Limitations of Anti-Angiogenic Treatment of Tumors

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Abstract

Clinical trials using anti-vascular endothelial growth factor /(VEGF) molecules induce a modest improvement in overall survival, measurable in weeks to just a few months, and tumors respond differently to these agents. In this review article, we have exposed some tumor characteristics and processes that may impair the effectiveness of anti-angiogenic approaches, including genotypic changes on endothelial cells, the vascular normalization phenomenon, and the vasculogenic mimicry. The usage of anti-angiogenic molecules leads to hypoxic tumor microenvironment which enhances tumor invasiveness. The role of tumor-infiltrating cells, including tumor associated macrophages and fibroblasts (TAMs and TAFs) in the therapeutic response to anti-angiogenic settings was also highlighted. Finally, among the new therapeutic approaches to target tumor vasculature, anti-PD-1 or anti-PD-L1 therapy sensitizing and prolonging the efficacy of anti-angiogenic therapy, have been discussed.

Anti-Angiogenesis in Clinical Use

Anti-angiogenesis therapy started in 2004 (Table 1), with the approval by Food and Drug Administration (FDA) of the first anti-angiogenic drug, bevacizumab (Avastin), a humanized monoclonal antibody anti- VEGF-A, approved for the treatment of previously untreated metastatic colorectal cancer in combination with chemotherapy [1]. Bevacizumab can be given safely for long periods, with manageable toxicity when added to chemotherapy, even if it induces severe side effects, including lethal hemoptysis and intestinal perforations [2,3]. The small molecule tyrosine kinase inhibitors (TKIs), including sunitinib, sorafenib, and pazopanib, are another class of anti-angiogenic drugs, able to interfere with vascular endothelial growth factor receptors-1 and -2 (VEGFR-1 and VEGFR-2), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptors (FGFRs), and Tie signaling (Table 2) [4]. VEGFR-trap protein aflibercept, formed by fusion of the VEGF binding domain of VEGFR-1 and VEGFR-2 has been approved for metastatic colorectal cancer [5].

Therapeutic inhibition of angiogenesis has been associated with increased local invasiveness and distant metastasis, as shown for the first time in 2009 [6,7]. Sunitinib and the anti VEGFR-2 antibody DC101 stimulated the invasive behavior of tumor cells despite their inhibition of tumor growth [6,7]. Increased invasiveness might result from enhanced expression of angiogenic cytokines induced by the treatment, including VEGF and placental growth factor (PlGF), or recruitment of endothelial progenitor cells (EPCs), that promote the formation of a pre-metastatic niche [8]. Inherent or acquired resistance to anti-VEGF molecules can occur leading to a lack of response and to disease recurrence, although discontinuation of the therapy is the principal factor limiting the effectiveness of anti-angiogenic therapies [9]. Moreover, anti-angiogenic treatment reduces drugs delivery to tumors, restricting their efficacy [10]. Tumors use multiple pathways for recruiting vessels and blocking VEGF alone has incomplete effects on tumor vasculature, and tumors

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Table 1. Approved anti-angiogenic agents for cancer and their indications

Monoclonal antibodies	Bevacizumab	- metastatic colorectal cancer
		- non-small cell lung cancer
		- glioblastoma multiforme
		- renal cell carcinoma
		- epithelial ovarian cancer
		- fallopian tube cancer
		- primary peritoneal cancer
		- cervical cancer
	Ramucirumab	- metastatic colorectal cancer
		- non-small cell lung cancer
		- gastric or gastroesophageal junction adenocarcinoma
Tyrosine kinase inhibitors (See Table 2)		
Fusion protein	Ziv-aflibercept	- metastatic colorectal cancer

may switch from one mechanism to another. Breast cancer cells express several angiogenic factors including VEGF, transforming growth factor beta (TGFβ), PlGF and platelet derived growth factor (PDGF) [11]. Other factors involved in compensatory angiogenic signaling are FGF-2, hepatocyte growth factor/scatter factor (HGF/SF), angiopoietins (Angs), and interleukin-8 (IL-8) [12–16].

Long-Term Anti-Angiogenic Therapy Leads to Tumor Hypoxia

Tumor hypoxia is a consequence of inadequate oxygen supply caused by extensive abnormalities in the vascular network, including elongated and tortuous vessels, aberrant vessel diameters, and vessel wall abnormalities (i.e., incomplete endothelial lining, interrupted basement membrane, and lack of pericytes and contractile vessel wall components) [17].

Anti-angiogenesis is responsible for a temporary decrease in tumor hypoxia, which parallels the maturation of the vasculature. VEGF inhibition could temporarily normalize the function of tumor-associated vasculature, decreasing vascular permeability in conjunction with restoration of sustained pressure gradients, thereby enhancing systemic delivery of oxygen or perfusion of cytotoxic agents to intratumoral sites [18]. In combination with chemotherapy, such vascular normalization strategies can improve drug delivery and cancer growth control [19].

The window of vascular normalization occurs transiently during the first days of treatment. When the tumor blood vessels lose their maturation [20] hypoxia re-increases and prolonged VEGF inhibition further increases local hypoxia [21], which in turn induces systemic secretion of other angiogenic cytokines [7,22–24]. Hypoxia within tumor increases during treatment with an anti-angiogenic agent, inducing pH drop and consequent acidosis [19]. Hypoxic conditions could also select more malignant cells, i.e. those able to grow in hypoxic conditions [25]. Moreover, hypoxia, produces a pressure mechanism that selects tumor cells with increased aggressiveness and lower sensitivity to anti-angiogenic therapy [23]. In fact, blood supply to the tumor remains heterogeneous and hypoxia increases while the permeability of tumor blood vessels is reduced, favoring disappointing results of clinical trials where anti-angiogenic therapies have been combined with systemic delivery of chemotherapeutic agents.

Hypoxia promotes the differentiation of tumor infiltrating myeloid cells to M2-pro-angiogenic tumor associated macrophages (TAMs) [26], and neovascularization through the mobilization of bone

Table 2. Anti-angiogenic tyrosine kinase inhibitors in clinical development

Agent	Target	Clinical activity and/or study
Sunitinib (SU11248; Sutent)	• VEGFR-1, -2, -3 • PDGFR • KIT • FLT3 • CSF-1R • RET	Kidney, breast, prostate, lung, liver, ovarian, colorectal, thyroid, head and neck, gastric, bladder, cervical and pancreatic cancer, GIST, melanoma, glioblastoma, myeloma, lymphoma
Sorafenib (BAY439006; Nexavar)	• VEGFR-2, -3 • PDGFR • Raf • KIT	Kidney, liver, breast, prostate, lung, ovarian, colorectal, thyroid, head and neck, gastric and pancreatic cancer, GIST, melanoma, glioblastoma, lymphoma, leukemia
Pazopanib (GW786034; Votrient)	• VEGFR-1, -2, -3 • PDGFR • KIT	Kidney, breast, lung, cervical, liver, thyroid, prostate and colorectal cancer, melanoma, glioblastoma
Vandetanib (ZD6474; Zactima)	• VEGFR-2 • EGFR • KIT • RET	Lung, kidney, thyroid, head and neck, prostate, ovarian, breast and colorectal cancer, glioma, neuroblastoma
Axitinib (AG013736)	• VEGFR-1, -2, -3 • PDGFR-β • KIT	Kidney, lung, thyroid, pancreatic, colorectal and breast cancer, melanoma
Cediranib (AZD2171; Recentin)	• VEGFR-1, -2, -3 • PDGFR-β • KIT	Kidney, breast, lung, liver, ovarian, head and neck, prostate and colorectal cancer, GIST, glioblastoma, melanoma
Vatalanib (PTK787; ZK222584)	• VEGFR-1, -2, -3 • PDGFR-β • KIT	Prostate, colorectal, kidney and pancreatic cancer, melanoma, lymphoma, leukemia
Motesanib (AMG706)	• VEGFR-1, -2, -3 • PDGFR • KIT • RET	Lung, thyroid, gallbladder, breast and colorectal cancer, GIST

CSF-1R colony stimulating factor-1 receptor, EGFR epidermal growth factor receptor, FLT3 fms-related tyrosine kinase 3, GIST gastro-intestinal stromal tumor, PDGFR platelet-derived growth factor receptor, VEGFR vascular endothelial growth factor receptor, RET, rearranged during treatment, RAF, rapid accelerated fibrosarcoma

marrow-derived endothelial precursor cells [27]. Hypoxia also promotes genetic instability in tumor endothelial cells [28], and induces the selection of more invasive metastatic clones of the cancer cells that are resistant to anti-angiogenic agents [29], through the production of pro-migratory proteins, such as stromal cell derived factor 1 alpha (SDF1-α) and HGF/SF and pro-invasive extracellular matrix proteins [30,31], which may stimulate mobilization and recruitment of EPCs and other bone marrow-derived cells [32,33].

Genotype Alterations

The cells of the tumor vasculature are more genetically stable than tumor cells, express specific antigens [34] and are more accessible for therapeutic molecules and immune cells, even if gene and chromosomal abnormalities have been documented in subpopulations of tumor endothelial cells, including aneuploidy, abnormal multiple chromosomes, and aberrant chromosomse [35].

Colorectal cancer endothelial cells overexpress specific transcripts as a result of qualitative differences in gene profiling compared with endothelial cells of the normal colorectal mucosa [36]. A distinct gene expression pattern related to extracellular matrix and surface proteins characteristic of proliferating and migrating endothelial cells has been demonstrated in glioma and invasive breast carcinoma [37,38].

Tumor endothelial cells isolated from human renal cell carcinoma are resistant to apoptotic stimuli with enhanced Akt activation and decreased expression of the tumor suppressor phosphatase and tensin homolog deleted from chromosome 10 (PTEN) [39]. Cytogenetic abnormalities are an expression of genetic instability and could explain the resistance of tumor endothelial cells to chemotherapeutic agents, including vincristine [39], 5-fluorouracil, adriamycin, and paclitaxel [40,41].

Cells of the Microenvironment

TAMs have been considered as primary cause of resistance to anti-VEGF agents [42]. PlGF mediated recruitment of pro-angiogenic TAMs might be a mechanism for tumor resistance [43]. Refractoriness to anti-angiogenic therapies in glioblastoma multiforme patient is associated with higher number of CD68^{+} TAMs and CD11b^{+} myeloid cells [44]. Blocking the recruitment of TAMs may be a mechanism to overcome resistance to anti-angiogenic therapy. VEGFR-2 inhibition in RIP1-Tag2 pancreatic neuroendocrine tumors upregulates Ang-2,

enhances infiltration of Tie2 expressing macrophages, and suppresses revascularization and tumor progression [45].

Tumor associated fibroblasts (TAFs) secrete several angiogenic growth factors, including epidermal growth factor (EGF), HGF, insulin-like growth factor, and FGF-2 [46]. TAFs are involved in resistance to anti-angiogenic therapy. In particular, CAFs generated PDGF-C, which is a key factor in sustaining angiogenesis and tumor growth under anti-VEGF treatment [13]. Pericytes can protect endothelial cells from VEGF withdrawal by activating compensatory pro-angiogenic pathway in anti-VEGF therapy [47].

Cancer Stem cells (CSCs) generate angiogenic factors to stimulate tumor angiogenesis, and tumor vasculature, in turn, supports CSC self-renewal and maintaining [48]. Moreover, CSCs recruit endothelial precursors for revascularization and tumor re-growth [49], and may be involved in tumor resistance as a consequence of their capability to produce much higher levels of VEGF in both anoxic and hypoxic environments than non-CSC population [50].

Bone marrow derived cells (BMDCs) are a major reserve of EPCs, which play an important role in tumor angiogenesis independent of VEGF [51]. The immature myeloid cell [52] and EPCs [53] infiltrate the tumor and mediate the resistance by incorporating themselves into vessels or by releasing pro-angiogenic growth factors [54].

Tumor Blood Vessels Normalization (Table 3)

Tumor vascular normalization is accompanied by increased pericyte coverage, while pericyte deficiency could be partly responsible for vessel abnormalities in tumor blood vessels [55] and partial dissociation of pericytes contribute to increased tumor vascular permeability [56]. Pericyte coverage promotes resistance through direct support or paracrine interactions with endothelial cells and tumor vessels covered by pericytes are less sensitive to VEGF blockade [57].

Vascular normalization improves T cell extravasation and promotes the conversion of pro-angiogenic (M2-like) into angiostatic (M1-like) TAMs [58]. TAMs usually exhibit M2-like phenotype, secreting immunosuppressive cytokines, such as IL-10, CCL17 and CCL22 and producing pro-angiogenic and tissue remodeling factors, such as VEGF, PlGF and matrix metalloproteinase-9 (MMP9) [59]. The increase of oxygenation in histidine-rich glycoprotein (HRG)-positive tumors caused by vascular normalization seems to provide a stimulus for polarizing TAMs away from M2-like type, which could further

Table 3. Events in tumor blood vessels normalizaton
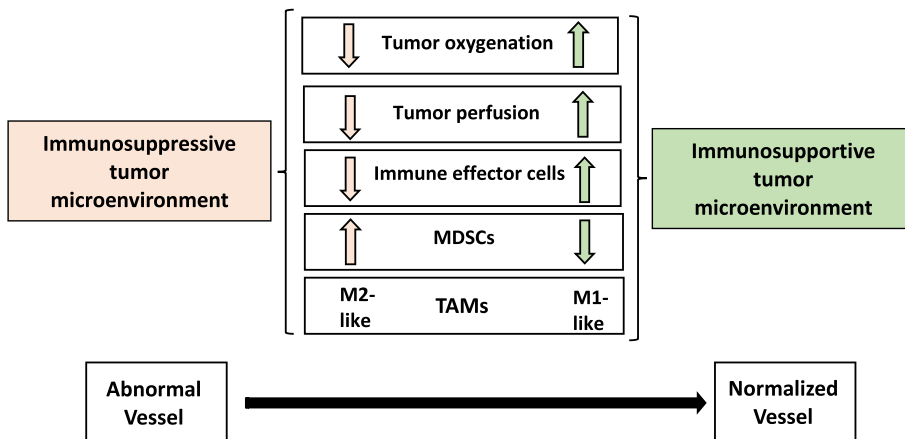
sustain the normalized vasculature, leading to decreased tumor growth and metastasis [60].

In the case of cerebral tumors, the process of normalization may induce a re-establishment of the low permeability characteristics of normal brain microvasculature, preventing the delivery of chemotherapeutics [61]. In the meantime, vascular normalization induces a drop in interstitial fluid pressure and reduction in hypoxia, which provide an improvement of the penetration and activity of cytotoxic drugs [62].

The state of vascular normalization generally is transient and in this context tumor is more receptive to delivery of chemotherapeutic drug and immune cell populations [63]. However, there appears to be a high degree of variation in temporal window of vascular normalization for various anti-angiogenic agents [64].

Alternative Mechanisms of Formation of Tumor Vasculature

Other modes of tumor vascularization may be less sensitive to anti-angiogenic therapies. Intussusceptive microvascular growth (IMG) ("intussusception, known also as or non-sprouting or splitting angiogenesis") is a concept of microvascular growth relevant for many vascular systems, which constitutes an additional and alternative mechanism to endothelial sprouting [65]. In IMG, the capillary network increased its complexity and vascular surface by insertion of a multitude of transcapillary pillars, a process they called "intussusception" (meaning "in-itself" growth) [65]. IMG generates vessels more rapidly with a less metabolic demand as compared to sprouting angiogenesis and is a strategy that tumors can use for rapid adaptation to milieu changes [65]. IMG has been observed in several human tumors, including melanoma, colon, mammary carcinomas, B-cell non-Hodgkin's lymphoma and glioblastoma [65].

In the course of the so called "vasculogenic mimicry," blood vessels are generated without the participation of endothelial cells through a differentiation of tumor cells into endothelial-like cells, and independent of classical angiogenic factors, including FGF-2 and VEGF [66]. Vasculogenic mimicry has been observed in different human malignant tumors, including breast, melanoma, bladder, kidney, glioblastoma, prostate, ovarian, lung, sarcomas, cell renal cell carcinoma and astrocytoma [67]. An increase in vasculogenic mimicry has been demonstrated after anti-angiogenic treatment with Bevacizumab [68].

Vascular co-option occurs in site of metastases or in densely vascularized organs. Tumor cells co-opt and grow as cuffs around adjacent vessels [69]. The prevalence of vessel co-option in in liver metastasis of breast and colorectal cancer [70] may explain why anti-angiogenic therapies are poorly effective in these pathological conditions. Vessel co-option could explain the onset of resistance to anti-angiogenic regimens in glioblastoma multiforme, hepatocellular carcinoma, and in metastasis to lung [71–73]. Moreover, anti-VEGF antibody treatment is responsible of an increased vessel co-option [73]. Simultaneous inhibition of angiogenesis and vessel co-option may represent a further improvement of the therapeutic approach [70].

Therapeutic Vaccines Promoting Immune Targeting of Tumor Vascular Cells

The programmed death protein 1 (PD-1), its ligand the programmed death ligand 1 (PD-L1) and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are negative regulators of T-cell immune function. Direct stimulation of the immune system with immune check-point inhibitors, such as antibody against PD-1/PD-L1 and CTLA-4 has been reported in multiple cancers. Antibodies against

CTLA-4 and PD-1/PDL-1 have proven efficacy in different tumor types and five drugs of this class have been approved by the FDA [74].

The production of angiogenic molecules by tumor cells inhibits the expression of adhesion molecules involved in leukocyte interactions with blood vessels, including intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and CD34, preventing adhesion and extravasation of effector T cells into the tumor [63]. The anergic phenotype of tumor endothelial cells can be reversed by anti-angiogenic therapy, which upregulates the expression of endothelial adhesion molecules in the tumor vasculature [75]. Abrogating VEGF signaling thus looks like an immunotherapeutic strategy.

Vaccination based on tumor blood vessel associated antigens (TBVA) normalizes the vasculature, induces T cell effector response, and inhibits tumor growth in murine models [76]. Two major types of vaccines are being developed, namely vaccines against defined angiogenesis-associated antigens, and whole endothelial cell vaccines, expressing numerous angiogenic antigens, using whole endothelial cells or isolated proteins from endothelial cell membranes [77]. Moreover, VEGF decreased in patients with metastatic melanoma responding to sequential anti-CTLA-4 and anti-PD-1 therapy, but increased in non-responders, indicating a mechanism of therapeutic resistance [78].

A clinical study of a combination therapy using antibody-anti-CTLA-4 with bevacizumab reported efficacy in patients with metastatic melanoma with a median overall survival of more than 2 years [79].

Anti-PD-1 or anti-PD-L1 therapy sensitized and prolonged the efficacy of anti-angiogenic therapy and improved anti-PD-L1 favoring vascular changes such as vascular normalization that facilitate enhanced cytotoxic T cell infiltration [80]. Moreover, CD4$^+$ T cell activation by immune-checkpoint blockade increased vessel normalization, as indicated by increased pericyte coverage, improved tumor vessel perfusion and reduced vascular permeability [81].

Concluding Remarks and Perspectives

Different mechanisms are involved in anti-angiogenic therapy resistance, in both pre-clinical and clinical setting (Table 4). The fact that tumors may grow without angiogenesis, through alternative mode of vasculature neo-formation, make them less likely to respond to anti-angiogenic drugs.

Alternative therapeutic strategies may be used to overcome resistance to anti-angiogenic therapy, including the association of multiple anti-angiogenic compounds or a combination of anti-angiogenic drugs with other treatment regimens. Effectiveness of the combination therapy should be monitored during disease progression with the aim of optimize the therapy and counteract the development of further resistance.

Table 4. Mechanisms are involved in anti-angiogenic therapy resistance.

Redundancy in growth factor signaling
Recruitment of bone marrow-derived cells
Stromal cells
Intussusceptive microvascular growth, vasculogenic mimicry, and vessel co-option
Redundancy in growth factor signaling
Increased invasiveness and metastasis
Endothelial heterogeneity

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