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Targeting Angiogenesis and the Tumor Microenvironment

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Synopsis

The role of the microenvironment during the initiation and progression of malignancy is appreciated to be of critical importance for improved molecular diagnostics and therapeutics. The tumor microenvironment is the product of a crosstalk between different cells types. Critical elements in the microenvironment include tumor associated fibroblasts, which provide an essential communication network via secretion of growth factors and chemokines, inducing an altered extracellular matrix (ECM), thereby providing additional oncogenic signals that enhance cancer-cell proliferation and invasion. Active contribution of tumor-associated stromal cells to cancer progression has been recognized. Stromal elements consist of the ECM, fibroblasts of various phenotypes, and a scaffold composed of immune and inflammatory cells, blood and lymph vessels, and nerves. This review will focus on therapeutic targets in the microenvironment related to tumor endothelium, tumor associated fibroblasts and the extracellular matrix.

Keywords

Angiogenesis; tumor microenvironment; tumor associated fibroblast; integrins; growth factors

Tumor microenvironment

The role of the microenvironment during the initiation and progression of malignancy is appreciated to be of critical importance for improved molecular diagnostics and therapeutics(1). The tumor microenvironment is the product of a crosstalk between different

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cells types. For instance, in epithelial tumors these cells include the invasive carcinoma and its stromal elements. Critical elements in the microenvironment include tumor associated fibroblasts, which provide an essential communication network via secretion of growth factors and chemokines, inducing an altered extracellular matrix (ECM), thereby providing additional oncogenic signals that enhance cancer-cell proliferation and invasion. Active contribution of tumor-associated stromal cells to cancer progression has been recognized(1). Stromal elements consist of the ECM, fibroblasts of various phenotypes, and a scaffold composed of immune and inflammatory cells, blood and lymph vessels, and nerves. This review will focus on therapeutic targets in the microenvironment related to tumor endothelium, tumor associated fibroblasts and the extracellular matrix, while the immune targets will be covered in the immunotherapy article in this issue.

Angiogenesis is necessary for tumor growth

The initial evidence that angiogenesis is necessary for tumor growth came from studies transplanting cancer cells into the avascular corneas of rabbits(2). In these studies, tumors did not grow in rabbit corneas before sprouting vessels were able to grow to connect to the tumor. Additionally, inhibiting blood vessel formation prevented tumor growth beyond 0.4 mm(2). Similar studies found that tumors placed in chicken embryo chorioallantoic membranes shrank during the first 3 days after placement(3). However, new vessel formation was seen to form from existing vessels after the tumors were placed. When these new vessels connected to the tumor, tumor growth continued. These studies not only identified a significant role of vessel formation in tumor progression, but they identified that tumors elicited the growth of vessels from existing vessels. This suggested that tumors released diffusible factors that initiate angiogenesis to continue and maintain their growth.

There are four known strategies by which tumors can augment their blood supply. They can stimulate angiogenesis; utilize existing vessels directly; induce vasculogenesis; or form vasculogenic networks without vascular cells. The secretion of pro-angiogenic factors and inhibition of anti-angiogenic factors induce vascular sprouting from pre-existing capillaries and venules, which constitutes the process of angiogenesis. This is the most common way tumors gain access to the vasculature. Tumors may also utilize existing vessels by growing alongside them, as is the case in astrocytomas(4). The strategy of vasculogenesis involves the formation of blood vessels from bone marrow precursors. This differs markedly from angiogenesis in that the sources of the cells that make up the vessels are from the bone marrow and not from pre-existing vessels. However, many of the soluble mediators that initiate vasculogenesis, notably VEGF, parallel those found in angiogenesis(5). Lastly, tumors themselves can form lumens which can be used to transport blood, but lack endothelial cells or other vascular components(6, 7). Multiple strategies may be in play in a particular tumor, depending on their tumor type or stage. In this review we will focus on the major pathways of angiogenesis as its contribution has been applicable to therapeutic intervention.

Growth factors/receptor tyrosine kinases

Vascular Endothelial Growth Factor Family

An essential mediator of angiogenesis is the vascular endothelial growth factor (VEGF) family, which consists of five family members of secreted proteins (VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, and platelet derived growth factor (PDGF)(8), that bind and activate three receptor tyrosine kinases (VEGFR-1, -2 and -3). VEGFA promotes endothelial cell migration, proliferation, vascular permeability, and tube formation. VEGFB was identified as an endothelial cell growth factor expressed in skeletal muscle and heart(9), however its role as an angiogenesis factor is not clearly defined. VEGFC and VEGFD play

critical roles in lymphangiogenesis, and their expression has been correlated with the development of lymph node metastases(10). Placental growth factor (PGF) promotes the survival of endothelial cells and regulates the activity of VEGF signaling(11).

Regulation of VEGF family genes expression is under the control of stresses such as hypoxia, acidity and hypoglycemia, which stimulate transcription and increase mRNA stability, resulting in increased protein expression. Under normoxic conditions, prolyl residues in hypoxia inducible factors (HIF) proteins are hydroxylated by prolyl hydroxylase in a reaction that uses molecular oxygen. Hydroxylation of HIF proteins targets them for ubiquitin-mediated proteolysis. Decreased oxygen concentration reduces the efficiency of this process: HIF proteins are stabilized and become available to bind to hypoxia-response elements in the promoters of target genes, thereby activating transcription(12).

VEGF proteins bind to receptor tyrosine kinases: VEGF receptor-1, -2 and -3 (VEGFR-1, -2, -3)(13), which mediate cell signaling resulting in the biologic effects of VEGF. VEGFR-1 (Flt-1) binds three of the VEGF family ligands, VEGF-A, VEGF-B and PGF. Activation of VEGFR-1 results in hematopoiesis, embryonic vessel development, macrophage chemotaxis and recruitment of endothelial progenitor cells to tumor blood vessels from the bone marrow(14). VEGFR-2 (Flk-1/KDR) is the central mediator of VEGF-stimulated tumor angiogenesis and is essential in embryonic vascular development. When VEGF binds to VEGFR-2, the receptor is phosphorylated and activates downstream signaling molecules including protein kinase C, phospholipase C, MAP kinase, Raf, PI3K and FAK pathways, resulting in endothelial cell migration, proliferation, tube formation, and anti-apoptosis(15). VEGFR-3 binds VEGF-C and -D and is involved in the formation of lymphatics in tumors and normal tissue(16).

A humanized version of a monoclonal antibody to VEGFA, bevacizumab (Avastin; Genentech), became the first Food and Drug Administration (FDA)–approved antiangiogenic drug in the United States in 2004(10). It was approved as a first-line treatment agent for metastatic colorectal cancer, in combination with 5-fluorouracil(17) and was later approved for treatment of metastatic non–squamous-cell lung cancer, breast cancer, and glioblastoma multiforme(18).

Additional FDA-approved drugs that block VEGF signaling are sunitinib and sorafenib, both receptor tyrosine kinase inhibitors that are administered orally. In addition to blocking VEGFR signaling, sorafenib, also blocks PDGFRB, FLT3, and KIT signaling(19). Similarly, sunitinib blocks signaling from VEGFR1-3, PDGFRA, PDGFRB, FLT3, and RET(19). Sorafenib has been approved for unresectable hepatocellular carcinoma and advanced renal cell carcinoma, whereas sunitinib has been approved for metastatic renal cell carcinoma, gastrointestinal stromal tumors (10) and neuroendocrine tumors(20).

TGF-β

TGF-β is a paracrine polypeptide with three homologous forms (TGFβ1, TGFβ2, and TGFβ3). TGF-β is produced in latent form as a zymogen, and after secretion a latency associated peptide is proteolytically cleaved to release active TGF-β. Active TGF-β binds to constitutively active type 2 receptors (TGFBR2) to activate type 1 receptors (TGFBR1) in a heteromeric complex that controls transcription through the action of a family of SMAD proteins (21). TGF-β is a proangiogenic agent despite the fact that, in vitro, TGF-β causes apoptosis and growth arrest of endothelial cells. This paradoxical behavior may be explained by the fact that TGF-β activates the secretion of fibroblast growth factor 2, which acts to stimulate the expression of VEGF. VEGF in turn acts in an autocrine manner through its receptor VEGFR2 to activate the MAPK pathway. However, TGF-β will reverse the protective action of VEGF, promoting apoptosis, which occurs in the pruning process, to

form the final vascular network(22). Therapeutic approaches for targeting TGF-β signaling include antagonism of TGF-β ligand binding to the heteromeric receptor complex with isoform-selective antibodies, such as lerdelimumab (TGF-β_2) and metelimumab (TGF-β_1) or the pan-neutralizing antibody GC-1008, and intracellular inhibition of the type I TGF-β receptor kinase with small-molecule inhibitors, such as LY550410, SB-505124 or SD-208(23).

Fibroblast Growth Factor

Fibroblast growth factors (FGFs) maintain endothelial cell function. FGF1 and FGF2 promote endothelial cell proliferation and migration and stimulate angiogenesis(24). FGFs produce their biological effects by binding to transmembrane tyrosine kinase receptors, FGFR1 through FGFR4(25). FGFR can activate PLC-γ thereby stimulating production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). This, in turn, releases intracellular calcium and activates Ca^{2+}-dependent PKCs. The activation of the PI3K-Akt cell survival pathway is one of the important biological responses induced by FGF2 in endothelial cells(26).

There are several inhibitors of FGF signaling that are undergoing clinical trials. FP-1039 (FGFR1:Fc) is a soluble fusion protein consisting of the extracellular domain of human fibroblast growth factor receptor 1c (FGFR1) linked to the Fc portion of human IgG1. FP-1039 prevents FGFR1 ligands from binding to any of their related receptors within the family of seven FGF receptors, and may mediate both anti-tumor and antiangiogenic effects. E-3810 and TKI258 are dual VEGFR and FGFR tyrosine kinase inhibitors in clinical trial(24).

Notch

The notch signaling pathway involves gene regulation mechanisms that control multiple cell differentiation processes during embryonic and adult life and is essential for cell-cell communication. This pathway has been directly linked to tumor angiogenesis and in the process of activating dormant tumors. VEGFA induces expression of the endothelium-specific Notch ligand delta-like 4 (DLL4): when DLL4 activates the Notch signaling pathway in neighboring cells, the effect is to inhibit dorsal sprouting of endothelial tubes. When expressed in tumor cells DLL4 can activate Notch signaling in host stromal cells, thereby improving vascular function(27). Inhibition of DLL4-mediated Notch signaling promotes a hyperproliferative response in endothelial cells, a process that leads to an increase in angiogenic sprouting and branching. Despite this increase in vascularity, tumors are poorly perfused, hypoxia increases, and tumor growth is inhibited. Neutralizing anti-Dll4 antibodies have been demonstrated to inhibit tumor growth in vivo(28). These findings point to the Notch pathway as a potential therapeutic target.

Angiopoiten/Tie receptors

Angiopoietins belong to a family of endothelial cell-specific molecules that play an important role in endothelial growth, maintenance, and stabilization by binding to Tie receptors(29). There are four types of angiopoietins: Ang-1, -2, -3 and -4. The Tie1 receptor is highly expressed in angioblasts, embryonic vascular endothelium, and endocardium, and in adult tissues is expressed in lung capillaries (30). The Tie2 receptor mediates survival signals for endothelial cells resulting in vessel maturation. Ang-2 is an autocrine antagonist that induces vascular destabilization, while Ang-1 is an agonist that promotes vessel stabilization in a paracrine fashion. Ang-2 is implicated in tumor-induced angiogenesis and progression and is increased during vascular remodeling (31)http://www.ejinme.com/article/PIIS0953620509001393/fulltext-bib16. AMG 386 is an investigational peptide-Fc fusion

protein that inhibits angiogenesis by preventing the interaction of Ang-1 and Ang-2 with their receptor, Tie2, and is being studied in clinical trials(32, 33).

Epidermal Growth Factor

The epidermal growth factor (EGF) family consists of eleven members which bind to one of four epidermal growth factor receptors (EGFR)(34, 35). All of the receptors, except HER3, contain an intracellular tyrosine kinase domain(36). In xenograft models activation of EGFR contributes to angiogenesis (37), in addition to cellular proliferation, survival, migration, adhesion, differentiation, and tumor metastasis(36). The EGFR pathway is more of an indirect regulator of angiogenesis by upregulating the production of pro-angiogenic factors such as VEGF. There are three FDA-approved EGFR inhibitors: cetuximab and panitumumab, which are monoclonal antibodies, and erlotinib, a tyrosine kinase inhibitor that specifically targets EGFR (10).

Insulin Like Growth Factor Pathway

The insulin-like growth factor pathway plays a major role in cancer cell proliferation, survival and resistance to anti-cancer therapies in many human tumors(38). Insulin like growth factor-1 (IGF-1) contributes to the promotion of angiogenesis through increasing VEGF expression via HIF-1a(39). The two main strategies in development for blocking IGF signaling as an anti-cancer therapeutic are receptor blockade and tyrosine kinase inhibition(40). Receptor blockade with the use of monoclonal antibody therapies against the IGF-1R (such as Figitumumab)(41) are being investigated. Tyrosine kinase inhibition in general will indiscriminately inhibit the kinase domains of all IGF system receptors. The exception to this is the NVP-AEW541 and NVP-ADW742, which has 15–30 fold increased potency for IGF-1R kinase inhibition compared to IR kinase inhibition in cellular assays(41).

Calcium signaling

One of the important intracellular pathways stimulated by a variety of angiogenic growth factors, including VEGF, FGF, and a novel angiogenesis factor secreted frizzled related protein 2 (SFRP2)(42) is activation of calcium signaling. Calcium signaling is mediated through transient increases in cytoplasmic free calcium which activates the phosphatase calcineurin. Activated calcineurin dephosphorylates nuclear factor of activated T-cells (NFAT), which then translocates from the cytoplasm to the nucleus(43). NFAT plays a critical role of in mediating angiogenic responses(44, 45). NFAT activation was identified as a critical component of SFRP2(42, 46) and VEGF-induced angiogenesis and linked to the induction of cyclooxygenase-2(47). Activation of the Ca^{2+} pathway induces cell proliferation and inhibits apoptosis in cultured endothelial cells, suggesting a proangiogenic activity.[41,42] FK506 is a calcineurin inhibitor that blocks NFAT activation, and has been shown to inhibit angiogenesis in vitro and tumor growth in vivo(42, 46). In pre-clinical models, a monoclonal antibody to SFRP2 reduced tumor growth in vivo, and inhibited endothelial and tumor cell NFAT activation in vitro(48).

Endogenous Angiogenesis Inhibitors

The activities of a variety of endogenous angiogenic inhibitors have been described to regulate tumor endothelial growth. These include thrombospondin-1(49), angiostatin(50) and endostatin(51). One of the most extraordinary developments in the discovery of endogenous inhibitors came again from the Folkman laboratory(50) via a Lewis Lung Carcinoma mouse model in which lung micrometastases seeded from subcutaneous primary tumors did not develop further when the tumor was intact, but grew rapidly after the primary tumor had been surgically removed. It was hypothesized that the primary tumor itself was

producing a circulating antiangiogenic agent that inhibited blood vessel growth in the lung micrometastases. After resection of the primary tumor the source of the endogenous angiogenesis inhibitor was removed and the lung metastases therefore grew rapidly. O'Reilly and Folkman isolated of a protein they called angiostatin from the urine of mice with intact primary tumors. Angiostatin is a fragment of plasminogen that occurs normally in the circulation, and the cleavage of plasminogen to produce angiostatin occurs in the tumor or itself(50). Purified angiostatin given daily to mice after resection of the primary tumor completely prevented the development of micrometastases. Angiostatin was subsequently shown to be active against primary tumors established in mice from inoculated human tumor cells, and it also inhibits the proliferation of endothelial cells in culture, but had no effect on tumor cell proliferation. Additional proteolytically activated antiangiogenic proteins have been isolated, including endostatin derived from collagen XVIII (51).

Tumor Endothelial Markers

A recent strategy to discovery novel angiogenesis targets is to compare differences in gene expression profiles between tumor and normal endothelium. St Croix isolated endothelial cells using magnetic bead selection from a human colon cancer and adjacent normal colon(52). Among the novel genes identified to be overexpressed by tumor endothelium was TEM8. TEM8 is the anthrax toxin receptor and preclinical targeting of this receptor in tumor models have been successful, make this molecule a candidate for future vascular targeting studies(53). Subsequent studies have used laser capture microdissection of blood vessels from breast tumors and normal breast tissue(54), or ovarian cancer and normal ovarian tissue(55), to identify novel targets that are presently under investigation. One target, SFRP2 has been shown to induce angiogenesis(56), and therapeutic targeting with a monoclonal antibody reduces tumor growth in pre-clinical models(48).

Cell Adhesion to the Extracellular Membrane

Integrins are heterodimer transmembrane receptors for the extracellular matrix composed of an alpha and beta subunits(57). Integrins bind ligands by recognizing short amino acid stretches on exposed loops, particularly the arginine-glycine-aspartic acid (RGD) sequence. Upon ligation integrins mediate signaling events that regulate angiogenesis, cell adhesion, proliferation, survival, and migration. Pathways that are activated include protein kinase B (PKB/Akt), integrin-linked kinase, mitogen-activated protein kinase (MAPK), Rac or nuclear factor kappa B (NF-κB). In inactive vessels, integrins interact with the basal membrane, thereby maintaining vascular quiescence. During angiogenesis, integrins are essential for endothelial cell migration, proliferation, and survival. In preclinical studies, inhibition of integrin function suppresses angiogenesis and tumor growth. Of the 24 known integrin heterodimers, αVβ3(58) and αVβ5(59) were the first vascular integrins targeted to suppress tumor angiogenesis. Three classes of integrin inhibitors are currently in preclinical and clinical development: synthetic peptides containing an RGD sequence (cilengitide; Merck), monoclonal antibodies targeting the extracellular domain of the heterodimer (Vitaxin; MedImmune), and peptidomimetics (S247; Pfizer), which are orally bioavailable nonpeptidic molecules mimicking the RGD sequence(60).

Focal Adhesion Kinase (FAK) is a protein that plays a critical role in intracellular processes of cell spreading, adhesion, motility, survival and cell cycle progression, and has shown to play a role in tumor angiogenesis(61). Pharmacologic blockade of FAK autophosphorylation reduces tumor growth in vivo(62) The FAK gene encodes a non-receptor tyrosine kinase that localizes at contact points of cells with extracellular matrix, and is activated by integrin (cell surface receptor) signaling. Recently Novartis Inc. developed FAK inhibitors down-regulation its kinase activity(63). The novel Novartis FAK inhibitor, TAE-226 recently was

employed in brain cancer and effectively inhibited FAK signaling and caused apoptosis in these cells (64). Another, ATP-targeting site inhibitor of FAK, Pfizer-PF-573,228 has been recently described(65).

Tumor Activated Fibroblasts

Fibroblasts in the tumor stroma synthesize fibroblast activation protein (FAP), a type II transmembrane protein that functions as a serine protease. FAP must be assembled into a dimer to become an active protease.(66) More than 90% of human epithelial cancers overexpress FAP including colon, breast, lung and ovarian cancers. Expression of FAP is highly restricted to cancer associated fibroblasts but its actual function has yet to be fully identified.(67). This enzyme was reported to cleave gelatin and collagen type I and has therefore been implicated in ECM remodeling. It is theorized that FAP has the ability to alter to tumor microenvironment and partially drive angiogenesis as it is overexpressed in tumors demonstrating increased microvessel density, and is overexpressed in human tumor microvessels compared to normal vessles(bhati). FAP mRNA is upregulated in endothelial cells that are undergoing capillary morphogenesis and reorganization. Sibrotuxumab (mAb F19) is a humanized monoclonal antibody to FAP developed for imaging purposes. It is well tolerated and localized to tumor cells but proved to have no effect on metastatic colorectal cancer in phase II trial(68). When conjugated to maytansinoid, the antibody did show long lasting tumor inhibition in multiple xenograft models.(69) Several small molecule inhibitors that block the enzymatic activity of FAP are under development. However, val-prolineboronic acid (PT-100, talabostat), a selective inhibitor both FAP and DPPIV, failed to demonstrate any clinical benefit in phase II trials of metastatic colorectal cancer, NSCLC, stage IV melanoma or chronic lymphocytic leukemia(70).

Conclusion

In summary, the tumor microenvironment involves complex biological signaling pathways with contributions from endothelial cells, tumor associated fibroblasts, and the extra-cellular matrix contributing to tumor growth. Anti-angiogenic therapy has been shown to increase survival in human tumors, but further research is needed to inhibit tumors that are not responsive to, or become resistant to current anti-angiogenic therapy. Further research targeting tumor associated fibroblasts is needed to validate if this will be a therapeutic approach for treating cancer.

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Key points

- The role of the microenvironment during the initiation and progression of malignancy is appreciated to be of critical importance for improved molecular diagnostics and therapeutics.
- The tumor microenvironment is the product of a crosstalk between different cells types. Critical elements in the microenvironment include tumor associated fibroblasts, which provide an essential communication network via secretion of growth factors and chemokines, inducing an altered extracellular matrix (ECM), thereby providing additional oncogenic signals that enhance cancer-cell proliferation and invasion.
- Therapeutic targeting of angiogenesis factors, tumor associated fibroblasts, and cell adhesion molecules compose are an active area of clinical trial investigation to determine efficacy of these approaches.
- Some angiogenesis inhibitors are FDA approved for cancer, yet further research is needed to improve efficacy in resistant tumors.
- Clinical trials targeting integrins and tumor activated fibroblasts are being conducted.