

Biological Aspects and Binding Strategies of Vascular Endothelial Growth Factor in Renal Cell Carcinoma

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Abstract Vascular endothelial growth factor (VEGF) is a key mediator in the pathogenesis of renal cell carcinoma (RCC). VEGF is up-regulated in clear cell RCC as a result of loss of the *von Hippel-Lindau* tumor suppressor gene and subsequent activation of the hypoxia response pathway. VEGF expression drives the migration and proliferation of endothelial cells to support the extensive angiogenesis in RCC. Strategies have been developed to bind and neutralize VEGF and have been investigated in RCC with promising results. Bevacizumab, a VEGF ligand-binding antibody, has shown prolonged time-to-progression versus placebo in treatment-refractory RCC patients and is being investigated currently in multiple RCC settings. VEGF-Trap is also a VEGF binding molecule with ongoing investigation in RCC.

A growing understanding of the underlying molecular biology of renal cell carcinoma (RCC) has identified several pathways pertinent to the pathophysiology of clear cell RCC. Activation of the hypoxia response pathway by mutations of the *von Hippel-Lindau* (*VHL*) tumor suppressor gene results in the transcriptional activation of a variety of genes important in tumor progression, including vascular endothelial growth factor (VEGF). VEGF is the most potent promoter of tumor-associated angiogenesis and as such has been exploited as a target for therapeutic inhibition. Inhibitors that target the VEGF protein have shown activity in metastatic RCC and are undergoing additional testing in many clinical settings for this disease.

RCC Biology Leading to VEGF Overexpression

In sporadic (noninherited) RCC, *VHL* gene allele deletion (loss of heterozygosity) has been shown in 84% to 98% of sporadic renal tumors, and mutation in the remaining *VHL* allele has been observed in ~50% of clear cell RCC tumors (1–5). *VHL* gene inactivation in RCC may also occur through gene silencing by methylation, which occurs in an additional 10% of clear cell RCC tumors (3, 6–8). Taken together, these data suggest that biallelic *VHL* gene inactivation

occurs in most clear cell RCC tumors and, in fact, suggests a role for this event in renal tumor initiation. There is no evidence that non-clear cell RCC tumors harbor mutations in the *VHL* gene (9).

The *VHL* gene encodes a 213-amino acid protein (pVHL), which plays an integral role in regulating the normal cellular response to hypoxia. In conditions of normoxia and normal *VHL* gene function, pVHL is the substrate recognition component of an ubiquitin ligase complex that targets a family of protein transcription factors, the hypoxia-inducible factors (HIF; HIF1 α and HIF2 α) for proteolysis (10–12). In conditions of cellular hypoxia, the pVHL-HIF interaction is disrupted as a result of loss of oxygen-dependent hydroxylation on HIF, leading to stabilization of the HIF transcription factors (13–15). In the absence of proper *VHL* function, one or both HIF factors are constitutively stabilized. These HIF factors promote the transcription of a large repertoire hypoxia-inducible genes, most notably VEGF (16, 17).

Vascular Endothelial Growth Factor

VEGF (also referred to as vascular permeability factor or VEGF-A) functions as an important regulator of endothelial cell physiology. VEGF is a secreted mitogen and the dominant factor that regulates angiogenesis in both normal development and tumor growth (18, 19). This factor exerts its growth-promoting influence not on the tumor cells themselves (except in rare circumstances) but instead on the vascular endothelial cells, promoting both proliferation and new vessel formation (Table 1). This ligand-mediated mitogenic response occurs in many physiologic processes, in which expansion of blood and nutrient delivery is necessary, including development, wound healing, maternal-fetal placenta formation, and uterine decidual formation as well as pathologic processes, such as diabetic retinopathy, tissue recovery from ischemic insult or injury, and cancer. The process of tumor angiogenesis, like other processes that integrate endothelial cell vascular network expansion, is dependent on secreted VEGF to promote existing vessel ingrowth into the tumor and expansion of vascular networks by neovascularization.

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Received 8/22/06; revised 10/17/06; accepted 10/23/06.

Presented at the Second Cambridge Conference on Innovations and Challenges in Renal Cancer, March 24–25, 2006, Cambridge, Massachusetts.

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doi:10.1158/1078-0432.CCR-06-2110

Table 1. Activity of VEGF and functional effect on endothelial cells

Activity	Functional effect
Mitogen-mediated cell proliferation	Expansion of vascular surface
Increased cell permeability	Generation of a primitive leaky vasculature
Endothelial cell migration	Mobilizing endothelial networks to areas of need
Inhibition of apoptosis	Promote cell survival during vascular network expansion
Endothelial cell migration	Preparation of cells for vascular network expansion

VEGF gene expression is regulated by a variety of factors, including growth factors, p53 mutation, estrogen receptor, thyroid stimulating hormone, nitric oxide, and hypoxia. Inappropriate activation of the hypoxia response pathway, as discussed elsewhere, is a major mechanism of VEGF transcriptional regulation in RCC (20, 21).

VEGF Function

Secreted VEGF protein is actually composed of a large family of alternatively spliced isoforms (22). The primary activity of these molecules is as a potent mitogen for capillary and vascular endothelial cells (Table 1; refs. 18, 23). VEGF acts additionally to stimulate elongation, network formation, and branching morphogenesis (24). It is perhaps the only chemokine with direct activity to stimulate proliferation of endothelial cells, promoted through binding and dimerization of cell surface transmembrane receptors. The receptors FLT1 [VEGF receptor (VEGFR) 1] and KDR/FLK1 (VEGFR2) are expressed on endothelial cells and comprise the major classifications of receptors that bind VEGF. The addition of an anti-VEGF neutralizing antibody abolished vascular network formation, thus establishing the role of this important protein in network formation (24).

In addition to its role as a mitogen and chemokine, the VEGF family regulates the permeability of both mature and developing vessels (25–30). VEGF increases the permeability of endothelial cells in a dose-dependent manner. These activities are additionally mediated through interactions with the FLT1 and KDR/FLK1 receptor family. Receptor activation and signal transduction via the phosphoinositol-3 kinase signaling cascade were required for the permeabilization of these cells (31). Microvessel hyperpermeability is the critical step for the abnormal transport of molecules and cells across the blood vessel wall and, therefore, the crucial step for many diseases, including tumor growth and metastasis (32).

VEGF and Cancer

As alluded to above, the vascularization of tumors provides a provocative target for therapy for several reasons. First, although new vessel formation is an important factor in development and wound healing, in the mature animal, the formation of new vessels is an event essentially relegated to pathologic conditions. Cancer cells require access to blood vessels for continued growth and the establishment of further

metastatic sites. To obtain nutrients for their growth, cancer cells co-opt host vessels, sprout new vessels from existing ones, and/or recruit endothelial cells from the bone marrow (33). In addition, the resulting vasculature is structurally and functionally abnormal, typically dilated, and highly permeable with global disorganization. Therefore, this unique vasculature may provide a unique target for inhibitory therapy (20).

A large amount of data has supported the importance of VEGF and its cognate receptors in tumor angiogenesis. VEGF is elevated in the serum of patients with non-small cell lung, colorectal, breast, ovarian, uterine, and RCC (34–39). As detailed herein, a variety of mechanisms account for the increase in VEGF, with activation of the hypoxic response pathway via the transcription factors HIF1 α and HIF2 α as the classic mechanism of induction.

RCC presents a unique clinical setting, in which a tumor type nearly universally usurps a proangiogenic cellular homeostatic mechanism. Through mutations in *VHL* and the subsequent dysregulated expression of the hypoxia-inducible transcription factors HIF1 α and/or HIF2 α , a large cohort of hypoxia-responsive genes is induced, including VEGF as one of the classic transcriptional targets (21). Additionally, RCC cells, in which *VHL* is mutant, express abundant levels of VEGF mRNA and protein, and reconstitution of these cells with a wild-type *VHL* cDNA restores predicted patterns of VEGF hypoxia-responsive regulation (16). Thus, increased expression of VEGF and the consequences of that increased expression are expected and predictable events in the development of most RCC.

Clinical Approach to VEGF Inhibition in RCC

Anti-VEGF antibody (bevacizumab). A recombinant humanized monoclonal antibody against VEGF (rhuMab VEGF, bevacizumab, Avastin, Genentech, South San Francisco, CA) was developed, which binds and neutralizes all biologically active isoforms of VEGF (but not other members of the VEGF family, such as VEGF-B and VEGF-C; ref. 40). *In vitro* studies have shown that bevacizumab causes decreased survival of human umbilical vascular endothelial cells and decreases VEGF-induced human umbilical vascular endothelial cell permeability (41). This humanized antibody was shown to inhibit bovine capillary endothelial cell proliferation in response to VEGF and has showed antitumor effects in sarcoma and breast cancer cell lines (40). In addition, preclinical studies have shown that bevacizumab has activity against metastases (42). For example, the murine antibody (A4.6.1) prevented lung metastases from Wilms' tumors implanted into kidneys of nude mice (43).

Bevacizumab in metastatic RCC was initially investigated in a randomized phase 2 trial, in which 116 patients with treatment-refractory, metastatic clear cell RCC were randomized to receive placebo, low-dose (3 mg/kg) bevacizumab, or high-dose (10 mg/kg) bevacizumab every 2 weeks (44). There were four (10%) partial responses in the high-dose bevacizumab arm. A substantial proportion of additional patients had tumor shrinkage not meeting objective response criteria, and the strict progression criteria resulted in several patients declared as disease progression with a lower total tumor burden than baseline. Nonetheless, an intent-to-treat analysis showed a significant prolongation of time-to-progression in the high-dose bevacizumab arm compared with placebo (4.8 versus

2.5 months; $P < 0.001$). In the high-dose bevacizumab arm, hypertension of any grade occurred in 36% of patients, and grade 3 hypertension was observed in 21% of patients. Asymptomatic proteinuria without renal insufficiency was observed in 64% of patients. All toxic effects were reversible with cessation of therapy. Preclinical studies show that VEGF maintains tumor vessel morphology even in established vascularized tumors, prompting a rationale for patients to continue receiving anti-VEGF therapy (45). Follow-up from the above trial showed four patients who have continued with therapy without progression for 3 to 5 years (46). Toxic effects in these patients were limited to proteinuria with normal renal function.

Bevacizumab has been further investigated in RCC in combination with an anti-epidermal growth factor receptor strategy. Preclinical investigation in human RCC xenograft models of bevacizumab and erlotinib, a small-molecule epidermal growth factor receptor inhibitor, has shown a potential benefit of combination therapy on tumor growth inhibition (47). A clinical trial in metastatic RCC with bevacizumab, 10 mg/kg i.v. every 2 weeks, in combination with erlotinib, 150 mg/d, reported a 25% partial response rate (48). Although they were generally mild to moderate, the most common adverse events were rash and diarrhea. However, recent findings from a randomized, multicenter, double-blind phase 2 trial further evaluating bevacizumab with or without erlotinib as first-line therapy for metastatic RCC showed that patients in both arms had similar progression-free survival (PFS) and response rates (49). The bevacizumab monotherapy arm showed a 14% objective response rate and an 8.5 median PFS. These data suggest that erlotinib does not add to the efficacy of bevacizumab and perhaps that the single-agent activity of bevacizumab has been underestimated. Of note, bevacizumab trials have shown a substantial PFS despite a low objective response rate. This observation is accounted for by a large percentage of patients with tumor shrinkage not meeting the objective response threshold. Further development of bevacizumab and other similar agents must account for this phenomenon and investigate tumor shrinkage and PFS as primary end points.

An intergroup phase 3 trial has investigated the addition of bevacizumab to an initial systemic therapy, IFN alfa, in RCC (50). Patients with metastatic clear cell RCC without prior systemic therapy were randomized to either low-dose IFN alfa 2b (Intron A, Schering-Plough, Kenilworth, NJ), 9 MU s.c. thrice weekly, or the same dose and schedule of IFN alfa 2b in combination with bevacizumab, 10 mg/kg i.v. every 2 weeks. The primary end point of the trial is overall survival, designed to detect an improvement in median survival from 13 months for IFN alfa alone to 17 months for the combination. Importantly, this trial collected baseline tumor samples from the vast majority of patients. Analysis of tumor genomic changes (e.g., *VHL* status) and RNA/protein expression is planned and will be associated with clinical outcome. Such an analysis is vital to further understand the molecular features of tumors that make them respond to a specific therapeutic targeting approach. A similarly designed phase 3 trial is under way in Europe using IFN alfa 2a (Roferon, Hoffmann-LaRoche, Grenzach-Wyhlen, Germany) instead of IFN alfa 2b. Both trials are nearing planned analysis, and results are eagerly awaited to determine the effect of adding bevacizumab to front-line therapy in RCC.

Clinical trials of another approach to initial therapy in metastatic RCC, high-dose interleukin 2 (IL-2), in combination with bevacizumab, are also under way. The rationale for this combination includes the suggestion that bevacizumab may prevent much of the tumor-induced immunosuppression attributed to VEGF and thereby enrich the immune-enhancing effects of IL-2 (51, 52). In addition, IL-2 toxicity may be reduced by the vascular effects of bevacizumab. Bevacizumab may decrease the significant vascular leak syndrome associated with IL-2 and allow more IL-2 doses to be administered with less toxicity. Bevacizumab, 10 mg/kg i.v. every 2 weeks, will be integrated with standard high-dose IL-2 regimens with both PFS and overall survival as primary end points. Bevacizumab in combination with low-dose IL-2 is also being investigated in a separate trial.

Combination studies are also evaluating the feasibility of combining bevacizumab with an inhibitor of the tyrosine kinase portion of the VEGFR, sorafenib (Nexavar, Bayer Pharmaceuticals, West Haven, CT, and Onyx Pharmaceuticals, Richmond, CA). The rationale for this type of 'vertical inhibition' of the VEGF axis is more complete VEGF blockade. Two separate trials are under way (53). Preliminary results suggest that less than full monotherapy doses are tolerable with notable hand foot syndrome and hypertension limiting dose escalation. Other toxicities include fatigue, diarrhea, elevated lipase levels, proteinuria, and thrombocytopenia. Two phase 1 trials of bevacizumab in combination with another VEGFR inhibitor, sunitinib malate (Sutent, Pfizer, Inc., New York, NY), are also under way.

VEGF-Trap. VEGF-Trap (Regeneron Pharmaceuticals, Tarrytown, NY, and Sanofi-Aventis, Bridgewater, NJ) is a fusion protein composed of the human VEGFR VEGFR1 (FLT1) extracellular immunoglobulin domain 2 and the VEGFR2 (KDR) extracellular immunoglobulin domain 3 fused to human IgG1 Fc molecule (Fig. 1). VEGF-Trap thus acts as a soluble decoy receptor to bind VEGF and disrupt subsequent VEGF signaling. VEGF-Trap binds to VEGF with great affinity ($K_d \approx 1$ pmol/L) and also binds another angiogenic protein,

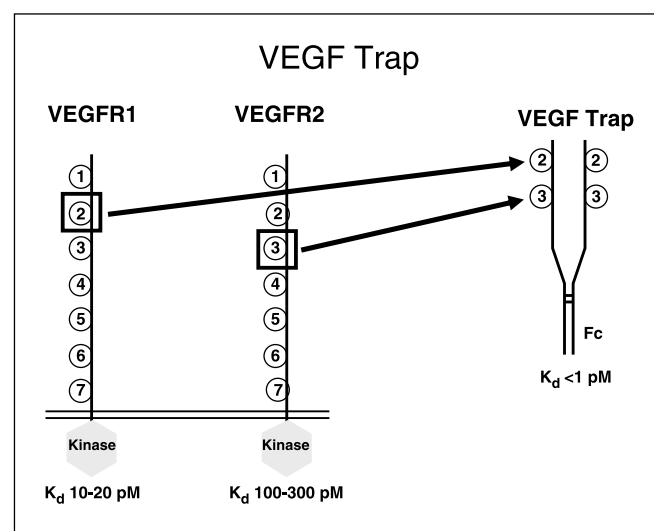


Fig. 1. VEGF-Trap structure. VEGF-Trap is a fusion protein composed of the human VEGFR VEGFR1 (FLT1) extracellular immunoglobulin domain 2 and the VEGFR2 (KDR) extracellular immunoglobulin domain 3 fused to human IgG1 Fc molecule.

placental growth factor. In cultured endothelial cell assays, VEGF-Trap showed inhibition of VEGF-induced VEGFR2 phosphorylation and endothelial cell proliferation. In xenograft models, VEGF-Trap-treated mice exhibited significant inhibition of tumor growth and tumor-associated angiogenesis in implanted rat C6 glioma, human A673 rhabdomyosarcoma, and mouse B16 melanoma tumors compared with vehicle-treated controls (54, 55). A murine xenograft model that used SK-NEP-1 cells (human Wilms' tumor cell line derived from embryonic renal cells) showed involution of existing mature vasculature and apoptosis of endothelial cells coincident with regression of established tumors after VEGF-Trap treatment (56, 57). VEGF-Trap-treated animals showed a significant regression of established tumors (mean tumor weight decreased by 79.3% by day 36; $P < 0.0002$).

Two phase 1 studies with VEGF-Trap have been reported in patients with refractory solid tumors. In the first trial (58), 38 patients, including 9 patients with metastatic RCC, received one (or two) s.c. dose(s) of VEGF-Trap followed 4 weeks later by 6 weekly injections (escalating dose levels of 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mg/kg) or 6 twice weekly (0.8 mg/kg) injections. Drug-related grade 3 adverse events included hypertension and proteinuria without a maximum tolerated dose determined. No anti-VEGF-Trap antibodies were detected. No objective responses have been observed in this trial. Fourteen of 24 assessable patients, including 5 of 6 patients at the highest dose level, have maintained stable disease for 10 weeks.

In the second trial (59, 60), 30 patients have been treated to date with i.v. VEGF-Trap every 2 weeks at one of five dose levels (0.3, 1.0, 2.0, 3.0, and 4.0 mg/kg). Drug-related grade 3 adverse events included arthralgia and fatigue. One patient with metastatic RCC has maintained stable disease for >11 months (at the 1.0 mg/kg dose level). Objective antitumor activity included a partial response in an advanced ovarian cancer patient and minor responses in metastatic bladder cancer and uterine leiomyosarcoma. Vascular imaging with dynamic contrast-enhanced magnetic resonance imaging done at baseline and after 24 h indicated effective inhibition of tumor perfusion at higher dose levels (≥ 2.0 mg/kg). Complete binding of circulating VEGF was documented at higher dose levels (≥ 2.0 mg/kg) with the observation of free in excess of bound VEGF-Trap in plasma. Further investigation is ongoing through an Eastern Cooperative Oncology Group trial randomizing metastatic RCC patients resistant to prior sunitinib or sorafenib to one of two doses of VEGF-Trap with a primary end point of PFS at 8 weeks.

Future Directions

Several ongoing clinical trials with VEGF binding strategies are noted above, which will further define the activity of this approach as monotherapy and combination therapy in metastatic RCC. As the clinical development of these active agents has far outpaced the ability to study them in preclinical models and/or with correlate studies, the understanding of the exact anti-tumor mechanism(s) is imprecise. It is believed that inhibition of VEGF results in decreased angiogenesis (i.e., reducing the growth of new blood vessels to prevent tumor growth), yet the sometimes dramatic tumor shrinkage observed may imply an additional effect on the tumor cell itself. In

addition, the effect of these agents on existing blood vessel structure and permeability is not well characterized. Clearly, additional studies involving blood and tissue-based correlates, along with functional imaging of tumor blood flow, is needed.

Conclusions

VHL gene inactivation is a frequent event in clear cell RCC, leading indirectly to overexpression of VEGF. Strategies to bind VEGF protein have undergone investigation in metastatic RCC. Bevacizumab has shown prolongation of time-to-progression versus placebo and is being investigated in multiple RCC settings. VEGF-Trap is a distinct VEGF binding molecule with evidence of effects on vasculature leading to tumor regression. Results from ongoing and future trials will more precisely define the role of VEGF binding strategies in RCC.

Open Discussion

Dr. Atkins: If the Cancer and Leukemia Group B (CALGB) intergroup trial is a positive trial for bevacizumab and interferon and if there is a role for anti-vascular endothelial growth factor-targeted therapies as first-line single agents from other trials, what will be the conclusion about bevacizumab and interferon?

Dr. Rini: I believe that most will interpret this data as confirming that bevacizumab has activity in the front-line setting.

Dr. Flaherty: My interpretation at the moment is that we have no real information to suggest that there is synergy between interferon and bevacizumab or, for that matter, interferon and any of these antiangiogenic drugs. Until evidence is more compelling that there is a real interaction, I would extrapolate single-agent data from these trials. The CALGB trial may show that bevacizumab is acting in an additive way with interferon, but these results should not be interpreted literally to mean that you can only get benefit from this agent with interferon. In other words, we will have enough single-agent data with bevacizumab to support the idea that the drug has single-agent activity.

Dr. Rini: There are probably a lot of immunomodulatory effects to bevacizumab and tyrosine kinase inhibitors (TKIs). Further study of bevacizumab and other TKIs with immunotherapy would certainly be reasonable to pursue.

Dr. Figlin: We might very well be left with four drugs having efficacy in untreated renal cell carcinoma with no direct comparison of the four drugs. We will make assumptions from the randomized phase 2 trial of bevacizumab with or without erlotinib. We will have the randomized phase 3 trial of sunitinib, the randomized phase 2 trial of sorafenib and interferon, and we may even have CCI interferon. The place where the cooperative groups really serve as a forum would be to compare drugs A, B, C, and D. I would encourage some group to take that challenge because how else are we going to move the field forward?

Dr. Atkins: It is not just oncologists who want this answer. Every patient coming in to see a doctor is going to want to know the answer to the question, "Which treatment should I get?"

Dr. George: Regarding von Hippel-Lindau (VHL), now that you are ready to move to the phase 3 level, in terms of

analyzing the mutational status, are you confident that we are comprehensive enough with mutational status and sequencing or do we need to be able to do analyses at the protein level to validate that?

Dr. Rini: I am confident in terms of sequencing the VHL gene and sequencing multiple tumors from the same patient and always getting the same results. There were false positives or negatives in the patients we have examined. I agree additional pathway examination (e.g., HIF expression, VHL protein expression, etc.) is needed. Further, prospective examination of these pathway elements in relation to association with treatment outcome is sorely needed.

Dr. George: Have you looked at CA9 staining or any kind of downstream readout to see if it correlates?

Dr. Rini: We just completed some immunohistochemistry analyses, and we are waiting for the results.

Dr. Atkins: We have looked at CA9 staining and VHL mutational status and did not see much of a correlation, although CA9 high expression tended to be more frequent than VHL mutations. This makes us wonder whether there is another mechanism for activating hypoxia inducible factor (HIF) other than VHL loss.

Dr. George: That is why staining HIF would be really important. You need to know CA9, HIF, and VHL status.

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