Pathways Mediating Resistance to Vascular Endothelial Growth Factor–Targeted Therapy

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Abstract Vascular endothelial growth factor (VEGF)–targeted therapy has become an important treatment option for the management of a number of human malignancies. Unfortunately, a significant number of patients do not respond to VEGF-targeted therapy when used as a single agent or in combination with chemotherapy. Furthermore, the duration of benefit from VEGF-targeted therapy can be relatively short (weeks to months). Ultimately, the vast majority of patients who initially respond to therapy will develop resistance. To date, the molecular and cellular mechanisms associated with resistance to VEGF-targeted agents are poorly understood. The mechanisms of action of anti-VEGF therapy are diverse, and it is entirely possible that resistance mechanisms are similarly diverse and depend on the tumor type. A better understanding of these mechanisms will help in the selection of those patients that are more likely to benefit from VEGF-targeted therapy and also provide for the rational development of therapies that circumvent or overcome resistance.

Background

Research in the field of tumor angiogenesis has provided the foundation for a radical change in the management and treatment of human cancer. A number of antiangiogenic approaches have been studied in the clinic, but thus far only drugs that target the vascular endothelial growth factor (VEGF)/VEGF receptor pathway have been validated based on phase III clinical trials to provide clinical benefit. VEGF was initially described as a vascular permeability factor by Dvorak and colleagues in the late 1970s and was later cloned and found to be homologous to VEGF by Ferrara in 1989. Since then, a number of randomized trials have shown the clinical benefit of various VEGF-targeted agents in patients with metastatic colorectal cancer, advanced non–small cell lung cancer, renal cell carcinoma, hepatocellular carcinoma, and metastatic breast cancer. VEGF-targeted agents have thus become a mainstay in the treatment of malignant disease.

Despite these important advances, it is well recognized that many patients do not respond to VEGF-targeted therapy, and for those that do, responses are short-lived, and resistance develops in the majority of patients. For example, the increase in overall survival in patients with advanced stage non–small cell lung cancer or colorectal cancer who receive chemotherapy plus the anti-VEGF monoclonal antibody (mAb) bevacizumab is relatively short, averaging less than two months in the most recent trials (1, 2). It is also important to point out that there are a number of negative phase III studies with VEGF-targeted therapy. The initial or eventual failure of VEGF-targeted therapy suggests that mechanisms of *inherent* and *acquired* resistance play a major role in the clinical progression of disease in patients treated with these agents. In the context of discussing resistance to this class of agents, it is important to consider their activity in certain tumor types as a monotherapy or in combination with other antineoplastic agents. In this regard, significant clinical benefit of VEGF-targeted therapy as a single agent has only been observed in renal cell carcinoma and hepatocellular carcinoma, and resistance is thought to be primarily related to the VEGF pathway. In colorectal cancer, non–small cell lung cancer, and breast cancer, VEGF-targeted therapy is administered with various cytotoxic regimens, and resistance is likely to be more complex. Thus, the mechanisms of action of anti-VEGF therapy are likely to be different in diseases where single-agent activity is observed, in contrast to diseases where efficacy has only been shown in combination with chemotherapy.

Although the selection of patients likely to benefit from VEGF-targeted therapy has been an area of intense investigation, thus far, no predictive factors for VEGF-targeted therapy have been identified. Therefore, acquired resistance remains a major challenge for oncologists treating patients with advanced or metastatic disease. This review will highlight preclinical studies that investigate resistance to VEGF-targeted therapy, with clinical vignettes to illustrate individual teaching points.

VEGF Biology

To better understand resistance to VEGF-targeted therapy, it is imperative to understand the biology of the VEGF ligand/

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Received 7/1/08; accepted 7/2/08.

Grant support: NIH grant CA112390 (L.M. Ellis) and The William C. Liedtke, Jr., Fund for Cancer Research (L.M. Ellis).

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doi:10.1158/1078-0432.CCR-07-5287

receptor system. The mammalian VEGF family of ligands consists of five glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF; refs. 3, 4). VEGF-A (commonly referred to as VEGF) is expressed as various isoforms secondary to alternative splicing. The VEGF ligands bind to and activate three structurally similar type III receptor tyrosine kinases, designated as VEGFR-1, VEGFR-2, and VEGFR-3. The VEGF family of ligands has distinctive binding specificities for each of these tyrosine kinase receptors, which contribute to their diversity of function.

In response to ligand binding, the VEGFR tyrosine kinases activate a network of distinct downstream signaling pathways (5). VEGFR-2 expression is restricted primarily to the vasculature and is the key mediator of VEGF-induced angiogenesis. VEGFR-1 is also expressed on the vasculature and certain hematopoietic cell populations (6). VEGFR-1 has been implicated in the homing of bone marrow–derived progenitor cells to sites of active angiogenesis (7), although the role of such cells has recently been challenged (8). VEGFR-3 preferentially binds VEGF-C and VEGF-D, and its expression in the adult is primary on lymphatic endothelial cells (with the rare exception of expression on tumor cells). More recent data have shown the expression and function of VEGFR-3 on vascular endothelial cells (9). VEGFR-3 plays a crucial role in postnatal lymphangiogenesis (10, 11).

The neuropilins (NRP-1 and NRP-2) act as coreceptors for the VEGFRs, increasing the binding affinity of VEGF to VEGF tyrosine kinase receptors (12–17). NRP-1 and NRP-2 have been postulated to signal independent of their association with VEGF tyrosine kinase receptors, but the role of VEGF activation of NRP-mediated signaling is not fully understood. In fact, recent studies suggest that dual targeting of the vasculature with antibodies to VEGF and NRP-1 is more effective than single agent therapy (18).

VEGF-targeted agents include neutralizing antibodies to VEGF or VEGFRs, soluble VEGF receptors or receptor hybrids, and tyrosine kinase inhibitors (TKI; ref. 4). The neutralization of VEGF-A by an antibody or soluble receptor construct can prevent its binding to, and activation of, VEGFR-1, VEGFR-2, NRP-1, and NRP-2. TKIs designed to target VEGF receptors are actually considered "multikinase" inhibitors because their mode of action is at the ATP binding pocket, which is similar among most tyrosine kinases. Notably, some TKIs significantly inhibit the activity of platelet-derived growth factor receptors (PDGFR), and thus resistance to anti-VEGF therapy may be more complex than that of more selective therapies such as mAbs (19).

Clinical Translational Advances

Mechanisms of resistance to anti-VEGF therapy

Compensatory angiogenic signaling pathways. Although VEGF is likely the predominant angiogenic factor in human tumors, it is clear from preclinical and clinical studies with VEGF-targeted agents that tumors can grow despite inhibition of the VEGF pathway. These observations led to the hypothesis that compensatory mechanisms for tumor angiogenesis may account for inherent or acquired resistance to VEGF-targeted therapy. The fibroblast growth factor (FGF) family of ligands was the first resistance mechanism identified. In an elegant study done in the Hanahan laboratory, the investigators showed that treatment with an anti-VEGF R-2 mAb was associated with a decrease in vascular density after 10 days of treatment with an anti-VEGFR2 antibody (20). However, these investigators noted an angiogenic rebound in tumors at 4 weeks that was associated with an increase in expression of redundant angiogenic factors, including members of the FGF family. By using a FGF-trap, these investigators were able to show that blocking FGF signaling minimized the acquired resistance to VEGF-targeted therapy and decreased tumor burden. These studies are especially interesting in light of recent clinical findings by Batchelor and associates that noted an increase in the circulating levels of bFGF when tumors progressed on VEGF-targeted therapy (21).

More recently, the cell membrane bound notch ligand/receptor system has been proposed as a resistance pathway for anti-VEGF therapy. This complex signaling system has been described in prior a *CCR Molecular Pathway* (22). In short, the activation of notch signaling by one of the five notch ligands can complement VEGF signaling by contributing to a more mature tumor vasculature network. Inhibition of notch signaling with an anti-DLL4 antibody or soluble FC chimeric construct can block notch signaling, which paradoxically leads to an increase in vessel count (22, 23). However, the function of these newly developed vessels is poor, and the overall result is a paradoxical decrease in tumor tissue perfusion. Several studies have shown that tumors that are inherently resistant to anti-VEGF therapy are nonetheless responsive to the inhibition of DLL4/notch signaling (22–24). Thus, combination therapies that target the notch signaling pathway may be a means of overcoming inherent resistance to VEGF-targeted therapy.

Tumor microenvironment and resistance to VEGF-targeted therapy. As stated above, the inhibition of VEGF signaling can lead to a presumed compensatory increase in expression of other angiogenic factors. In both preclinical studies and clinical trials, PlGF has been shown to be increased in plasma following blockade of VEGF signaling (21, 25). Carmeliet and colleagues developed a mAb that targets PlGF in attempts to block tumor angiogenesis. Utilizing this mAb, these investigators showed that both VEGF-sensitive and VEGF-resistant tumors respond to PlGF neutralization (26). One intriguing aspect of this study was the fact that antibodies to PlGF can inhibit recruitment of macrophages that are thought to play an important role in contributing to the angiogenic response. In this regard, the effects of anti-PlGF therapy were similar to therapy that depletes tumors of macrophages, suggesting that PlGF signaling may inhibit angiogenesis via indirect effects on the microenvironment. Lastly, anti-PlGF therapy leads to less of a hypoxic response with less induction of compensatory angiogenic mediators. Anti-PlGF strategies may play a complementary role with anti-VEGF therapy, although more studies are necessary to test this hypothesis. Interestingly, VEGF-trap binds both VEGF-A and PlGF.[3] The clinical development of this agent is far behind that of bevacizumab, but preliminary studies do not suggest that the dual targeting of VEGF-A and PlGF will be better than targeting VEGF-A alone. In addition, TKIs to VEGFRs would theoretically block the function of PlGF by blocking the activation of VEGFR-1. As always, well-conducted

[3] http://www.regeneron.com/vegftrap.cancer.html

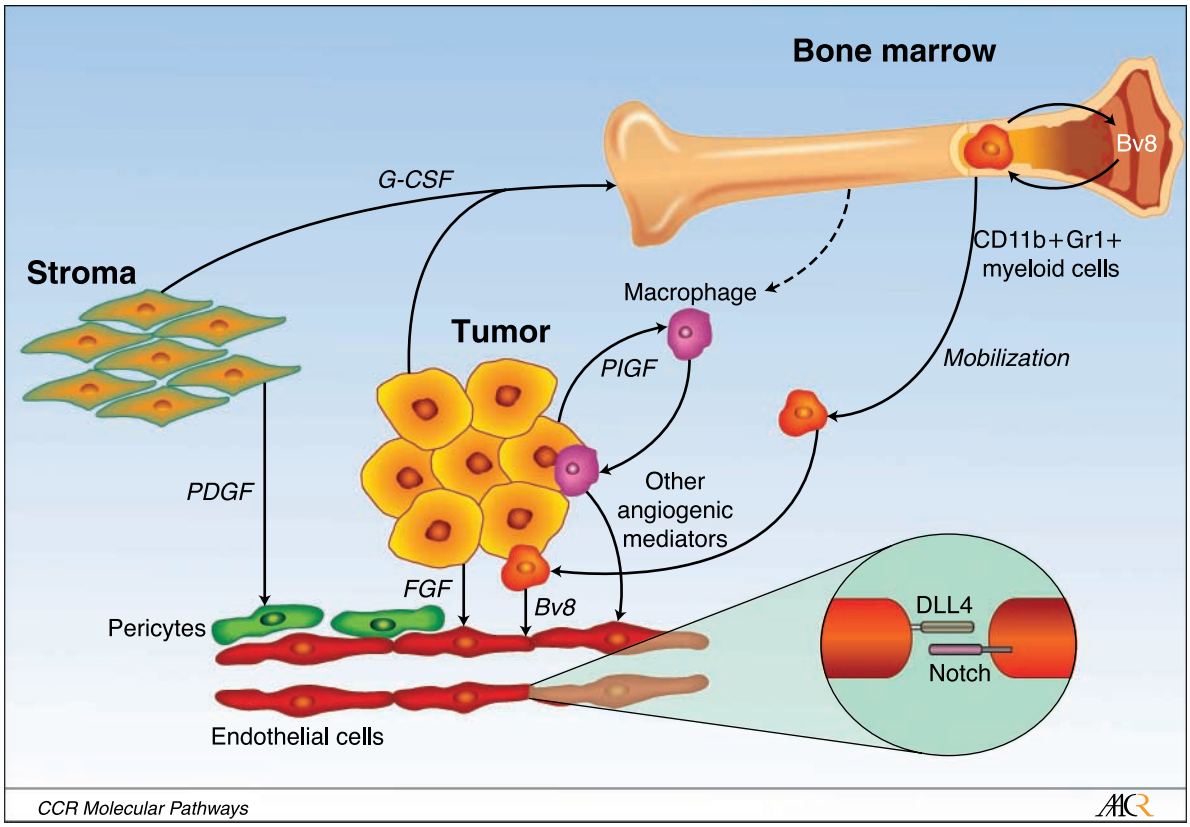

Fig. 1. Resistance pathways to VEGF-targeted therapy. Although VEGF signaling plays a central role in tumor angiogenesis, numerous compensatory angiogenic factors and cell types contribute to resistance to VEGF-targeted therapy. Mediators of resistance to VEGF-targeted therapy include soluble angiogenic factors such as FGF, PlGF, and Bv8; cell-bound DLL4 that can activate Notch on adjacent endothelial cells; pericytes that directly support endothelial cell survival; macrophages that secrete numerous angiogenic factors; and bone marrow – derived myeloid cells that also secrete soluble angiogenic factors.

clinical trials will be necessary to determine the relative importance of targeting PlGF or VEGF/PlGF in cancer therapy.

Although most studies in angiogenesis research focus on endothelial cell function within the tumor vasculature, it is important to recognize that pericytes provide survival signals to endothelial cells and play an important functional role in mediating blood flow and endothelial cell permeability. Direct contact between pericytes and endothelial cells enhances endothelial cell survival (27). In addition, pericytes secrete paracrine factors that mediate endothelial cell survival signaling via Akt activation (28). In the developing retina, Benjamin showed that the withdrawal of VEGF signaling leads to the selective survival of endothelial cells that have pericyte coverage (29); pericyte coverage was termed "mature vessels," a term widely used in tumor biology to describe pericyte coverage of endothelial cells. The migration and proliferation of pericytes is predominantly mediated by PDGF-BB (secreted primarily by endothelial cells) interacting with the PDGF β receptor on pericytes (28). Inhibition of VEGF signaling can lead to the pruning of "immature" (without pericyte coverage) tumor microvasculature, increasing the *relative* percentage of vessels with pericyte coverage (mature vessels). Jain and associates, however, have done studies showing that blockade of VEGF signaling in tumors can lead to the up-regulation of angiopoietin-1 that, in turn, increases the pericyte coverage of vessels, reversing the effects of VEGF on pericyte detachment (30). A number of elegant studies from the Bergers and Hanahan laboratories, as well as others, have shown that targeting both pericytes and endothelial cells (i.e., PDGF-R and VEGFR inhibitors) leads to greater efficacy than either agent alone (31). Due to the homology between VEGFRs and PDGFRs, many kinase inhibitors targeting VEGFRs also inhibit PDGFR signaling. As stated above, the clinical benefit of dual targeting of the vasculature (PDGFR and VEGFR inhibition) remains to be determined.

More recently, Hanahan and colleagues have shown that tumor-derived PDGF (squamous cell carcinoma) can stimulate secretion of FGFs from cancer-associated fibroblasts that, in turn, supports angiogenesis (32). These studies show the importance of the microenvironment in regulating tumor angiogenesis, and provide additional targets in attempts to abrogate inherent or acquired resistance.

CD11b$^+$Gr1$^+$ myeloid cells. Recent work from Ferrara and associates have identified a specific myeloid cell population that migrates to tumors and mediates tumor angiogenesis and resistance to anti-VEGF therapy (33). These investigators found that G-CSF (and IL-6 and SDF1) secreted by tumor cells and tumor stroma stimulates the mobilization of CD11b$^+$Gr1$^+$ myeloid cells in the bone marrow (Fig. 1). Once mobilized, these CD11b$^+$Gr1$^+$ cells are recruited to the tumor and stimulate angiogenesis, conferring resistance to VEGF-targeted therapy. These myeloid cells did not colocalize with endothelial cells as has been described for other bone marrow – derived progenitor cells (a point of controversy at the present time; ref. 8). Interestingly, some tumors

intrinsically recruit $CD11b^+Gr1^+$ cells from the bone marrow to the tumor site, whereas others do this only in response to VEGF-targeted therapy (34). Depletion of $CD11b^+Gr1^+$ myeloid cell populations in preclinical models sensitized tumors to anti-VEGF therapy, showing the role of these infiltrating cells in mediating resistance therapy.

Ferrara and colleagues have recently shown that $CD11b^+Gr1^+$ cells express the angiogenic factor Bv8, a protein related to endocrine-gland–derived-VEGF (34, 35). Bv8 was originally identified as a secreted protein from the skin of the frog *Bombina variegata* (36). Bv8 facilitates the mobilization of $CD11b^+Gr1^+$cells in the bone marrow and stimulates angiogenesis in tumors independent of VEGF. Treatment with anti-Bv8 neutralizing antibodies in preclinical models reduces granulocyte colony-stimulating factor-mediated $CD11b^+Gr1^+$ cell mobilization and inhibits tumor growth and angiogenesis (35).

Clinical observations with continuation of VEGF-targeted therapy following progression. As stated above, exclusive of patients with renal cell carcinoma and hepatocellular carcinoma, VEGF-targeted therapy is most efficacious when combined with chemotherapy. This holds true for metastatic colorectal cancer, non–small cell lung cancer, and metastatic breast cancer. This raises the important question of whether therapy failure is due to resistance to the VEGF-targeted agent or chemotherapy, or both. An interesting observational study from data collected in a registry (BRiTE registry), Grothey et al. examined the use of bevacizumab throughout the course of treatment in patients with metastatic colorectal cancer (37). In those patients who progressed on frontline therapy with chemotherapy plus bevacizumab and received further therapy without continuation of bevacizumab, overall survival was 20 months. For those patients who progressed on frontline chemotherapy plus bevacizumab and were continued on bevacizumab while changing the chemotherapeutic regimen, the overall survival was 32 months. These data suggest that resistance to combination regimens of anti-VEGF therapy and chemotherapy may be due to the chemotherapy component rather than the anti-VEGF component. Since this is an observational study, and not a randomized trial, these findings must be considered hypothesis-generating (i.e., taken with a "grain of salt"). This interesting observation has led to clinical trials studying the efficacy of continued VEGF inhibition through multiple lines of therapy.

Another variation of this approach is being explored with VEGFR-selective TKIs. As discussed earlier, VEGFR TKIs have distinct target inhibition profiles with activities against various tyrosine kinases (19). Therefore, it is possible that after a patient progresses on one VEGFR TKI inhibitor, the patient could respond to "tweaking" the therapy by switching to another VEGF pathway inhibitor. In very preliminary studies in patients with renal cell carcinoma, those patients who had progressed on frontline therapy with either sorafenib or sunitinib were treated with the alternative TKI. Interestingly, many of these patients who had become resistant to one TKI remained sensitive to another. These are small clinical studies that will require validation in larger patient populations. The unique target profiles of the various TKIs, however, may be used to our advantage to block compensatory resistance pathways as described above or as yet unidentified.

Perspective

Despite major advances in the clinical development of VEGF-targeted therapy, most patients' tumors are inherently resistant. When response is obtained, the benefit of VEGF inhibitors is transient, lasting weeks to months. Therefore, nearly all tumors are either inherently resistant or develop acquired resistance to agents targeting the VEGF/VEGFR pathway. To add to the complexity, the mechanisms of action of this class of drugs vary from tumor type to type. For example, single-agent anti-VEGF therapy is efficacious in renal cell carcinoma (38–40), but has not shown activity as a single agent in colorectal cancer (where combination with chemotherapy is necessary to show benefit). Therefore, the resistance mechanisms outlined in this review are likely to be tumor-type specific. Understanding resistance mechanisms will benefit clinical development in several ways. First, we will be able to identify predictive markers of response, as at the current time there are no known biomarkers for the efficacy of anti-VEGF/VEGFR therapy. Second, understanding resistance mechanisms will provide for the development of effective therapy in patients who have become refractory to frontline VEGF/R inhibition, as single-agent therapy or in combination with chemotherapy. Lastly, understanding resistance mechanisms will provide insight into mechanisms of action of this class of drugs, as this area remains a subject of controversy in the field (41).

Disclosure of Potential Conflicts of Interest

L. Ellis has major commercial research grants with ImClone Systems, Amgen, and Sanofi-Aventis, and is a consultant to Genentech.

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