

Proteins Expressed on Tumor Endothelial Cells as Potential Targets for Anti-Angiogenic Therapy

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The fact that solid tumors are dependent on blood supply has inspired many researchers to search for anti-angiogenic molecules that could be used therapeutically to halt the blood flow and rob tumors of nourishment and oxygen. Some promising preclinical and clinical results further confirmed the advantages of angiogenesis inhibitors. However, toxicity side effects and development of drug resistance in cancer cells have suggested the necessity to develop more specific and potent anti-angiogenic targets. Tumor blood vessels express some molecules that are not present in resting blood vessels in normal tissues. Many of these proteins, such as vascular endothelial growth factor receptor, integrins, delta-like ligand 4, EphB4, ephrin-B2, tumor endothelial markers 5 and 8, annexin A1 and fibronectin extra-domain B, are functionally important to tumor angiogenesis, and therefore can be taken as potential targets for anti-angiogenic therapy. In this review article, we discuss these potential target proteins expressed on the tumor endothelial cells and their possible therapeutic use.

Journal of Cancer Molecules 4(1): 17-22, 2008.

Keywords:

angiogenesis
tumor endothelial cells
tumor endothelial marker
VEGF

Introduction

The growth of solid tumors is dependent on their capacity to induce the growth of blood vessels to supply them with oxygen and nutrients. The critical role of angiogenesis in cancer, the formation of new vessels from the pre-existing vasculature, was first proposed 37 years ago by Folkman [1]. Angiogenesis and the nutrient supply by new blood vessels is essential for tumors to grow beyond 1-2 mm [2]. The new blood vessels also offer a path for tumor cells to enter the circulation, enabling the metastasis of cancer cells to multiple organs. It is no wonder that, since 1971, it has been found to be a crucial prognostic factor in many types of cancer and had frequently been correlated with tumor progression, disease severity and capacity for metastasis [3].

Tumor angiogenesis involves an intricate interplay between the tumor and supportive cells, such as tumor-associated endothelial cells, pericytes, smooth muscle cells, fibroblasts, and macrophages. Tumor angiogenesis activates endothelial cell proliferation, recruits migrating endothelial cells and pericytes, and forms new blood vessels through vascular remodeling and maturation. Considering the side effects associated with traditional chemotherapies and the possibility of interrupting a tumor's supply of oxy-

gen and nutrients, there has been great interest take in the targeting of tumor vasculature and much effort has been directed towards the development of anti-angiogenic agents. It has been proposed that tumor-stimulated endothelial cells have a unique proliferating and migrating phenotype compared with quiescent endothelial cells, and that targeting this phenotype would be so specific that no major side effects could occur, except for during wound healing and the menstrual cycle when most endothelial cells are more active. However, toxicities may be caused and depend on the duration and different rates of anti-angiogenic onset. For example, anthracycline chemotherapy may reduce the left ventricular ejection fraction, which can ultimately be life threatening [4,5]; meanwhile, the anti-angiogenic agent bevacizumab has been associated with gastrointestinal perforations and wound-healing complications and found to possibly cause acute life-threatening problems [6]. Moreover, tumors eventually become resistant to angiogenesis inhibitors in almost all treated patients [7]. A more detailed understanding of the molecular mechanisms involved in tumor angiogenesis and interaction between tumor and vascular system should allow more specific and potent strategies to be developed.

Angiogenesis in cancer

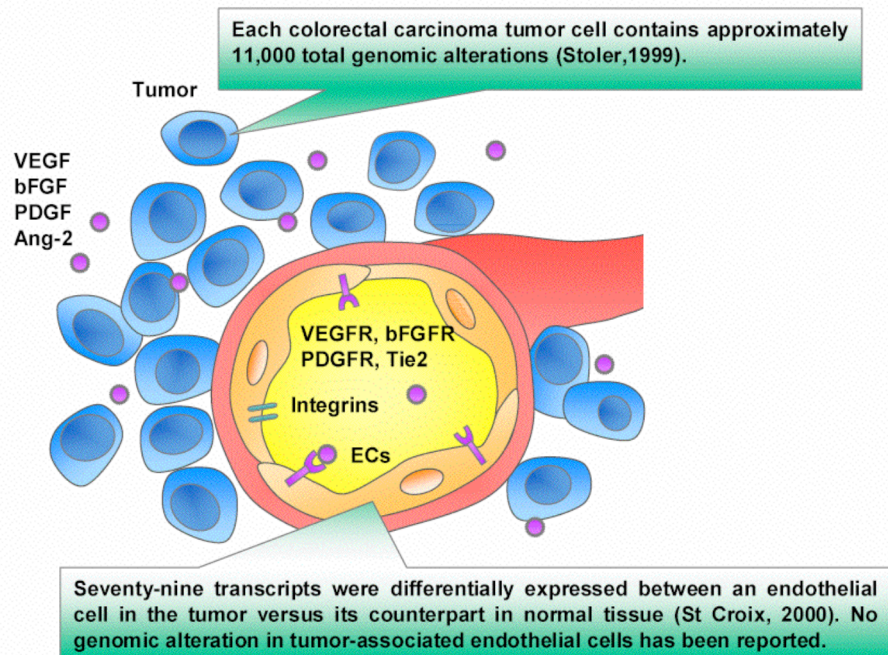
Tumor vessels are structurally and functionally abnormal. Compared with the vasculature in normal tissues, the tumor vasculature is highly disorganized, vessels are strikingly tortuous and leaky architectures [8-11], their diameter is irregular and their walls are thin [12,13]. The flow of blood through the tumor capillaries is frequently sluggish, and at times might be stationary or even experience a reversal in the direction of flow [14,15]. A relative deficiency of pericytes, or pericyte function, could be responsible for these morphological features in tumor vasculature [16].

Received 3/17/08; Revised 3/27/08; Accepted 3/27/08.

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²Abbreviations: VEGF, vascular endothelial growth factor; PLGF, placenta-like growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; Ang, angiopoietin; HIF-1 α , hypoxia-inducible factor-1 α ; CEPs, circulating endothelial progenitor cells; PIGF, placenta growth factor; CECs, circulating endothelial cells; VEGFR, VEGF receptor; ECM, extracellular matrix; DLL4, delta-like ligand 4; TEMs, tumor endothelial markers; EDB, fibronectin extra-domain B.

Figure 1: Angiogenesis inhibitors target genetically stable endothelial cells. Cancer cells produce various angiogenic factors, such as VEGF, PDGF, bFGF and Ang-2, to promote angiogenesis within the tumor. However, in contrast to the cancer cells housing instable genome that could develop resistance to chemotherapeutic regimen, the endothelial cells possess mutation-free genome so that no refractory clone will be selected out after target therapy.



Angiogenesis is orchestrated by a variety of activators and inhibitors. Angiogenic switch is “off” when the effect of pro-angiogenic molecules is balanced by anti-angiogenic molecules, and is “on” when the net balance is favor of angiogenesis [9,17,18]. Pro- and anti-angiogenic molecules can emanate from cancer cells, endothelial cells, stromal cells, blood, and the extracellular matrix [19]. Expression of pro-angiogenic factors can be induced by tumor-associated hypoxia, activation of oncogenes, inactivation of tumor-suppressor genes, and secretion of various growth factors and cytokines. It has been reported that oncogene-driven tumor expression of pro-angiogenic proteins include vascular endothelial growth factor (VEGF²), placenta-like growth factor (PLGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietin (Ang)-2, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), pleiotrophin, and others [20]. However, when stimulated by PDGF, not only tumor cells but also tumor stroma and pericytes produce angiogenic factors such as Ang-1 [21]. Tumor-associated hypoxic conditions also activate transcription factor hypoxia-inducible factor-1 α (HIF-1 α) [22], which induces expression of several angiogenic factors including VEGF, nitric oxide synthase (NOS), PDGF, Ang-2 [23]. Recent studies have shown that tumor-associated macrophages respond to hypoxia in tumors by upregulating the expression of HIFs [24]. HIF-1 α also induces expression of epithelial-mesenchymal transition (EMT) regulators, such as Snail, Zeb1, SIP1, E47, LOX (lysyl oxidase) and TWIST, and promotes metastasis in response to intratumoral hypoxia [25-27].

Bone marrow-derived circulating endothelial progenitor cells (CEPs) also contribute to some forms of angiogenesis, such as development of the vasculature in early embryonic development as well as tumor angiogenesis [28,29]. These cells can be mobilized from the bone marrow and then enter the peripheral-blood circulation, migrate to sites of ongoing angiogenesis and differentiate into mature endothelial cells [30,31]. This process is triggered by the increased availability of pro-angiogenic factors such as VEGF, placenta growth factor (PlGF) and Ang-1 [32]. Once at the site of neovascularization, the endothelial progenitor cells themselves can

secrete further pro-angiogenic factors. Increases in the number of CEPs and circulating endothelial cells (CECs) have been reported in various pathological conditions including cancer. At the clinical level, evidence is emerging that CEC kinetics and viability might correlate with clinical outcomes in cancer patients who undergo anti-angiogenic treatment. Therefore, the measurement of CEC and CEP may potentially serve a surrogate marker for monitoring anti-angiogenic treatment and drug activity, and could help to determine the optimal biological dose of anti-angiogenic drugs [33,34].

Potential targets for anti-angiogenic therapy

Endothelial cells that make up tumor vasculature are genetically stable and non-malignant and rarely develop drug resistance, in contrast to tumor cells that often acquire drug resistance from genetic and epigenetic mechanisms (Figure 1). For example, a human colorectal cancer cell may have as many as 11,000 total genomic alterations [35]. In contrast, there are only few differences in gene expression between an endothelial cell in a tumor bed and its counterpart in normal tissue. One study reported 79 transcripts were differentially expressed in tumor-associated endothelium, 46 of which were significantly elevated [36]. However, there has been no report of genomic alterations in human endothelial cells in the tumor bed. As a result, these abnormal gene expressions can be potential targets in cancer therapy, and angiogenesis inhibitors are emerging as a new class of therapeutic agents.

VEGFs and VEGF receptors

The VEGFs-VEGF receptors (VEGFRs) signaling pathways are important in angiogenesis. Angiogenic vessels express elevated levels of VEGFRs for binding to VEGFs. For example, the expression of VEGFR1 (FLT1) and VEGFR2 (FLK1/KDR) is elevated in the endothelial cells of developing tumor blood vessels [37]. VEGFR3 (FLT4) is involved in the development of blood vessels in the embryo, but becomes restricted to the endothelia of lymphatic vessels in the adult. The VEGF family includes the prototype member VEGF-A,

Table 1: VEGF signaling as targets in anti-angiogenic therapy

Molecular target	Drug	Current status	Reference
VEGF	Bevacizumab (antibody)	FDA approved	[6]
VEGFR-2	IMC-1C11 (antibody)	Phase I	[91]
VEGFR-3	mF4-31C1 (antibody)	Preclinical trial	[92]
VEGFR-1, -2, and -3	Vatalanib (small molecule)	Phase III	[93]

PIGF [38], VEGF-B [39], VEGF-C [40], VEGF-D [41], VEGF-E [42], and VEGF-F [43]. It is now known that VEGF, also referred to as VEGF-A, is one such key regulators of angiogenesis produced by tumor cells and tumor-associated stroma cells. It acts predominantly on VEGFR2 to stimulate endothelial cell migration and proliferation and to increase vascular permeability [44,45].

The gene encoding human VEGF-A is organized in eight exons, separated by seven introns [46,47]. Alternative exon splicing results in the generation of four principal isoforms - VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆, which have 121, 165, 189 and 206 amino acids, respectively [48]. VEGF₁₆₅ is the predominant isoform. VEGF-A action constitutes a rate-limiting step in normal and pathological blood vessel growth. VEGF-B interacts with VEGFR1. Under some circumstances, VEGFR1 might function as a “decoy” receptor that sequesters VEGF and prevents its interaction with VEGFR2 [49]. VEGF-C and VEGF-D bind VEGFR3 thus regulating lymphatic angiogenesis [50]. VEGF mRNA transcription and stability are also affected by other growth factors and oncogenes, including estrogen, nitric oxide, FGF, PDGF, tumor necrosis factor- α (TNF- α), epidermal growth factor, interleukin (IL)-1 α , IL-6, Ras, and Wnt [51]. There is growing evidence that VEGFR1 plays an important role in hematopoiesis and that VEGFR2 is the major mediator of the mitogenic, migratory, angiogenic, and vascular permeability-enhancing effects of VEGF on endothelial cells [51].

VEGF signaling can be inhibited by antibodies and small molecules that inactivate VEGF or its receptor (Table 1). For example, bevacizumab (Avastin; Genentech and Roche) is a humanized monoclonal antibody that binds the VEGF ligand and prevents receptor binding and signal transduction [51]. Bevacizumab has been approved as an adjunct to first-line treatment of metastatic colorectal cancer [6], and is currently being evaluated in other malignancies.

Integrins

The integrins, which consist of α - and β -subunits, are a large family of cell-surface receptors that bind the extracellular matrix (ECM) components. They transmit signals from the ECM to cells that regulate cell growth and survival [52]. Endothelial cells in angiogenic vessels express their own integrins, which differs from the integrin repertoire of resting endothelial cells. The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are upregulated in endothelial cells undergoing angiogenesis [53,54]. The $\alpha_5\beta_1$ integrin, which is a fibronectin receptor, is also selectively expressed in angiogenic vasculature [55]. Blockage of these integrins interferes with angiogenesis [56,57]. A high-affinity humanized anti- $\alpha_v\beta_3$ antibody (Vitaxin) is recently been developed as an anti-angiogenic drug [58]. Ruoslahti and co-workers, using *in vivo* panning of phage-displayed peptide libraries, identified integrin-binding peptides containing an arginine-glycine-asparagine (RGD) motif [59]. In biodistribution studies with animals and scintigraphic studies in humans, these peptides have not yet been shown to preferentially localize to tumor blood vessels, though other RGD-based peptides and peptidomimetics have been extensively tested in preclinical studies [60,61].

Delta-like ligand 4 (DLL4)

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Delta-like ligand 4 (DLL4) is a member of the Notch/Delta family of signaling molecules and, like other Delta molecules, is located in the plasma membrane. DLL4 is expressed on arterial endothelium in the developing embryos; in adults it is usually detected at sites where there is active angiogenesis, including tumors [62]. DLL4 is induced by both hypoxia [62] and VEGF [63], a negative-feedback regulator of vascular growth. VEGF blockade results in a loss of many tumor vessels and an apparent “normalization” of the remaining tumor vessels. However, DLL4 blockade results in a striking increase in tumor vascularity. These vessels function poorly causing a decrease in tumor perfusion and decrease in tumor growth [64,65].

Ephrins and Eph

The ephrin ligands and Eph receptors constitute a large family of signaling molecules that are also involved in angiogenesis. The ephrins bind to two families of transmembrane EphA and EphB receptor tyrosine kinases. Several ephrins and Eph receptors have been shown to be present on vascular endothelium. These include ephrin-A1, which plays a role in TNF- α -induced inflammatory angiogenesis [66], and ephrin-B1, which promotes endothelial capillary-like assembly and attachment *in vivo* [67]. Ephrin-B2 and the Eph receptors EphB3 and EphB4 are also present in vascular endothelium. During embryonic development, ephrin-B2 and EphB4 are expressed in arteries and veins, respectively, and are required for vascular remodeling during blood vessel maturation [68]. However, the ephrin-A1 ligand and its EphA2 receptor are expressed in tumor angiogenesis [69]. Antibody inhibition experiments show that Eph/ephrin signaling is required for angiogenesis to proceed [66]. Further studies have shown that soluble EphA2-Fc and EphA3-Fc constructs inhibit tumor angiogenesis and growth *in vivo* [70], providing the first functional evidence for EphA receptor in the regulation of tumor angiogenesis. It has also been reported that tumor cell expression of EphB4 promotes angiogenesis through interactions with ephrin-B2 presented on endothelium [71]. Consistent with an important role of EphB4/ephrin-B2 interactions in tumors, soluble fusion proteins containing the extracellular domain of EphB4 (sEphB4) reduce the growth and vascularization of tumors by antagonizing endogenous receptor-ligand interactions [72,73]. The abrogation of the function of both ephrins and Ephs may provide novel antiangiogenic and antitumor activities.

Tumor Endothelial Markers (TEMs)

Using endothelium isolated from normal and cancerous colon, serial analysis of gene expression (SAGE) libraries were constructed and a direct comparison identified genes that were upregulated in the tumor endothelium [36]. This led to the identification of several novel tumor endothelial markers (TEMs). TEM5 and TEM8 showed strong tumor endothelial expression but were essentially absent from normal tissue [74]. TEM5 is a seven-pass transmembrane G-protein-coupled receptor (GPCR) with a long extracellular amino-terminal domain that contains five leucine-rich regions, an immunoglobulin domain, a hormone-receptor domain, and a proteolytic cleavage site that is adjacent to

the first transmembrane stretch [75]. A soluble TEM5 (sTEM5) fragment is shed by endothelial cells during angiogenesis and binds to glycosaminoglycans of ECM and proteoglycans present on cell surface. Further proteolytic processing of sTEM5 leads to exposure of its RGD motif, mediating endothelial cell survival by linking integrin $\alpha_v\beta_3$ to glycosaminoglycans [76]. TEM8 has a single-pass transmembrane domain. TEM8 has been shown to be an anthrax-toxin receptor, and the binding of the toxin to TEM8 expressed on tumor endothelium is followed by endothelial death, which might explain the antitumor activity of the toxin [77]. TEM8-Fc, the protective antigen-binding domain of human TEM8 linked to the Fc portion of human immunoglobulin, has been found to suppress the growth and metastasis of human tumor xenograft in athymic nude mice [78]. Furthermore, TEM8 has been found to interact with the M2 isoenzyme of pyruvate kinase (M2-PK), which has an important role in tumor growth and metastasis. TEM8-Fc may be used as a therapeutic agent to trap M2-PK [78].

Annexin A

Schnitzer and co-workers have used a subtractive proteomic mapping strategy to identify proteins that are differentially expressed on the endothelial surface in normal and tumor tissues [79]. They compared the endothelium from rat normal lung tissue with that from lungs with metastatic breast adenocarcinoma and identified annexin A1, which was preferentially expressed on tumor endothelial cells. Furthermore, relatively low radioactive doses of the annexin A1 antibody labeled with iodine-125 have been shown to have a therapeutic benefit in rats [79].

Other potential targets in the tumor endothelium

Endothelial-specific protein disulphide isomerase is a hypoxia-induced gene that is predominantly expressed by endothelial cells [80]. It has been shown to protect the endothelium from apoptosis during hypoxia-induced stress.

Roundabout-4 (Robo4) is a member of the roundabout receptor family and has a smaller extracellular domain than the neuronal roundabout receptors. Roundabout-4 is endothelial-specific and strongly expressed in the developing embryo [81] and at sites such as tumors where active angiogenesis is occurring [82].

Fibronectin extra-domain B (EDB) is a 91-amino acid type III homology domain that is inserted into the fibronectin molecule under tissue remodeling conditions by a mechanism involving alternative splicing at the level of the primary transcript [83]. EDB is essentially undetectable in healthy adult individuals. However, EDB-containing fibronectin is abundant in many aggressive solid tumors, and displays either predominantly vascular or diffuse stromal patterns of expression, depending on the tumor type [84-86]. Anti-EDB antibodies stain tumor blood vessels in grade III-IV astrocytomas, but less than 10% of the vessels are stained in grade I-II astrocytomas and no staining is detectable in normal tissues [85].

Challenge and perspective

The development of targeted therapeutics against cancer, with improved discrimination between tumor cells and non-malignant counterparts, is one of the major goals of anticancer research. The current angiogenesis inhibitors have been found to induce toxicity side effects including bleeding, disturbed wound healing, thrombosis, hypertension, hypothyroidism, proteinuria, skin toxicity, leucopenia, immunomodulation, and so on. We expect that a better understanding of the molecular mechanisms involved in the toxicity of angiogenesis inhibition should allow more specific and

potent inhibitors to be developed. Moreover, a more detailed understanding of the complex parameters that govern the interactions between the tumor and vascular compartments will help to improve anti-angiogenic strategies – not only for the treatment of cancer, but also for the prevention of recurrence. Anti-angiogenic therapy is a new modality for which many traditional parameters for cytotoxic chemotherapy do not apply. Surrogate biomarkers are urgently needed to evaluate the activity of angiogenesis inhibitors and help determine appropriate doses because, unlike conventional cytotoxic agents, their lack of severe side effects precludes the use of maximum tolerated dose and some angiogenesis inhibitors produce a U-shaped dose-response curve. Reaching a prolonged, stable disease state without detrimental adverse effects may be a meaningful goal for angiogenesis inhibitors. In conclusion, vascular markers selectively expressed on tumor endothelial cells seem to be ideally suited for tumor-targeting strategies, opening new possibilities for the imaging and the therapy of cancer [87-90].

Acknowledgments

This work is supported by Academia Sinica and National Science Council, Taiwan.

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