REVIEW

Tumor Angiogenesis and Anti-angiogenic Therapy

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Anti-angiogenic therapy is an anti-cancer strategy that targets the new vessels that grow to provide oxygen and nutrients to actively proliferating tumor cells. Most of the current anti-cancer reagents used in the clinical setting indiscriminately target all rapidly dividing cells, resulting in severe adverse effects such as immunosuppression, intestinal problems and hair loss. In comparison, anti-angiogenic reagents theoretically have fewer side effects because, except in the uterine endometrium, neoangiogenesis rarely occurs in healthy adults. Currently, the most established approach for limiting tumor angiogenesis is blockade of the vascular endothelial growth factor (VEGF) pathway. In line with the results of preclinical studies, significant therapeutic effects of VEGF blockers have been reported in various types of human cancers, even in patients with progressive/recurrent cancer who could not otherwise be treated. However, some patients are refractory to this treatment or acquire resistance to VEGF inhibitors. Moreover, several studies have shown that VEGF blockade damages healthy vessels and results in adverse effects such as hemorrhagic and thrombotic events. In recent research that indicated possible ways to overcome these problems, several VEGF-independent and tumor-selective pro-angiogenic mechanisms were discovered that could be targeted in combination with or without conventional VEGF blockade. These findings offer opportunities to greatly improve current anti-angiogenic treatment for cancer. (Keio J Med 61 (2) : 47–56, June 2012)

***Keywords*: angiogenesis, tumor angiogenesis, VEGF, macrophage, anti-angiogenic therapy**

Introduction

In mammalian development, a vascular network is formed throughout the body (**Fig. 1**), except in avascular tissues (e.g., cornea and intervertebral disks), to meet the tissue requirements for oxygen and nutrients.[1] Three major processes are necessary to form a complete vascular network: vasculogenesis, angiogenesis and vascular remodeling.[2] Vasculogenesis denotes *de novo* blood vessel formation, in which vascular precursor cells (angioblasts) migrate to sites of vascularization, differentiate into endothelial cells and coalesce to form the initial vascular plexus. Angiogenesis refers to the budding of new capillary branches from existing blood vessels, whereas vascular remodeling describes a later phase when a newly formed vessel increases its luminal diameter in response to increased blood flow and acquires identity as an artery, vein or capillary.[3] Once these three processes are completed during postnatal development, adult vasculature is stable and rarely proliferates under physiological conditions. However, in pathological situations such as ocular neovascular diseases and cancer, existing vessels again start to grow to meet the abnormal requirements for oxygen and nutrients of the pathologically expanding tissues.

Anti-angiogenic therapy targets vascular growth within tumors, with the aim of suppressing tumor growth and metastasis. Most current anti-cancer chemotherapeutic agents used in the clinical setting indiscriminately target all rapidly dividing cells (e.g., at the level of DNA replication and protein synthesis) and therefore can cause severe adverse effects such as immunosuppression, intestinal problems and hair loss. In comparison, anti-angiogenic reagents theoretically have fewer side effects because, except in the uterine endometrium during the menstrual cy-

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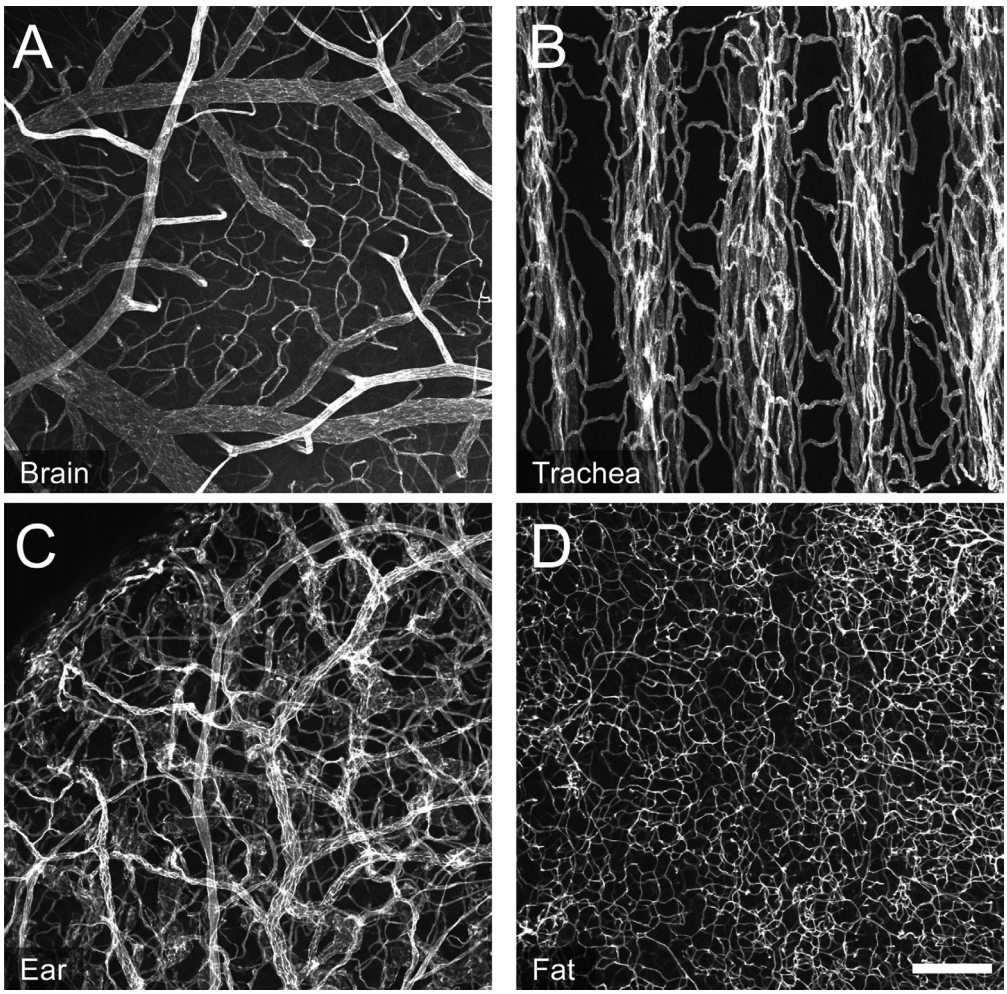

Fig. 1 A well-organized vascular network is formed throughout the body.
Whole-mount CD31 immunostaining of endothelial cells in normal mouse tissues [brain (A), trachea (B), ear (C) and fat (D)] at postnatal day 30. Scale bar: 200 μm.

cle, neoangiogenesis rarely occurs in healthy adults. This review discusses the history and current understanding of the molecular and cellular bases of tumor angiogenesis and anti-angiogenic therapy.

Tumor Angiogenesis and Anti-VEGF Therapy

More than a century ago, early pioneering researchers observed that dense vascular networks often accompanied human tumors. The existence of tumor-derived angiogenic factors was postulated more than 70 years ago, and it was proposed that vascular growth in the tumor relies on such factors.[4,5] In 1971, Judah Folkman, who became known as the "father of tumor angiogenesis," first emphasized the importance of tumor vascularity for tumor growth.[6] He described how, if a tumor could be stopped from growing its own blood supply, it would wither and die (**Fig. 2**). Since then, various studies have led to the discovery of a growing number of anti-angiogenic molecules that limit tumor angiogenesis. Vascular endothelial growth factor (VEGF) was first discovered by Senger and colleagues as a vascular permeability factor secreted by a guinea pig tumor cell line.[7] Various *in vitro* and *in vivo* studies have since uncovered the role of VEGF as a central player in both physiological and pathological angiogenesis.[8] Pathologically expanding tumor tissues rapidly exhaust the available oxygen supply and become hypoxic. The activation of hypoxia-inducible factor (HIF) signaling in hypoxia-sensing cells triggers VEGF expression.[9,10] VEGF is secreted not only by tumor cells but also by tumor-associated stromal cells.[11] In turn, secreted VEGF stimulates vascular growth into hypoxic tumor tissues to meet the tumor's oxygen requirements (**Fig. 2**).

The use of VEGF blockers to prevent this process is the most established of the anti-angiogenic modalities.

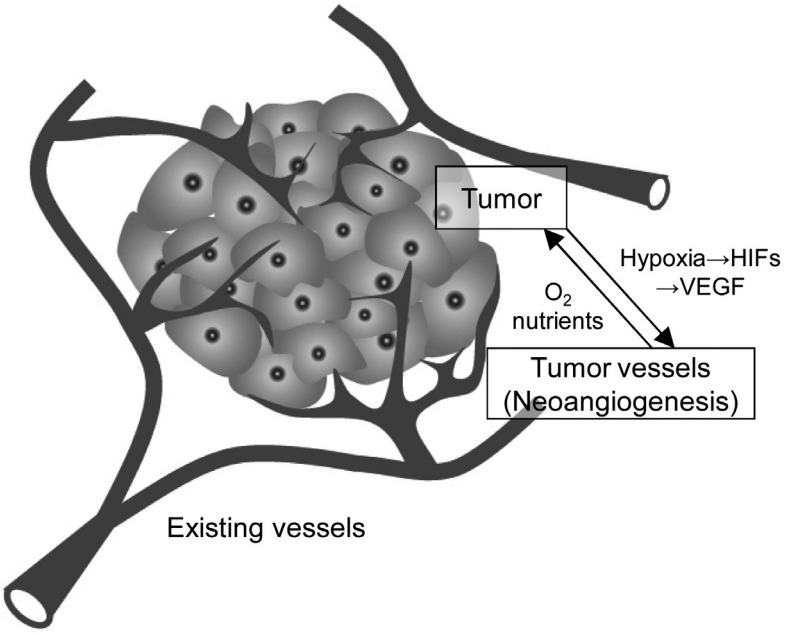

Fig. 2 Association between tumor growth and its vascularity.
Adult vasculature is stable and rarely proliferates under physiological conditions. However, in cancer, existing vessels again start to grow (neoangiogenesis) in response to hypoxia inducible factor (HIF)-driven VEGF expression in tumors. Newly formed vessels provide oxygen and nutrients to rapidly expanding tumors.

A number of preclinical studies of angiogenesis inhibition by administration of VEGF blockers have demonstrated significant tumor-suppression effects in various types of cancers.[12,13] In a phase III study of patients with metastatic colorectal cancer, bevacizumab, a humanized anti-VEGF monoclonal antibody, showed significant benefits in combination with 5-fluorouracil (5-FU)-based chemotherapy. In response, the U.S. Food and Drug Administration (FDA) approved bevacizumab for the treatment of metastatic colorectal cancer in combination with 5-FU-based regimens in 2004. Almost simultaneously, the FDA approved pegaptanib, an aptamer that blocks the 165-amino-acid isoform of VEGF-A, for the treatment of the wet form of age-related macular degeneration.[14] Thereafter, bevacizumab and multi-targeted tyrosine kinase inhibitors (e.g., sorafenib, sunitinib or pazopanib), which block the signaling of pathways such as VEGF, were approved for clinical use in various types of cancers including metastatic non-squamous non-small cell lung cancer, metastatic breast cancer, recurrent glioblastoma multiforme and metastatic renal cell carcinoma. A number of studies have reported their significant therapeutic efficacy.[8,15] In 2007, three years after the approval of bevacizumab by the FDA, the Ministry of Health, Labour and Welfare in Japan approved the use of bevacizumab for patients with progressive/recurrent colorectal cancer that cannot be surgically resected.

Problems with Anti-VEGF Therapy

Currently, as described above, the most established approach for limiting tumor angiogenesis is blockade of the VEGF pathway. In line with preclinical studies, administration of VEGF blockers to patients with various types of cancers has had significant therapeutic effect. However, some patients are refractory or acquire resistance to VEGF inhibitors.[16] This may be explained, at least in part, by the administration of often higher doses and different schedules in preclinical mouse studies (10 mg/kg body weight, twice a week) compared to humans (5 mg/kg body weight, once every 2 weeks) resulting in more pronounced effects in mice. Very modest doses are given to patients, as is usual for other anti-cancer reagents, to avoid possible off-target toxicities. In fact, animal studies have shown that interrupting VEGF blockade induces rapid vascular regrowth in tumors because the persisting empty sleeves of the basement membrane provide a scaffold for rapid revascularization.[17] This result indicates that insufficient doses of VEGF blockers can easily result in vascular regrowth and limit the tumor-suppression effects.

More importantly, several studies have shown that

Table 1. Known adverse effects of VEGF blockade

Adverse effect	Possible mechanism
Bleeding	Endothelial cell apoptosis; loss of integrity of the endothelial vessel lining
Thrombotic events	Increased platelet activation; exposure of the prothrombotic basement membrane to the circulating blood
Hypertension	Inappropriate balance between arteries and veins; reduced levels of prostaglandin I-2 and nitric oxide
Proteinuria	Podocyte dysfunction
Leukopenia, lymphopenia	Disturbance of hematopoiesis
Hypothyroidism	Regression of capillaries around thyroid follicles

VEGF blockade damages not only tumor vessels but also healthy vessels and results in severe problems such as hemorrhagic and thrombotic events (**Table 1**).[18,19] Mice studies indicate that constitutive signaling through VEGF in both a paracrine and an autocrine manner is required for the homeostasis of adult vessels.[20–22] VEGF blockade induces abnormal endothelial apoptosis, resulting in the loss of integrity of the endothelial vessel lining and increased platelet activation due to exposure of the circulating blood to the prothrombotic basement membrane. VEGF signaling is required not only for endothelial function but also for the function of podocytes and hematopoietic cells.[23,24] Accordingly, VEGF blockade causes moderate proteinuria, leukopenia and lymphopenia.[18,25] At the end of 2010, an advisory panel to the FDA announced that bevacizumab is neither safe nor effective against breast cancer, and the FDA decided to revoke market approval and re-label this drug. Researchers are now seeking novel anti-angiogenic targets specific for tumor vessels that avoid signaling pathways essential for the maintenance of healthy vessels.

Potential Mechanisms of Resistance to Anti-VEGF Therapy

Various mechanisms are thought to underlie the resistance to VEGF blockade observed in some patients with cancer. The extent of each mechanism responsible is highly variable from one cancer to another and differs depending on the type of VEGF blocker used. Understanding the molecular bases of these cancer type-dependent resistance mechanisms against VEGF blockade offers opportunities to improve anti-angiogenic treatment. Compensation by other pro-angiogenic mechanisms, such as fibroblast growth factors (FGFs) and angiopoietins, is a major factor that may contribute to poor responsiveness to VEGF blockade.[16,26–29] Recent preclinical studies have shown that these VEGF-independent pro-angiogenic mechanisms exert significant effects, particularly in the absence of VEGF signaling.

VEGF blockade-induced vessel regression causes hypoxia in tumor tissues, and the high rate of tumor cell proliferation in the absence of sufficient oxygen can result in the elimination of a major population of tumor cells. However, a proportion of hypoxia-tolerant cancer cells, which are likely to be so-called cancer stem cells,[30] survive in poorly oxygenated niches and elicit tumor adaptation to anti-angiogenesis. Some reports suggest that the resultant selection of tumor cells renders tumors even more invasive and metastatic.[31,32] They suggest that the administration of VEGF blockers alone can aggravate tumor hypoxia and worsen malignancy. However, contradictory results from other preclinical and clinical studies indicate that the concept of cancer aggravation by VEGF blockade is still unproven.[33,34] Randomized, placebo-controlled phase III studies in 4205 cancer patients did not support a decreased time to disease progression, increased mortality or altered disease progression pattern after cessation of bevacizumab therapy.[33]

Pericytes are essential components of blood vessels and are necessary for normal development, homeostasis and the integrity of the blood–brain barrier. Pericyte recruitment is mainly regulated by platelet-derived growth factor (PDGF)-B/PDGF receptor-β, transforming growth factor-β (TGF-β) and angiopoietin/Tie signaling.[35] In general, blood vessels found in tumors are immature with a sparse covering of pericytes.[3] However, in some cancers, tumor vessels are covered with a dense pericyte coat, and these vessels are less sensitive to VEGF blockers.[36] Interestingly, glioblastoma stem-like cells undergo a vasculogenic process: they differentiate into endothelial cells and cooperate with the neoangiogenic vessels (vasculogenic mimicry). These tumor cell-derived endothelial cells are less sensitive to VEGF blockade and thus conceivably contribute to the blockade resistance observed in certain types of cancer.[37,38] Myeloid cell-driven angiogenesis is also known to contribute to refractoriness or resistance to VEGF blockade. In particular, $CD11b^{+}Gr1^{+}$ myeloid cells have been shown to render tumors refractory to angiogenic blockade by anti-VEGF antibodies. This effect is mediated by the secreted protein Bv8, which is upregulated by granulocyte colony-stimulating factor.[39] $CD11b^{+}Gr1^{-}$ macrophages are also known to have a role in the VEGF-independent pro-angiogenic mechanism. Macrophages are currently one of the most promising targets for anti-angiogenic cancer therapy (see below).

Chronic exposure of endothelial cells to anti-VEGF drugs may lead to a stable epigenetic mechanism of resis-

tance. Epigenetic modulation of expression of anti-apoptotic genes such as *bcl-2* and *survivin* could conceivably lead to resistance to various inhibitors of angiogenesis.[40] Indeed, absence of the proapoptotic Bcl-2 homology 3 (BH3)-only Bcl-2 family member *Bim* in endothelial cells almost completely abolishes the effect of VEGF blockade.[41] Enhancer of zeste homolog 2 (EZH2), a member of the polycomb protein group that contributes to the epigenetic silencing of target genes, is highly upregulated in tumor endothelial cells.[42] EZH2 promotes tumor angiogenesis by methylating and silencing the *vasohibin1* gene, an intrinsic negative regulator of angiogenesis.[43,44]

Macrophages and Tumor Angiogenesis

Macrophages are white blood cells produced through colony stimulating factor-1 (CSF-1)-dependent differentiation of monocytes.[45,46] They have a role in both non-specific (innate immunity) and specific (adaptive immunity) defense mechanisms through phagocytosis of cellular debris and pathogens. Recent evidence demonstrates that macrophages are also involved in vascular development by promoting angiogenic branching and anastomosis.[47,48] Macrophages stimulate vessel sprouting via a soluble factor other than VEGF, rather than through direct contact with endothelial cells.[49] These data suggest that macrophages promote angiogenesis independently of VEGF signaling. Histological examination of various cancers reveals a vast accumulation of macrophages, known as tumor-associated macrophages (TAMs). TAMs, visualized by antibodies against macrophage markers such as CD11b, F4/80 and CD68, have oval to typical stellate morphology and are found in both avascular and vascularized areas throughout tumors (**Fig. 3A**).

TAMs orchestrate various aspects of cancer progression, including the diversion and skewing of adaptive responses, cell growth, matrix deposition and remodeling, and the construction of metastatic niches where they prepare the tissue for the arrival of tumor cells.[50] One current focus in macrophage research is the phenotypic shift observed during cancer progression; this shift is called "macrophage polarization" and links molecular pathways involved in inflammation and cancer (**Fig. 3B**). Classically activated (M1) macrophages, following exposure to interferon γ (IFNγ), are tumoricidal, as characterized by proinflammatory activity, antigen presentation and tumor lysis. During cancer progression, macrophages acquire features similar to alternatively activated macrophages (M2), which increase angiogenesis, tumor growth and invasion into surrounding tissues and suppress the activation of dendritic cells, cytotoxic T lymphocytes and natural killer cells.[51] IL-10 signaling interferes with NF-κB activation and M1 macrophage-induced inflammation. The balance between the activation of M1 macrophage-associated STAT1 and M2 macrophage-associated STAT3/6 regulates macrophage polarization.[52] Recent evidence demonstrates that histidine-rich glycoprotein (HRG) and HIF-1α suppress M2 polarization.[53,54] Conversely, Krüppel-like factor 4 (KLF4) and suppressor of cytokine signaling1 (SOCS1) have been shown to promote M2 polarization.[55,56]

Promotion of tumor angiogenesis is one of the most important roles of TAMs. Jeffrey Pollard and his colleagues first established the role of TAMs in the "angiogenic switch," which is identified as the formation of a high-density vessel network and promotes the malignant state of tumors.[57] Blockers of CSF-1 eliminate large populations of macrophages. Our recent study found that M2 macrophages expressed CSF-1 receptor more abundantly than M1 macrophages did, suggesting that CSF-1 blockade would be more effective on M2 (tumor-promoting) than on M1 (tumor-suppressive) macrophages. Accordingly, in a mouse osteosarcoma model, CSF-1 inhibition effectively suppressed tumor angiogenesis and disorganized empty sleeves of extracellular matrices.[47] Therefore, in contrast to VEGF blockade, interruption of CSF-1 inhibition did not promote rapid vascular regrowth. Continuous CSF-1 inhibition did not affect healthy vascular and lymphatic systems outside the tumor.[47] These results indicate that CSF-1-targeted therapy is a realistic strategy for limiting tumor angiogenesis. Currently, several drugs that inhibit CSF-1 signaling are in early clinical trials.[50] However, it should be noted that possible side effects of CSF-1 inhibition are disturbed wound healing and impaired macrophage-related immunity.[58]

Tumor Endothelium-specific Molecular Signatures

As described above, VEGF signaling is required for the maintenance of healthy vessels, and its blockade leads to side effects resulting from endothelial damage throughout the body (**Table 1**). The effects of VEGF blockade in healthy vessels are apparently more prominent in humans than in mice. This reflects the considerable differences in doses and schedules used for mice and humans; the doses of bevacizumab used in preclinical mouse studies were much higher than those given to patients. Nevertheless, preclinical mouse studies did not reveal the toxic effects apparent in humans. Therefore, to limit toxicity, it is important to find tumor endothelium-specific molecular targets.

It has been observed that tumor blood vessels differ morphologically from their normal counterparts; for example, they display inappropriate growth and regression due to abnormal proliferation and apoptosis in endothelial cells, insufficient pericyte recruitment, enhanced leakiness, decreased blood flow and abnormalities in the basement membrane.[3,59] These abnormal structures lead to reduced drug delivery into tumors. Inhibitors of neuronal NO synthase (nNOS) or prolyl hydroxylase domain protein 2 (PHD2) have been reported to normalize these structures, thereby enhancing the efficacy of con-

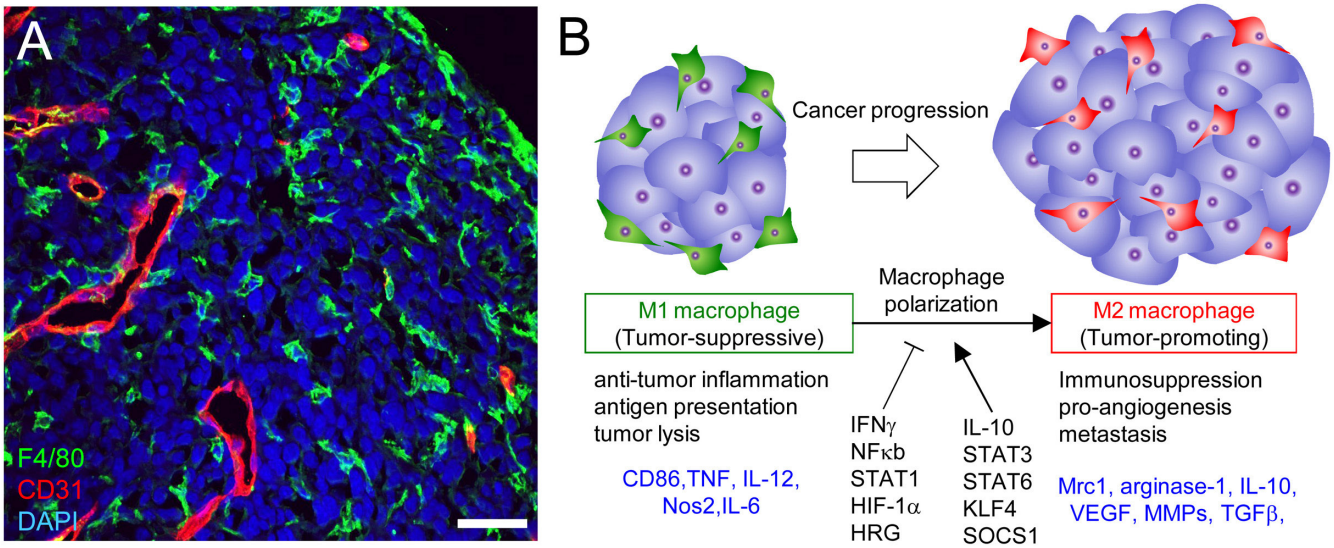

Fig. 3 Tumor-associated macrophages and their "polarization."
(A) Sectional immunohistochemistry of a B16 melanoma 10 days after cell transplantation into the skin on the back of a syngeneic mouse. Tumor-associated macrophages (TAMs), visualized by antibodies against F4/80, were found in both avascular and vascularized areas with oval to typical stellate morphology. (B) A scheme for macrophage "polarization." Classically activated (M1) macrophages have tumoricidal activity. During cancer progression, macrophages shift to alternatively activated macrophages (M2), which in turn suppress tumor immunity and increase angiogenesis and metastasis. The typical M1 macrophage expression profile includes CD86, tumor necrosis factor (TNF), IL-12, Nos2 and IL-6, whereas M2 macrophages preferentially express macrophage mannose receptor (Mrc1), arginase-1, IL-10, VEGF, matrix metalloproteinases (MMPs) and transforming growth factor-β (TGFβ). Several exogenous and intracellular signaling pathways are known to positively or negatively regulate transition from M1 to M2 macrophages. Scale bar: 50 μm.

ventional anti-cancer therapies.[60–62] Gene expression analysis of endothelial cells derived from human blood vessels of normal and malignant colorectal tissues revealed forty-six genes that were specifically elevated in tumor-associated endothelium.[63] A pioneering study by Hida and colleagues reported genotypic alterations in tumor endothelial cells: tumor endothelial cells show chromosome abnormalities characterized by aneuploidy and abnormal multiple centrosomes,[64] which may lead to genomic instability and activation of the DNA damage response (DDR). In this context, Chavakis and colleagues reported that a major DDR molecule, H_2AX, is specifically activated in pathological angiogenesis of an ocular neovascular model and a tumor-xenograft model.[65] Loss of H_2AX strongly impairs pathological angiogenesis but does not affect healthy vessels. An antibody against placental growth factor (PlGF), a member of the VEGF family that selectively binds VEGFR-1 and its co-receptors neuropilin-1 and -2, inhibits growth of VEGF blockade-resistant tumors without affecting healthy vessels.[66] Targeting PlGF inhibits not only angiogenesis but also the recruitment of angiogenic macrophages. Macrophage depletion by CSF-1 blockade can have tumor-selective anti-angiogenic effects;[47] therefore, the main anti-cancer effects of anti-PlGF therapy could be the result of its impact on macrophages.

Future Directions and Perspectives

The evidence demonstrates that tumor vascularity mainly depends on VEGF signaling.[12,13] However, recent preclinical and clinical studies show that a remarkable degree of compensation by VEGF-independent pro-angiogenic mechanisms occurs, and this compensation works, in particular, in the absence of VEGF signaling. It has also been demonstrated that combining conventional anti-VEGF therapy with blockade of VEGF-independent pro-angiogenesis pathways greatly enhances tumor suppression (**Fig. 4**). For example, blockade of FGFs or angiopoietins enhances and prolongs the anti-angiogenic effects of VEGF blockade.[28,67] Some types of perivascular cells such as macrophages (Kubota *et al*. 2009; Qian and Pollard, 2010), pericytes,[35] cancer cell-derived endothelial cells[37,38] and tissue-resident vascular precursors[68] are known to contribute significantly to tumor angiogenesis and refractoriness to VEGF blockade. Although further analysis of the molecular and cellular bases is required, these cells show promise as targets in combination therapy with VEGF blockade.

So far, the efficacy of the anti-angiogenic strategy has

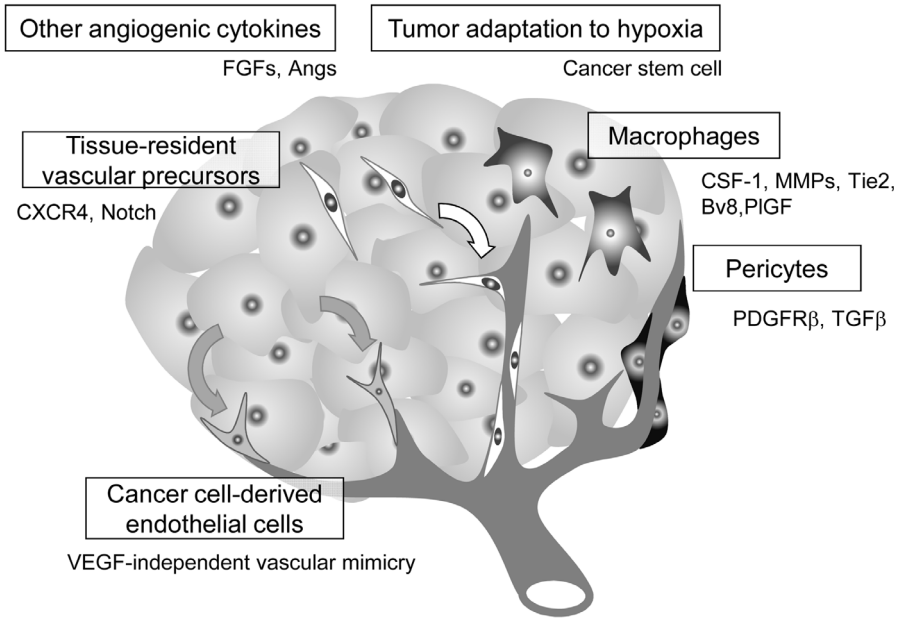

Fig. 4 VEGF-independent pro-angiogenic mechanisms: novel targets for anti-angiogenic therapy.
VEGF blockade enhances the expression in tumors of other pro-angiogenic cytokines such as fibroblast growth factors (FGFs) and angiopoietins (Angs). Selection of hypoxia-tolerant tumor cells may elicit tumor adaptation to anti-angiogenesis. Some types of perivascular/vessel-associated cells such as macrophages, pericytes, cancer stem-like cells and tissue-resident vascular precursors contribute to tumor angiogenesis and refractoriness to VEGF blockade. Inhibition of macrophage function could be achieved by targeting colony-stimulating factor 1 (CSF-1), matrix metalloproteinases (MMPs), Tie-2, Bv8 or placental growth factor (PlGF). To suppress pericyte recruitment, PDGFRβ or TGFβ signaling could be targeted. The recruitment and function of tissue-resident vascular precursors are mediated by CXCR4 and Notch signaling.

been validated in solid tumors. Recently, it has gained increasing attention in leukemia therapy. Chronic myeloid leukemia (CML) is highly vascularized in the bone marrow, and the vessel density has potential as a prognostic indicator, which suggests that angiogenesis contributes to leukemogenesis.[69] Although anti-VEGF therapy has limited effectiveness in the treatment of CML,[70] a recent preclinical study indicated that anti-PlGF treatment prolonged survival of patients with imatinib-resistant CML. This treatment works by inhibiting bone marrow angiogenesis and directly suppressing CML proliferation, in part independently of oncogenic Bcr-Abl1 signaling.[71] In mouse models of T-cell acute lymphoblastic lymphoma and pro-B-cell leukemia, the anti-angiogenic effects of thrombospondins secreted by $CD4^+$ T cells were sufficient to elicit spontaneous oncogene inactivation leading to tumor regression.[72]

Overall, the evidence demonstrates that anti-angiogenic therapy (typically blockade of VEGF signaling) has remarkable therapeutic effects in various types of human cancers. However, the molecular bases of cancer type-dependent resistance mechanisms against VEGF blockade, especially VEGF-independent pro-angiogenic mechanisms, now need to be clarified. Targeting these mechanisms would greatly enhance the effects and minimize the required doses of VEGF blockers. There is no doubt that effort in this area will yield opportunities to greatly improve anti-angiogenic treatment.

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