Cancer Cell
Perspective

Antiangiogenesis Strategies Revisited: From Starving Tumors to Alleviating Hypoxia

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Ten antiangiogenic drugs targeting VEGF or its receptors are approved for cancer treatment. However, these agents, intended to block tumors' blood supply, may cause hypoxia, which may fuel tumor progression and treatment resistance. Emerging clinical data suggest that patients whose tumor perfusion or oxygenation increases in response to these agents may actually survive longer. Hence, strategies aimed at alleviating tumor hypoxia while improving perfusion may enhance the outcome of radiotherapy, chemotherapy, and immunotherapy. Here I summarize lessons learned from preclinical and clinical studies over the past decade and propose strategies for improving antiangiogenic therapy outcomes for malignant and nonmalignant diseases.

Tumors acquire blood supply via multiple mechanisms: angiogenesis (sprouting new vessels from existing vessels), co-option, intussusception, vasculogenesis, vascular mimicry, and trans-differentiation of cancer cells into endothelial cells (Carmeliet and Jain, 2011). More than 40 molecules have been identified to play a critical role in blood vessel recruitment, but most studies to date have focused on vascular endothelial growth factor (VEGF) and its receptors. In fact, since 2004, ten drugs that target VEGF or its receptors have been approved for the treatment of various malignant diseases (Table 1), with many more in clinical trials. Unfortunately, these agents—used as monotherapy or in combination with chemotherapy—have only provided survival benefits on the order of weeks to months in some tumor types and have not been efficacious at all in others. Multiple mechanisms underlie these incremental benefits. In this Perspective, I will discuss these mechanisms and speculate on how we can better utilize current antiangiogenic (AA) agents and develop new ones to improve the benefit to patients with cancer or other diseases with abnormal vasculature. Instead of reviewing the entire literature, I will focus on the underlying principles, inspired by the works of many in this field, but rely heavily on insights gained from our own preclinical and clinical studies.

Solid Tumors Develop Resistance to Targeted Therapies, Including AA Therapies

Millions of advanced cancer patients worldwide have benefitted from molecularly targeted therapeutics, whether these agents target oncogenic pathways in cancer cells, angiogenic pathways in blood vessels, or both. However, some tumors are intrinsically resistant to these agents, whereas others develop resistance after an initial response, therefore limiting overall survival benefits to months (Table 1). An important feature that distinguishes AA drugs from other targeted therapies is that AA agents are typically given to unselected patients for the approved indications, whereas cancer cell-targeted therapeutics are given to only subsets of patients selected on the basis of biomarkers. Therefore, the informed selection of patients likely to benefit from AA drugs could significantly improve the benefits derived from these agents. For example, recent studies show that recurrent and newly diagnosed glioblastoma (GBM) patients whose tumor blood perfusion or oxygenation increases after the initiation of AA therapy survive 6–9 months longer than those whose tumor perfusion does not change or, instead, decreases (Batchelor et al., 2013; Emblem et al., 2013; Sorensen et al., 2012). These emerging data suggest that we should be able to improve overall survival with a more personalized use of existing AA agents and by developing novel hypoxia-alleviating agents.

Why Alleviating Hypoxia Is Critical for Improving Cancer Treatment

The imbalance between pro- and antiangiogenic signaling as well as physical compression leads to abnormal vessels and impaired blood perfusion in tumors (Jain 2005, 2013). The degree of blood flow impairment varies with tumor growth stage and location and can differ among tumor regions (Movie S1 available online, embedded in Figure 1) or between a primary tumor and its metastases. This progressively worsening heterogeneity in blood perfusion as tumors grow raises an interesting conundrum. If a tumor needs blood vessels to grow and to metastasize, how does it keep growing when growth impairs the very blood supply that brings the required nutrients and removes waste products? This apparent paradox can be understood by thinking about how a reduced blood supply can impart a survival advantage to these renegade cells by creating an abnormal microenvironment characterized by hypoxia and acidosis (Figure 1).

My hypothesis is that impaired blood supply and the resulting abnormal tumor microenvironment help cancer cells evade the immune system, increase their invasive and metastatic potential, and apply selective survival pressures to which cancer cell populations adapt (Figure 1). Under physiological conditions, immune cells constantly patrol tissues to identify and destroy pathogens, foreign antigens, and abnormal cells. However, a hypoxic and acidic microenvironment reprograms the resident macrophages (phagocytes)—whose job is to recognize, engulf and remove dying cells—into a protumorigenic and immunosuppressive phenotype (Casazza et al., 2014; Colegio et al., 2014; Finger and Giaccia, 2010; Hanahan and Coussens, 2012; Keith et al., 2012; Motz and Coukos, 2013; Noy and Pollard, 2014;

Table 1. Survival Benefits from Anti-VEGF/VEGFR Drugs

Drug	Approved Indication	Improvement in Response Rate (%)	Improvement in PFS (Months)	Improvement in OS (Months)	Reference
Bevacizumab	metastatic colorectal cancer (with chemotherapy)	10	4.4	4.7	Kindler et al., 2010
		0	1.4	1.4	Saltz et al., 2008
		14.1	2.6	2.1	Giantonio et al., 2007
	metastatic nonsquamous NSCLC (with chemotherapy)	20	1.7	2	Sandler et al., 2006
		16.2 and 13	0.4 and 0.6	NS	Reck et al., 2010
	metastatic breast cancer (with chemotherapy)[a]	15.7	5.9	NS	Miller et al., 2007
		9 and 18	0.8 and 1.9	NS	Miles et al., 2010
		11.8 and 13.4	1.2 and 2.9	NS	Robert et al., 2011
		9.9	2.1	NS	Brufsky et al., 2011
	metastatic renal cell carcinoma (RCC) (with IFN-α)	18	4.8	NS	Rini et al., 2010
		12.4	3.3	NS	Escudier et al., 2010
	advanced cervical cancer (with chemotherapy)	12	2.3	3.7	Tewari et al., 2014
Sunitinib	metastatic RCC	35	6	4.6	Motzer et al., 2007
	GIST	6.8	4.5	NS	Demetri et al., 2006
	PNET	9.3	4.8	?	Raymond et al., 2011
Sorafenib	metastatic RCC	8	2.7	NS	Escudier et al., 2007
	unresectable HCC	1	NS	2.8	Llovet et al., 2008
	unresectable HCC	2	1.4	2.3	Cheng et al., 2009
Pazopanib	metastatic RCC	27	5	NA	Sternberg et al., 2010
	advanced soft tissue sarcoma	6	3	NS	van der Graaf et al., 2012
Vandetanib	advanced medullary thyroid cancer	43	6.2	NA	Wells et al., 2012
Axitinib	advanced RCC	10	2	NA	Motzer et al., 2013
Regorafenib	chemorefractory metastatic colorectal cancer	0.6	0.2	1.4	Grothey et al., 2013
Aflibercept	chemorefractory metastatic colorectal cancer	8.7	2.2	1.4	Van Cutsem et al., 2012
Cabozantinib	advanced medullary thyroid cancer	25	7.2	NS	Elisei et al., 2013
Ramucirumab	metastatic gastric and GEJ cancers	0.8	0.8	1.4	Fuchs et al., 2014
	metastatic GEJ cancers (with chemotherapy)	12	1.5	2.3	Wilke et al., 2014
	metastatic NSCLC (with chemotherapy)	NA	NA	NA	M. Perol, 2014, AACR Annual Meeting Proceedings, abstract

NS, not significant; NA, not available.
[a]No longer approved in the United States.

Palazón et al., 2012; Semenza, 2014; Wilson and Hay, 2011). Hypoxia and acidosis can also attenuate the killing potential of immune effector cells within the tumor microenvironment. Specifically, growth factors and cytokines (e.g., transforming growth factor β [TGF-β] and VEGF) induced by hypoxia or acidosis suppress the activity of T lymphocytes and inhibit the ability of dendritic cells to process tumor antigens and present them to lymphocytes (Barsoum et al., 2014; Calcinotto et al., 2012; Gabrilovich et al., 2012; Palazón et al., 2012). In addition, hypoxia can directly upregulate, via HIF1α activation, the expression of the immune checkpoint protein PD-L1 by myeloid-derived suppressor cells, dendritic cells, and cancer cells to aid immune suppression and evasion (Noman et al., 2014).

In addition to protection from the immune system, hypoxia may select for more malignant cells because cells that respond to physiological cues normally undergo apoptosis under hypoxic conditions (Wilson and Hay, 2011). Hypoxia can increase the invasive potential of cancer cells by inducing the production of promigratory proteins (e.g., SDF1α and HGF) and proinvasive extracellular matrix molecules (Finger and Giaccia, 2010; Semenza, 2014). Hypoxia also provides a niche for so-called cancer stem cells and facilitates inflammation while also conferring resistance to radiation and many widely used therapeutic agents (Wilson and Hay, 2011). Collectively, these observations may explain why intratumoral hypoxia correlates with a poor prognosis in many human cancers (Wilson and Hay, 2011).

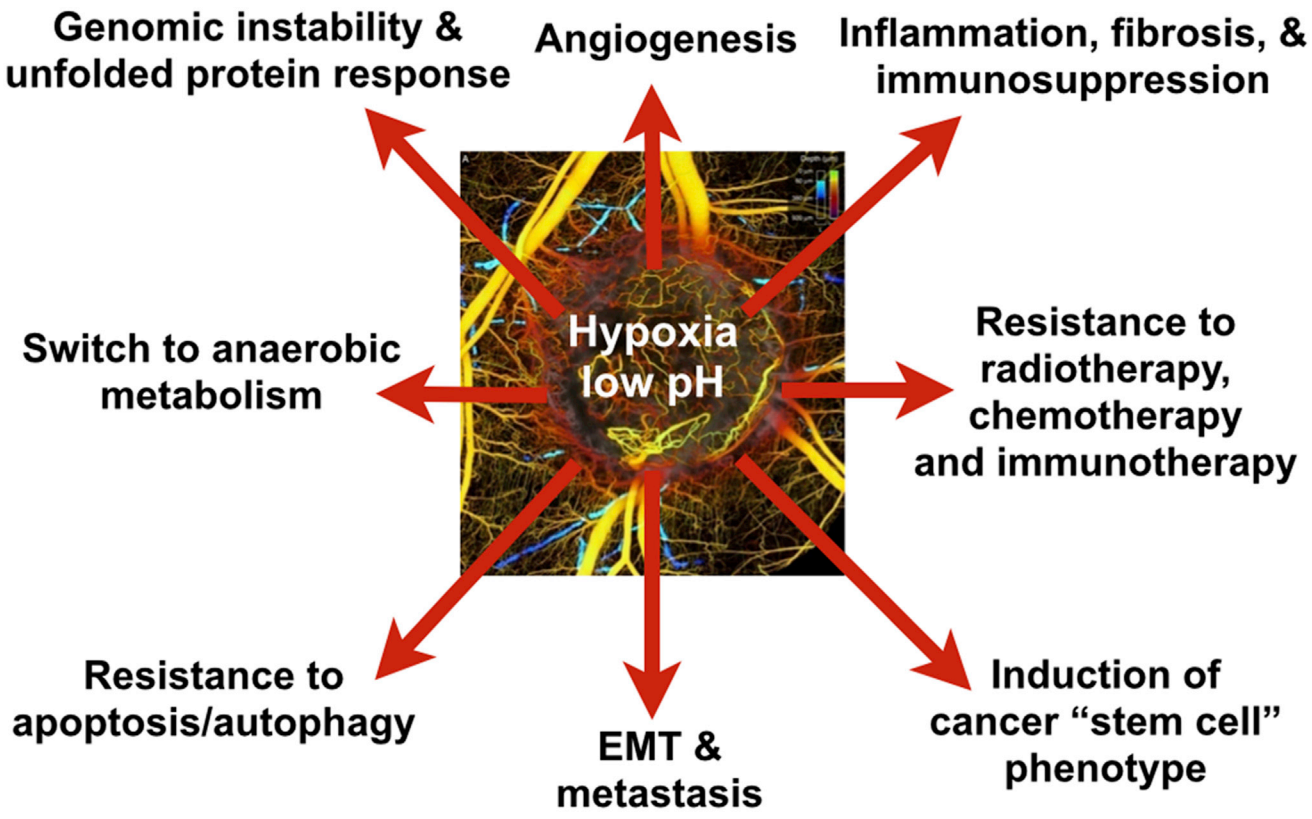

Figure 1. Hypoxia and Acidosis Resulting from Impaired Perfusion Fuel Tumor Progression and Treatment Resistance
Shown are adverse consequences of hypoxia and acidosis and some of the molecular players contributing to these: induction of the cancer "stem cell" phenotype (e.g., Akt/β-catenin and OCT4) (Lee and Simon, 2012); resistance to radiotherapy, chemotherapy, and immunotherapy (e.g., fewer oxygen radicals and cell cycle arrest) (Huang et al., 2013; Neri and Supuran, 2011; Wilson and Hay, 2011); tumor growth and genomic instability and expression of growth factors (e.g., IGF1 and TGF-α), oncogenes, and tumor suppressor genes (Bindra et al., 2007; Bristow and Hill, 2008; Wilson and Hay, 2011); EMT, invasion, and metastasis (e.g., CXCR4, Snail, Lox, and cMET) (Finger and Giaccia, 2010; Semenza, 2014); inflammation, immunosuppression, and fibrosis (e.g., IL-6, TGF-β, SDF1α, tumor-associated macrophage [TAM] polarization, Tregs, and myeloid-derived suppressor cells [MDSCs]) (Casazza et al., 2014; Chen et al., 2014; Colegio et al., 2014; Motz and Coukos, 2013; Palazón et al., 2012; Semenza, 2014); abnormal angiogenesis (e.g., HIFs/VEGF, and Ang2) (Carmeliet and Jain, 2011); resistance to apoptosis/autophagy (e.g., BNIP3) (Semenza, 2014); and switch to anaerobic metabolism (e.g., Glut1, LDHA, and PGK1) (Semenza, 2014; Vander Heiden et al., 2009). Many of these consequences are dependent on HIF1α, whereas others are not. Therefore, improving tumor perfusion may alleviate these adverse consequences. The inset shows heterogeneous perfusion in a tumor, leading to hypoxic and acidic regions (reproduced from Vakoc et al., 2009). See also Movie S1 of heterogeneous perfusion in real time.

Elevated interstitial fluid pressure (IFP) resulting from the compression of lymphatic vessels also fuels tumor progression and resistance to treatment but via distinct mechanisms (Jain, 2013). Tumor vessel leakiness worsens interstitial hypertension, causes edema, and leads to sluggish blood flow because of clogging of red blood cells concentrated by the leakage of plasma (Jain, 1988; Netti et al., 1996; Sevick and Jain, 1989). Tumor vessel leakiness and compression thereby collaborate in creating a vicious cycle responsible for both acute and chronic hypoxia as well as acidosis. Therefore, normalizing the tumor microenvironment by repairing the function of tumor vessels may be a promising strategy to slow tumor progression and enhance cancer treatment.

Antiangiogenic Agents Can Normalize the Tumor Vasculature and Alleviate Hypoxia

The role of blood vessels in tumor progression has been investigated for more than a century (for a review, see Carmeliet and Jain, 2000). However, the development of AA agents was catapulted by the groundbreaking hypothesis—put forward in 1971 by the late Dr. Judah Folkman—that starving tumors by blocking angiogenesis would slow tumor progression and improve patient survival (Folkman, 1971). The cloning of VEGF by Napoleone Ferrara and his team was a turning point for the field (Ferrara, 2002). Initially discovered as vascular permeability factor by Harold Dvorak and colleagues (Dvorak, 2002), VEGF turned out to be a key survival factor for endothelial cells. This discovery propelled the development of both small (e.g., tyrosine kinase inhibitors [TKIs] and peptides] and large molecular weight (e.g., antibodies and receptor bodies) inhibitors of VEGF or its downstream signaling (Ferrara, 2002). As anticipated, these inhibitors alone decreased both blood vessel density and growth of tumors.

In contrast to most preclinical studies, monotherapy with bevacizumab, an anti-VEGF monoclonal antibody, failed to show an overall survival benefit in patients (Jain, 2005). In multiple randomized phase III trials, bevacizumab conferred a survival benefit only when given in combination with chemotherapy (Table 1). These clinical findings seemed paradoxical. Why do drugs designed to destroy tumor blood vessels benefit patients

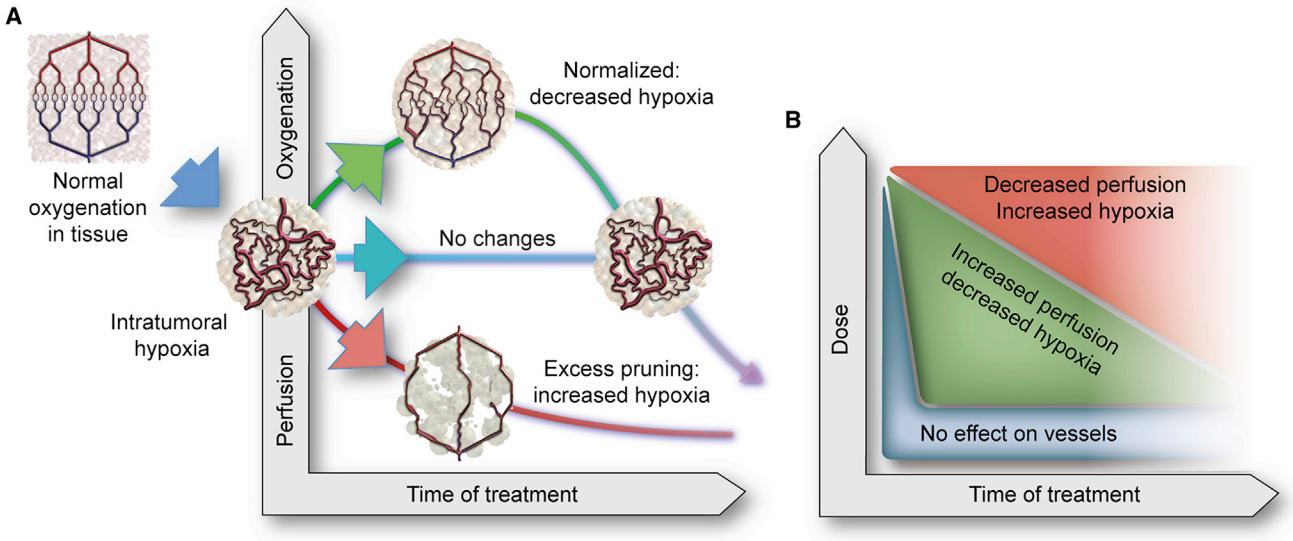

Figure 2. Effect of Vascular Normalization on Tumor Perfusion/Oxygenation

(A) In normal tissue, the blood vessels have normal structure and function because of balance of the signals downstream of the proangiogenic molecules (e.g., VEGF and Ang2) and antiangiogenic molecules (e.g., sVEGFR1, thrombospondins, and semaphorins). In contrast, tumor vessels are structurally and functionally abnormal because of an imbalance between pro- and antiangiogenic signals. This creates an abnormal microenvironment in tumors, characterized by hypoxia, acidosis, and elevated fluid pressure, that fuels tumor progression and treatment resistance via multiple mechanisms as shown in Figure 1. Inhibiting proangiogenic signaling or enhancing antiangiogenic signaling can prune some abnormal vessels and remodel the rest, resulting in a normalized vasculature. Depending upon the extent of normalization versus pruning, tumor perfusion/oxygenation may increase, remain unchanged, or decrease. Some tumors might be intrinsically resistant to a given AA agent, and others may switch to nonsprouting mechanisms of vessels recruitment (e.g., vessel co-option) that are refractory to the given AA agent and continue to make abnormal vessels again. Adapted and updated from Jain (2001) and Sorensen et al. (2012). See also Movie S2 of vessel co-option.

(B) The window of increased perfusion from vascular normalization depends on the dose and potency of antiangiogenic therapy. High doses may cause excessive pruning of tumor vessels, resulting in a shorter window, and may starve a tumor of oxygen and other nutrients. High doses may also increase toxicity, including some fatal. Adapted and updated from Jain (2013).

only by improving the efficacy of therapeutics that rely on those very blood vessels to reach their target?

I tried to resolve this paradox by proposing that judicious use of AA agents could transiently "normalize" the abnormal tumor vasculature, resulting in improved blood perfusion (Figure 2A). The latter would decrease hypoxia (known to confer resistance to radio-, chemo-, and immune therapies) and increase drug accessibility. Therefore, therapies given during the window of normalization might achieve greater efficacy (Jain, 2001). The normalized vessels would also resist shedding of cancer cells from the primary tumor, potentially decreasing metastases (Jain, 2005). This hypothesis, although controversial, offered a potential explanation for why bevacizumab can improve the outcome of chemotherapy and, more importantly, offered guidelines to improve such combination therapies (Jain et al., 2006). Other hypotheses, although not mutually exclusive, also offered potential reasons for combining AA agents with chemotherapies (Carmeliet and Jain, 2011). For example, some antiangiogenic agents may directly kill cancer cells and sensitize endothelial cells to cytotoxic drugs. Additionally, anticancer agents may also directly kill endothelial cells. Finally, killing of tumor and other stromal cells by cytotoxic and/or antiangiogenic agents may transiently decompress blood vessels, resulting in improved perfusion. Alternatively, decreased perfusion caused by pruning excess vessels could block the clearance of anticancer drugs accumulated in a tumor. This strategy would be especially effective for drugs that are more toxic under hypoxic and/or acidic conditions.

A variety of preclinical studies using direct and indirect AA agents supported the normalization hypothesis (Izumi et al., 2002; Jain et al., 1998; Tong et al., 2004; Winkler et al., 2004; Yuan et al., 1996). These studies also revealed that blockade of VEGF signaling or upregulation of thrombospondin transiently pruned the immature and leaky vessels of tumors in mice and actively remodeled the remaining vasculature so that it more closely resembled the normal vasculature. The morphological changes were accompanied by functional improvements: decreased IFP, decreased tumor hypoxia, and improved penetration of macromolecules from these vessels in these tumors. Radiation therapy had a better outcome when given during the normalization window compared with prior to or after the normalization window (Winkler et al., 2004). We also discovered that Tie-2 activation contributed to the increased pericyte coverage, and that an increase in matrix metalloproteinase (MMP) activity repaired the basement membrane (Winkler et al., 2004).

Although the focus of this Perspective is primarily on VEGF inhibition because of its clinical relevance, a number of other molecular targets, present in cancer and a variety of host cells, that facilitate or hinder vascular normalization have also been investigated (Goel et al., 2011) (Figure 3 and Table 2). Several agents that target these pathways are now in clinical trials (e.g., Ang-2/Tie-2 and FGFR) (Vasudev and Reynolds, 2014).

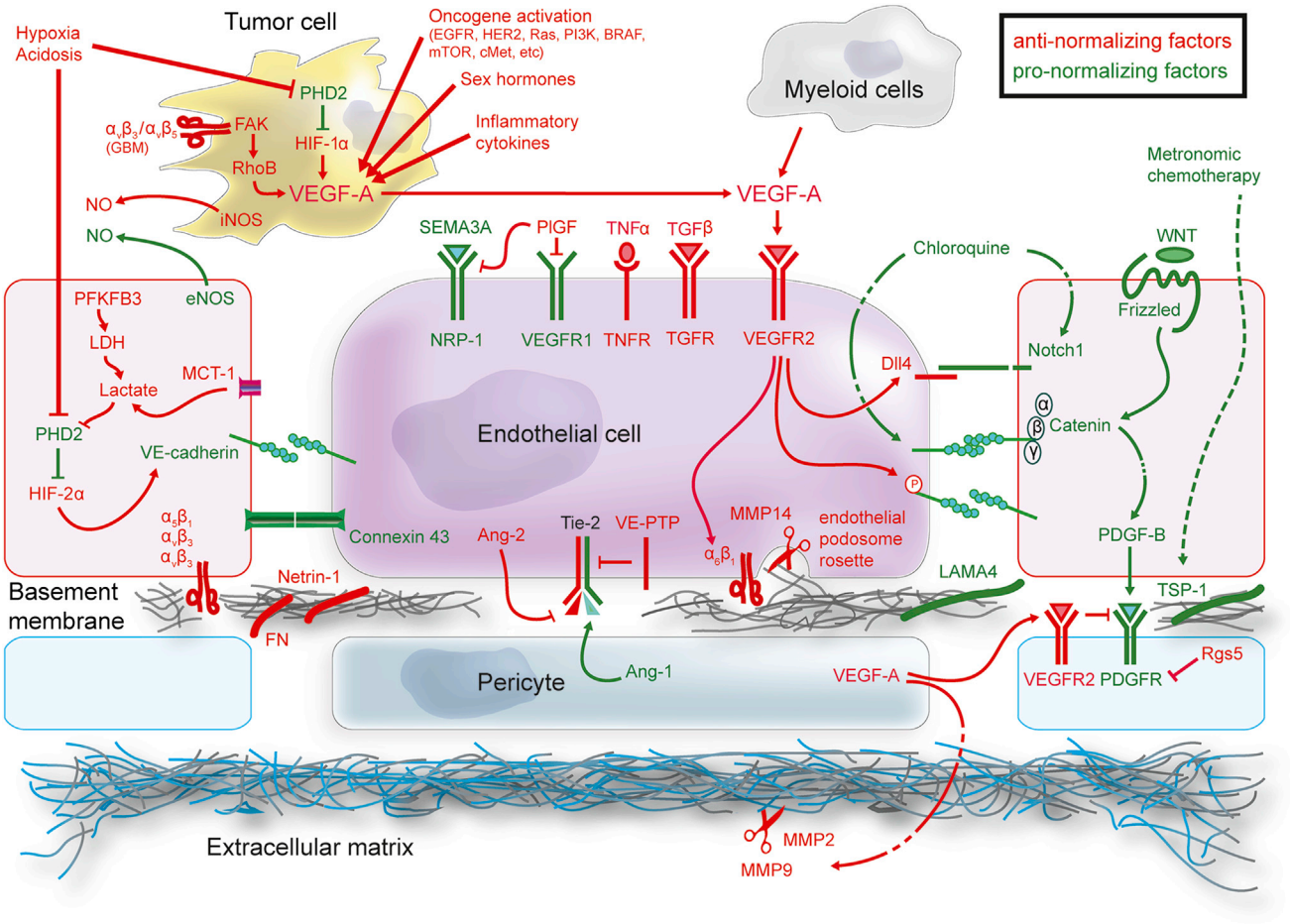

Figure 3. Pathways that Facilitate or Hinder Vascular Normalization
Although most studies on vascular normalization have focused on VEGF, a number of molecular players can facilitate (green) or hinder normalization (red). Note that the outcome may be dose- and context-dependent. Table 2 provides further details about each of these molecular players. The tumor cell is depicted near endothelial cells to save space. Adapted and updated from Goel et al. (2011).

Most recently, chloroquine, an antimalarial drug being tested in cancer patients, has been shown to induce vascular normalization by enhancing Notch signaling in endothelial cells, leading to improved blood perfusion in tumors and reduced metastasis (Figure 3) (Maes et al., 2014).

Increased Tumor Perfusion/Oxygenation Appears to Confer Survival Benefits

A phase I/II trial in rectal carcinoma patients revealed that a single dose of bevacizumab decreased vessel density, increased pericyte coverage, and lowered the interstitial fluid pressure in tumors (Willett et al., 2004). A subsequent trial in recurrent GBM patients demonstrated that cediranib, an oral pan-VEGFR TKI, normalized tumor vessels and alleviated edema (Batchelor et al., 2007). However, the improvement in progression-free survival (PFS) and overall survival (OS) correlated with the extent of normalization (Sorensen et al., 2009). Notably, cediranib transiently increased perfusion and oxygenation in a subset of recurrent and newly diagnosed GBM patients, and only these patients survived longer (Batchelor et al., 2013; Emblem et al., 2013; Sorensen et al., 2012). Similar findings on increased oxygenation and pathological response have been reported with bevacizumab in patients with locally advanced breast cancer (J. Garcia-Foncillas et al., 2012, J. Clin. Oncol., abstract).

These clinical trials suggest that, for a given dose/schedule of AA agents, some patients whose tumor perfusion/oxygenation increases benefit, whereas others do not. However, these findings need to be tested in prospective randomized trials. If validated, increased perfusion/oxygenation early in treatment could serve as a potential predictive biomarker for AA therapy. The next important challenge is to determine why, for the same dose of AA agents, tumor perfusion goes up only in some patients and not in others.

Benefits of Vascular Normalization Are AA Dose- and Drug Size-Dependent

As originally hypothesized, the extent of vascular normalization in a primary or metastatic lesion is likely dependent on the dose of anti-VEGF/R drugs relative to the level of VEGF in that lesion (Figure 2B). Very high doses of anti-VEGF/R agents could cause a rapid reduction in blood perfusion for a tumor with a certain level of VEGF by excessive vessel pruning and might not improve the outcome of concurrent therapies. High doses may increase hypoxia, resulting in increased metastasis and

Table 2. Molecules Linked with Tumor Angiogenesis and Vascular Normalization

Molecule	Complete Name	Cell Type	Localization	References
Ang1	angiopoietin 1	pericyte	soluble factor	Huang et al., 2010; Winkler et al., 2004
Ang2	angiopoietin 2	endothelial	soluble factor	Huang et al., 2010; Nasarre et al., 2009
BRAF	proto-oncogene B-Raf	tumor	intracellular	Bottos et al., 2012; Goel et al., 2012
c-Met	hepatocyte growth factor receptor (HGFR)	tumor	transmembrane	Goel et al., 2012; You et al., 2011
Connexin-43	connexin 43	endothelial	transmembrane	Zhang et al., 2014
Dll4	delta-like ligand 4	endothelial	transmembrane	Hellström et al., 2007
EGFR	epidermal growth factor receptor	tumor	transmembrane	Izumi et al., 2002
eNOS	endothelial NOS	endothelial	intracellular	Fukumura et al., 2006; Goel et al., 2013
FAK	focal adhesion kinase	tumor, endothelial	intracellular	Skuli et al., 2009
FN	fibronectin	extracellular matrix (ECM)	extracellular	Chiang et al., 2009
Frizzled	frizzled	endothelial	transmembrane	Elisei et al., 2013
HIF	hypoxia-inducible factor	tumor, endothelial	intracellular	Semenza, 2014
i/nNOS	inducible/neuronal nitric oxide synthases	tumor cells	intracellular	Fukumura et al., 2006; Kashiwagi et al., 2008
Lactate	lactate	endothelial	intracellular	Goveia et al., 2014; Végran et al., 2011
LAMA4	laminin α4	ECM	extracellular	Seano et al., 2014a; Zhou et al., 2004
LDH	lactate dehydrogenase	endothelial	intracellular	Goveia et al., 2014; van Beijnum et al., 2006
MCP-1	monocarboxylate transporter 1	endothelial	transmembrane	Goveia et al., 2014
MMP14	matrix metalloproteinase 14	tumor, endothelial	transmembrane	E.I. Ager, S.V. Kozin, N.D. Kirkpatrick, G. Seano, D.P. Kodack, V. Askoxylakis, Y. Huang, S. Goel, M. Snuderl, A. Mizikansky, D.M. Finkelstein, D.T. Dransfeld, L. Devy, Y. Boucher, D. Fukumura, and R.K.J., unpublished data; Seano et al., 2014b
MMP2	matrix metalloproteinase 2	tumor, endothelial	soluble factor	Fang et al., 2000; Winkler et al., 2004
MMP9	matrix metalloproteinase 9	tumor, endothelial	soluble factor	Jodele et al., 2005; Winkler et al., 2004
Netrin-1	netrin 1	ECM	extracellular	Castets and Mehlen, 2010
Notch1	notch homolog 1	endothelial	transmembrane	Hellström et al., 2007; Phng and Gerhardt, 2009
NRP-1	neuropilin 1	endothelial	transmembrane	Maes et al., 2014; Maione et al., 2009
PDGF-B,C	platelet-derived growth factor B, C	endothelial	soluble factor	Abramsson et al., 2003; di Tomaso et al., 2009
PDGFR	PDGF receptor	pericyte	transmembrane	Abramsson et al., 2003; di Tomaso et al., 2009
PFKFB3	6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3	endothelial	intracellular	De Bock et al., 2013; Goveia et al., 2014
PHD2	HIF-prolyl hydroxylase 2	tumor, endothelial	intracellular	Mazzone et al., 2009
PI3K	phosphatidylinositol bisphosphate 3-kinase	tumor	intracellular	Karar and Maity, 2011
PlGF	placental growth factor	endothelial	soluble factor	Fischer et al., 2008
Ras	Ras family	tumor	intracellular	Karar and Maity, 2011; Zhang et al., 2001
Rgs5	regulator of G-protein signaling 5	pericyte	intracellular	Hamzah et al., 2008
RhoB	ras homolog gene family, member B	tumor cells	intracellular	Skuli et al., 2009
SEMA3A	semaphorin 3A	endothelial	transmembrane	Maione et al., 2009
TGFR	TGF receptors	endothelial	transmembrane	Liu et al., 2012
TGFβ	transforming growth factor β	tumor	soluble factor	Liu et al., 2012
Tie2	TIE 2	endothelial	transmembrane	Huang et al., 2010; Winkler et al., 2004
TNFR	TNF receptors	endothelial	transmembrane	Johansson et al., 2012
TNFα	tumor necrosis factor α	tumor	soluble factor	Calcinotto et al., 2012; Johansson et al., 2012
TSP-1	thrombospondin 1	ECM	extracellular	Lawler and Lawler, 2012
VE-cadherin	vascular endothelial cadherin	endothelial	transmembrane	Maes et al., 2014; Orsenigo et al., 2012
VEGF-A	vascular endothelial growth factor A	tumor, endothelial	soluble factor	Batchelor et al., 2007; Ferrara, 2002; Winkler et al., 2004

(*Continued on next page*)

Table 2. *Continued*

Molecule	Complete Name	Cell Type	Localization	References
VEGFR2	VEGF receptor	endothelial	transmembrane	Ferrara, 2002; Sitohy et al., 2012; Winkler et al., 2004
VE-PTP	VE-protein tyrosine phosphatase	endothelial	transmembrane	Goel et al., 2013
WNT	WNT family	endothelial	soluble factor	Reis et al., 2012; Zhang et al., 2001
α5β1 integrin	α5β1 integrin	endothelial	transmembrane	Magnussen et al., 2005
α6β1 integrin	α6β1 integrin	endothelial	transmembrane	Seano et al., 2014a, 2014b
αvβ3 integrin	αvβ3 integrin	tumor, endothelial	transmembrane	Seano et al., 2014b; Skuli et al., 2009
αvβ5 integrin	αvβ5 integrin	tumor, endothelial	transmembrane	Skuli et al., 2009
β-catenin	β-catenin	endothelial	intracellular	Reis et al., 2012

For additional references, see Goel et al. (2011). The effects of many of these molecules are context- and dose-dependent.

enrichment of cancer stem cells (Chung et al., 2012). In contrast, lower doses might improve perfusion and outcome. Indeed, low doses of an anti-VEGFR2 antibody (10 or 20 mg/kg) increased perfusion compared with a high dose (40 mg/kg) or control immunoglobulin G (IgG) in a breast cancer model (Huang et al., 2012).

There are no clinical data directly comparing the dose effect of anti-VEGF agents on perfusion or oxygen levels. However, a study showed decreased perfusion and uptake of docetaxel in the non-small-cell lung cancer in patients after administration of 15 mg/kg bevacizumab (Van der Veldt et al., 2012). Unfortunately, unlike the GBM trials (Batchelor et al., 2013; Sorensen et al., 2012), this study did not look at the time course of perfusion or drug uptake, nor did it examine whether these two parameters correlated with the treatment outcome. So, although this clinical study does show a decrease in perfusion with a 15 mg/kg dose, it does not reveal whether this reduction in perfusion translated into a survival benefit for these patients. If starving tumors were the key mechanism of benefit from bevacizumab, as generally hypothesized, one would expect lower PFS and OS with a lower dose of bevacizumab, assuming the lower dose will not saturate the VEGF target. However, there was no statistically significant difference in PFS or OS in non-small-cell lung cancer patients treated with 15 or 7.5 mg/kg bevacizumab with chemotherapy (Reck et al., 2010). Whether lowering the dose below 7.5 mg would have increased PFS or OS in these patients is not known. These results collectively argue for tailoring the dose and schedule of anti-VEGF agents for individual patients using imaging or other biomarkers, including the levels of VEGF and its receptors in the primary tumor and metastatic lesions.

The size of concurrently administered drugs is also important because anti-VEGF therapy is likely to lower the size of pores in tumor vessels, therefore lowering the extravasation of drugs (Hobbs et al., 1998). For example, in a number of preclinical studies and a clinical study, bevacizumab has been shown to decrease the uptake of antibodies (e.g., trastuzumab and cetuximab) (S. Oosting et al., 2012, J. Clin. Oncol., abstract; Arjaans et al., 2013; Heskamp et al., 2013; Pastuskovas et al., 2012). In contrast, in a breast cancer model in mice, low-dose anti-VEGFR2 antibody (5 or 10 mg/kg compared with the standard dose of 40 mg/kg) improved the delivery of antibody-sized nanoparticles (~12 nm) but not larger nanoparticles (60 or 120 nm), whereas higher doses did not improve the delivery of either (Chauhan et al., 2012). Furthermore, combination therapy with a low dose of anti-VEGFR2 antibody (5 mg/kg) improved the efficacy of 10 nm nab-paclitaxel (Abraxane) but not 100 nm liposomal doxorubicin (Doxil) (Chauhan et al., 2012). Therefore, the dose of anti-VEGF agents may need to be tailored for the size of concurrently administered therapeutics and may compromise the delivery of therapeutics beyond a certain size for a given tumor. All this, of course, makes the optimal use of AA agents more complex.

Although a number of preclinical studies have shown increased perfusion and drug uptake in tumors with a variety of AA agents (Goel et al., 2011; Maes et al., 2014), there are no clinical studies to date that have measured both the time course of perfusion and drug uptake along with the survival benefit. The closest to drug uptake kinetics has been the measurement of oxygen supply, which, similar to perfusion kinetics, seems to support the benefit of vascular normalization (Emblem et al., 2013; Batchelor et al., 2013). It is possible that the benefit of vascular normalization may come primarily from improved tumor oxygenation resulting from improved perfusion, rather than improved drug uptake, for various reasons discussed earlier (Figure 1).

Overcoming Resistance to AA Therapy Using Biomarkers

Because tumors use multiple pathways for recruiting vessels, it is not surprising that blocking VEGF/R alone has inconsistent or incomplete effects on tumor vasculature. For example, non-sprouting mechanisms may be predominant in some treatment-naive tumors (e.g., vessel co-option in the metastatic legions in the lungs, liver, and lymph nodes) or may become operative as a means of escape from anti-VEGF therapy in other tumors (e.g., vessel co-option in GBM) (Movie S2). Some of these mechanisms may be triggered by molecules produced in response to increased hypoxia (e.g., HGF, SDF1α, and Ang2) resulting from excessive pruning by longer treatment duration and/or higher doses of anti-VEGF agents. Some treatment-naive tumors may have a majority of blood vessels invested in pericytes and, therefore, remain resistant to VEGF blockade (Sitohy et al., 2012). Other tumors may have excessive amounts of endogenous anti-VEGF molecules (e.g., sVEGFR1, NRP1, and thrombospondin) and may therefore not respond to exogenous VEGF blockade (Duda et al., 2010). Finally, cellular mechanisms involving various immune cell populations (Gr-1$^+$ myeloid cells

and T helper 17 cells) and fibroblasts have been implicated in resistance to AA therapy (Chung et al., 2013; Hanahan and Coussens, 2012; Noy and Pollard, 2014; Öhlund et al., 2014).

To gain insights into the molecular players involved in intrinsic and evasive resistance to anti-VEGF agents, we and other investigators have measured a panel of molecules in the tumor-tissue or circulation of patients undergoing AA therapies. Correlating the levels of these tissue/circulating biomarker candidates with treatment outcome has revealed candidate pathways potentially involved in treatment resistance to anti-VEGF therapies.

For example, elevated levels of sVEGFR1 prior to treatment were associated with a poor outcome from bevacizumab in rectal carcinoma, hepatocellular carcinoma (HCC), and metastatic colorectal carcinoma patients (Duda et al., 2010; Meyerhardt et al., 2012; Raut et al., 2012; Willett et al., 2009; Zhu et al., 2013). Additionally, high levels of sVEGFR1 were also associated with fewer side effects in rectal and liver cancer patients (Duda et al., 2010; Zhu et al., 2013). Finally, a retrospective analysis has shown that a genetic polymorphism in the *VEGFR1* gene correlates with increased VEGFR1 expression and a poor outcome of bevacizumab treatment in metastatic renal cell carcinoma and pancreatic ductal adenocarcinoma patients (Lambrechts et al., 2012). Similarly, elevated levels of NRP1 were associated with a poor outcome in some trials (Lambrechts et al., 2013). It is possible that VEGFR1 and NRP1 function as endogenous VEGF traps. Therefore, adding an external anti-VEGF agent may not have significant biologic effects in patients with high sVEGFR1/NRP1 levels (Jain, 2013). Additionally, increased VEGFR1 levels may induce increased proangiogenic signaling by PlGF when VEGF is blocked (Lambrechts et al., 2012). These hypothesis-generating findings need to be tested prospectively, and, if validated, alternate pathways need to be targeted in patients with elevated sVEGFR1/NRP1 levels. Along these lines, the baseline level of the short form of VEGF (VEGF-A121) is a predictive biomarker in some studies but not in others (Lambrechts et al., 2013) and is being prospectively examined in a breast cancer trial (ClinicalTrials.gov identifier NCT01663727).

As an example of evasive resistance, circulating levels of the chemokine SDF1α rise in patients who evade various anti-VEGF therapies, including rectal carcinoma with bevacizumab, GBM with cediranib, HCC with sunitinib, and soft tissue sarcoma with sorafenib (Duda et al., 2011). The SDF1α/CXCR4 pathway is involved in vessel co-option, vasculogenesis, fibrosis, lymphocyte trafficking, and cancer cell invasion, depending on the tumor and treatment. For example, in HCC, this pathway appears to increase fibrosis, whereas, in GBM, it appears to facilitate the invasion of cancer cells and co-option of host vessels by invading cancer cells (Chen et al., 2014; N.D. Kirkpatrick and R.K. Jain, 2010, American Association for Cancer Research Proceedings, abstract; Movie S2). The latter finding has led to a clinical trial with AMD3100 (an anti-CXCR4 drug) plus bevacizumab in recurrent GBM patients (ClinicalTrials.gov identifier NCT01339039).

Other evasive pathways include Ang2/Tie2/VE-PTP and HGF/cMET, which play important roles in vascular structure and function (e.g., pericyte coverage and permeability) and cell invasion (Figure 3) (Sennino et al., 2012; Vasudev and Reynolds, 2014; M. Hidalgo et al., 2014, ASCO Annual Meeting Proceedings, abstract). In addition, cellular mechanisms of resistance involve the participation of local or bone marrow-derived populations of immune cells (e.g., Gr-1$^+$ myeloid cells and Th17 cells) or pericyte coverage, which promote resistance through direct support of paracrine interactions with the endothelial cells (Carmeliet and Jain, 2011; Chung et al., 2013). A number of agents that target these evasive pathways are now in clinical trials (Vasudev and Reynolds, 2014).

Targeting VEGF versus VEGFR2 May Have a Different Outcome

Because VEGFR2 is thought to be the main receptor conveying the proangiogenic signals downstream of VEGF, it is generally assumed that targeting VEGFR2 would have similar biological effects as targeting the ligand. However, this is not the case in some malignancies. For example, although bevacizumab monotherapy has not improved overall survival in any phase III trial, the anti-VEGFR2 antibody ramucirumab led to an OS advantage of 1.4 months in advanced gastric or gastresophageal junction (GEJ) adenocarcinomas (Table 1). Interestingly, when added to paclitaxel, ramucirumab also increased OS by 2.3 months in patients with GEJ tumors (Table 1). When combined with chemotherapy, both bevacizumab and ramucirumab failed to improve OS in metastatic breast cancer, but both improved survival in non-small-cell lung cancer (NSCLC) (Table 1). It is tempting to assume that blood vessels of GEJ tumors are highly or even exclusively dependent on VEGFR2 signaling for their survival, and, hence, ramucirumab's benefits result from starving these tumors, which is in support of the original antiangiogenesis hypothesis. However, the starvation hypothesis does not explain the failure of bevacizumab in the same tumor type.

Blood Vessels Are Not the Only Target of Antiangiogenic Agents

As pointed out above, the targets of AA agents include not only blood vessels but also subsets of cancer and stromal cells. For example, VEGF can serve as a survival factor, promote epithelial-mesenchymal transition (EMT), and support the stem cell phenotype in cancer cells. VEGF can also block the maturation of dendritic cells (Goel and Mercurio, 2013). Similarly, PlGF—a member of the VEGF family—functions as a survival factor for medulloblastoma cells and facilitates their spread through the cerebrospinal fluid via neuropilin 1 (NRP1) signaling (Snuderl et al., 2013). Other angiogenic molecules, including angiocrines such as platelet-derived growth factor (PDGF), angiopoietins, SDF1α, and TGF-β, also support the survival, proliferation, and migration of various types of cancer and stromal cells (Butler et al., 2010). Similarly, sunitinib targets both VEGFR and c-KIT, which is commonly mutated in gastrointestinal stromal tumor (GIST) cells. Therefore, AA agents, and especially multireceptor TKIs, may affect tumor growth and metastasis by multiple mechanisms, making the task of deciphering their primary mechanism of action or identifying predictive biomarkers more complex. Future studies that incorporate tissue, circulating, and imaging biomarkers are needed to resolve these outstanding issues.

Agents Targeting Oncogenic Pathways Can Also Normalize Tumor Vessels

Although agents targeting endothelial or perivascular cells can directly induce vascular normalization, inhibition of oncogenic signaling can have the same effect indirectly. In 1998, we

showed that the initial effects of castration upon androgen-dependent carcinoma are primarily vascular (preceding tumor cell death) because of an indirect mechanism of hormone depletion that suppresses tumor cell production of angiogenic factors (Jain et al., 1998). We subsequently showed that inhibition of human epidermal growth factor receptor 2 (HER2) signaling in breast cancer cells using trastuzumab normalizes breast tumor vessels by modulating the expression of at least four pro- and antiangiogenic molecules (Izumi et al., 2002). Moreover, several other reports describe similar effects from inhibiting oncogenic pathways (e.g., Ras, phosphatidylinositol bisphosphate 3-kinase [PI3K], AKT, epidermal growth factor receptor [EGFR], and BRAF), which can lower the expression of VEGF and other proangiogenic molecules (Goel et al., 2012). Hence, such agents have the potential to improve tumor oxygenation via dual mechanisms: improved perfusion through normalization of vessels and reduced oxygen consumption by dying cancer cells.

Combining Antiangiogenic Agents with Drugs that Target Oncogenic Pathways

Combining AA agents with agents targeting oncogenic pathways, similar to chemotherapeutic agents, has led to some unexpected results. For example, despite promising preclinical results from combining VEGF- and EGFR-targeted agents in colorectal and NSCLC models, all phase III trials combining these targeted agents failed (Tol et al., 2009). Similarly, phase III trials combining VEGF and HER2-targted therapies in HER2+ breast cancer patients also failed (Gianni et al., 2013). A potential mechanism for these failures, as suggested above, is that the dose of bevacizumab used may have decreased the size of pores in the tumor vessel walls and compromised the delivery of antibodies (Chauhan et al., 2012). This hypothesis is consistent with elevated baseline plasma VEGF concentrations being associated with a greater bevacizumab benefit. It is also consistent with the recent randomized phase II trial showing the benefit of combining bevacizumab with a smaller drug, erlotinib, in EGFR-mutant NSCLC patients (Seto et al., 2014).

Unfortunately, in all of these trials, patients with CNS metastases were excluded. We discovered that treatment of HER2+ breast tumors in the mouse brain with trastuzumab leads to increased VEGF production by host cells in the brain (Izumi et al., 2002). To this end, we combined HER2-targeted drugs (trastuzumab and lapatinib) with an anti-VEGFR2 antibody and demonstrated a significant improvement in survival of mice bearing HER2+ tumors in the brain (Kodack et al., 2012). Moreover, a phase II clinical trial with dual HER2 blockade and bevacizumab showed encouraging results in heavily pretreated HER2+ breast cancer patients with brain metastases (Falchook et al., 2013). Whether this will translate into increased OS in brain metastasis patients in a phase III trial remains to be seen.

Combining Antiangiogenic Agents with Vessel-Decompressing Agents

Diminished blood perfusion and hypoxia in tumors results not only from the abnormal structure and leakiness of tumor vessels, but also from the compression of vessels by extravascular components in tumors (Chauhan et al., 2013, 2014; Jain, 1988, 2014; Padera et al., 2004; Stylianopoulos et al., 2012). This is evident in highly desmoplastic tumors where a large fraction of vessels is compressed and may contribute to the failure of AA therapies in these patients (e.g., pancreatic ductal adenocarcinomas, a subset of breast cancers) (Kindler et al., 2010). Moreover, some tumors begin to produce more extracellular matrix in response to VEGF blockade, partly from increased hypoxia, and become treatment-resistant (Aguilera et al., 2014; Chen et al., 2014). Therefore, strategies that can alleviate compressive forces exerted by stromal cells and/or the extracellular matrix in desmoplastic tumors should decompress tumor vessels and sensitize these tumors to AA agents. In fact, when Shh blockade improves perfusion in desmoplastic pancreatic ductal adenocarcinomas in mice, these tumors become responsive to an anti-VEGFR2 antibody (Rhim et al., 2014). Additionally, agents that can normalize both desmoplastic stroma and abnormal blood vessels may be effective in these treatment-resistant tumors (Stylianopoulos and Jain, 2013).

Our laboratory has recently discovered that widely prescribed antihypertensive drugs—angiotensin receptor blockers and ACE inhibitors, collectively known as renin-angiotensin system (RAS) inhibitors—can inhibit cancer-associated fibroblasts' activity to decrease the production of collagen I and hyaluronan, reduce compressive forces in tumors, open up blood vessels in desmoplastic breast and pancreatic ductal adenocarcinomas in mice, and improve the delivery and efficacy of chemotherapeutics (Chauhan et al., 2013). We are currently developing agents that can realize this goal without significantly lowering blood pressure. Other antifibrotic agents (e.g., pirfenidone, PEGPH20) may also benefit the treatment of desmoplastic tumors (Chauhan et al., 2014; Kozono et al., 2013). However, given the heterogeneity of stromal cells and the pro- and antitumor signals they impart, the choice of molecular target for depleting stroma is critical. For example, a recent study utilized the genetic ablation of SMA+ cells to deplete stroma in desmoplastic pancreatic tumors (Özdemir et al., 2014). Because pericytes, required to maintain vessel integrity, are also SMA+, this strategy destroyed many blood vessels and increased hypoxia. As expected (Figure 1), this genetic SMA+ cell depletion approach induced EMT, stem cell phenotype, invasion, metastasis, inflammation, and immunosuppression by increasing hypoxia in these tumors.

Although there are no prospective clinical data on the combination of RAS inhibitors with standard therapies, retrospective studies show that metastatic renal cell carcinoma patients survive 7 months longer when they receive RAS inhibitors in combination with sunitinib compared with sunitinib alone (30 versus 23 months) (Keizman et al., 2011). Similarly, another retrospective study of 2,277 advanced lung cancer patients showed better overall survival when RAS inhibitors were given concurrently with chemotherapy alone or chemotherapy and bevacizumab (A.R. Menter et al., 2014, ASCO Annual Meeting Proceedings, abstract). Because a significant fraction of cancer patients develop hypertension during the course of AA therapy, and because RAS inhibitors are fairly safe and relatively inexpensive, it would be worthwhile to test this hypothesis prospectively. Besides RAS inhibitors, it would also be of interest to test whether alleviation of desmoplasia by nintedanib, an AA agent that targets VEGFR, FGFR, and PDGF receptor (PDGFR) and proven to be effective in idiopathic pulmonary fibrosis, contributed to the survival benefit in combination with docetaxel in lung adenocarcinoma patients (Reck et al., 2014; Richeldi et al., 2014).

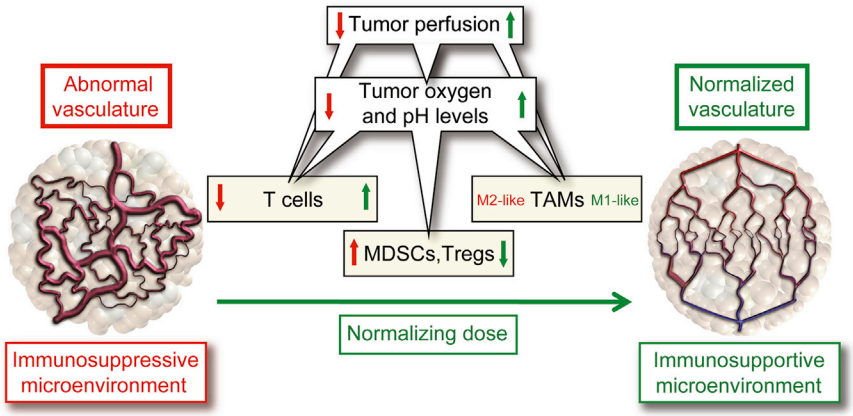

Figure 4. Vascular Normalization Can Reprogram the Tumor Microenvironment from Immunosuppressive to Immunosupportive

The abnormal tumor vasculature can impede T effector cell infiltration into tumors and create a hypoxic and acidic tumor microenvironment that upregulates PD-L1 on MDSCs, dendritic cells, and cancer cells; increases the accumulation of regulatory T cells (Tregs); impairs T effector cells; and polarizes TAMs to the immune inhibitory M2-like phenotype to suppress T effector cell function. Hypoxia can also upregulate multiple immune-suppressive growth factors and cytokines (e.g., VEGF and TGF-β). Vascular normalization with an appropriate dose and schedule of antiangiogenic treatment can normalize the tumor vasculature and generate a more homogeneous distribution of perfused tumor vessels, facilitating the infiltration of T effector cells while reducing MDSC and Treg accumulation. In addition, alleviation of hypoxia and acidity by improved vascular perfusion polarizes TAMs to an immunostimulatory M1-like phenotype. Adapted and updated from Huang et al. (2013).

Vascular Normalization Can Improve Immunotherapy

Recently approved immune checkpoint inhibitors have led to unprecedented improvements in overall survival in melanoma patients (Wolchok et al., 2013). However, a subset of patients even in this highly responsive disease does not benefit. Additionally, the first Food and Drug Administration (FDA)-approved therapeutic vaccine, sipuleucel-T, where autologous dendritic cells are exposed to a fusion protein consisting of GM-CSF and prostatic acidic phosphatase and then infused back into the body, demonstrated a modest survival benefit of a few months. Finally, various vaccine and adoptive T cell therapies, including with chimeric antibody receptors (CAR), have shown promise in various malignancies (Ogino et al., 2011). We hypothesize that normalizing the tumor microenvironment will improve the outcome of all of these different immunotherapies and potentially allow lowering the dose of immunotherapeutic agents, which, in turn, may decrease their toxicity (Huang et al., 2013).

As stated earlier, the abnormal microenvironment of tumors helps tumors evade the immune response through multiple mechanisms, including impairment of lymphocyte infiltration, upregulation of immune checkpoint protein expression via hypoxia, recruitment of Tregs, and establishment of an immunosuppressive tumor microenvironment that impairs the function of the resident and transiting immune effector cells (Figure 4) (Huang et al., 2013; Motz et al., 2014). Our laboratory has demonstrated that normalizing doses of anti-VEGFR2 antibody (DC101) can alleviate hypoxia, improve the delivery of immune effector cells into the tumor, convert the immunosuppressive microenvironment of tumors into an immunostimulatory one, and improve survival from a vaccine therapy (Huang et al., 2012). There are also a number of preclinical studies that show the benefit of combining AA agents with various immunotherapies (Table 3). Whether vascular normalization played any role in these results remains unknown. Regardless, clinical trials combining immune checkpoint blockers and other immunotherapies with AA agents have begun (Table 3).

Targeting Endothelial Cell Metabolism Can Inhibit Tumor Angiogenesis

Until recently, only molecular signals such as VEGF and others have been demonstrated to regulate angiogenesis. However, endothelial cells require energy and new biomass to proliferate and migrate during new vessel formation. Carmeliet and colleagues have recently discovered increased glycolysis via the glycolytic activator PFKFB3 in leading endothelial cells, known as the tip cells, during sprouting angiogenesis (De Bock et al., 2013; Schoors et al., 2014). PFKFB3-driven glycolysis is also required for proliferating stalk endothelial cells that elongate the vascular sprout. These studies show that endothelial cell metabolism plays a pivotal role in vessel sprouting. Furthermore, pharmacological blockade of PFKFB3 inhibited pathological angiogenesis with modest side effects. These studies provide a paradigm shift in previous antiglycolytic strategies that aimed to block glycolysis maximally and permanently but at the cost of causing severe toxicity. Therefore, targeting tumor endothelial cell metabolism opens up new possibilities for AA therapy. Whether targeting PFKFB3 also blocks nonsprouting modes of vessel recruitment is not known.

Antiangiogenic Agents Cause Toxicities

Similar to most cancer therapeutics, AA agents may lead to cardiovascular and noncardiovascular adverse effects, some of which may be fatal in ~1.5%–2.5% of patients. These toxicities are dependent not only on the class of AA agents (targeting VEGF versus VEGFR versus VEGFR-TKI), but also on the specific AA agent. Cardiovascular toxicities include hypertension, thromboembolic disease, left ventricular dysfunction, myocardial ischemia, and prolongation of the QTc interval. Noncardiovascular toxicities include proteinuria, bleeding, delayed wound healing, gastrointestinal perforation, fatigue, thyroid dysfunction, stomatitis, myelosuppression, cutaneous effects (including hand-foot syndrome), and dysphonia. Other rare and AA agent-specific adverse effects include reversible posterior leukoencephalopathy, osteonecrosis of the jaw, microangiopathic hemolysis, pancreatic enzyme elevations, and hypoglycemia. Some of these adverse effects are dose-dependent and are even reversible, whereas others are not. Retrospective studies have shown an association between some of these (e.g., hypertension) and the survival benefit, but none have been proven prospectively. Some of the adverse effects appear contradictory, such as hemorrhage and thrombosis, which makes their management challenging

Table 3. Combination of Immunotherapies with Antiangiogenic Agents

Antiangiogenic	Immunotherapy	Tumor Models	Results
Preclinical Studies			
Anti-VEGFR2 monoclonal antibody (mAb)	whole tumor cell vaccine (secreting GM-CSF)	breast carcinoma (Neu-expressing)	↑ trafficking of $CD8^+$ T cells
			↑ regression of tumor in FVB mice (Manning et al., 2007)
Anti-VEGFR2 mAb	whole tumor cell vaccine (mitomycin-treated)	breast carcinoma	↑ recruitment of $CD4^+$ and $CD8^+$ T cells
			↓ MDSCs and Tregs
			↑ survival (Huang et al., 2012)
Adenoviral delivery of sVEGFR1/R2	whole tumor cell vaccine (secreting GM-CSF)	colon carcinoma, melanoma	↑ infiltration of $CD4^+$ and $CD8^+$ T cells
			↓ MDSCs and Tregs
			↑ survival (Li et al., 2006)
VEGF peptide mimic	HER-2 B cell epitope vaccine	breast carcinoma	↑ High affinity HER-2 native antibodies
			↑ antitumor and antiangiogenic effects
			↓ tumor growth (Foy et al., 2012)
SU 6668	whole tumor cell vaccine (irradiated) and recombinant B7.2-IgG fusion protein	breast carcinoma	↑ recruitment of $CD8^+$ T cells, tumor growth delay (Huang et al., 2002)
Sunitinib	Pox virus-based vaccine expressing carcinoembryonic antigen and costimulatory molecules	colon carcinoma	↑ intratumoral T cells
			↓ MDSCs and Tregs
			↓ tumor volume and ↑ survival (Farsaci et al., 2012)
Sorafenib	anti-PD-1 antibody with a CXCR4 inhibitor (AMD3100)	hepatocellular carcinoma	↑ intratumoral T cells
			↓ MDSCs and Tregs
			↓ primary and metastatic tumor volume and ↑ apoptosis (T. Reiberger et al, 2014, Hepatology, abstract)
Anti-mouse VEGF mAb	peptide-pulsed dendritic cells (DCs)	sarcoma	↑ DC number and function.
			↑ tumor growth delay (Gabrilovich et al., 1999)
Anti-mouse VEGF mAb-	anti-gp100 pmel-1 T cells, gp100 vaccine, IL-2 after lymphodepletion	melanoma	↑ immune cell infiltration
			↑ tumor growth delay
			↑ survival (Shrimali et al., 2010)
	VEGFR-1 CAR-modified T cells	lung carcinoma	↓ endothelial tube formation in vitro
			↑ tumor growth delay and ↓ metastasis (Wang et al., 2013)
Anti-VEGFR2	anti-PD-1 antibody	colon carcinoma	↑ inhibition of tumor neovascularization
			↑ T cell infiltration
			↑ expression of cytokines (Yasuda et al., 2013)
Clinical Studies			
NA	peptide vaccine (VEGFR1, VEGFR2, URLC10, TTK, or CDCA1)	NSCLC	↑ T cell response
			↑ Stable disease for 2 months (Suzuki et al., 2013)
NA	antiangiogenic peptide vaccine	different solid tumors	↑ activation of T cells
			Antitumor activity being evaluated (Hayashi et al., 2013)
Sunitinib		RCC	↓ number and function of MDSCs and Tregs (Ko et al., 2009)
Bevacizumab	IFN-α2A	metastatic RCC	↑ progression-free survival (Escudier et al., 2010)
Bevacizumab	ipilimumab	advanced melanoma	↑ T cell infiltration (Hodi et al., 2014)
Bevacizumab	nivolumab	NSCLC	ClinicalTrials.gov identifier NCT01454102

and deciphering the underlying mechanisms complex. (For a comprehensive recent review on these adverse effects and their management, please see http://www.uptodate.com/contents/toxicity-of-molecularly-targeted-antiangiogenic-agents-cardiovascular-effects and http://www.uptodate.com/contents/toxicity-of-molecularly-targeted-antiangiogenic-agents-non-cardiovascular-effects.) It is tempting to postulate that reducing the dose of AA agents would not only reduce toxicity but may also increase the extent of normalization and delay the onset of hypoxia with all its negative consequences. However, there are no phase III randomized trials to date that compare the effect of a high versus a low dose of the same AA agent on efficacy or toxicity.

Animal Models of Cancer and Experimental Design Need to Be Improved

A major challenge in the AA therapy of cancer has been the discordance between the preclinical and clinical results. There are many potential reasons for this, including limitations of available animal models as well as the experimental design. First, the preclinical tumor models used generally grow rapidly and are more sensitive to anti-VEGF agents than their human counterparts (with the notable exception of renal cell carcinomas). Genetically engineered mouse models (GEMMs) are less genetically complex than human tumors and rarely metastasize similarly to the human disease. Patient-derived xenograft models are improving with the development of mice with a more humanized immune system. Second, almost all AA agents have been approved for metastatic disease, whereas most preclinical studies examine the effect on primary tumors (corresponding to the neoadjuvant setting in the clinic). Although better murine models of advanced disease are now being developed, preclinical studies are rarely carried out in the adjuvant or metastatic settings (Francia et al., 2011). It is worth noting that the FDA will provide accelerated approval upon demonstration of a substantial increase in the pathological complete response rate for patients with aggressive breast cancers (e.g., triple-negative), with full approval conditional on the eventual demonstration of improvements in disease-free and OS rates as well as acceptable toxicity (Prowell and Pazdur, 2012). Third, although both bevacizumab and aflibercept have shown improved OS only when combined with chemo- or immune therapies, most preclinical studies tested these agents as monotherapies. Fourth, although bevacizumab does not recognize mouse VEGF, many investigators use bevacizumab in their murine studies, therefore not addressing the contribution of host VEGF in the outcome. Fifth, in many preclinical studies, the dose of AA agents has been unusually high, potentially leading to misleading results (Chung et al., 2012). Sixth, many studies using GEMMs in which the relevant gene is knocked out in the embryo represent prevention rather than intervention studies. Hence, the findings may not translate to the treatment setting and can even derail treatment strategies. Seventh, murine models tend to underestimate toxicity. We need to take these limitations into account in both experimental design and data interpretation.

The Normalization Strategy Can Benefit Patients with Nonmalignant Diseases

Abnormal vessels are a hallmark of not only cancer but also of a number of nonmalignant diseases that afflict more than half a billion people worldwide. These include wet age-related macular degeneration (AMD) and diabetic macular edema. Vascular normalization seems to be a major mechanism of benefit from the approved anti-VEGF agents (Jain, 2005). Neurofibromatosis 2 (NF2)-associated schwannomas also harbor abnormal vessels causing edema, which may contribute to hearing loss by disrupting auditory nerve function. Additionally, inflammatory molecules produced as a result of hypoxia may also trigger hearing loss (Roosli et al., 2012). Indeed, low-dose bevacizumab improved hearing in 60% of NF2 patients treated on a compassionate-use basis (Plotkin et al., 2009). A follow-up phase II study showed a durable hearing benefit in approximately 36% of patients (S. Plotkin, personal communication). Bevacizumab is now approved for NF2-related schwannomas in the United Kingdom. Other potential applications of vascular normalization include controlling plaque rupture, neurovascular complications stemming from radiation therapy, and tuberculosis (Jain et al., 2007; Solano et al., 2007). Unfortunately, similar to cancer, the nonmalignant diseases also become resistant to anti-VEGF therapies. Fortunately, the benefit may last years in the latter compared with only a couple of months in the former (Table 1). One cause of failure may be fibrosis, presumably instigated by hypoxia resulting from prolonged VEGF blockade. Additionally, some vessels may be refractory to VEGF blockade because of pericyte coverage. Phase II data suggest that prevention of fibrosis and pruning of resistant vessels with a PDGF inhibitor may prolong the benefit of ranibizumab in patients with wet AMD and has led to a phase III trial (ClinicalTrials.gov identifier NCT01940900). A number of trials that target PDGF or Ang2 along with VEGF to prolong the benefit to patients are planned or ongoing (Ratner, 2014).

Summary and Perspective

In conclusion, AA therapy, despite being given to unselected patients, has benefitted numerous patients worldwide who had no other alternative treatment options. Similar to various therapeutic approaches that looked straightforward in the beginning, antiangiogenesis has turned out to be more complex and nuanced than originally envisaged for multiple reasons.

First, a major part of the complexity in AA therapy stems from multiple mechanisms employed by tumors to recruit blood vessels. These mechanisms seem to vary not only spatially and temporally within a tumor but also between a primary tumor and its metastases and among tumor types. Moreover, tumors may switch from one mechanism to another during growth and in response to treatment. Although VEGF seems to be a central player in sprouting angiogenesis, our knowledge of the molecular players in other mechanisms is still in its infancy. Understanding these mechanisms in more detail will allow the development of novel agents to target all types of tumor vessels and enhance the treatment outcomes from these agents via vascular normalization and/or starvation.

Second, the initial focus of antiangiogenic therapy was to target endothelial cells, pericytes, and/or the basement membrane in which they are invested. Now we know that these cells interact not only with each other and cancer cells but also with the extracellular matrix and other stromal cells in the tumor microenvironment, including resident and transiting immune cells, cancer stem cells, as well as fibroblasts/myofibroblasts.

This interaction is not only chemical in nature but also physical. Although our understanding of the biochemical crosstalk between the stroma and cancer cells has grown exponentially, our understanding of the physical microenvironment is in its early stages (Jain et al., 2014). The physical forces exerted by the tumor stroma can directly induce cancer cell invasion (Tse et al., 2012) and compress blood and lymphatic vessels. As discussed earlier, the resulting hypoxia, acidosis, and interstitial hypertension can fuel tumor heterogeneity, progression, and treatment resistance. Furthermore, forces exerted by plasma and interstitial fluid can also affect vessel formation and function (Song and Munn, 2011). Therefore, strategies to control these forces are likely to yield new ways of alleviating hypoxia, slowing tumor progression and reducing treatment resistance.

Third, not only blood vessels but also other components of the tumor microenvironment are abnormal, and all of these abnormalities in concert seem to fuel tumor progression and treatment resistance. Therefore, we need to develop therapeutic agents that normalize the entire tumor microenvironment, including immune and other stromal cells, and not just the tumor blood vessels. Limited preclinical as well as retrospective clinical studies suggest targeting the rennin-angiotensin system as a promising approach for normalizing CAFs in desmoplastic tumors, which account for about 25% of human tumors. Similarly, a number of agents that aim to normalize the immune microenvironment have been approved, and others are being tested. In the long run, a judicious combination of these agents is likely to yield a significant benefit to patients while reducing their toxicity.

Finally, unlike cancer cell-targeted therapies, many AA agents are not directly cytotoxic but directly affect vascular permeability. This makes interpreting contrast-enhanced images complicated. Hence, the search for biomarkers has been challenging. Most biomarker studies have focused on circulating biomarkers that are unable separate the response of the host from that of neoplastic lesions. Tissue biomarkers, based generally on limited samples of tumors, do not account for the heterogeneity inherent in all malignancies. Advanced imaging techniques can provide both spatial and temporal information but are expensive and use protocols that may not be standardized across multiple institutions. A limited number of correlative trials have used all three approaches and have provided powerful insights into the mechanisms of response and resistance. However, these trials have been small. Hence, these findings need to be validated in prospective, randomized trials. Importantly, future trials with novel agents need to integrate all three types of biomarkers.

Addressing these challenges and judiciously using existing and newly developed AA agents, alone or with other emerging therapeutic approaches, is likely to increase the survival benefits in selected patients while sparing other patients from unnecessary and expensive treatments.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two movies and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2014.10.006.

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