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 pathophysiological 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
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. © 2012 Elsevier Inc.

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.
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

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**Table 1** Functions, Binding Properties, and Biological Implications of VEGFs

<table>
<thead>
<tr>
<th>VEGF isoform</th>
<th>Receptor</th>
<th>Coreceptor</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA165</td>
<td>VEGFR1, VEGFR2</td>
<td>NRP1, NRP2</td>
<td>Angiogenesis (permeability, survival, migration of EC)</td>
</tr>
<tr>
<td>VEGFA121</td>
<td>VEGFR1, VEGFR2</td>
<td>NRP1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Angiogenic/antiangiogenic properties&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VEGFA145</td>
<td>VEGFR1, VEGFR2</td>
<td>NRP2</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>VEGFA&lt;xxx&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>VEGFR1, VEGFR2</td>
<td>NRP1</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>VEGFB</td>
<td>VEGFR1</td>
<td>NRP1</td>
<td>Fatty acid uptake in EC of the heart</td>
</tr>
<tr>
<td>VEGFC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>VEGFR3 (VEGFR2)</td>
<td>NRP2</td>
<td>Lymphangiogenesis</td>
</tr>
<tr>
<td>VEGFD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>VEGFR3 (VEGFR2)</td>
<td>NRP2</td>
<td>Lymphangiogenesis</td>
</tr>
<tr>
<td>PIGF</td>
<td>VEGFR1</td>
<td>NRP1, NRP2</td>
<td>Inflammatory cell recruitment</td>
</tr>
</tbody>
</table>

Abbreviations: EC, endothelial cells.
<sup>a</sup>VEGFA121 binds NRP1 but does not bridge to VEGFRs (Pan et al., 2007).
<sup>b</sup>VEGFA121 has been described as antiangiogenic (Nowak et al., 2008).
<sup>c</sup>Processed.
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 NH2-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

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

I. 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>
<thead>
<tr>
<th>Therapeutic agent</th>
<th>Type</th>
<th>Target</th>
<th>Clinical development</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ramucirumab/IMC-1121B</td>
<td>mAb</td>
<td>VEGFR2</td>
<td>Phase II/III</td>
<td>Spratlin (2011)</td>
</tr>
<tr>
<td>MF-1/IMC-18F1</td>
<td>mAb</td>
<td>VEGFR1</td>
<td>Phase I</td>
<td>Wu et al. (2006b)</td>
</tr>
<tr>
<td>CDP791</td>
<td>PEG di-Fab conjugate</td>
<td>VEGFR2</td>
<td>Phase II</td>
<td>Youssoufian et al. (2007)</td>
</tr>
<tr>
<td>VEGF-Trap/aflibercept</td>
<td>Fusion protein</td>
<td>VEGFA, PIGF</td>
<td>Phase II/III</td>
<td>Teng et al. (2010)</td>
</tr>
<tr>
<td>VEGFAS/Veglin SU11248/sunitinib (Sutent)</td>
<td>Oligonucleotide</td>
<td>VEGFA, VEGFC, VEGFD</td>
<td>Phase I</td>
<td>Approved in 2006 (GIST and RCC)</td>
</tr>
<tr>
<td>Pazopanib (Votrient)</td>
<td>RTKI</td>
<td>VEGFR1–3, PDGFR, Flt-3, c-kit</td>
<td>Approved in 2009 (RCC)</td>
<td>Sternberg et al. (2010)</td>
</tr>
<tr>
<td>AG013736/axitinib</td>
<td>RTKI</td>
<td>VEGFR1–3, PDGFR, c-kit</td>
<td>Phase II/III</td>
<td></td>
</tr>
<tr>
<td>AZD6474/vandetanib (Zactima)</td>
<td>RTKI</td>
<td>VEGFR1–3, EGFR, RET</td>
<td>Phase II/III</td>
<td></td>
</tr>
<tr>
<td>AZD2171/cediranib (Resentin)</td>
<td>RTKI</td>
<td>VEGFR1–3, c-kit</td>
<td>Phase II/III</td>
<td></td>
</tr>
<tr>
<td>Brivanib alanilate</td>
<td>RTKI</td>
<td>VEGFR2, FGFR1</td>
<td>Phase II/III</td>
<td>Diaz-Padilla and Siu (2011)</td>
</tr>
<tr>
<td>AV-951/tivozanib</td>
<td>RTKI</td>
<td>VEGFR1–3, PDGFR</td>
<td>Phase II/III</td>
<td>De Luca and Normanno (2010)</td>
</tr>
<tr>
<td>PTK787/vatalanib</td>
<td>RTKI</td>
<td>VEGFR1–3, PDGFR, c-kit</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>AE941/Neovastat</td>
<td>Shark cartilage component</td>
<td>VEGF/VEGFR binding, MMP2, MMP9</td>
<td>Phase II/III</td>
<td></td>
</tr>
</tbody>
</table>

**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.

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.

I. 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
et al., 2009, 2010), (5) invasion/migration, (6) unfolded protein response (UPR) (Wouters and Koritzinsky, 2008), (7) autophagy (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 bevacizumab 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,

<table>
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<tr>
<th>Pathway</th>
<th>Target</th>
<th>Agents</th>
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<tr>
<td>Hypoxia</td>
<td>Hypoxia-activated cytotoxin</td>
<td>Tirapazamine</td>
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<tr>
<td>HIF-1 inhibitors</td>
<td>HIF-1α mRNA expression</td>
<td>EZN-2968, Aminoflavone</td>
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<td>HIF-1α protein synthesis</td>
<td>Topotecan, EZN-2208, Cardiac glycosides, PX-478, Temsirolimus, Everolimus</td>
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<td></td>
<td>HIF-1α degradation</td>
<td>17AAG/17DMAG, HDAC inhibitors</td>
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<td>HIF-1α DNA binding</td>
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<td>HIF-1α transcriptional activity</td>
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<td>Metabolism</td>
<td>Hexokinase 2</td>
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<td>Invasion and migration</td>
<td>Met/ALK</td>
<td>Crizotinib</td>
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<td>MET/VEGF</td>
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<td>UPR and autophagy</td>
<td>HSP90</td>
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**Table III** Examples of Pharmacological Strategies to Target Hypoxic Cells
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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REFERENCES


