Refractoriness to Antivascular Endothelial Growth Factor Treatment: Role of Myeloid Cells

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Abstract

CD11b+Gr1+ cells, which include neutrophils, macrophages, and myeloid-derived suppressor cells, have been shown to contribute to tumor angiogenesis. Recently, we found that accumulation of CD11b+Gr1+ in tumors renders them refractory to angiogenic blockade by vascular endothelial growth factor (VEGF) antibodies. This effect was traced to a pathway of CD11b+Gr1+-mediated angiogenesis that is, at least in part, driven by the secreted protein Bv8, which is up-regulated by the important myeloid growth factor granulocyte colony-stimulating factor (G-CSF). Thus, G-CSF may promote tumor angiogenesis through a Bv8-dependent pathway that bypasses VEGF and renders tumors refractory to anti-VEGF therapy. [Cancer Res 2008;68(14):5501–4]

Vascular Endothelial Growth Factor A Is a Key Regulator of Physiologic and Pathologic Angiogenesis

Physiologic angiogenesis, the formation of new blood vessels, is a fundamental process in embryonic and adult life and is also important for tissue repair (1). Vascular endothelial growth factor (VEGF)-A is a pivotal regulator of angiogenesis, and loss of a single VEGF-A allele will result in embryonic lethality (2). Similar to normal tissues, solid tumors require new blood vessels for growth and survival. Therefore, agents that block angiogenesis may offer attractive modalities for cancer therapy.

In mammals, the VEGF family comprises of five members including VEGF-A (thereafter called VEGF), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF; ref. 3). There are also several isoforms of VEGF-A termed VEGF121, VEGF165, VEGF189, and VEGF206, which are produced by alternative exon splicing. Members of VEGF family bind to and signal through three tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR), and VEGFR-3. Moreover, activation of VEGFRs may be facilitated by coreceptors including heparan sulfate proteoglycans and neuropilins (reviewed in ref. 3). Although VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells, monocytes, macrophages, and hematopoietic stem cells, the expression of VEGFR-3 is largely restricted to lymphatic endothelial cells consistent with a role of this receptor in lymphangiogenesis (4).

Early experiments with anti-VEGF neutralizing antibodies showed that blockade of VEGF-A alone may substantially suppress tumor growth and angiogenesis in several models (5). These proof-of-concept investigations prompted efforts to develop therapies to inhibit the VEGF signaling pathway by targeting either the ligands (mainly VEGF-A) or the receptors (VEGFR-1 and VEGFR-2; ref. 3). Bevacizumab (Avastin), a humanized variant of an anti-VEGF neutralizing monoclonal antibody, is the first antiangiogenic agent to be approved by the Food and Drug Administration for the treatment of metastatic colorectal cancer (6) and non–small-cell lung cancer (7) in combination with cytotoxic chemotherapy. In addition to strategies aimed at blocking VEGF, a variety of small molecule receptor tyrosine kinase (RTK) inhibitors targeting VEGF receptor signaling pathway including Bay 43-9006 (Nexavar or sorafenib) and SU11248 (Sutent or sunitinib) have been developed. Sorafenib, initially developed as a Raf kinase inhibitor, was later shown to inhibit several RTKs including VEGFRs and to show efficacy in renal cell cancer. SU11248 inhibits VEGFRs, PDGFR, c-kit, and Flt-3 and has efficacy in imatinib-resistant gastrointestinal stromal tumor (8) and renal cell carcinoma.

An overview of current clinical trials with anti-VEGF agents and other angiogenesis inhibitors can be found at Cancer.gov.1

In summary, characterization of the VEGF signaling pathway led to the identification of several target molecules with promising therapeutic potentials.

Myeloid Cells and Tumor Growth

In addition to tumor cells, stromal cells are thought to be a source of various proangiogenic factors. Tumor stroma includes a variety of cell types including fibroblasts and bone marrow–derived cells (BMDC; ref. 9). Tumor-associated fibroblasts (TAF) are an important source of stromal-derived factor 1, leading to the recruitment of endothelial progenitor cells or myeloid cells into the tumor microenvironment (10). Also, various tumor-infiltrating hematopoietic cells such as T- and B- lymphocytes, and monocytes/macrophages have been suggested to secrete proangiogenic factors including VEGF-A (11). Tumor-associated BMDCs may also contribute to tumor angiogenesis by secreting proangiogenic factors or by direct incorporation into the tumor vasculature (12). Tie2-expressing monocytes have also been reported to promote tumor growth through the secretion of proangiogenic factors (13).

Recently, much research has focused on a population of myeloid cells, identified by the expression of the cell surface markers CD11b and Gr1, which include a variety of cell types such as neutrophils, immature dendritic cells, monocytes, and early myeloid progenitors. In cancer, interest in these cells, which includes, among others, a class of immune suppressive cells termed myeloid-derived suppressor cells (MDSC), relates to their ability to promote tumor progression. For a discussion of the terminology and controversies surrounding it, see (14) and (15). The controversies stem to a large extent to the lack of a precise and uniform definition of these cells.

1 http://www.cancer.gov/clinicaltrials/developments/anti-angio-table
degree from the heterogeneity of this cell population. Because we have no evidence that the role in refractoriness to anti-VEGF therapy of myeloid cells observed in our models relates to immunosuppression (see next section), we prefer to use the term "CD11b+Gr1+ cells" because it is devoid of such functional implications. Recent studies suggest that neutrophils play an important role in the induction of the angiogenic switch in cancer, as illustrated in a transgenic mouse model of pancreatic β-cell carcinogenesis (16). This induction coincides with the secretion of matrix metalloproteinase (MMP)-9, which has been associated with increased bioavailability of VEGF-A for its receptors. Neutrophils express VEGF-A especially in response to granulocyte colony-stimulating factor (G-CSF; ref. 17). Besides being a source of angiogenic factors, tumor-associated CD11b+Gr1+ cells suppress the immune response in different subtypes of T cells including CD4+ and CD8+ cells (reviewed in ref. 18). Several mechanisms have been proposed for the immunosuppressive effects of CD11b+Gr1+ cells and MDSCs, including secretion of immunosuppressive cytokines and up-regulation of nitric oxide (NO), reactive oxygen species, and the arginine catabolic enzyme arginase (ARG1). In this context, activation of cyclooxygenase-2 (COX2) seems to be a critical factor for activation of CD11b+Gr1+ cells (19). COX2 generates prostaglandin E2 (PGE2) and also up-regulates the expression of ARG1 and NO synthase (NOS2). PGE2 promotes the differentiation of bone marrow progenitor cells into CD11b+Gr1+ MDSCs (20). ARG1 mediates suppression of CD3+ T cells and also restricts expression of interleukin (IL)-12 and IFN-γ, which promote the development of cytotoxic T-effector cells. NO generated by NOS2 also suppresses T-cell activation through effects on the IL-2 signaling cascade. In addition, high concentrations of NO can induce apoptosis in T cells through the CD95 signaling pathway as well as caspase-independent pathway(s). Together, these studies suggest that CD11b+Gr1+ cells such as MDSCs play a role in tumor growth through induction of angiogenesis and suppression of T-cell–mediated immune responses.

**Resistance/Refractoriness of Tumors to Anti-VEGF Treatment: Role of CD11b+Gr1+ Cells**

Several hypotheses have been proposed to explain tumor growth in the absence of VEGF. For example, other angiogenic...
factors may compensate for the lack of VEGF or selection for a population of tumor cell that are “hypoxia resistant” may lead to their preferential outgrowth (21). Alternatively, remodeling of tumor vasculature may result in the generation of mature, stabilized vessels that are less responsive to angiogenesis inhibitors. However, detailed mechanisms of resistance to anti-VEGF treatment in cancer patients remain to be determined.

To gain insight into the role of CD11b+Gr1+ cells in refractoriness to anti-VEGF, we screened a series of murine cell lines to establish experimental tumor models that are responsive/refractory to the treatment (22). Interestingly, refractory tumors were associated with a significant increase in the frequency of tumor-infiltrating CD11b+Gr1+ cells, compared with sensitive ones. This was consistent with a greater frequency of these cells in the bone marrow and spleen of mice bearing refractory tumors. Experiments in which tumor cells were mixed with CD11b+Gr1+ cells before host introduction elucidated the functional relevance of these cells. CD11b+Gr1+ cells isolated from refractory tumors, but not from sensitive tumors, were able to mediate refractoriness to anti-VEGF treatment. These studies suggested that CD11b+Gr1+ cells could facilitate tumor angiogenesis. Indeed, mixing sensitive tumors with CD11b+Gr1+ cells from refractory tumors resulted in a more pronounced neo-vascular supply compared with that resulting from mixture with CD11b−Gr1− cells. Moreover, the combination of an anti-Gr1 antibody with anti-VEGF delayed the onset of refractoriness (22).

It is noteworthy that anti-VEGF refractoriness was observed in immunocompetent C57BL/6 or in immunocompromised XID mice, suggesting that the accumulation of CD11b+Gr1+ cells and their role in mediating refractoriness were not dependent on the adaptive immune system (Fig. 1; ref. 22).

To further investigate the role of CD11b+Gr1+ cells in refractoriness to anti-VEGF, we also treated the same tumors with mFlt(1-3)-IgG, a high affinity chimeric-soluble VEGFR-1 variant that not only neutralizes VEGF-A but also PlGF and VEGF-B (23). We saw no difference between anti-VEGF or mFlt(1-3)-IgG-treated groups in the efficiency of tumor formation or in the accumulation of CD11b+Gr1+ cells, suggesting that VEGFR-1 selective ligands do not compensate for the lack of VEGF-A, at least under these experimental circumstances. In apparent conflict with these conclusions, Fisher and colleagues (24) recently reported that targeting PlGF alone with neutralizing antibodies elicits significant antitumor effects in some anti-VEGF refractory models. Elucidating the reasons for such discrepancy will require further investigation. In addition, to address whether a lack of response to anti-VEGF might reflect a general refractoriness to other antitumor agents, we treated tumor-bearing mice with several cytotoxic agents. No correlation was seen between response to anti-VEGF therapy or to cytotoxic chemotherapy. Therefore, refractoriness to anti-VEGF did not predict a lack of response to other antitumor agents (22).

To elucidate the molecular basis of refractoriness to anti-VEGF, we performed array analysis in the tumors as well as in CD11b+Gr1+ cells isolated from the bone marrow of mice bearing refractory or sensitive tumors. Gene array analysis in anti-VEGF–treated tumors revealed a unique profile of gene expression in each tumor type. Analysis of differentially expressed genes in refractory versus sensitive tumors identified several cytokines, such as G-CSF, and MCP-1, known to be involved in the mobilization of bone marrow–derived myeloid cells to the peripheral blood. In addition, proinflammatory factors such as MIP-2 and IL-1R were found to be highly expressed in refractory tumors (22). Therefore, gene array analysis identified factors that might enable tumors to instruct the bone marrow to generate more myeloid cells, induce mobilization of hematopoietic cells to the peripheral blood, and perhaps help them home to homing to the tumors. These investigations provided a potential cellular and molecular basis to explain refractoriness to anti-VEGF treatment, at least in the tumor models examined.

In evaluating the mechanism of angiogenesis mediated by CD11b+Gr1+ cells, we identified the orthologue of the secreted protein Bv8 as a critical mediator (25). Bv8, also called prokineticin-2, belongs to a larger class of peptides that are defined by a five disulfide bridge motif called a colipase fold (26). Both Bv8 and the related factor EG-VEGF bind two highly homologous G-protein-coupled receptors termed EG-VEGFR/PKR-1 and EG-VEGFR/PKR-2 (27). Bv8 is up-regulated in CD11b+Gr1+ cells after implantation of tumor cells (25). Earlier studies showed that Bv8 may mobilize hematopoietic cells to the peripheral blood and also stimulate production of granulocytic and monocytic colonies in vitro (28). Notably, anti-Bv8 treatment of mice implanted with a variety of human tumors resulted in a significant reduction in tumor growth, arguing that Bv8 had a critical role. Bv8 was found to be dramatically up-regulated by G-CSF, both in vitro and in vivo (Fig. 1). This upregulation seemed to reflect the operation of a crosstalk between the tumor and CD11b+Gr1+ cells because neutralizing anti-G-CSF antibodies ablated the increase in Bv8 expression in CD11b+Gr1+ cells after tumor implantation (25). More recently, we found that Bv8 plays a role in myeloid cell–dependent angiogenesis not only in tumor xenografts but also in the RIP-Tag transgenic model of pancreatic β-cell tumorigenesis (29).

Concluding Remarks

It is now generally accepted that targeting angiogenesis in general and the VEGF pathway in particular has a significant effect in cancer therapy. However, angiogenesis is a highly degenerate process mechanistically, and the heterogeneity and genomic plasticity of tumors allow them to evolve alternate pathways when a negative growth selection is applied. Indeed, recent studies indicate that tumors can use alternative angiogenic pathways when treated with VEGF-blocking agents. Our recent investigations suggest that recruitment of CD11b+Gr1+ cells, which occurs inherently and independently of treatment, represents a cellular mechanism underlying the inherent refractoriness that tumors can develop in response to anti-VEGF therapy. These data are consistent with the observation that antiangiogenic agents are most efficacious when combined with cytotoxic agents and/or therapies targeted toward ablating myeloid cells (reviewed in 30). In particular, the characterization of Bv8 as a myeloid cell–derived mediator of angiogenesis and mobilization of myeloid cells may provide a novel target for cancer therapy.

Disclosure of Potential Conflicts of Interest

E. Shojaei and N. Ferrara: Genentech, Inc. employees and shareholders.

Acknowledgments

Received 3/11/2008; accepted 3/12/2008.
References
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