Role of the VEGF/VEGFR Axis in Cancer Biology and Therapy

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New vessel formation (angiogenesis) is an essential physiological process for embryologic development, normal growth, and tissue repair. Angiogenesis is tightly regulated at the molecular level; however, this process is dysregulated in several pathological conditions such as cancer. The imbalance between pro- and antiangiogenic signaling molecules within tumors creates an abnormal vascular network that is characterized by dilated, tortuous, and leaky vessels. The pathoph ysiological consequences of these vascular abnormalities include temporal and spatial heterogeneity in tumor blood flow, oxygenation, and increased tumor interstitial fluid pressure. The resultant microenvironment deeply impacts on tumor progression, and also leads to a reduction in therapy efficacy. The discovery of vascular endothelial growth factor (VEGF) as a major driver of tumor angiogenesis has led to efforts to develop novel therapeutics aimed at inhibiting its

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activity. Anti-VEGF therapy has become an important option for the management of several human malignancies; however, a significant number of patients do not respond to anti-VEGF therapy when used either as single agent or in combination with chemotherapy. In addition, the benefit of antiangiogenic therapy is relatively short lived and the majority of patients relapse and progress. An increasing amount of reports suggest several potential mechanisms of resistance to antiangiogenic therapy including, but not limited to, tumor hypoxia. This chapter discusses the role of the VEGF axis in tumor biology and highlights the clinical application of anti-VEGF therapies elaborating on pitfalls and strategies to improve clinical outcome.

I. VASCULAR ENDOTHELIAL GROWTH FACTORS AND THEIR RECEPTORS IN CANCER BIOLOGY

A. Vascular Endothelial Growth Factors

There are five structurally related Vascular Endothelial Growth Factors (VEGF) ligands (VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PIGF)). VEGFs are disulfide-bonded homodimers, although VEGFA and PIGF heterodimers have also been described (DiSalvo *et al.*, 1995). Each VEGF ligand is expressed as several different variants due to alternative splicing or posttranslational processing. Each variant binds differently to VEGF receptors (VEGFRs) and their coreceptors and therefore induces different biological responses, such as angiogenesis, lymphangiogenesis, permeability, inflammatory cell recruitment, and fatty acid uptake (see Table I). VEGFs are produced by many different cell types and act in an autocrine and paracrine manner. Knockout mice lacking expression of different VEGF ligands have demonstrated the critical role of VEGFs in vessel formation and function. The most striking effects are seen for VEGFA, where even one deleted allele is lethal (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). VEGFA is critical for development of endothelial cells during embryogenesis and for organization of the vasculature, as well as for their survival.

B. VEGF Receptors

VEGFs bind to three structurally related receptor tyrosine kinases (RTKs), VEGFR1, VEGFR2, and VEGFR3. In addition, a number of coreceptors (such as neuropilins, NRPs) that lack intrinsic catalytic activity bind VEGF and modulate the effect of the VEGFRs. VEGFRs have a high degree of homology within the kinase domain; however, their signaling properties greatly differ.

Table I Functions, Binding Properties, and Biological Implications of VEGFs

VEGF isoform	Receptor	Coreceptor	Biological function
VEGFA165	VEGFR1, VEGFR2	NRP1, NRP2	Angiogenesis (permeability, survival, migration of EC)
VEGFA121	VEGFR1, VEGFR2	NRP1[a]	Angiogenic/antiangiogenic properties[b]
VEGFA145	VEGFR1, VEGFR2	NRP2	Angiogenesis
VEGFA189	VEGFR1, VEGFR2	NRP1	Angiogenesis
VEGFA(xxx)b	VEGFR1, VEGFR2	No	Antiangiogenic properties
VEGFB	VEGFR1	NRP1	Fatty acid uptake in EC of the heart
VEGFC[c]	VEGFR3 (VEGFR2)	NRP2	Lymphangiogenesis
VEGFD[c]	VEGFR3 (VEGFR2)	NRP2	Lymphangiogenesis
PIGF	VEGFR1	NRP1, NRP2	Inflammatory cell recruitment

Abbreviations: EC, endothelial cells.
[a]VEGFA121 binds NRP1 but does not bridge to VEGFRs (Pan *et al.*, 2007).
[b]VEGFA121 has been described as antiangiogenic (Nowak *et al.*, 2008).
[c]Processed.

1. VEGFR1

VEGFR1 (alternatively denoted as Fms-like tyrosine kinase 1, Flt1, in the mouse) is a single-transmembrane glycoprotein. Interestingly, VEGFR1 binds VEGFA with at least 10-fold higher affinity than VEGFR2, yet it is poorly activated (Ferrara and Davis-Smyth, 1997). A study by Gille *et al.* (2000) of chimeric VEGFR1 and VEGFR2 revealed that the juxtamembrane domain of VEGFR1 plays an inhibitory role in VEGFR1 signaling pathways, although the precise mechanism requires further investigation. Accumulating evidence indicates that a soluble form of VEGFR1 (sVEGFR1) has a negative regulatory role in human physiology, presumably by trapping VEGFA (Kendall *et al.*, 1994). Moreover, sVEGFR1 levels are elevated in patients with breast cancer, pancreatic cancer, leukemia, and colorectal cancer, where it is associated with a favorable prognosis (Scheufler *et al.*, 2003; Toi *et al.*, 2002). VEGFR1 is expressed not only in vascular endothelial cells but also in other cell types (monocytes and macrophages, human trophoblasts, renal mesangial cells, vascular smooth muscle cells, dendritic cells, and various types of cancer cells) (Shibuya and Claesson-Welsh, 2006). The fact that VEGFR1 is usually expressed at low levels has limited the progress in elucidating its signal transduction pathways (Fig. 1).

Notably, VEGFR1 plays a role in tumor progression and dissemination. Indeed, the rate of tumor growth of melanoma and glioma tumor models is considerably reduced in VEGFR1 $TK^{-/-}$ mice (Kerbel, 2008; Muramatsu *et al.*, 2010). In addition, VEGFR1 activity has been shown to play a role in

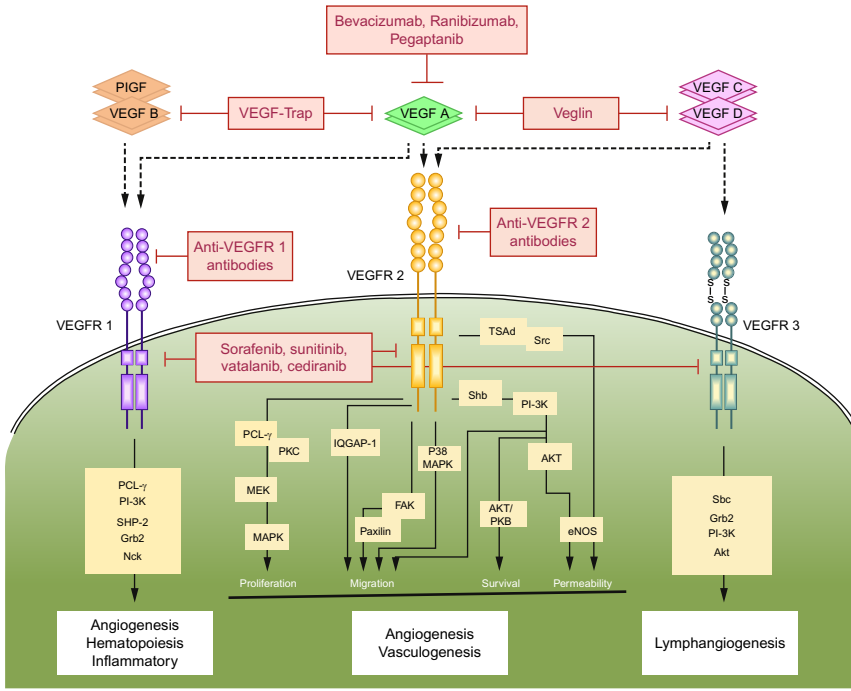

Fig. 1 Signaling and biological processes mediated by the VEGF/VEGFRs axis and therapeutic agents.

metastatic dissemination, and expression of VEGFR1 in tumor cells seems to increase tumor invasiveness (Mylona *et al.*, 2007; Seto *et al.*, 2006). Furthermore, VEGFR1 has been shown to activate extracellular signal-regulated kinase 1/2, stress-activated protein kinase/c-Jun NH_2-terminal kinase (Fan *et al.*, 2005), and Src family kinases (Lesslie *et al.*, 2006) to mediate growth and migration of human colorectal carcinoma cells. Finally, activation of VEGFR1 in breast cancer cells supports their growth and survival (Wu *et al.*, 2006a), strongly arguing in favor of the importance of VEGFR1-mediated signaling in these models.

Regulation of inflammatory cell recruitment by VEGFR1 appears to be exerted mainly through PIGF. Notably, the expression of PIGF is very low under physiological conditions, but it may be strongly upregulated in various cell types by different pathological stimuli such as hypoxia, inflammatory cytokines, or oncogenes (Failla *et al.*, 2000; Green *et al.*, 2001; Larcher *et al.*, 2003). PIGF has been regarded as an attractive candidate for anti-angiogenic therapy. Indeed, it has been shown that PIGF plays a key role in

promoting pathological angiogenesis associated with tumor progression (Carmeliet *et al.*, 2001), and overexpression of PIGF in a mouse melanoma model resulted in increased tumor growth and metastasis (Li *et al.*, 2006).

2. VEGFR2

There is much evidence that VEGFR2 (KDR) is the major mediator of VEGFA-driven responses in endothelial cells and it is considered to be a crucial signal transducer in both physiologic and pathologic angiogenesis (Hicklin and Ellis, 2005). In addition, VEGFR2 binds proteolytically processed VEGFC and VEGFD (McColl *et al.*, 2003). The signaling pathways triggered by engagement of VEGFR2 are relatively well understood (see Fig. 1).

VEGFR2 is expressed in most if not all adult vascular endothelial cells, as well as in circulating endothelial progenitor cells, pancreatic duct cells, retinal progenitor cells, megakaryocytes, and hematopoietic cells (Hicklin and Ellis, 2005). VEGFR2, often in combination with VEGFR3, is significantly upregulated in the tumor vascular endothelium in most common human solid tumor types (Smith *et al.*, 2010). Tumor cells may also express VEGFR2, although epithelial and mesenchymal tumor cells typically express VEGFR1 rather than VEGFR2 (Hicklin and Ellis, 2005; Podar and Anderson, 2005). Nevertheless, increased expression of VEGFR2 on tumor cells has been noted for melanoma and hematological malignancies (Youssoufian *et al.*, 2007). It has been shown that VEGFR2-mediated signaling led to survival of cancer cells under chronic hypoxic conditions and might contribute to a more aggressive phenotype (Calvani *et al.*, 2006). Growing evidence supports an important link between chronic inflammation and tumor development. Induction of VEGFR2 expression in tumor cells, and also in intestinal epithelium during colitis, is mediated by the proinflammatory cytokine interleukin 6, which is a strong promoter of tumor growth in experimental colitis-associated colon cancer (Waldner *et al.*, 2010). sVEGFR2 has been described and may have important biological roles. sVEGFR2 binds VEGFC and thus prevents activation of VEGFR3, consequently inhibiting lymphatic endothelial cell proliferation (Albuquerque *et al.*, 2009). Notably, it has been recently shown that downregulation of sVEGFR2 in advanced metastatic neuroblastoma may promote lymphogenic spread of metastases (Becker *et al.*, 2010).

3. VEGFR3

VEGFR3 (alternatively denoted Fms-like tyrosine kinase 4, Flt4, in the mouse) is activated by the binding of VEGFC or VEGFD. VEGFR3 and its ligands are key players in the regulation of normal and tumor lymphangiogenesis (Shibuya and Claesson-Welsh, 2006). Indeed, gene inactivation to

eliminate expression of VEGFC alone, or combined deletion of VEGFC and VEGFD, unexpectedly resulted in defects mainly in lymphatic vessels, while blood vessels remained unaffected in mouse models (Haiko *et al*., 2008). In adult tissues, VEGFR3 has an essential role in lymphatic endothelial cells, but its expression is also induced in endothelial cells engaged in active angiogenesis (Carmeliet *et al*., 2009), such as in tumor vessels (Laakkonen *et al*., 2007). The expression of VEGFR3 in tumor cells is controversial (Petrova *et al*., 2008); however, it has been clearly demonstrated that inhibition of VEGFR3 activity arrests tumor vascularization, leading to decreased vascular density in several tumor models (Laakkonen *et al*., 2007). The axis VEGFC/VEGFR3 plays a fundamental role in the tumor microenvironment by promoting the formation of new lymphatic vessels from preexisting ones (He *et al*., 2004). VEGFC, produced by tumor cells, induces lymphatic endothelial destabilization, resulting in endothelial sprouting as well as leakage and enlargement of the vessels. These changes facilitate entry of tumor cells into the lymphatics and further dissemination of metastasis to sentinel lymph nodes (Achen and Stacker, 2008; He *et al*., 2005).

4. NEUROPILINS

There are two NRP homologues, NRP1 and NRP2. The NRPs were first identified as receptors for class 3 semaphorins, a family of soluble molecules with neuronal guidance functions, and are now implicated in the development of the nervous and vascular systems (Hicklin and Ellis, 2005). Importantly, NRPs are also coreceptors for VEGF ligands and are being investigated as possible therapeutic targets to arrest angiogenesis as well as lymphangiogenesis in cancer. Interestingly, increased NRP expression in human leukemia and lymphoma (Karjalainen *et al*., 2011) and in many solid tumors is associated with increased metastasis (Cohen *et al*., 2002; Hansel *et al*., 2004; Kawakami *et al*., 2002; Lantuejoul *et al*., 2003; Latil *et al*., 2000; Stephenson *et al*., 2002; Vanveldhuizen *et al*., 2003). However, it is still controversial whether, and to which extent, cancer cells express NRPs.

C. VEGF/VEGFR Axis and the Tumor Microenvironment

The fine balance between the supply of oxygen and nutrients by blood vessels and the proliferation of cancer cells determines the onset of intratumor hypoxia and contributes to the angiogenic switch. Tumors that fail to activate the angiogenic pathway remain dormant and do not progress. The key regulator of hypoxia-induced angiogenesis is the transcription factor hypoxia-inducible factor (HIF)-1. Multiple HIF-1 target genes are involved in different steps of angiogenesis: arterial destabilization (VEGFA, PIGF,

VEGFR1), increased vascular permeability (VEGFA, VEGFR1, angiopoietin 2, Tie-2), extracellular matrix remodeling (MMPs, collagen prolyl-4-hydroxylase, uPAR), migration and proliferation of endothelial cells (VEGFA, PIGF, FGF2, angiopoietin 1, MCP-1, PDGF, SDF-1, CXCR4), endothelial cells sprouting (angiopoietin 2, Tie-2), endothelial tube formation and cell-to-cell interaction (VEGFA, PIGF, angiopoietin 1, integrins), and recruitment of and interaction with pericytes (PDGF, PAI-1, angiopoietin 1, Tie-2) (Hirota and Semenza, 2006). VEGFA exerts multiple effects within the tumor microenvironment, which aggravates tumor growth and metastatic spread and reduces treatment efficacy. Antibodies that bind VEGF and thereby prevent its binding to VEGFRs inhibit angiogenesis and have been exploited clinically for cancer therapy (Ferrara, 2005).

II. TARGETING VEGF/VEGFR FOR CANCER THERAPY

Despite the existence of many pathways that contribute to the angiogenic process, the VEGF/VEGFRs pathway is considered a key regulator of angiogenesis and this realization has led to considerable interest and efforts to exploit this pathway for cancer therapy. It is, therefore, not surprising that most of the antiangiogenic agents currently in preclinical and clinical development focus on inhibition of the VEGF pathway (Fig. 1). Several anti-VEGF strategies have been developed, including neutralizing antibodies to VEGF or VEGFRs, soluble VEGFR/VEGFR hybrids, and tyrosine kinase inhibitors of VEGFRs (Ellis *et al*., 1996; Gerber *et al*., 2000; Kim *et al*., 1993; Klohs and Hamby, 1999; Prewett *et al*., 1999). Table II summarizes some of the principal antiangiogenic molecules that are currently being used in clinical trials to target VEGF signaling.

A. Antibodies and Decoy Receptor-Based Therapies

1. BEVACIZUMAB

One of the earliest strategies used to inhibit VEGF activity has involved neutralizing antibodies to VEGF. In preclinical studies, a murine anti-VEGF monoclonal antibody inhibited angiogenesis and growth of human tumor xenografts (Gerber *et al*., 2000; Kim *et al*., 1993; Prewett *et al*., 1999). Notably, the anti-VEGF antibody bevacizumab (Avastin®; Genentech Inc.) has been the first antiangiogenic agent to be approved for cancer therapy, in combination with chemotherapy, by the U.S. Food and Drug Administration. It was initially approved for the treatment of metastatic colorectal

Table II Antiangiogenic Agents in Advanced Clinical Development

Therapeutic agent	Type	Target	Clinical development	References
Bevacizumab/Avastin	mAb	VEGFA	Approved in 2004 (CC), 2006 (NSCLC), 2008 (BC), 2009 (RCC, glioblastoma)	Van Meter and Kim (2010)
Ramucirumab/IMC-1121B	mAb	VEGFR2	Phase II/III	Spratlin (2011)
MF-1/IMC-18F1	mAb	VEGFR1	Phase I	Wu *et al.* (2006b)
CDP791	PEG di-Fab conjugate	VEGFR2	Phase II	Youssoufian *et al.* (2007)
VEGF-Trap/aflibercept	Fusion protein	VEGFA, PIGF	Phase II/III	Teng *et al.* (2010)
VEGFAS/Veglin	Oligonucleotide	VEGFA, VEGFC, VEGFD	Phase I	Levine *et al.* (2006)
SU11248/sunitinib (Sutent)	RTKI	VEGFR1–3, PDGFR, c-kit, Flt3	Approved in 2006 (GIST and RCC)	Sulkes (2010)
Sorafenib (Nexavar)	RTKI	VEGFR2–3, PDGFR, Raf-1, Flt-3, c-kit	Approved in 2005 (RCC), 2008 (HCC)	Sulkes (2010)
Pazopanib (Votrient)	RTKI	VEGFR1–3, PDGFR, Flt-3, c-kit	Approved in 2009 (RCC)	Sternberg *et al.* (2010)
AG013736/axitinib	RTKI	VEGFR1–3, PDGFR, c-kit	Phase II/III	Kelly *et al.* (2010)
AZD6474/vandetanib (Zactima)	RTKI	VEGFR1–3, EGFR, RET	Phase II/III	Morabito *et al.* (2009)
AZD2171/cediranib (Resentin)	RTKI	VEGFR1–3, c-kit	Phase II/III	Lindsay *et al.* (2009)
Brivanib alanitate	RTKI	VEGFR2, FGFR1	Phase II/III	Diaz-Padilla and Siu (2011)
AV-951/tivozanib	RTKI	VEGFR1–3, PDGFR	Phase II/III	De Luca and Normanno (2010)
PTK787/vatalanib	RTKI	VEGFR1–3, PDGFR, c-kit	Phase II	Scott *et al.* (2007)
AE941/Neovastat	Shark cartilage component	VEGF/VEGFR binding, MMP2, MMP9	Phase II/III	White (2010)

Abbreviations: BC, breast cancer; CC, colorectal carcinoma; HCC, hepatocellular carcinoma; mAb, monoclonal antibody; NSCLC, nonsmall cell lung carcinoma; RCC, renal cell cancer; RTKI, receptor tyrosine kinase inhibitor.

cancer in combination with intravenous 5-fluorouracil-based chemotherapy (Hurwitz *et al*., 2004). Subsequently, bevacizumab has been approved for various indications in nonsquamous cell lung carcinoma (NSCLC), metastatic renal cell carcinoma, and glioblastoma multiforme (Escudier *et al*., 2010; Friedman *et al*., 2009; Kreisl *et al*., 2009; Rini *et al*., 2008; Sandler *et al*., 2006; Van Meter and Kim, 2010). The antitumor activity of bevacizumab is primarily manifested in combination with chemotherapy, except for renal cell carcinoma, where it has shown efficacy as a single agent (Yang *et al*., 2003). Presently, bevacizumab is being used in nearly 1000 clinical trials, and despite promising results, its effects in many types of cancer are modest or even irrelevant (Van Meter and Kim, 2010). Furthermore, recent studies have raised the possibility that treatment with bevacizumab is associated with a more aggressive invasive tumor phenotype, particularly in glioblastoma (Keunen *et al*., 2011). Although the clinical impact of these results is far from clear, it is obvious that antiangiogenic therapy will have to be closely evaluated depending on disease stage and molecular profile.

2. VEGFR2-TARGETING ANTIBODIES

Preclinical data with anti-VEGFR2 antibodies have demonstrated a reduction in VEGF-induced signaling as well as angiogenesis and primary or metastatic growth in a variety of different tumor models (Bruns *et al*., 2002; Prewett *et al*., 1999; Shaheen *et al*., 2001; Zhu *et al*., 1999); therefore, the specific, antibody-based blockade of VEGFR2 has also received special attention in clinical trials. Ramucirumab (IMC-1121B; Imclone Systems) is currently being tested in several clinical trials, including breast cancer, gastric cancer, and HCC (Spratlin, 2011). Based on preliminary results, this antibody has shown activity in patients previously treated with other antiangiogenic agents, suggesting a more efficient antitumor response with direct targeting of VEGFR2.

3. VEGF–PIGF DECOY RECEPTOR

After showing a remarkable inhibitory activity in different experimental models, VEGF-Trap (aflibercept; Sanofi-Aventi, Regeneron), the soluble decoy receptor with very high affinity for VEGFA and PIGF, entered clinical trials. Phase 3 trials with aflibercept in metastatic colon cancer and prostate cancer are still underway; however, studies in patients with NSCLC failed to reach the primary endpoint of improvement in overall survival (OS).

B. RTKs Small Molecule Inhibitors

Small molecule inhibitors of VEGFR tyrosine kinase activity represent another major approach to blocking VEGF-mediated angiogenesis. Several tyrosine kinase inhibitors have been developed to selectively inhibit VEGFR2, but they have also activity on other VEGFRs and tyrosine kinase receptors, including basic fibroblast growth factor (FGF) receptor, EGFR family members, PDGFR-α, PDGFR-β, c-kit, and Flt3.

1. SUNITINIB AND SORAFENIB

Sunitinib was approved in 2006 for its clinical use in imatinib-resistant gastrointestinal stromal tumors and advanced metastatic renal cell carcinoma (Demetri *et al*., 2006; Motzer *et al*., 2007), whereas sorafenib received FDA approval for the treatment of metastatic renal cell carcinoma (Escudier *et al*., 2007) and HCC (Llovet *et al*., 2008). Notably, sunitinib and sorafenib have shown clinical efficacy as single agents, possibly due to their ability to inhibit multiple RTKs and in particular those regulating tumor angiogenesis. Additional clinical trials aimed to evaluate combinations of sorafenib and sunitinib with different chemotherapeutic agents and other antiangiogenic agents are ongoing.

It is important to point out that preclinical studies have challenged the classic schedule of administration currently used for sunitinib in clinical trials. Indeed, short-term treatment with sunitinib was associated with an accelerated metastatic tumor growth and invasiveness in different tumor models (Ebos *et al*., 2009), stressing the importance of fully understanding the potential responses to antiangiogenic therapies and optimizing dose and schedule in clinical trials. Interestingly, continuous daily administration of sunitinib in patients with advanced pancreatic neuroendocrine tumors showed clear improvement in both progression free and OS in a phase 3 trial (Raymond *et al*., 2011), which led to FDA approval of sunitinib for the treatment of pancreatic neuroendocrine tumors.

2. PAZOPANIB

Pazopanib (Votrient), a pan-VEGFR inhibitor developed by GlaxoSmithKline, is currently being tested in a broad clinical program across multiple tumor types. It received approval by the FDA for use in advanced renal cell carcinoma (Sternberg *et al*., 2010). A phase 3 clinical is being conducted to compare pazopanib with sunitinib for treatment of metastatic renal cell carcinoma based on the potential better toxicity profile associated with administration of pazopanib.

III. CHALLENGES OF VEGF/VEGFR TARGETED THERAPY: LIMITED THERAPEUTIC RESPONSE AND DEVELOPMENT OF RESISTANCE

Antiangiogenic therapy has become an important option for the treatment of cancer. However, its systematic application remains problematic because of poor understanding of mechanisms of action and occurrence of resistance (Jain *et al*., 2006). Indeed, a significant fraction of patients do not respond to antiangiogenic therapy (Burris III and Rocha-Lima, 2008), whereas those who respond have relatively modest benefits, mostly in progression-free survival rather than in OS. In addition, a number of significant toxicities have been observed in patients treated with antiangiogenic agents, emphasizing that a careful assessment of the risk-benefit ratio needs to be conducted in individual patients. Despite disease stabilization and an increase in the proportion of patients with progression-free survival, tumors eventually become resistant to antiangiogenic agents and relapse (Bergers and Hanahan, 2008; Ellis and Hicklin, 2008a; Kerbel, 2008; Shojaei and Ferrara, 2008b). Ultimately, which patients may potentially benefit from the addition of an antiangiogenic agent to the therapeutic regimen remains poorly understood.

Multiple mechanisms may account for the activity of anti-VEGF agents in cancer patients including, but not limited to, their effect on tumor vasculature (Ellis and Hicklin, 2008b). Evidence has been provided supporting both a vascular regression, which is presumably associated with increased intratumor hypoxia (Kerbel and Folkman, 2002), and a so-called normalization of tumor vasculature, with a consequent decrease in interstitial pressure and better delivery of chemotherapy (Jain, 2005b). These conflicting and still largely controversial observations emphasize how important it is to better understand the effects of antiangiogenic agents on the tumor microenvironment to eventually better characterize the mechanisms that mediate resistance.

A. Intrinsic Resistance to VEGF-Targeted Therapies

A substantial fraction of patients treated with antiangiogenic agents, including bevacizumab, sorafenib, or sunitinib, fail to show even a transient clinical benefit (Batchelor *et al*., 2007; Burris III and Rocha-Lima, 2008). This lack of clinical benefit could be interpreted as a rapid adaptation to and escape from the effects of antiangiogenic agents. Alternatively, in some cases, there may be preexisting resistance. It is conceivable that a number of pathways may be activated in human cancers that eventually confer

intrinsic resistance to antiangiogenic therapy, such as redundancy of angiogenic factors (FGFs, PDGFs, PIGF) (Fischer *et al.*, 2007; Relf *et al.*, 1997), increased metastatic and invasive potential without an angiogenic switch (Casanovas *et al.*, 2005), high levels of infiltrating inflammatory cells that produce a number of proangiogenic factors (Shojaei and Ferrara, 2008b) or hypovascularity, such as in pancreatic ductal adenocarcinoma (Saif, 2007).

B. Acquired Resistance to Antiangiogenic Agents

Considering the results of both preclinical and clinical research showing modest effects of antiangiogenic therapy in patients with solid tumors, it is now widely recognized that tumors rapidly adapt to the effects of anti-VEGF agents to resume growth. Apart from instances of intrinsic resistance, most tumors acquire resistance to antiangiogenic therapies by upregulating pathways that sustain tumor growth and progression. Acquired resistance to antiangiogenic agents has been attributed to a number of potential mechanisms, including upregulation of alternative proangiogenic signals, increased production of proangiogenic factors by stromal cells, recruitment of bone marrow-derived proangiogenic cells, increased vascular pericyte coverage, and activation of an invasive phenotype. In addition, hypoxia-dependent responses may also play a role in several of these adaptive mechanisms. For instance, elevated CA9 (a HIF-1 target gene) and HIF-2α levels are inversely correlated with response to bevacizumab and irinotecan in malignant astrocytoma (Sathornsumetee *et al.*, 2008), suggesting that intra-tumor hypoxia may be an important factor in mediating resistance to antiangiogenic agents.

1. UPREGULATION OF COMPENSATORY PROANGIOGENIC PATHWAYS

A compensatory increase of FGFs was one of the first mechanisms of resistance identified in preclinical models (Casanovas *et al.*, 2005). The potential relevance of these findings is supported by clinical data that reported the induction of FGF2 in serum of patients that progressed on anti-VEGF therapy (Batchelor *et al.*, 2007). In addition, both in preclinical and clinical studies, PIGF was shown to be upregulated following anti-VEGF therapy (Batchelor *et al.*, 2007), while blockade of PIGF using monoclonal antibodies reduced tumor angiogenesis and metastasis in mouse models, regardless of whether tumors were sensitive or resistant to anti-VEGF therapy (Fischer *et al.*, 2007). Anti-PIGF therapies might play a complementary role to anti-VEGF therapy; however, clinical development

of VEGF-Trap (that binds both VEGF and PIGF) has not shown any additional benefit compared to bevacizumab.

Recent data emphasize the role of the cell membrane-bound Notch ligand/receptor system in the development of resistance to antiangiogenic therapy (Li *et al*., 2011). Moreover, tumors that have an intrinsic resistance to anti-VEGF agents appear to be sensitive to inhibition of Dll4 (Delta-like ligand 4; Yan and Plowman, 2007).

2. PRODUCTION OF PROANGIOGENIC FACTORS BY STROMAL CELLS

Reduced efficacy of antiangiogenic therapy may be due to the involvement of the stromal compartment in tumor angiogenesis. In particular, tumor-associated fibroblasts (TAFs) are thought to play a major role in tumor growth and possibly in resistance to antiangiogenic therapy (Liang *et al*., 2006). Notably, it has been shown that TAFs from tumors resistant to anti-VEGF therapy can support tumor growth and angiogenesis by producing PDGF-C, proposing yet another potential mechanism of resistance (Crawford *et al*., 2009). Indeed, these observations emphasize the role that the tumor microenvironment plays in drug resistance in general and to antiangiogenic agents in particular, strongly suggesting that the stromal cellular component needs to be understood in order to improve efficacy of anticancer therapies.

3. RECRUITMENT OF BONE MARROW-DERIVED PROANGIOGENIC CELLS

Induction of intratumor hypoxia during therapy with antiangiogenic agents may lead not only to an increase in the production of proangiogenic factors by tumor and stromal cells but also to recruitment of bone marrow-derived cells (BMDCs) that have the capacity to elicit angiogenesis and tumor growth. Proangiogenic BMDCs consist of vascular progenitors (such as endothelial and pericytes progenitors) and vascular modulators (such as tumor-associated macrophages, immature monocytic cells, myeloid cells) (Kerbel, 2008). Indeed, a marked mobilization of circulating BMDCs occurs rapidly after treatment of tumor-bearing mice with vascular disrupting agents, along with massive induction of tumor hypoxia (Shaked *et al*., 2006). Moreover, circulating endothelial cells (CECs) have been shown to contribute to the rapid regrowth of tumors. Of interest, an increase in FGF2, SDF-1, and viable CECs was observed when tumors progressed following treatment with the VEGF RTK inhibitor AZD2171 in glioblastoma patients (Batchelor *et al*., 2007).

More recently, it has been suggested that a specific myeloid cell population migrates to tumors and mediates tumor angiogenesis and resistance to anti-VEGF agents (Shojaei *et al*., 2007). Interestingly, tumor and stromal cell production of G-CSF, IL6, and SDF-1 mediates the mobilization of $CD11b^{+}Gr1^{+}$ myeloid cells to the tumor, where they elicit angiogenesis and confer resistance to anti-VEGF therapy (Shojaei and Ferrara, 2008a; Shojaei *et al*., 2007).

4. INCREASED PERICYTE COVERAGE OF THE VASCULATURE

Pericytes are involved in vascular stability and provide survival signals to endothelial cells. Inhibition of VEGF signaling may spare endothelial cells that are in strict contact with pericytes in "mature vessels" (Benjamin *et al*., 1999). Conversely, anti-VEGF therapy not only may lead to endothelial cell apoptosis and pruning of immature tumor vasculature (without pericyte coverage) but also may increase angiopoietin 1 that enhances pericyte recruitment to the vessels, thereby reversing the effect of anti-VEGF therapy (Winkler *et al*., 2004). Indeed, a number of studies have shown that targeting both pericytes and endothelial cells (PDGFR and VEGFR inhibitors) may lead to synergistic inhibition of tumor growth (Bergers *et al*., 2003). Conversely, recent evidence suggests that targeting pericytes in the tumor vasculature may lead to disruption of vessel integrity, enabling tumor cells to transit into the circulation system and metastasize (Xian *et al*., 2006). Moreover, a negative rather than a positive effect of VEGF on pericyte function and vessel maturation has also been recently suggested, adding complexity to the potential effects of VEGF/PDGF modulation (Greenberg *et al*., 2008). Due to the similarities between VEGFRs and PDGFRs, many RTK inhibitors that target VEGFRs also inhibit PDGFRs functions. The clinical benefit of targeting both endothelial cells and pericytes remains to be determined.

C. Role of the Hypoxic Tumor Microenvironment in the Resistance to Antiangiogenic Therapies

The functional consequences of antiangiogenic therapies on the tumor microenvironment are still poorly understood and controversial. Indeed, at least two hypotheses have been proposed: (1) "normalization" of the vasculature, with a consequent decrease in intratumor hypoxia and interstitial pressure, which would be associated with a better delivery of chemotherapy; (2) vascular "regression," resulting in an increase of intratumor hypoxia, selection of more metastatic clones, and resistance to therapy (Jain, 2005a;

Kerbel and Folkman, 2002). Several lines of evidence in preclinical models support the hypothesis that antiangiogenic therapy might be associated with an increase in intratumor hypoxia and selection of a more malignant phenotype (Bergers and Hanahan, 2008; Bottaro and Liotta, 2003; Casanovas *et al*., 2005; Ebos *et al*., 2009; Franco *et al*., 2006; Keunen *et al*., 2011; Paez-Ribes *et al*., 2009; Pennacchietti *et al*., 2003; Steeg, 2003). Moreover, these preclinical data appear to be consistent with clinical findings demonstrating increased intratumor hypoxia in patients with nonsmall cell lung cancer and primary liver following treatment with bevacizumab (Smit *et al*., 2011; Yopp *et al*., 2011). Notably, it has been recently shown that administration of antiangiogenic agents, such as sunitinib and bevacizumab, increases the cancer stem cell (CSC) population in breast cancer xenografts as a consequence of the generation of tumor hypoxia (Conley *et al*., 2012). This study strongly indicates that hypoxia-driven CSC stimulation limits the effectiveness of antiangiogenic agents and suggests that, to improve patient outcome, antiangiogenic therapies might have to be combined with CSC-targeting drugs. Interestingly, several studies have demonstrated the acquisition of an invasive phenotype in glioblastoma patients who have developed multifocal recurrence of tumors during the course of antiangiogenic therapy (Narayana *et al*., 2009, 2011; Norden *et al*., 2008). This data strongly suggests that reduction of tumor vasculature and increase in intratumor hypoxia might result in enhanced tumor cell invasiveness. In addition, intratumor hypoxia has been implicated not only in the increased metastatic phenotype of tumors in response to antiangiogenic agents but also in a number of mechanisms of resistance that have been described so far (Rapisarda and Melillo, 2009). Indeed, hypoxia plays an important role in the regulation of angiogenic factors (FGFs, PDGFs, PIGF) (Fischer *et al*., 2007; Relf *et al*., 1997), such as regulation of Notch/Dll-4 signaling (Diez *et al*., 2007), recruitment of BMDCs (Ceradini *et al*., 2004) (that have the capacity to elicit tumor growth and angiogenesis; Kerbel, 2008), recruitment of $CD11b^{+}Gr1^{+}$ myeloid cells (triggered by G-CSF, IL6, and SDF-1 secreted by tumor and stromal cells) (Shojaei and Ferrara, 2008a), recruitment of $CD11b^{+}$ myeloid cells at the premetastatic sites (in response to SDF-1 and LOX gradients) (Erler *et al*., 2009; Yang *et al*., 2008) and pericyte recruitment to vessels (Winkler *et al*., 2004) (in response to the HIF-1 regulated genes PDGF, PAI-1, angiopoietin 1, and Tie-2; Hirota and Semenza, 2006).

The hypoxic tumor microenvironment may also be an important predictive factor to identify tumors that may be more sensitive or resistant to anti-VEGF therapy (Dang *et al*., 2008). For example, treatment with antiangiogenic agents has been shown to increase plasma levels of VEGF in cancer patients, and such an increase has been proposed to be a potential predictive biomarker for tumor response (Bertolini *et al*., 2006, 2007; Bocci *et al*., 2004). These observations underline the complexity of the relationship

between antiangiogenic therapies and the tumor microenvironment and they emphasize the need to identify biomarkers that may guide the selection of patients in which combined targeting of tumor hypoxia and angiogenesis may be more beneficial.

IV. IMPROVING THE THERAPEUTIC OUTCOME OF VEGF-TARGETING AGENTS BY COMBINATION STRATEGIES

Considering the complexity of pathways regulating tumor angiogenesis and the limited activity observed by targeting VEGF-dependent responses, combination strategies that target multiple pathways involved in angiogenesis might be beneficial. Hence, combining VEGFR2 inhibitors with a blockade of PDGFR-β (Bergers *et al*., 2003), VEGFR1 (Gille *et al*., 2007), MMPs (Mancuso *et al*., 2006), and other growth factors (e.g., EGF) shows additive antitumor activity in preclinical models (Ciardiello *et al*., 2004; Wedge *et al*., 2002). In addition, combinatorial therapies are being conducted that target VEGFA and stroma-derived growth factors, such as EGF or FGF. A preclinical study by Cascone *et al*. showed that dual targeting of VEGFR and EGFR increased progression-free survival and delayed the appearance of resistance associated with antiangiogenic therapy (Cascone *et al*., 2011). Brivanib, a dual inhibitor of VEGFR and fibroblast growth factor receptor-1 (FGFR1) is already being evaluated in about 20 clinical trials, including hepatocellular carcinoma and colorectal carcinoma (Diaz-Padilla and Siu, 2011).

A. Can Intratumor Hypoxia be Exploited in Combination Strategies with AntiAngiogenic Agents?

The potential therapeutic relevance of hypoxia in the development of resistance to antiangiogenic agents argues in favor of the development of combination strategies aimed to thwart adaptive hypoxia-dependent responses during anti-VEGF treatment. Indeed, a number of therapeutic strategies have been devised to target the hypoxic microenvironment: (1) targeting hypoxic cells by using bioreductive prodrugs that are converted to cytotoxins under hypoxic conditions (Wilson and Hay, 2011), (2) development of inhibitors of HIF-1 activity (Melillo, 2006; Onnis *et al*., 2009), (3) inhibition of downstream pathways activated by hypoxia such as metabolism (Denko, 2008; Papandreou *et al*., 2011), (4) pH homeostasis (Chiche

Table III Examples of Pharmacological Strategies to Target Hypoxic Cells

Pathway	Target	Agents
Hypoxia	Hypoxia-activated cytotoxin	Tirapazamine
HIF-1 inhibitors	HIF-1α mRNA expression	EZN-2968, Aminoflavone
	HIF-1α protein synthesis	Topotecan, EZN-2208, Cardiac glycosides, PX-478, Temsirolimus, Everolimus
	HIF-1α degradation	17AAG/17DMAG, HDAC inhibitors
	HIF-1-DNA binding	Anthracyclines
	HIF-1α transcriptional activity	Bortezomib
Metabolism	Hexokinase 2	2DG, Lonidamine
	PDK1-4	DCA
Invasion and migration	Met/ALK	Crizotinib
	MET/VEGF	XL-880/XL-184
UPR and autophagy	HSP90	17AAG/17DMAG
	IRE1	Salicaldehydes
	Proteasome	Bortezomib
	Autophagy	Chloroquine

et al., 2009, 2010), (5) invasion/migration, (6) unfolded protein response (UPR) (Wouters and Koritzinsky, 2008), (7) authopagy (Rouschop and Wouters, 2009), and (8) DNA damage response and repair pathways (Olcina *et al*., 2010; Table III).

Several studies have already addressed the question of whether combining inhibition of hypoxic targets with anti-VEGF agents might result in a therapeutic advantage. In this regard, evidence has been provided that combination of bevazizumab with low-dose daily topotecan, a camptothecin analog Top1 poison that inhibits HIF-1α protein synthesis *in vitro* and *in vivo* (Rapisarda *et al*., 2004a,b), results in increased antitumor activity relative to either agent alone in xenografts models (Rapisarda *et al*., 2009). Consistent with these findings, combination of bevacizumab with irinotecan (a topoisomerase I inhibitor that also inhibits HIF-1) has shown clinical benefit in glioblastoma patients with a 6-month OS of 62–77% (Chen *et al*., 2007; Vredenburgh *et al*., 2007). Given that HIF-1-dependent genes may play key roles in multiple mechanisms implicated in the resistance to anti-VEGF therapies, a combination of these agents with HIF-1 inhibitors might result in inhibition of adaptive pathways and increased therapeutic efficacy. Likewise, activity of HIF-1 inhibitors might be maximized in the presence of therapy-induced intratumor hypoxia.

Recent work from the McDonald laboratory has combined a blockade of VEGFR with that of c-Met, an RTK that binds hepatocyte growth factor and has been shown to play an important role in angiogenesis,

epithelial–mesenchymal transformation, drug resistance, invasion, and metastasis. This combinatorial blockade improves antitumor activity in the RIP-Tag2 pancreatic islet cancer model when compared to an agent that targets only VEGFR. VEGFR and c-Met inhibition reduced pericyte vascular coverage, induced intratumor hypoxia and tumor cell apoptosis, slowed tumor vasculature regrowth after treatment, and reduced invasiveness of primary tumors and metastasis. These results suggest that combining VEGFRs and c-Met inhibition is a viable option to achieve a better therapeutic outcome (You *et al.*, 2011).

V. THE IMPORTANCE OF BIOMARKERS FOR PATIENTS' SELECTION

Profiling tumors from individual patients has the potential to radically change therapeutic strategies by identifying patients that will most likely benefit from a particular agent or combination. Despite the obvious benefits potentially provided by this approach, identification of predictive biomarkers to efficiently select patients remains elusive at this time. Several biomarkers that might predict sensitivity to antiangiogenic therapies have been evaluated, including VEGF levels and polymorphisms, VEGFR expression and imaging parameters, but with mixed results (Murukesh *et al.*, 2010).

A. VEGF/VEGFRs Expression and Polymorphisms

One of the first biomarkers to be evaluated has been the plasma concentration of VEGFA. Of the many trials, only results with E4599 indicated that the pretreatment plasma concentration of VEGF was of prognostic significance in nonsmall cell lung cancer patients (Dowlati *et al.*, 2008). Intuitively, one would predict that the pretreatment plasma concentration of VEGF would be most helpful in diseases that respond to single-agent VEGF inhibitors (e.g., renal, ovarian, and hepatic cancer), however, this hypothesis hasn't been fully investigated. The increase in plasma VEGF concentration in patients treated with anti-VEGF antibodies has also been seen in those receiving low-molecular-weight RTKIs. A VEGFR inhibitor biomarker signature has emerged in which the drugs induce an increase in plasma VEGF and PIGF, as well as reductions in soluble VEGFR2 and VEGFR3. Presumably, this biomarker signature reflects the larger repertoire of receptors targeted by RTKIs compared with anti-VEGF antibodies. If true, one might not expect to see an increase in VEGFR3 concentrations in patients receiving bevacizumab, although this has not been formally reported.

Interestingly, in patients with upper gastrointestinal cancers, VEGFA and VEGFR2 appear to be potential predictive biomarkers to identify responders to a combination therapy of bevacizumab and erlotinib (Rohrberg *et al*., 2011). Moreover, in renal cell cancer (RCC), the ratio of VEGFA121/VEGFA165 mRNA levels seems to predict responsiveness to sunitinib (Paule *et al*., 2010).

Few studies have reported a potential association between clinical outcome and single-nucleotide polymorphisms (SNPs) in VEGF genes. When patients with metastatic breast cancer were treated with paclitaxel and bevacizumab (E2100 trial), SNP analysis demonstrated that VEGF-2578 AA and VEGF 1154-A genotypes were associated with better OS, but not response rate (RR) or PFS (Schneider *et al*., 2008). In contrast, those patients who received bevacizumab alone had a better RR and PFS but not OS, thereby challenging the pathophysiological role of these SNPs with regard to bevacizumab efficacy. Moreover, in patients with metastatic clear cell renal cell carcinoma treated with sunitinib, VEGF SNP-634 is associated with hypertension and a combination of VEGF SNP 936 and VEGFR2 SNP 889 genotypes is associated with OS (Kim *et al*., 2012).

Perhaps the most attractive tissue biomarker that could be used to predict sensitivity is phosphorylated VEGFR2. In patients with inflammatory breast carcinoma, administration of bevacizumab resulted in a significant reduction of phospho-VEGFR2, which was coupled with a marked increase in tumor cell apoptosis, but no significant change in proliferation (Wedam *et al*., 2006). In a phase I trial of a VEGFR2-binding di-Fab fragment, biopsy data were compatible with the proposed mechanism of action (Ton *et al*., 2007). However, such reports are very infrequent for at least two reasons: (a) detection of phosphorylated proteins requires extremely rapid tissue preservation to avoid dephosphorylation of receptors and (b) limited choice of antibodies that bind with sufficient specificity to phosphorylated VEGFR2. Whether a validated biomarker assay of antiphosphorylated VEGFR2 could be used successfully in a multisite study remains to be established.

B. Imaging as a Biomarker

Early clinical trials of VEGF inhibitors sought pharmacological proof of concept by examining changes in the tumor vasculature, predominantly through the use of MRI, which is a technology that is noninvasive, sensitive, and avoids ionizing radiation. Of all the biomarkers that have been tested in trials of VEGF inhibitors, the most consistent findings have been achieved with dynamic contrast-enhanced MRI (DCE-MRI). Although many of these studies were small and confounded by interpatient heterogeneity, overall data show that patients whose tumors undergo at least a 50% reduction in

DCE-MRI parameters attain stable disease or a better response (Murukesh *et al.*, 2010). Thus, DCE-MRI perhaps holds the greatest promise as a biomarker associated with responses to VEGF inhibitors.

Recent interest in MRI techniques that do not require contrast has highlighted blood oxygenation level-dependent (BOLD) imaging and arterial spin labeling (ASL). ASL is a technique in which protons entering the zone of interest are magnetized and was developed for imaging the vasculature of the brain. Although initial results with ASL in patients treated with VEGF inhibitors have shown promise as a potential biomarker (de Bazelaire *et al.*, 2008), ASL is technically challenging and usually requires 3T MRI machines. BOLD imaging, a technique that relies on the paramagnetic effects of deoxyhemoglobin, can be used to provide information on the oxygenation status of the patient's tumor and in particular the oxygen status in tumor vessels.

VI. CONCLUSION AND PERSPECTIVES

The identification of the VEGF/VEGFRs pathway as an important regulator of the angiogenesis process has prompted considerable research into its role in the pathogenesis of cancer. Continued progress has been made in the identification and characterization of new VEGF ligands and receptors, as well as their respective function, roles, and regulatory mechanisms. Clinical trials with anti-VEGF agents have initially generated great enthusiasm for the potential universal application of this novel therapeutic approach to human cancers. However, the premise that the efficacy of antiangiogenic agents would not be limited by the inevitable occurrence of drug resistance has turned out to be a hopeful but incorrect prediction. Clearly, a better understanding of the VEGF/VEGFR family and their role in tumor angiogenesis is necessary to improve treatment outcome and design appropriate combination strategies. Identification of biomarkers predictive of response is essential to select patients that might respond to therapy. The rapid translation of promising and validated hypothesis from preclinical models to the clinical setting may be another way to expedite the development of more effective and desperately needed therapeutic strategies.

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