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The VEGF family in cancer and antibody-based strategies for their inhibition

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Abbreviations: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; EC, endothelial cell; Nrp, neuropilin; VEGFR, vascular endothelial growth factor receptor; HSP, heparin-sulfate proteoglycan; ECM, extracellular matrix; uPA, urokinase type of plasminogen activator; Flt, fms-like tyrosyl kinase; Flk, fetal liver kinase; 5-FU, 5-fluorouracil; LV, leucovorin; ECOG, eastern cooperative oncology group; OS, overall survival; PFS, progression-free survival; CALGB, cancer and leukemia group B; Fab, fragment antigen binding

Angiogenesis is required in normal physiological processes, but is also involved in tumor growth, progression and metastasis. Vascular endothelial growth factor (VEGF), a primary mediator of angiogenesis in normal physiology and in disease, and other VEGF family members and their receptors provide targets that have been explored extensively for cancer therapy. Small molecule inhibitors and antibody/protein-based strategies that target the VEGF pathway have been studied in multiple types of cancer. This review will focus on VEGF pathway targeting antibodies that are currently being evaluated in pre-clinical and clinical studies.

Introduction

Angiogenesis is a tightly regulated process responsible for the development of new blood vessels from a pre-existing vascular network. During development and normal physiological processes such as wound healing and the menstrual cycle, angiogenesis is regulated by endogenous activators and inhibitors. Within adult animals, the levels of endogenous mediators are balanced and endothelial cells (ECs) are largely quiescent.[1,2] In pathological settings, such as age-related macular degeneration, rheumatoid arthritis, diabetic retinopathy and tumor growth and metastasis, angiogenesis is critical for disease progression.[2,3] A key step in tumor development, the 'angiogenic switch' occurs when endogenous activators of angiogenesis outweigh endogenous inhibitors, thereby shifting the balance of angiogenic mediators and stimulating angiogenesis. This results in increased blood vessel formation, which supplies growing tumors with necessary oxygen and nutrients for sustained growth;[4] however the resulting vasculature is disorganized and poorly structured, leading to chaotic blood flow and leaky blood vessels.[5,6] Although dysfunctional when compared to the hierarchical, well-structured vascular network found in normal tissue,[7,8] tumor vasculature is nonetheless essential for continued tumor growth. In the absence of a blood vascular network, tumors are restrained in size due to limits in the diffusion of oxygen.[9] In 1971, Judah Folkman was the first to hypothesize the potential therapeutic benefit of targeting tumor angiogenesis.[9] The vascular endothelial growth factor (VEGF) family of proteins are key regulators of normal and tumor angiogenesis and so provide attractive targets for anti-cancer therapies. This review will focus on antibody-based strategies to target the VEGF pathway in tumors.

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The VEGF Family

There are five members of the human VEGF family: VEGF-A (referred to in this review as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF).[2,3] In addition, multiple isoforms of VEGF, VEGF-B and PlGF are generated through alternative splicing of pre-mRNA.[1] The VEGF family ligands interact with the receptor tyrosine kinases VEGF receptor-1 (VEGFR1), VEGFR2 and VEGFR3. VEGF family interaction with VEGFRs is also regulated by the non-enzymatic co-receptors neuropilin (Nrp)-1 and Nrp2.[1]

The *VEGF* gene contains eight exons and seven introns.[10,11] VEGF binds to VEGFR1, VEGFR2, Nrp1 and Nrp2.[1] VEGF induces vascular permeability[12] and also functions as an EC mitogen and survival factor,[13-15] and an inducer of EC cell and monocyte migration.[16,17] Alternative splicing of *VEGF* yields nine different isoforms in total and four major isoforms: $VEGF_{121}$, $_{165}$, $_{189}$ and $_{206}$.[18] The bioavailability of the different VEGF isoforms is mediated by their expression of heparin sulfate proteoglycan (HSP)-binding domains, encoded on exons 6a, 6b and 7.[19,20] These domains have strong affinity for proteoglycans found on cell plasma membranes or within the extracellular matrix (ECM), thereby restricting the diffusion of larger isoforms of VEGF.[21] Release of VEGF from the ECM and cell membrane allows for VEGF-mediated activity and signaling. The proteolytic release

of VEGF is mediated by the extracellular proteases plasmin,[22] urokinase type of plasminogen activator (uPA)[23] and matrix metalloproteinases.[24-26] Proteolytic release of VEGF is induced by remodeling and microenvironment cues elicited during physiological and pathologic angiogenesis.[27]

The *VEGF-B* gene contains seven exons that undergo alternative splicing to produce two isoforms, VEGF-B_{167} and VEGF-B_{186}.[28] VEGF-B binds to both VEGFR1 and Nrp1.[1] The overall function of VEGF-B remains unclear, with suggested roles in heart function in adults, but not in developmental angiogenesis or cardiovascular development since *VEGF-B* null mice are viable despite some abnormalities in cardiac conduction.[29]

The *VEGF-C* gene is made up of eight exons, but does not undergo alternative splicing. Mature VEGF-C binds to VEGFR2 and VEGFR3 and is involved in developmental lymphangiogenesis and the maintenance of adult lymphatic vasculature.[30] *VEGF-C* null mice are embryonic lethal and heterozygous *VEGF-C* loss is characterized by lymphedema from defective development of the lymphatic vasculature.[31] Interestingly, VEGF-C is not required for blood vessel development since vessels appeared normal in *VEGF-C* null animals.[31]

VEGF-D is composed of seven exons and is found on the X chromosome.[32] Mature VEGF-D binds to both VEGFR2 and VEGFR3 as a non-covalent homodimer.[33] Knock out studies in mice suggest that VEGF-C, and perhaps other growth factors, are capable of substituting for VEGF-D function, as *VEGF-D* null mice are viable and have a normal lymphatic vasculature during development and in the adult.[34]

The last member of the human VEGF family is PlGF. The *PlGF* gene contains seven exons that generate four different isoforms by alternative splicing.[35-37] These isoforms are primarily expressed in the placenta, but are also found within the heart, retina, skin and skeletal muscle.[1] There is reduced vascularization of the corpus luteum and retina in *PlGF* null mice, but these animals are viable.[38]

The VEGF Receptors

There are three receptor tyrosine kinases that mediate the angiogenic functions of VEGF family members: VEGFR1, VEGFR2 and VEGFR3. Although these receptors potentiate diverse downstream functions, they are structurally very similar. The VEGF receptors each contain a seven member immunoglobulin-like domain extracellular region, a single transmembrane domain segment, a juxtamembrane segment, a split intracellular protein-tyrosine kinase domain, and a carboxyterminal tail.[1]

VEGFR1, also known as fms-like tyrosyl kinase-1 (Flt-1), binds VEGF, VEGF-B and PlGF.[39-42] Alternative splicing of *VEGFR1* produces a soluble form of the receptor (sVEGFR1) that contains the first six of the seven immunoglobulin domains, and binds to and inhibits the function of VEGF.[43] VEGFR1 can function as a decoy receptor, utilizing its strong affinity for VEGF (approximately 10 times stronger than that of VEGFR2 for VEGF) to sequester the ligand, preventing it from signaling through other receptors.[17] Despite the strong binding affinity of VEGFR1 to VEGF, the kinase activity of this receptor is weak making it difficult to evaluate levels of VEGFR1 auto-phosphorylation in cells that have not been engineered to express high levels of the receptor.[17] VEGFR1 is essential during development. *VEGFR1* null animals are embryonic lethal, characterized by ECs that do not form a structured, organized vascular network.[44] Interestingly, mice that do not express the tyrosine kinase domain of VEGFR1 but retain the ligand-binding extracellular domains and the transmembrane segment (*VEGFR1-TK*$^{-/-}$) are viable, emphasizing the importance of ligand sequestration in VEGFR1 function.[45] The mutant phenotype resulting from *VEGFR1* loss in embryonic stem cell-derived blood vessels can be rescued with VEGFR2 small molecule inhibitors.[46] Although VEGFR1 signaling remains unclear, there is support for the involvement of the receptor in hematopoiesis,[47,48] the migration of monocytes and the recruitment of bone marrow-derived progenitor cells.[16,49] VEGFR1 has also been implicated in the paracrine release of growth factors from ECs[50] and inducing VEGF-B-mediated EC expression of matrix metalloproteinase-9, uPA and plasminogen activator inhibitor-1, molecules important for ECM degradation that can potentiate VEGF release and cell migration.[41] In addition, VEGF binding to VEGFR1 has been shown to induce SHP-1 phosphatase activity that in turn reduced VEGFR2 phosphorylation levels.[51] These data support VEGFR1 functioning to negatively regulate activity of VEGFR2, which could have important implications for targeting the VEGF pathway within tumors.

VEGFR2 [Fetal liver kinase-1 (Flk-1)/Kinase Domain-containing Receptor (KDR)] is the predominant mediator of VEGF-induced angiogenic signaling.[52-54] Functions of VEGFR2 include EC survival, migration, proliferation and vascular permeability.[1,55,56] VEGFR2 binds all VEGF isoforms, VEGF-C and VEGF-D. Although VEGR2 has a lower affinity for VEGF than VEGFR1, it has a stronger kinase activity. When VEGF binds VEGFR2, it induces receptor dimerization and trans auto-phosphorylation.[1] The predominant phosphorylation sites on VEGFR2 occur on tyrosine 1175 and 1214, inducing signaling cascades through PI3K, AKT, PLCγ, p38 MAPK and p42/44 MAPK.[1,55,57] Ebos et al. identified a soluble, circulating form of VEGFR2, and Albuquerque et al. recently found that this soluble receptor is a distinct splice variant that inhibits lymphangiogenesis.[58,59] Recently, VEGFR2 expression by macrophages has been demonstrated to mediate macrophage infiltration in tumor bearing animals.[60] *VEGFR2* null mice are embryonic lethal between day E8.5-9.5. These animals have severe defects in endothelial and hematopoietic cell development with no organized blood vessel found at any point within the developing embryo or the yolk sac.[61]

VEGFR-3 (Flt-4) binds both VEGF-C and VEGF-D and functions in the remodeling of embryonic primary capillary plexus, with sustained roles in adult angiogenesis and lymphangiogenesis.[62-64] *VEGFR3* null mice are embryonic lethal at day E9.5 and display cardiovascular failure as a result of the abnormal structure and organization of large vessels that leads to defective vessel lumens and an accumulation of fluid within the pericardial cavity.[65] In humans, inactivating mutations within the catalytic loop of the VEGFR3 kinase domain cause Milroy disease, a hereditary

form of lymphedema where defective lymphatic vessels cause chronic swelling of the extremities.[66] The lymphatic abnormalities of Milroy disease and the phenotype of *VEGFR3* null mice, suggest that VEGFR3 functions first in the development of the cardiovascular system and later in the lymphatic vasculature in adults.[1]

The two non-enzymatic co-receptors for the VEGF family, Nrp1 and Nrp2 were first identified as receptors for semaphorins, which function during neurogenesis.[1,67,68] Structurally, the Nrps have a large extracellular region, a transmembrane segment and a short intracellular domain that apparently does not function catalytically, but may serve as a binding site for other co-receptors or downstream signaling molecules.[69] Nrp1 binds VEGF isoforms with HSP-binding domains, PlGF and both VEGF-B isoforms and potentiates signaling through VEGFR2.[70-72] Nrp1 functions during vascular development and in angiogenesis as demonstrated by genetic modifications in mouse models. *Nrp1* overexpressing mice are embryonic lethal due to hemorrhaging and excessive capillary and blood vessel formation.[73] *Nrp1* null mice are embryonic lethal between days E10.5-12.5 resulting from nervous system and cardiovascular abnormalities.[74] Nrp2 binds to $VEGF_{145,165}$, VEGF-C and PlGF.[75,76] Nrp2 functions during lymphatic development as indicated by *Nrp2* null mice that are normal and display phenotypically normal vasculature but have a severe reduction in small lymphatic vessels.[77,78] Additional work evaluating the role of Nrp1 and Nrp2 by Takashima et al. demonstrated that *Nrp1 Nrp2* double null mice have avascular yolk sacs and are embryonic lethal at day E8.5. Further, $Nrp1^{+/-}$ $Nrp2^{-/-}$ or $Nrp1^{-/-}$ $Nrp2^{+/-}$ mice are embryonic lethal at day E10-0.5 with severe angiogenic abnormalities in both the yolk sac and the embryo and an overall reduction in embryo size.[79] These mutant embryos had a similar phenotype to *VEGF* or *VEGFR2* null mice, highlighting the importance of the Nrps in embryonic blood vessel development.

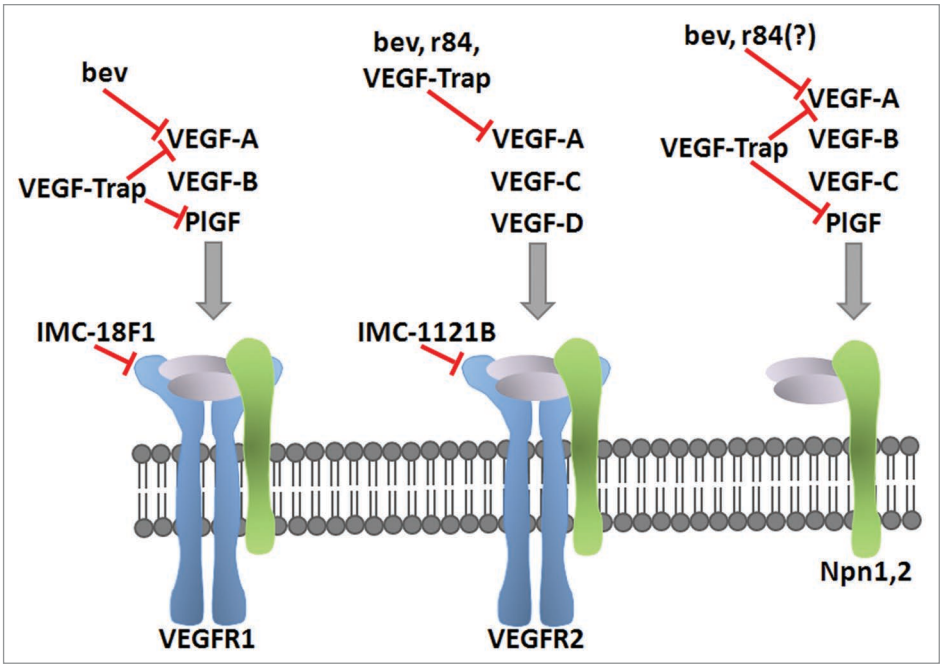

Figure 1. Blockade of the VEGF pathway with mAbs. The specificity of the VEGF family ligands for the VEGF receptors and coreceptors are shown. The clinically-relevant mAbs targeting the anti-VEGF pathway discussed in this review are placed based on their blockade of VEGF ligand or receptor. The ligand-binding antibodies bevacizumab (bev), r84, and VEGF-Trap inhibit ligand binding to the indicated receptor. IMC-18F1 and IMC-1121B bind VEGFR1 and VEGFR2 respectively, and prevent ligand binding.

Monoclonal Antibodies as Therapeutic Agents

The clinical use of monoclonal antibodies (mAbs) to treat cancer and other diseases is well established. There are currently more than 20 mAbs approved by the Food and Drug Administration (FDA) for therapeutic use.[80] Rituximab (Rituxan®, Genentech, Inc.), a chimeric mAb directed against CD20 used to treat patients with non-Hodgkin lymphoma, was the first anti-cancer mAb to gain FDA approval. Since then, mAbs targeting key pathways in tumor survival have been developed and successfully used clinically to treat patients including trastuzumab (Herceptin®, Genentech, Inc.) which recognizes the HER2/neu cell surface receptor expressed in 15–20% of breast cancers, and cetuximab (Erbitux®, ImClone Systems) which binds epidermal growth factor receptor (EGFR) and is approved for the treatment of metastatic colorectal cancer and head and neck cancer.[80]

Tumor angiogenesis is a hallmark of cancer, and is required for continued growth and metastasis.[81] VEGF is a major mediator of angiogenesis in normal physiology and in cancer. There is an upregulation of VEGF family members and the VEGF receptors in many different tumors,[1] providing a target for cancer therapy. This target has been utilized by investigators, leading to the development of anti-angiogenic small molecule tyrosine kinase inhibitors such as sorafenib (Nexavar®, BAY 43-9006, Bayer Pharmaceuticals Corp.) and sunitinib (Sutent®, SU11248, Pfizer, Inc.,),[1] and a number of mAbs against VEGF ligands and receptors. The latter strategy will be the focus of the rest of this review (refer to **Figs. 1 and 2** for a summary of the anti-VEGF pathway and the mAbs targeting this pathway discussed here).

Antibodies and Fusion Proteins Targeting VEGF

Bevacizumab (Avastin®, Genentech, Inc.). In 1993, the Ferrara et al. introduced a mouse anti-human VEGF mAb (A.4.6.1) that inhibited the growth of A673 rhabdomyosarcoma, G55 glioblastoma and SK-LMS-1 leyomiosarcoma tumor cell line xenograft models in vivo but not in vitro,[82] illustrating an indirect role of VEGF signaling in tumor survival. The efficacy of A.4.6.1 in controlling the growth of a number of tumor cell lines in

Drug	Target	Inhibition	Clinical Status
Bevacizumab hz mAb	hu VEGF	VEGF binding to VEGFR1,R2	Approved for mCRC, NSCLC, mBC, mRCC, glioblastoma
2C3 ms mAb	hu VEGF	VEGF binding to VEGFR2	Effective tool in pre-clinical studies
r84 hu mAb	hu,ms VEGF	VEGF binding to VEGFR2	Effective in pre-clinical studies; clinical trial candidate
VEGF-Trap hu Fc fp	hu,ms VEGF PlGF	VEGF,PlGF binding to VEGFR1,R2	Effective in pre-clinical studies; in clinical trials
MF1/IMC-18F1 rt/hu mAb	ms/hu VEGFR1	VEGFR1 activation by PlGF, VEGF,-B	Effective in pre-clinical studies; in clinical trials
DC101/IMC-1C11 rt/hz mAb	ms/hu VEGFR2	VEGFR2 activation by VEGF,-C,-D	Effective in pre-clinical studies; immunogenic in clinical trials
IMC-1121B hu mAb	hu VEGFR2	VEGFR2 activation by VEGF,-C,-D	Effective in pre-clinical studies; in clinical trials

Figure 2. Current anti-angiogenic mAbs with applications in cancer therapy. hz, humanized; hu, human; ms, mouse; rt, rat; fp, fusion protein; mCRC, metastatic colorectal cancer; NSCLC, non-small cell lung cancer; mBC, metastatic breast cancer; mRCC, metastatic renal cell carcinoma.

xenograft models in immunocompromised mice was described subsequently in a number of publications.[83-85] A.4.6.1 underwent site-directed mutagenesis resulting in the humanized mAb bevacizumab (Avastin®, Genentech Inc.) that binds human VEGF with an affinity similar to A.4.6.1 ($K_d \approx 0.5$ nM).[86] Structural analysis of VEGF bound to bevacizumab Fab gave insight to the specificity and mechanism of the mAb. Gly88 of human VEGF is required for bevacizumab to binding. In mouse and rat VEGF this residue is replaced by a serine, disrupting the binding of bevacizumab to rodent VEGF.[87] Further, bevacizumab Fab bound to VEGF does not induce structural conformational changes in the ligand, suggesting that bevacizumab is effective by sterically disrupting the ability of VEGF to interact with its receptors.[87-89] Preclinical safety evaluations of bevacizumab were performed in *Macaca fascicularis*. Some treatment-induced changes were observed, including suppressed angiogenesis within the female reproductive tract, but all adverse effects were reversible with the cessation of bevacizumab treatment.[90]

In 1997, bevacizumab entered Phase 1 clinical trials and was found to be relatively non-toxic and well-tolerated, without exacerbating toxicities related to patient chemotherapy treatment.[91,92] In subsequent Phase 2 and 3 trials, the primary toxicities induced following bevacizumab therapy were hypertension, proteinuria and gastrointestinal perforations, which occurred more frequently in patients with colorectal cancer or metastases within the gastrointestinal tract. Bleeding events are also a concern with bevacizumab therapy and can be fatal with some tumor histologies, such as in squamous non-small cell lung cancer.[93] These Phase 2 and 3 clinical trials led to the approval of bevacizumab, the first anti-angiogenic strategy approved for cancer therapy, for the treatment of five different cancers to date. In metastatic colorectal cancer (mCRC), bevacizumab in combination with IFL chemotherapy [ironotecan, 5-fluorouracil (5-FU) and leucovorin (LV)] significantly increased median duration of patient survival by 4.7 months as compared to IFL treatment alone, leading to the approval of this regimen in 2004 as a first-line treatment for mCRC.[94] Approval of bevacizumab as a second-line treatment for mCRC in 2006 was the result of the eastern Cooperative Oncology Group E3200 (ECOG E3200) study where bevacizumab plus FOLFOX4 (oxaliplatin, 5-FU and LV) significantly increased patient overall survival (OS) and progression-free survival (PFS) by 2.1 and 2.6 months, respectively as compared to FOLFOX4 treatment alone.[95] In 2006, bevacizumab was also approved as a first-line treatment for non-small cell lung cancer (NSCLC) based on the findings of the ECOG E4599 study where bevacizumab in combination with paclitaxel and carboplatin versus chemotherapy alone significantly increased OS by 2 months and PFS by 1.7 months.[96] The results of the ECOG E2100 study lead to the approval of bevacizumab in 2008 as a first-line therapy for HER2-negative metastatic breast cancer (mBC) where bevacizumab plus paclitaxel failed to increase OS versus paclitaxel alone treatment, but did significantly increase PFS by 5.9 months.[97]

Bevacizumab is not currently recommended for second- or third-line treatment of mBC that has progressed following anthracycline and taxane chemotherapy. This decision was made based on the findings of the AVF2119 study where mBC patients that had previously been treated with anthracycline and taxane had no increased in PFS or OS with bevacizumab in combination with capecitabine versus capecitabine alone.[98] FDA approval of single agent bevacizumab therapy for second-line treatment of glioblastoma in May 2009 was the result of AVF3708g and NCI 06-C-0064E trial that demonstrated durable objective response rates.[99,100] In July 2009, bevacizumab was approved for the treatment of metastatic renal cell carcinoma (mRCC) based on the Hoffmann-La-Roche BO17705 trial where bevacizumab plus interferon (IFN) alfa-2a resulted in a statistically significant 4.8 month increase in PFS versus IFN alfa-2a treatment alone,[101] and the Cancer and Leukemia Group B (CALGB) 90206 trial that demonstrated a statistically significant 3.3 month increase in PFS with combination bevacizumab plus IFN alfa therapy versus IFN alfa alone.[102] Bevacizumab has paved the way for subsequent anti-angiogenic therapies in cancer. A more detailed account of bevacizumab's progress through clinical trials in a number of tumor types is provided in a thorough review by Grothey and Galanis.[93] However, the survival benefits of bevacizumab therapy, although reaching statistical significance, are modest and as mentioned above, are measured in months. These somewhat disappointing results of bevacizumab's clinical efficacy in combination with bevacizumab-mediated toxicity highlight the need to re-evaluate how to best utilize anti-angiogenic therapies in the clinic.

2C3 and r84 (AT001, affitech AS). 2C3 is a murine monoclonal $IgG2_{a,}\kappa$ antibody that binds human VEGF and inhibits VEGF from interacting with VEGFR2, but not VEGFR1. 2C3-mediated blockade of VEGFR2 signaling blocks VEGF-mediated EC growth, VEGFR2 phosphorylation, vascular permeability and inhibits the growth of established human tumor xenografts in immunocompromised mice.[103,104] In addition, 2C3 localizes to pools of VEGF in the tumor stroma and within the perivascular connective tissue of solid human tumors.[103] Since its initial identification, 2C3 has been characterized in numerous tumor xenograft models as an effective inhibitor of tumor growth and angiogenesis and as a modulator of macrophage and immune cell infiltration.[60,105-108]

The success of 2C3 in preclinical models lead to the development of a phenotypically similar, fully human mAb, r84 (AT001, Affitech AS) that was generated by screening a human anti-VEGF single chain variable fragment library for specific 2C3-like properties. 2C3 and r84 cross-block each other in VEGF ELISA assays indicating that the epitope each mAb binds is very similar; however, the exact epitope on VEGF bound by each mAb has not been determined. Efforts are currently underway to solve the structure of VEGF bound by the Fab of r84, which is anticipated to uncover the epitope for r84. r84 binds mouse and human VEGF, but not other VEGF family members, and specifically inhibits VEGF from binding to VEGFR2, but allows VEGF to bind and activate VEGFR1. Through specific VEGF blockade, r84 inhibits VEGFR2-mediated endothelial cell migration and phosphorylation cascades in vitro and controls tumor growth, angiogenesis and immune cell infiltration in vivo.[108-110] Research with 2C3 and r84 demonstrate that inhibition of VEGF-mediated VEGFR2 activation is sufficient to control tumor growth and calls into question the pro-tumorigenic function of VEGFR1. In addition, because r84 binds mouse and human VEGF, it facilitates evaluation of the effects of blocking tumor-derived (human) and stromal (mouse) VEGF in preclinical xenografts models. Therefore r84 should more closely mirror the activity of selective VEGF inhibition in human patients. A mouse chimeric version of r84 (mcr84) has been generated and is a useful tool for studying angiogenesis in immunocompetent animals and in syngenic tumor models.[109] To date, treatment of tumor-bearing mice with r84 has not induced anti-VEGF-related toxicities that have been characterized in other preclinical models using different inhibitors of the VEGF pathway.[111-113] Therefore, the potential efficacy of r84 as a cancer therapeutic for treating cancer patients is highly anticipated.

VEGF-trap (aflibercept, Regeneron Pharmaceuticals, Inc.). The precursor of VEGF-Trap (aflibercept, Regeneron Pharmaceuticals, Inc.), the therapeutic drug currently in Phase 2 and 3 clinical trials, was first developed by trying to harness the strong affinity of VEGFR1 for VEGF to inhibit VEGF-mediated angiogenesis within tumors. A soluble decoy receptor was engineered by fusing the first three immunoglobulin domains of VEGFR1 to the Fc constant region of human IgG1 antibody, creating a forced homodimer, mFlt(1-3)-IgG that bound VEGF and PlGF with high affinity;[114,115] however, this fusion protein had poor pharmacokinetic properties in vivo that required large, frequent doses for efficacy.[116] To improve the half-life without losing affinity, mFlt(1-3)-IgG was modified to contain the second immunoglobulin domain of VEGFR1 and the third immunoglobulin domain of VEGFR2 fused to human IgG1 Fc region, creating the fusion protein known as VEGF-Trap. This fusion protein had improved pharmacokinetics and affinity for VEGF (approximately 1 pM) as compared to the parental mFlt(1-3)-IgG, and effectively inhibited tumor growth and angiogenesis in xenograft models.[117] Pre-clinical trials demonstrated the efficacy of VEGF-Trap in suppressing tumor growth and angiogenesis through its ability to bind both mouse and human VEGF,[118-120] and supported its entry into clinical trials were it is being currently evaluated in a number of cancers and in age-related macular degeneration.

Antibodies Targeting the VEGF Receptors

MF1/IMC-18F1 (ImClone Systems). The rat anti-mouse VEGFR1 IgG1 mAb, MF1 (ImClone Systems) was first shown to suppress tumor and ischemic retinal angiogenesis, as well as inflammation in an autoimmune arthritis model.[49] Kaplan et al. used MF1 to demonstrate VEGFR1 participates in the pre-metastatic niche in animal models, and that blockade of VEGFR1 with MF1 more effectively blocked tumor metastases than did inhibition of VEGFR2.[121] These studies led to the development of a fully human mAb directed against human VEGFR1 (mAb 6.12, IMC-18F1, ImClone Systems) that blocked VEGFR1 signaling in VEGFR1-expressing breast cancer cells lines in vitro,

and inhibited tumor growth in vivo.[122] The final characterization of IMC-18F1 came in 2006. IMC-18F1 bound to VEGFR1 with high-affinity and was able to block VEGFR1 from interacting with VEGF, VEGF-B and PlGF, preventing downstream VEGFR1 signaling and breast cancer cell line growth in vitro and in vivo. Targeting human and mouse VEGFR1 with IMC-18F1 and MF1, respectively, more effectively controlled tumor growth than either agent alone.[123] The safety and dosing of IMC-18F1 is currently being evaluated in a Phase 1 clinical trial.

DC101/IMC-1C11 (ImClone Systems). DC101 is a rat anti-mouse VEGFR2 (Flk-1) mAb that inhibits ligand-induced activation of VEGFR2.[124] In vivo syngenic and xenograft tumor models in mice demonstrated the ability of DC101 to control tumor growth and reduce tumor angiogenesis by targeting EC- or tumor-expressed VEGFR2.[125-129] Since DC101 recognizes only mouse VEGFR2 and therefore is not a candidate for clinical trials, a single chain variable fragment with human VEGFR2 reactivity that was also able to block in vitro VEGFR2 signaling was isolated from a single chain antibody phage display library.[130] This fragment became IMC-1C11 (ImClone Systems), a chimeric mAb that blocked tumor growth and angiogenesis in tumor xenografts and was tested in a Phase 1 clinical trial in colorectal carcinoma patients with liver metastases.[131,132] Although treatment with IMC-1C11 did not induce grade 3 or 4 toxicities, 50% of treated patients developed anti-chimeric antibodies that impeded the future progression of this antibody in the clinic.[132]

IMC-1121B (ramucirumab, ImClone Systems). To develop an anti-human VEGFR2 mAb that would not be immunogenic in clinical trials, ImClone Systems used a human antibody phage display library to isolate VEGFR2-specific human Fab fragments. The resulting best Fab bound to human VEGFR2 with high affinity, and inhibited ligand-induced VEGFR2 activation in endothelial cells.[133,134] Affinity maturation of the best Fab clone and subsequent synthesis of a full length antibody yielded IMC-1121B, a fully human IgG1 mAb with higher affinity for VEGFR2 that was a more potent inhibitor of VEGF-induced VEGFR2 signaling and EC migration in vitro. IMC-1121B also increased the survival of murine xenograft models in vivo more effectively than other anti-VEGFR2 antibodies, including IMC-1C11.[135,136] There are 13 current Phase 1 or 2 clinical trials with IMC-1121B to assess the safety and efficacy of this mAb.

Future Directions

The development of anti-angiogenic therapies was highly anticipated. This therapeutic strategy was hypothesized to avoid the tumor resistance pathways of traditional anti-cancer drugs by targeting the vasculature as opposed to the genetically instable and highly mutagenic tumor cell population. The pre-clinical success of targeting the VEGF pathway using mAb-based therapy further bolstered this hypothesis. However, clinical studies of anti-VEGF strategies in cancer patients have not delivered the level of efficacy anticipated. To date, bevacizumab is the most developed anti-VEGF pathway mAb. Bevacizumab is currently indicated as a first- or second-line treatment in five different tumor types, and is being evaluated in many clinical trials. Therefore, experience with bevacizumab in the clinic provides a working model for the benefits and pitfalls of anti-angiogenic mAb therapies, as well as a benchmark for other anti-angiogenic mAb discussed in this article.

As discussed previously, the results of bevacizumab in Phase 2 and 3 clinical trials have been modest when compared to the success of anti-VEGF therapy in pre-clinical models.[90] In the clinic, responses with bevacizumab as a single agent therapy (in glioblastoma) or in combination with standard chemotherapy (in non-small cell lung cancer, metastatic colorectal cancer, breast cancer and renal cell carcinoma) is measured in a few months correlating with small, albeit statistically significant increases in PFS, and rarely in OS.[93] This differs from some anti-angiogenic small molecule tyrosine kinase inhibitors (TKIs) that do not display improved efficacy when combined with chemotherapy.[137] The differences between anti-angiogenic TKIs and mAbs may be due to functional changes occurring within the tumor in response to the different drugs. Treating tumors with bevacizumab, or other anti-VEGF pathway mAbs counteracts the inherent disorganization and abnormalities of the tumor vasculature. This process has been termed "normalization."[138] The pruned, normalized tumor vasculature achieved with anti-angiogenic therapy has increased pericyte coverage and stability and a reduction in vessel leakiness and interstitial fluid pressure, which in combination improves the subsequent delivery of chemotherapy and other drugs. This process allows for cytostatic anti-angiogenic therapy (e.g., bevacizumab and other anti-VEGF pathway mAbs) combined with cytotoxic chemotherapy to have improved clinical response as compared to either agent alone. This absence of synergy with some VEGFR TKIs and chemotherapy is perhaps due to the targeting of other tyrosine kinase receptors such as PDGFR that can disrupt the normalization process.[137] A better understanding of the normalization process among tumor types would allow for optimization of anti-angiogenic and chemotherapeutic drug delivery schedules, perhaps improving the overall efficacy of these drugs in the clinic.

Bevacizumab therapy is associated with several adverse effects. The most common toxicities are hypertension, proteinuria and bleeding events that result from a loss of homeostatic VEGF signaling and vascular maintenance.[139] Certain histologies, such as squamous non-small cell lung cancer, were more prone to fatal bleeding events, leading to the exclusion of these patients from future studies.[93] In addition, perforations were more frequent in patients with metastatic colorectal cancer, ovarian cancer or metastases within the gastrointestinal tract. Inhibition of VEGFR signaling with sorafenib and sunitinib therapy is also associated with hypertension, proteinuria and bleeding events similar to treatment with bevacizumab. However, there are a host of off-target adverse effects with sorafenib and sunitinib therapy, including skin reactions, hand-foot syndrome, fatigue and diarrhea resulting from TKI inhibition of targets other than the VEGFRs.[139] Based on these patterns, specific blockade of VEGF, PlGF, VEGFR1 or VEGFR2 with r84, VEGF-Trap, IMC-18F1 or IMC-1121B might induce toxicities more similar to bevacizumab rather than sorafenib and sunitinib. It is also possible that VEGFR1 signaling is important for maintaining the homeostatic

function of VEGF, and thus therapies allowing for continued VEGFR1 signaling such as r84 and IMC-1121B may provide a less severe toxicity profile than bevacizumab. In support of this, pre-clinical studies in our laboratory with extended (12 week) treatment of tumor-bearing and non-tumor bearing mice with r84 failed to induce toxicity (unpublished data). Results from IMC-1121B on-going clinical trials and future studies with r84 in the clinic will ultimately answer these questions about differences in toxicities between the VEGF pathway antibodies. Alternatively, the severity or frequency of toxicities between mAbs targeting the VEGF pathway may depend more on the relative affinity of the drug for its target. In pre-clinical studies with mice engineered to express human VEGF, anti-VEGF antibodies of increasing affinity had a greater toxicity induction.[111] Additionally, chemotherapy regimens such as carboplatin and paclitaxel, in combination with bevacizumab or VEGFR TKIs can exacerbate the toxicities of these targeted therapies.[140] Therefore, carefully assessing both drug affinity for its target and chemotherapy doses and regimens is required to control toxicity in patients receiving drugs targeting the VEGF pathway. The distribution of toxicities within mAb strategy and patient groups should be taken into consideration as future anti-VEGF pathway therapies are introduced into the clinic to minimize adverse effects and to monitor for new patterns of toxicity.

With the expanding use of anti-angiogenic therapy in the clinic, it has become more apparent that not all tumors respond or will remain responsive to this treatment option. The inherent non-responsiveness of certain tumors and the development of acquired resistance to therapy following an initial response, has been termed intrinsic and evasive resistance, respectively.[141] Pre-clinical research from a number of investigators has identified several mechanisms mediating resistance to anti-angiogenic therapy. This includes a switch from a primary reliance on VEGF to an alternative growth factor such as fibroblast growth factor, interleukin-8 and ephrins, increased stabilization of existing vessels by improved pericyte coverage, stimulating infiltration of pro-angiogenic immune cells and the co-option of normal vasculature through enhanced tumor invasiveness and metastasis.[141] Patients with intrinsically resistant tumors would have no clinical benefit from anti-angiogenic therapy and therefore should be excluded from these treatments; however, we currently lack effective biomarkers that would allow for the stratification of intrinsically resistant or sensitive tumors, or for the monitoring of tumor response and the development of resistance to therapy.[142]

The identification of biomarkers of response to anti-angiogenic drugs is being actively pursued. In mBC, baseline levels of circulating VEGF have shown promise as a biomarker of response to bevacizumab therapy, but this has not translated to other tumor types such as mCRC or NSCLC.[143] In addition, the utility of baseline VEGF levels as a biomarker of response varies depending on tumor type and therapy regimen (mAb or VEGFR TKI), bringing into question the universal applicability of this marker. Genetic polymorphisms of VEGF and interleukin-8 have been indicated as predictive biomarkers of response to bevacizumab in mBC and ovarian cancer, respectively; however these markers have yet to be validated in other tumor types or in relation to other anti-VEGF pathway therapies.[143] In addition, there are increased levels of circulating PlGF following anti-VEGF therapy that have been positively correlated with improved outcome in bevacizumab-treated rectal cancer patients, although it remains to be seen if this increase in PlGF is predictive or prognostic of response to therapy.[143] A systemic increase in blood pressure has potential as a pharmacodynamic biomarker of the effectiveness of VEGF blockade by bevacizumab and VEGFR TKIs. Recent studies have implicated the degree of hypertension in patients as a diagnostic biomarker of response to VEGF targeted therapy, although this has yet to be validated in large studies.[143]

Although there are several candidates awaiting validation as pharmacodynamic or prognostic biomarkers of anti-angiogenic therapy, including circulating levels of VEGF and PlGF, genetic polymorphisms of angiogenic factors and the degree of hypertension following therapy, we still lack biomarkers predictive of response to therapy that exist for other targeted drugs such as the overexpression of human epidermal growth factor receptor 2 (HER2/*neu*) in BC as a predictive marker of response to trastuzumab therapy. Similar to HER2/*neu* overexpression, predictive markers of response to anti-angiogenic therapy may be tumor-dependent. Our laboratory and others are currently assessing tumor-specific factors in xenograft models while others are evaluating patient samples for potential biomarkers of response. Further studies are required to identify and validate predictive versus prognostic biomarkers and may require carefully designed clinical trials and more frequent collection of patient tumor and serum samples throughout treatment. A better understanding of what dictates patient response and how to monitor resistance could lead to improved efficacy of mAbs targeting the VEGF pathway.

The opportunity to better understand the function of individual components of the VEGF pathway in the tumor microenvironment is afforded by evaluation of the efficacy and biology of the anti-VEGF strategies outlined in this review. As there are very few studies that directly compare anti-VEGF pathway mAbs, it is difficult to say with certainty that one strategy is best or will work for every patient. All of the mAbs discussed in this review target the VEGF pathway; however the specificity of the different mAbs affects the function of these therapies within the tumor microenvironment and provides clues to the potential advantages and disadvantages among the different therapies. It is of particular interest to compare the strategies that are specific to VEGF (bevacizumab and r84) to VEGF-Trap and those that inhibit VEGFR1 (MF1, IMC-18F1) or VEGFR2 (DC101, IMC-1C11, IMC-1121B) directly.

Bevacizumab and r84 are both highly specific for VEGF-A and do not directly interfere with other VEGF family members. r84 is even more selective than bevacizumab due to the fact that it only inhibits VEGFR2 activation, leaving intact VEGFR1 signaling. In endothelial cells, VEGFR1 primarily functions as a negative inhibitor of VEGFR2 signaling by acting as a decoy receptor for VEGF and preventing VEGF from binding to and inducing angiogenesis through VEGFR2. This idea is supported by previously mentioned genetic experiments where loss of VEGFR1 leads to embryonic death due to too many endothelial cells, but

mice expressing only the extracellular domain of VEGFR1 are viable.[44,45] Additionally, VEGF binding to VEGFR1 can stimulate SHP-1 phosphatase to actively reduce levels of VEGFR2 phosphorylation.[51] Further, whereas VEGF activation of VEGFR1 does not alter gene expression, PlGF binding to VEGFR1 in vitro changes the gene expression of more than 50 genes.[144] PlGF may also provide an escape mechanism for anti-VEGF targeted therapy and blocking PlGF directly has been demonstrated to have anti-tumor effects.[145] Treatment with r84 would allow for regulatory signaling through VEGFR1 and could reduce PlGF activation of VEGFR1 as a result of competition with PlGF for VEGFR1 binding. Therefore, VEGF binding to VEGFR1 may be an important negative regulator of tumor angiogenesis that could be harnessed with r84, but not with bevacizumab. VEGF-Trap blocks VEGF from binding to VEGFR1 and VEGFR2 and blocks PlGF from binding to VEGFR1, thereby preventing negative regulation of VEGFR2 activity by VEGFR1 similar to bevacizumab and uniquely controlling VEGFR1 activation by PlGF. Thus the in vivo mechanisms of action of VEGF-Trap may fall somewhere between that of r84 and bevacizumab.

Alternatively, blocking VEGFR1 activity with mAbs has been very effective in VEGFR1 expressing tumors.[122] VEGFR1 activity has also been linked to tumor metastasis and blocking VEGFR1 with MF1/IMC-18F1 reduces tumor growth, demonstrating the potential importance of this receptor in tumor progression and the need to inhibit its function in patients. Despite these data, the overall functions of VEGFR1 remain unclear and the full effects of VEGFR1 blockade are uncertain. However, there is still a strong possibility that VEGFR1 functions as a negative regulator of VEGFR2 and direct targeting of VEGFR1 with MF1/IMC-18F1 may be in effect, inhibiting an inhibitor of angiogenesis, which may be therapeutically counterproductive. Therefore, the results of on-going IMC-18F1 and IMC-1121B clinical trials and entry of r84 into the clinical arena are highly anticipated to elucidate the importance of VEGFR1 signaling in tumor angiogenesis and progression.

As previously mentioned, VEGFR2 is the predominant mediator of VEGF-induced angiogenesis and consequently, blocking functional signaling of this receptor with neutralizing antibodies such as DC101, IMC-1C11 and IMC-1121B is effective at reducing angiogenesis and tumor growth. There is increased expression of VEGFR2 in the tumor microenvironment, which in turn increases the potential of anti-VEGFR2 therapies to specifically target the tumor and not normal tissues. Directly targeting VEGFR2 also prevents receptor activation by other VEGF family members (e.g., VEGF-C, -D), which are not blocked by bevacizumab, r84, or VEGF-Trap. Additionally, the anti-VEGFR2 mAbs do not inhibit VEGFR1 negative regulation of VEGFR2, potentially enhancing blockade of VEGFR2 function. However, the anti-VEGFR2 mAbs will also block soluble VEGFR2, a natural inhibitor of lymphangiogenesis.[59] This effect is a potential disadvantage of directly targeting VEGFR2, given the importance of the lymphatic vasculature in metastasis.[146] Infiltration of immune cells such as macrophages within tumors can promote tumor survival and progression.[147] Macrophages in tumor bearing animals express VEGFR2, and blockade of this receptor has been demonstrated to reduce macrophage migration and infiltration in tumors.[60,109] Therefore, targeting VEGFR2 directly can negatively affect tumor growth and metastasis by reducing the population of tumor-promoting immune cells within the tumor microenvironment.

The anti-VEGFR antibodies have a broader specificity profile given that these agents interfere with signaling pathways stimulated by multiple members of the VEGF family. Thus far, it is unclear if a broader specificity anti-VEGF agent is more effective than bevacizumab or r84. In fact, a recent direct comparison of mouse chimeric r84 to sunitinib and a peptoid that binds and inhibits VEGFR1 and VEGFR2 demonstrated that r84 was as or more effective in controlling tumor growth in two models of breast cancer in immunocompetent mice.[109] This study also evaluated the immunological phenotype of tumors under therapy and found that in general, treatment of r84 resulted in fewer immunosuppressive cells (e.g., myeloid-derived suppressor cells, T_{reg}, immature dendritic cells) in the tumor microenvironment. These cell based changes are likely the result of an altered cytokine profile after therapy.[109] To our knowledge similar studies have not been performed with the other anti-VEGF agents discussed here. Species specificity issues and other inherent challenges with pharmaceutical-based novel therapies preclude a head-to-head test of these leading anti-VEGF strategies in pre-clinical models. Thus we are forced to make assumptions regarding the potential superiority of one agent over another based on the efficacy observed in similar models and the biology of the therapy employed.

In reality, arguing the benefits and shortcomings of the individual mAb-based strategies available for targeting the VEGF pathway in cancer may be short-sighted. Selectively targeting angiogenesis in patients will most likely require an arsenal of therapeutics and the best strategy may very well depend upon the tumor type, stage, histology or may be entirely patient specific. This again highlights the need for biomarkers that can predict a patient's response to anti-angiogenic therapy, as well as the need for an array of selective therapies to improve patient survival by inhibiting tumor angiogenesis.

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Declaration of Interest

R.A.B. is a consultant for Peregrine Pharmaceuticals, which is developing the r84 antibody.

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