to manipulate gene expression, induce G1 cell cycle arrest, and increase necrotic cell death [75]. This acidity can result in a more aggressive phenotype of tumor cells, primarily through affecting their invasive capacity and metastatic potential [76]. Similar to hypoxic conditions, by maintaining a low pH, tumor cells are able to evade surveillance and destruction by immune cells [77]. Additionally, a low pH in the tumor microenvironment is a known contributor to more aggressive tumor phenotypes and chemoresistance [78].

1.6. Hypoxia and Drug Resistance

A number of cancer treatment strategies rely on the presence of oxygen in order to exert their anti-tumor effect. The basis of radiotherapy is generation of reactive oxygen species, which then damages tumor cell DNA resulting in cell death. This reaction in turn becomes permanent when oxygen reacts with the free electron of the free radical. In a pioneering study, Gray and colleagues demonstrated that the presence of oxygen conferred radiation sensitivity in tumors. In fact, killing hypoxic tumor cells requires a three-fold higher dose of radiation compared with killing normoxic tumor cells [79]. This is problematic for treatment success given that the dose of radiation cannot be safely increased to compensate for this difference in light of limited radiation tolerance of normal tissues. Indeed, normoxic tumors have a higher chance of radiotherapy success. Likewise, photodynamic therapy (PDT) relies on oxygen to induce photo-oxidation, and PDT resistance is common in hypoxic tumors [80,81].
The role of hypoxia in chemoresistance is well-documented and involves increased HIF production in response, which enhances the expression of membrane efflux pumps. The most common efflux pumps linked to multidrug resistance are the ATP Binding Cassette (ABC) family of transporters, which reduce intracellular accumulation of chemotherapy to sub-therapeutic levels [82]. Although constantly expressed, HIFs are degraded when there is normal oxygen tension, but are stabilized in the presence of hypoxia [18]. In colon cancer cells subjected to hypoxia, HIF-1 activation occurred, which resulted in overexpression of multidrug resistance 1 (MDR1; P-glycoprotein) [16,83]. MDR1 is an ATP-dependent efflux pump that can effectively transport chemotherapy drugs out of the cell and is one of the major mechanisms involved in chemotherapy failure [84,85]. HIF-1 upregulation of MDR1 is also associated with chemoresistance, enriched stem cell population, and aggressive phenotype in triple negative breast cancers [86]. In colon cancer cells, blocking HIF-1α has been shown to reverse multi-drug resistance via downregulation of P-glycoprotein [87].

In the presence of hypoxia, HIF-1α increases the activity of Snail and Twist, transcription factors that promote epithelial-to-mesenchymal transition (EMT) and are associated with resistance to chemotherapy [88]. Several groups have found that hypoxic tumor cells are less proliferative than their normoxic counterparts [89]. This becomes problematic given that chemotherapy targets highly proliferative cells, thereby selecting for the survival of the more aggressive hypoxic cells. Further, Saggar et al. found that chemotherapy repopulates hypoxic cells that contribute to treatment failure, possibly due to enhanced nutrient availability following clearance of rapidly proliferating cells [90]. The link between normoxia and chemotherapeutic success likely explains the benefits of hyperbaric oxygen therapy (HBOT) in improving their effectiveness [91–93]. The excess oxygen molecules provided by HBOT enhances chemotherapy-induced oxidative stress, thereby lowering the therapeutic dose and mitigating side effects [91–93].

1.7. Hypoxia and the Immune Environment

Immunotherapy has become the fourth pillar of cancer therapy, joining surgery, radiation, and chemotherapy. Hypoxia has been identified as a barrier to the success of immunotherapy due to its association with tumor escape from immune detection [93]. Through stabilization of HIF-1, hypoxia upregulates chemokines such as CCL28, which enhances tumor influx and function of myeloid-derived suppressor cells [94]. HIF-1α also increases the expression of forkhead box P3 (FoxP3), which is indispensable for the development of Tregs [95]. Hypoxia also promotes immune evasion by upregulating expression of checkpoint molecules such as the programmed death ligand 1 (PD-L1) [96] and involves binding of HIF-1 to a hypoxia response element in the PD-L1 proximal promoter [97]. Hypoxia is also reported to inhibit the antitumor immune response. HIF-1α stabilization prevents TCR-mediated Ca\(^{2+}\) signaling and prevents CD8+ T cell activation [98]. In mice, low oxygen availability led to the reduction of aggressive cellular activity and correlated with decreased pro-inflammatory cytokine production [99]. Likewise, areas of tumor hypoxia are associated with reduced T lymphocyte proliferation and enhanced apoptosis and are often relatively devoid of these cells [100]. In addition to the effects on T lymphocytes, hypoxia also affects the function of natural killer (NK) cells. There is substantial evidence that hypoxia suppresses the cytotoxic effect of NK cells in tumors [101]. Upregulation of HIF-1α within the tumor can lead to decreased expression of the natural killer group 2 member D (NKG2D) receptor on NK and T cells, leading to immune evasion and impaired tumor cell killing [101,102]. Hypoxia is also known to enhance the uptake of regulatory T-cells (Tregs), which lead to the activation of transforming growth factor-beta (TGF-β), further suppressing NK cell function [103]. TGF-β is also a key player in the recruitment of cancer-associated fibroblasts (CAFs) in solid tumors [104]. CAFs are responsible for production of cytokines and generation of fibrous material, which contributes to mechanical barriers to immune cell infiltration and function [105]. Dendritic cells, the main antigen-presenting cells, are critical in activating naïve T cells and generating a specific immune response [103]. Sustained HIF-1α expression led to DC expression of immunosuppressive
mediators such as iNOS and IL-10, which hindered CD8 T cell function [105]. Through gene knockout studies in mice, Weigert et al. demonstrated that HIF-1α hinders the generation of dendritic cells in the bone marrow [106].

1.8. Therapeutic Use of Vascular-Targeting Agents

The poor prognosis of treatment strategies in hypoxic tumors has prompted studies into identifying oxygenation status of tumors as a way to predict therapy efficacy [107]. Several strategies have been employed to use hypoxia as an advantage to therapy. For instance, hypoxia-activated prodrugs (HAPs) are enzymatically reduced in low oxygen levels to generate cytotoxic species [108]. Other strategies focus on reversing tumor hypoxia, such as enhancing the oxygen-carrying capacity of plasma through hyperbaric oxygen therapy [109]. Moreover, molecules that improve the rate of diffusion of oxygen from red blood cells to the vascular wall [110] and engineered oxygen transport molecules [111] are yielding promising results as combination therapies, preclinically [112].

Further strategies focus on targeting the source of tumor hypoxia: tumor vasculature. In 1993, Kim et al. formed murine tumor models of rhabdomyosarcoma, glioblastoma, and leiomyosarcoma and found that mice treated with VEGF monoclonal antibodies suppressed tumor growth [113]. Given that the antibodies had no effect on these cells in vitro, this pioneering study demonstrated that blocking the actions of an angiogenic mediator has direct effects on tumor growth by manipulating tumor vasculature. Early studies into anti-angiogenic agents were designed to induce destruction of the tumor vessels in hopes of starving the tumor. Although vascular disruption yields acute anti-tumor effects, this extensively reviewed strategy does not translate to long-lasting tumor suppression [113,114]. Several anti-angiogenic therapies have been approved clinically, although their benefit to overall survival has been modest likely due to the aggressiveness of cancer cells as they adapt to lower oxygen levels in their environment [115]. Godet et al. (2019) [92] demonstrated that lung cancer cells exposed to hypoxia in the primary tumor environment are six times more likely to become viable circulating cells compared with those in normoxic tumor areas [92,116]. Hypoxic cells develop a gene signature that includes changes in p53 and e-cadherin—ensuring their resistance to oxidative stress and fueling metastasis [117]. Even following re-oxygenation, the cells exhibited “hypoxic memory” and maintained this aggressive phenotype [117]. High-dose anti-angiogenic therapies also have undesirable effects such as further reduction of oxygen levels and decreased tumor delivery of chemotherapy due to further vessel destruction and greater impairment of tumor perfusion. These agents have instead been evaluated in low doses as adjuvants to chemotherapy, for which they have garnered success clinically, as demonstrated in Table 1. This efficacy is largely attributed to a phenomenon known as vascular normalization, proposed by Jain et al. in 2001 [118,119]. The process of vascular normalization involves improving overall morphology of vasculature by specifically destroying immature vessels while maintaining intact tumor vasculature that resembles normal vessels throughout the body. The reduced demand for blood supply brought about by reliable circulation reduces HIF-1, thereby re-establishing the balance between pro- and anti-angiogenic factors. Immature vessels—those that appear tortuous with expanded lumens, display decreased pericyte coverage, and require VEGF for survival—are highly dependent on circulating angiogenic factors [120]. Their dependence makes them susceptible to anti-angiogenic molecules and are therefore pruned in the process of vascular normalization. Similarly, vasculature with low pericyte investment is also more susceptible to anti-angiogenic therapy. This is likely as a result of paracrine signaling between endothelial cells and pericytes, which maintains stability of mature vessels [121].
Table 1. Clinical success of FDA-approved anti-angiogenic drugs alone and in combination, as measured by progression free survival (PFS) and overall survival (OS).

| Anti-Angiogenic Drug | FDA Approval | Mechanism | Indication | Combination Agent | Anti-angiogenic Drug + Combination Agent vs. Combination Agent Alone (*) | Ref. |
|----------------------|--------------|-----------|------------|-------------------|---------------------------------------------------------------------|------|
| Bevacizumab (Avastin®) | 2004 | Humanized monoclonal antibody that binds to and inhibits the activity of VEGF-A | Cervical | Paclitaxel + Cisplatin | 9.63 (* 6.67) | 17.51 (* 12.68) | [122] |
|                       |              |           |            | Paclitaxel + Topotecan | 7.36 (* 5.29) | 16.20 (* 12.68) | [122] |
|                       |              |           | Colorectal (metastatic) | 5-Fluorouracil | 8.8 (* 5.6) | 17.9 (* 14.6) | [123] |
|                       |              |           | NSCLC | Carboplatin + Paclitaxel | 6.2 (* 4.5) | 12.3 (* 10.3) | [124] |
|                       |              |           | Ovarian, Fallopian, primary peritoneal | Carboplatin + Paclitaxel | 18.1 (* 14.5) | 36.6 (* 28.8) | [125] |
|                       |              |           | Renal Cell | Interferon alfa | 10.2 (* 5.4) | 23.3 (* 21.3) | [126,127] |
| Axitinib (Inlyta®)    | 2012 | Tyrosine Kinase Inhibitor (VEGFR-1, VEGFR-2, VEGFR-3) | Renal Cell | Pembrolizumab | 15.1 (* 11.1) | - | [128] |
| Cabozantinib (Cometriq®) | 2012 | Tyrosine Kinase Inhibitor (VEGF, MET, AXL) | Hepatocellular | Placebo | 5.2 (* 1.9) | 10.2 (* 8.0) | [129] |
|                      |              |           | Medullary Thyroid | Placebo | 11.2 (* 4.0) | - | [130] |
| Everolimus (Afinitor®, Zortress®) | 2009 | mTOR inhibitor | Breast | Exemestane | 10.6 (* 4.1) | - | [131] |
|                      |              |           | Advanced Kidney | Lenvatinib | 14.6 (* 7.4) | - | [132] |
| Pazopanib (votrient®) | 2009 | Tyrosine Kinase Inhibitor (VEGFR-1,-2,-3, PDGFR-a, -b, c-KIT, FGFR-1, -3) | Renal Cell | placebo | 9.2 (* 4.2) | 22.9 (* 20.5) | [133] |
|                      |              |           | Soft Tissue Sarcoma | placebo | 4.6 (* 1.6) | 12.5 (* 10.7) | [134,135] |
| Regorafenib (Stivarga®) | 2012 | Tyrosine Kinase Inhibitor (VEGFR-1,-2,-3, TIE-2, PDGFR, FGFR, KIT, RET, RAF-1, BRAF) | Colorectal Cancer | Placebo | - | 6.4 (5.0) | [135] |
|                      |              |           | Gastrointestinal | Placebo | 4.8 (* 0.9) | - | [136] |
|                      |              |           | Hepatocellular | Placebo | - | 10.6 (* 7.8) | [137] |
Table 1. Cont.

| Anti-Angiogenic Drug | FDA Approval | Mechanism | Indication | Combination Agent | Anti-angiogenic Drug + Combination Agent vs. Combination Agent Alone (*) | Ref. |
|----------------------|--------------|-----------|------------|-------------------|------------------------------------------------------------------------|------|
| Sorafenib (Nexavar<sup>®</sup>) | 2005 | Tyrosine Kinase Inhibitor (Raf, PDGF, VEGFR-2, -3, c-KIT) | Renal Cell | placebo | PFS (mts) 5.5 (* 2.8) | OS (mts) 19.3 (* 15.9) | [138] |
| | | | Hepatocellular | placebo | 5.5 (* 2.8) | 10.7 (* 7.9) | [139] |
| | | | Advanced thyroid | placebo | 10.8 (* 5.8) | - | [140,141] |
| Sunitinib (Sutent<sup>®</sup>) | 2006 | Tyrosine Kinase Inhibitor (PDGF-a, b, VEGFR-1, -2, -3, KIT, FLT-3, CSF-1R) | Pancreatic Neuo-endocrine | Placebo | PFS (mts) 12.6 (* 5.8) | OS (mts) 38.6 (* 29.1) | [141] |
| | | | Gastrointestinal Stromal | Placebo | - | 18.5 (* 8.9) | [136] |
| Vandetanib | 2011 | Tyrosine Kinase Inhibitor (VEGFR-2, EGFR, RET) | Medullary Thyroid | Placebo | PFS (mts) 30.5 (* 19.2) | - | [142] |
| Ziv-aflibercept | 2012 | Fusion protein (two human VEGF receptors connected by Fc domain) | Colorectal | FOLFIRI chemo (Folinic Acid, Fluorouracil, irinotecan) | PFS (mts) 6.9 (* 4.7) | OS (mts) 13.5 (* 12.0) | [143] |
| Ramucirumab | 2014 | Human monoclonal antibody against VEGFR-2 | Gastric | Paclitaxel | - | 9.6 (* 7.4) | [144,145] |
| | | | NSCLC | Docetaxel | 4.5 (* 3.0) | 10.5 (* 9.1) | [145] |
| | | | Colorectal | Placebo | 2.8 (* 1.6) | 8.5 (* 7.3) | [146] |

* denotes overall survival (OS) or progression free survival (PFS) of the agents used alone whereas bolded numbers represent OS/PFS when used in combination with anti-angiogenic therapy.
2. Considerations for Success of Vascular Normalizing Agents

The observation of improved perfusion was initially counter-intuitive in that researchers expected that anti-angiogenic therapy would decrease tumor profusion. Evidence now suggests that the clinical success of vascular normalization appears to be a function of dose, duration of treatment, and tumor subtype or vascularization status. In early clinical trials with the anti-VEGF antibody, bevacizumab, those patients whose tumors demonstrated significant improvement in perfusion following anti-angiogenic therapy showed the greatest progress clinically [114]. In fact, higher doses of, or extended treatment with, VEGF pathway antagonists can return to vascular destruction and a subsequent decrease in perfusion, leading to the concept of a “normalization window” where optimal benefit is achieved [118]. The presence of abnormal vessels is a key indicator for response to anti-angiogenic therapy because this therapy can induce hypoxia of poorly vascularized tumors. As such, high microvascular density at the beginning of treatment correlates with response to bevacizumab treatment [147]. Whereas increased overall survival was not detected in the total population of 980 ovarian cancer patients after bevacizumab treatment, increased overall survival was increased in the subpopulation of patients with increased vessel density and higher amounts of VEGF prior to treatment. A correlation between baseline microvessel density and response to anti-angiogenic therapy is also seen in breast cancer, non-small-cell lung cancer, and colorectal cancer [121,148,149]. By contrast, no correlation between baseline vascular density and response to anti-VEGF therapy was observed in renal cancer [150]. These data indicate that the efficacy of vascular normalization varies between tumor types and between patients. In addition, as discussed below, vascular normalization differs between therapeutics. Thus, there is not a “one-size-fits-all” approach to normalizing the tumor vasculature.

In addition to pruning tumor vessels, the fortification of vessels by pericytes is a key component of vascular normalization. Thus, the relative number of vessels with pericycle coverage can be used to quantify vessel normalization and response to therapy. The recruitment of pericytes can help to limit excess pruning induced by anti-VEGF treatment. Therapeutics that favour fortification over pruning may have significant benefit in that their normalization window may be wider. Thrombospondin-1 (TSP-1)-based reagents may be well-suited to increase fortification in that TSP-1 has been shown to promote smooth muscle cell proliferation and migration while inducing apoptosis of endothelial cells [151,152]. A significant portion of the anti-angiogenic activity of TSP-1 resides within the type 1 repeats of TSP-1, designated 3TSR. As described below, 3TSR has potent normalizing effects of the tumor vasculature, which in turn promotes delivery of therapeutics and immune cells to tumors. Systemic upregulation of TSP-1 reportedly mediates the anti-angiogenic effect of metronomic dosing of chemotherapy [153].

Angiopoietin-1 and its receptor Tie-2, which is present on endothelial cells and pericytes, promotes vessel maturation and fortification, and thus, resistance to anti-angiogenic therapy. Tie-2 is inactivated by angiopoietin-2 and vascular endothelial protein tyrosine phosphatase (VE-PTP) [154]. Goel et al. (2013) have shown that inhibiting the activity of VE-PTP fortifies vessels and promotes the delivery of chemotherapeutics in mammary tumors. Similarly, blocking angiopoietin-2 can promote vascular normalization and prolong survival induced by anti-VEGF therapy in glioblastoma [155].

Upregulation of Tie-2 signaling or TSP-1 represent two examples of a wide range of reagents that can regulate vascular normalization through modulating metabolism, signal transduction, and extracellular matrix degradation [121]. Similarly, recently type 1 T helper cells have been shown to participate in vascular normalization through immune reprogramming [156]. A co-dependence of the immune and vascular systems was evident from the fact that vascular normalization was decreased by depletion or inactivation of CD4+ lymphocytes. Furthermore, adoptive transfer of T effector cells to immunodeficient tumor-bearing mice reduced hypoxia in immunodeficient mice. Taken together, the data indicate that vascular normalization through pruning and fortification can be achieved
in multiple ways. There must be excessive vascularization prior to treatment so that the anti-angiogenic therapy does not increase hypoxia and the anti-angiogenic therapy must promote fortification in order for there to be a therapeutic benefit. Identifying optimal strategies for optimizing vascular normalization is an important area for future research.

3. Vascular Normalizing Agents as Adjuvants to Traditional Cancer Therapeutics

Normalized tumor vessels have also been shown to re-program many other aspects of the tumor microenvironment known to limit delivery of cancer therapies discussed earlier in this review, giving rise to the term ‘microenvironment normalization’ [99]. Anti-angiogenic drugs have opened new avenues for combination therapy. In a humanized murine model of colorectal adenocarcinoma, combination therapy with anti-PDGFR and anti-VEGFR tyrosine kinase inhibitors decreased IFP in tumors, allowing for enhanced delivery of taxol therapy [157]. Improved delivery of chemotherapy through vascular normalization in solid tumors has been extensively reviewed [100]. We and others have extended the utility of vascular normalization to enhancing the delivery and functionality of agents beyond traditional chemotherapy. The vascular shutdown typical of oncolytic viruses (OV) was prevented using thrombospondin type-1 repeats in a mouse model of advanced stage ovarian cancer [158]. This led to enhanced intratumoral trafficking of immune cell subsets, thereby improving immunotherapeutic success [99,158,159]. In addition to enhancing vascular perfusion and providing a conduit for immune cells, the enhanced oxygenation of tumors as a result of low-dose anti-angiogenic therapy has improved immune cell function and reprogrammed immune cell subsets with greater anti-tumor capabilities [156]. Vascular normalizing therapies continue to be recognized for their oxygen-modulating function in sensitizing tumors to traditional therapies, which have often been met with resistance [160].

4. Conclusions

Dysregulated tumor vasculature creates tumor hypoxia, which encourages aggressive tumor cell adaptations, impedes immune surveillance, fuels metastasis, and promotes resistance to current standard of care treatments. Pre-clinical studies of novel therapeutic strategies often fail to account for the vascular density and oxygenation status of the tumor subtype, resulting in a lack of clinical efficacy in patients. Anti-angiogenic therapies have the potential to normalize the tumor microenvironment, allowing for significantly better anti-tumor results when used in combination with other therapeutics. Future studies should focus on optimal timing and dosing of these agents in candidate solid tumors to prevent over-normalizing or pruning back tumor vasculature when the intent is to improve perfusion for enhanced systemic therapy.

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