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REVIEW ARTICLE

Angiogenesis in cancer – general pathways and their therapeutic implications

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Summary

A vast amount of data shows that angiogenesis has a pivotal role in tumor growth, progression, invasiveness and metastasis. This is a complex process involving essential signaling pathways such as vascular endothelial growth factor (VEGF) and Notch in vasculature, as well as additional players such as bone marrow-derived endothelial progenitor cells. Primary tumor cells, stromal cells and cancer stem cells strongly influence vessel growth in tumors. Better understanding of the role of the different pathways and the crosstalk between different cells during tumor angiogenesis are crucial factors for developing more effective anticancer therapies. Targeting angiogenic factors from the VEGF family has become an effective strategy to inhibit tumor growth and so far the most successful results are seen in metastatic colorectal cancer (CRC), renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLL). Despite the initial enthusiasm, the angiogenesis inhibitors showed only moderate survival benefit as monotherapy, along with a high cost and many side effects. Obviously, other important pathways may affect the angiogenic switch, among them Notch signaling pathway attracted a large interest because its ubiquitous role in carcinogenesis and angiogenesis. Herein we present the basics for VEGF and Notch signaling pathways and current advances of targeting them in antiangiogenic, antitumor therapy.

Key words: *angiogenesis, anti-angiogenic therapy, cancer, Notch signaling, VEGF signaling*

Introduction

Angiogenesis is involved in many physiological processes, but also is a hallmark in the pathology of many diseases (cancer, ischemia, atherosclerosis, inflammatory diseases), in wound healing and in tissue regeneration. It is of crucial importance for the survival of cancer cells, for tumor growth and spreading [1]. Angiogenesis is a complex process, tightly regulated at molecular level by involving growth factors, receptors, extracellular matrix (ECM) proteins and the humoral factors [2]. Well defined types of cells participate in the process of angiogenesis and most of them have specific molecular markers. The primary driver of angiogenesis is hypoxia, leading to secretion of VEGF and other pro-angiogenic molecules from hypoxic cells [3]. The proper angiogenesis is a stepwise process [4], beginning with degradation of the ECM and sprouting of the endothelial cells (expressing VEGF receptors) toward the gradient of VEGF. The next step is differentiation of the endothelial cells into several cell types (tip, stalk and tube cells) with subsequent tube formation and maturation [5]. The tip cells (expressing DLL4) are non-proliferative cells, located at the top of the new vessels and account for the direction of the new vessel. Stalk cells (expressing Notch-1) are highly proliferative and form the trunk of the new vessels. The tube cells are non-proliferating, lumen containing cells that shape the final appearance of the vessels. Specialized support cell type, the pericytes expressing platelet-derived growth factor receptor beta (PDGFRβ) and alpha-smooth-muscle actin (α-SMA), cover the capillaries by coupling to endothelial cells with direct cell-to-cell and gap junctional contacts [6]. Other cells, mentioned later on, from

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circulation and from the tumor environment contribute to tumor angiogenesis as well.

VEGF signaling in angiogenesis

VEGF family members are the most important factors that induce angiogenesis [7]. A number of essential properties are attributed to VEGF with direct effect on promoting angiogenesis. First, it is its his proliferative effect on target endothelial cells, which start growing under its influence, increasing their survival and decreasing the apoptotic rate. Second, it enhances vascular permeability which is connected to extravasation and migration of different cells from/into circulation. Inducing vasodilatation is another property of VEGF in regard to its potent angiogenic effect. VEGF family includes VEGF A, B, C, D, and placental growth factor (PIGF). VEGF-A is the best characterized for its role in angiogenesis; it acts through tyrosine kinase VEGF receptor 2 (VEGFR2), whereas the role of VEGF receptor 1 (VEGFR1) in angiogenesis is still vague. Most cancers exhibit a high expression of VEGF [8]. Immunohistochemical analysis for VEGF expression in CRC (compilation data from 17 independent studies) showed association of the degree of its immunoreactivity with poor survival [9]. Increased expression of VEGF has been found in NSCLC [10] and RCC [11], and in many instances it is associated with increased risk of recurrence, metastasis, and death. Preclinical studies provided evidence that angiogenesis also plays a central role in breast cancer carcinogenesis and metastatic potential. A variety of pathophysiological circumstances can induce VEGF expression. One of the most important is low oxygen tension which triggers hypoxic response by mediation of hypoxia-inducible factor 1α (HIF1α) [12]. Paracrine or autocrine release of some major growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF) α and β, platelet-derived growth factor (PDGF), insulin-like growth factor 1 (ILGF-1) and fibroblast growth factor (FGF) upregulate VEGF levels as well [13]. Somatic genetic mutations could also induce activation of VEGF [14]. All the abovementioned events show off the central role of angiogenesis in tumor development and spreading. Tumors over 2 mm^3 are unlikely to survive without proper vascularisation. In contrast to the normal vessels, tumor vessels have a different morphology, characterized by higher leakage, arbitrary branching and blind ending.

VEGF in tumors can act through three mechanisms. Tumor cells produce VEGF, which binds VEGFR2 on the endothelial cells (paracrine mechanism). However, elevated levels of VEGF are not useful as predictive markers, because various cells, such as platelets, muscle cells and tumor stromal cells, also produce it [15]. Some tumors express both VEGF and VEGFR2 (autocrine mechanism). In some cases, like in breast cancer, VEGF receptor is expressed within the cells (intracrine mechanism). VEGF can also bind neuropilin receptor (NRP), which lacks tyrosine kinase activity and may serve as co-receptor of VEGFR2, accounting for the regulation of angiogenesis [16]. Binding of VEGF-C to VEGFR3 promotes lymphangiogenesis. There are different signaling pathways after binding VEGF with its receptor, which promote migration, survival and proliferation of the endothelial cells. MAPK pathway is related to DNA synthesis and the cell growth, whereas PI3K pathway promotes their survival. The activation of *src* leads to cell migration [17].

Folkman first proposed that targeting the vessels formation in the tumor can prevent its growth [18]. During the past decades, intensive research led to the creation of a new type of therapy for cancer, aiming at the disruption of tumor vasculature by targeting the VEGF family members (Table 1). Bevacizumab is a monoclonal antibody against VEGF, whereas Sorafenib and Sunitinib are inhibitors of tyrosine kinase receptors (TKRs). Bevacizumab is effective in advanced CRC, especially in combination with the conventional chemotherapy [30,31], Sorafenib is approved as monotherapy of hepatocellular carcinoma and Sunitinib for metastatic RCC. The most encouraging results have been obtained for Bevacizumab in the treatment of metastatic CRC, RCC, breast cancer, and NSCLC patients.

The antitumor activity of Bevacizumab has been extensively demonstrated in preclinical studies in several tumor models. These studies found that single-agent therapy with bevacizumab or its murine equivalent resulted in tumor growth inhibition of a number of human tumor cell lines xenografted in rodent models, irrespective of the route of administration or tumor location. The assayed tumor types included rhabdomyosarcoma, glioblastoma, leiomyosarcoma, colon adenocarcinoma, hepatoblastoma, neuroblastoma, Wilms'tumor, and ovarian, prostate or breast carcinomas [32]. Studies with *in vitro* cell lines did not reveal a direct effect of the antibody on the growth of tumor cells, suggesting that tumor suppression is mediated through inhibition of neovascularization. Several studies have shown that anti-VEGF therapy also prevented the growth of tumor cells at metastatic sites [33].

Table 1. Anti-VEGF signaling therapy in clinical trials

Targeted factor	*Agent*	*Activity*	*Clinical trials*
VEGF	*Bevacizumab* (*Avastin*); Genentech, South San Francisco, CA; www.genentech.com)	Monoclonal antibody - neutralizes the biologically active forms of VEGF that interact with VEGF receptors 1 and 2	Phase II and III trials in, RCC, breast cancer, Kaposi's sarcoma, pancreatic cancer, ovarian cancer, lymphoma, myeloma, melanoma, head and neck cancer, leukemia, liver cancer, sarcoma, mesothelioma, and lung cancer [19,20]
VEGF	*VEGF-Trap* (Aventis and Regeneron Pharmaceuticals, Tarrytown, NY www.regeneron.com)	Derivative of VEGFR1, comprising portions of the extracellular domains of both VEGFR1 and VEGFR2	Phase I trial in patients with solid tumors and non-Hodgkin's lymphomas [21]
VEGF receptors and other tyrosine kinases	*Sorafenib* (BAY 43-9006, Bayer Pharmaceuticals Corporation, Leverkusen, Germany, www.pharma.bayer.com, and Onyx Pharmaceuticals, Inc., Emerville,CA, www.onyx-pharm.com)	Targets several receptor tyrosine kinases involved in neovascularization	Phase I study in advanced refractory solid tumors [22]; Phase III study in patients with RCC [23]
VEGFR tyrosine kinases	*Vatalanib PTK787/ZK22258,* Novartis Pharma AG, Basel, Switzerland, www.novartis.com, co-developed with Schering AG, Berlin, www.schering.de	Inhibits all known VEGFR tyrosine kinases	Phase I/II study in patients with recurrent glioblastoma multiforme [24]; Phase III trial in patients with newly diagnosed or relapsed advanced stage CRC [25].
VEGFR2-TIE2 tyrosine kinases	*Regorafenib* (BAY 73-4506, commercial name Stivarga, Bayer)	Multi-kinase inhibitor	Phase III trial in metastatic CRC [26]
Angiogenic receptors	*Sutent (Sunitinib malate), (SU11248* Pfizer, Inc., New York, NY, www.pfizer.com*)*	Inhibits the tyrosine kinase activity of a number of receptors including PDGFR, VEGFR2, Flt-3, and c-Kit	Phase II clinical trial involving CRC patients [27]; Phase II clinical trials in patients with previously treated metastatic breast cancer and in patients with unresectable neuroendocrine tumors [28,29]

CRC: colorectal cancer, RCC: reual cell carcinoma

However, the initial enthusiasm regarding anti-VEGF signaling therapy was cooled by subsequent trials, which could not confirm benefit of angiogenesis inhibitors as monotherapy. There are several problems that need to be solved. During treatment, many patients become resistant to antiangiogenic drugs, while no useful clinical marker exists that can predict which individuals will benefit better from treatment. Besides, antiangiogenic agents are expensive and have toxic side effects [17].

The key to these questions is a more thorough understanding of the mechanism of tumor angiogenesis, accounting for other angiogenic pathways. Among them, Notch signaling is the best candidate because of its ubiquitous role in cell patterning and differentiation.

Notch/Delta-like ligand 4 (Dll4) signaling in carcinogenesis and angiogenesis

The Notch family includes 4 receptors (Notch 1-4) and 5 ligands (Jagged 1, 2 and Delta-like (Dll 1,3,4) [34]. Notch receptors are single transmembrane proteins located on the cell surface, which interact with ligands expressed on the adjacent cells. This leads to splitting of the receptor by TACE and γ-secretase and subsequent translocation of its intracellular portion (NICD) into the nucleus. The subsequent interaction with RBP-Jk/CBF-1 protein results in expression of genes normally silent in the absence of Notch signal such as basic helix-loop-helix genes Hes 1,5,7 and Hesr/Hey 1,2,L. These are regulatory genes involved in cell differentiation and their expression is associated with uncontrolled proliferation of tumor cells [35]. Notch mutations have been found in approximately 50% of the cases of T-cell acute lymphoblastic leukemia (T-ALL) [36]. High expression of Jagged-1 and Notch-1 has been found in breast cancer [35], RCC [37], bladder cancer [38], pancreatic cancer [39] and metastatic prostate cancer [40]. Approximately 4.7% of all solid tumors bear Notch-1 mutations, whereas Notch-2 and -3 are affected in 1.5 and 1.3%, re-

spectively. Notch-1 mutations are more frequent in squamous cell lung cancer and breast cancer and range between 5 and 15% [41]. Notch signaling is essential for the colonic cell proliferation and differentiation and also plays a role in colonic carcinogenesis. A striking feature of the human CRC is the absence of goblet cells and the resemblance to the colonic stem cells, located at the base of the crypts. These unique, self-renewing cells are undifferentiated and can produce all differentiated cells in the crypts. This process is regulated by Notch receptors [41]. Reedijk and colleagues reported an elevated expression of Jagged-2 and Notch-1 in cases of CRC and concluded that CRC cells resemble the crypt cells. In a cohort of 60 CRC samples, Veenendaal et al. found a high expression of Notch [42]. The final step of the activation of Notch pathway is the expression of HES1, which can be used as a surrogate marker instead of Notch. It was also found that HES1 is expressed in all cases of CRC, but did not correlate with prognosis in a series of 130 cases. It results in suppression of cyclin-dependent kinase (CDK) inhibitors p27 and p57, with subsequent stimulation of cell proliferation and suppression of cell differentiation in the colon [43].

Several authors have demonstrated the dual role of Notch signaling in carcinogenesis [44-47]. Depending on the cellular context, it may act as oncogene or as tumor suppressor. Notch acts as oncogene in sporadic colon cancer [43], T-acute lymphoblastic leukemia and breast cancer [47], prostatic [40], bladder [38] and pancreatic cancer [39], but as a tumor suppressor in skin cancer, cervical cancer and Ewing's sarcoma [44,46,48], B-cell acute lymphoblastic leukemia, chronic myelomonocytic leukemia [45], and hepatocellular carcinoma [49]. Better understanding of this dual role of Notch, depending on cellular context and the elucidation of crosstalk between Notch and the other signaling pathways is of a paramount importance for developing of new therapies and also for understanding all possible consequences of Notch inhibition.

Of note, Notch signaling plays a significant regulatory role in angiogenesis, of which Notch1 receptors, Dll4 and Jagged1 ligands are the best studied. Notch-1 receptor plays an important role in endothelial cell differentiation [50]. In response to VEGF, tip cells express a large amount of Dll4, which binds Notch-1 in the stalk cells, leading to downregulation of VEGF receptors with subsequent inhibition of sprout formation. Jagged-1 is an antagonist of Dll4 and promotes angiogenesis [51]. The role of Notch-1 and Dll4 in tumor angiogenesis is of particular interest. Dll4 has shown strong expression in endothelial cells of diverse solid tumors [52,53].

Regulation of the Notch receptors is of paramount importance since they are involved in cell fate, differentiation and proliferation through cell-to-cell interplay, thus it is obvious that they regulate the complex tumor-stromal-endothelial cells interactions. The different expression of Fringe genes (Lfng, Mfng, Rfng) secures the specificity of these interactions [43]. Glycosyltransferase or Fringe can modify Notch in the Golgi apparatus. Delta ligands prefer Fringe-modified Notch, whereas Jagged prefer unmodified Notch. This results in different activation of Notch at the border of Fringe-expressing and non-expressing cells [34].

Despite of endogenous Notch regulation, the DLL4/Notch signaling can be targeted in anticancer treatment. Its ubiquitous role in carcinogenesis and tumor angiogenesis makes it a challenging target. Unlike the VEGF antibodies, the treatment with Dll4 antibodies increases the vascularisation of the tumor. However, the new vessels are abnormal with increased leakage, hy-

Table 2. Anti-DLL4/Notch signaling therapy in clinical trials

Targeted factor	*Agent*	*Activity*	*Clinical trials*
DLL4	*Demcizumab* (OMP-21M18; OncoMed Pharmaceuticals)	Humanized monoclonal antibody that inhibits DLL4 in the Notch signaling pathway	Phase I trials in advanced pancreatic cancer patients; Phase I trials in advanced NSCLC patients [http://oncozine.com/profiles/blogs/clinical-data-on-oncomed-s-anti-notch2-3-and-demcizumab-programs]
Notch	*Gamma-secretase inhibitor* RO4929097 (National Cancer Institute)	Oral small molecule inhibitor of gamma-secretase, blocks the processing (activation) of Notch	Phase I/II trials in children with relapsed/refractory solid or CNS tumors, lymphoma, or T-Cell leukemia [54-56]

NSCLC: non small cell lung cancer, CNS: central nervous system

persprouting, loss of organization and impaired tube formation, which leads to hypoxia and tumor shrinkage. There are reports suggesting beneficial effect in tumors resistant to VEGF antibodies. Similar to VEGF antibodies, improved efficacy of Dll4 antibodies was observed when they are combined with chemo- and radiotherapy [53]. Moreover, the simultaneous administration of VEGF and Dll4 antibodies seems to be more effective [54,55]. The therapy against DLL4/Notch signaling in tumors is still in its infancy (Table 2).

The exact mechanism of action of anti-DLL4/Notch therapy is still unknown. Yamanda and colleagues, suggest a possible role of VEGF-Dll4-Ephrins axis. Ephrins are tyrosine kinase receptors (A and B) with two ligands – Ephrin A and B. They found that Dll4 blockade suppresses ephrinB2 expression in tumors. On other hand, Dll4 antibodies and soluble ephrinB2 lead to suppression of tumor growth, suggesting they are antagonists. In addition, the knockdown of ephrinB2 mimicked the effect of Dll4 [38].

Before introducing anti-Notch therapy there are several questions to be answered:

- Notch may act not only as an oncogene, but also as a tumor suppressor, probably depending on the cellular environment and interaction with other signaling pathways [32, 39].
- The exact mechanism of action is still elusive.
- Notch signals play an important role in T-cell differentiation. Although they are over-expressed in the tumor vessels, they also can be found in smaller extent in arterioles and the gastrointestinal tract. The non-specific inhibition of Notch pathways via γ-secretase inhibitors shows beneficial effect in the treatment of neuroblastoma, but is accompanied with gastrointestinal adverse effects [12]. These effects are not observed in the treatment with Dll4 antibodies, but Dll4 can induce vascular neoplasms, atrophy of the thymus and subcutaneous tumors [12,39].
- From the therapeutic standpoint, it is important to be proved which type of tumors will benefit most from such treatment and to validate the most appropriate combination and dosage regimen [40].

Circulating bone marrow cells in angiogenesis

A large number of bone marrow cells were found in circulating blood with a possible influence on angiogenesis. They are mainly hematopoietic cells ($CD45^+$) such as monocytes myeloid cells, neutrophils, macrophages, but also $CD45^-$ endothelial progenitor cells (EPCs). First discovered by Asahara in 1997, they were found to play a role in vascular repair in cases of myocardial infarction, limb ischemia, atherosclerosis, but also in ocular and tumor angiogenesis [41]. Although the phenotype of EPCs is still under investigation, in vivo they are characterized as $CD34^+$, $CD133^+$, $CD45^-$, $VEGRF\text{-}2^+$, but their isolation from the blood is still problematic [42]. They are mobilized from the bone marrow in response to tumor cytokines, including VEGF and through the vessels they are recruited into the tumor with incorporation into the sprouting vessels [43].

Experimental evidence showed that inoculation of only 12% EPCs led to progression of microscopic metastatic lesions to macroscopic ones [44]. Treatment with vascular disruptive agents leads to increase of their number and infiltration of the periphery of the tumor, which results in a rapid tumor progression [45]. Firstly, this effect is described with administration of maximal doses of cyclophosphamide. These observations are the rationale for the so-called metronomic therapy – regular administration of low doses of chemotherapeutic agents. It seems to prevent recruitment of the EPCs and their differentiation during angiogenesis [46].

EPCs represent a promising novel target for anticancer therapy. The precise understanding of their activation, mobilization and recruitment in the tumor vessels is of paramount importance.

Cancer stem cells (CSCs) and angiogenic pathways

The heterogeneity of tumor cells is well-known, which is usually explained by their genetic instability. In relation to colon cancer – and according to the cancer stem cell theory - the tumor originates from colonic undifferentiated and multipotent stem cells at the base of the colonic crypts as a result of accumulated genetic and epigenetic changes. The subsequent dysregulation of Wnt, Notch, Hedgehog and TGF-β signaling pathways transforms CSC into colonic cancer stem cell (CCSC). CSCs are $CD133^+$ and $CD44^+$ positive and have been found in CRC. Moreover, when they were transferred into mice they were able to produce a tumor with identical phenotype [47-50]. It has been reported that Notch signaling prevents apoptosis of CSCs and is 10-30 fold higher in them in comparison to the other cell types [51]. A recent work of Lu et al., demonstrated that endothelial

cells are also involved in promoting the CSCs by secreting soluble Notch ligand Jagged 1 [52].

It is thought, that conventional chemotherapy does not affect them in contrast to other tumor cells. That led to the hypothesis that they are responsible for the resistance to treatment and recurrences. This population has a high expression of VEGF, which suggests possible effect of anti-VEGF antibodies. A possible practical implication is that the combination of metronomic chemotherapy and anti-VEGF antibodies can be a more effective treatment [53].

In conclusion, tumor growth results from tumor angiogenesis. A comprehensive study of angiogenic signaling pathways and their complex orchestration in tumor cell environment, taking into account the effects of endothelial progenitor and stem cells, will give the most accurate information to adequately influence tumor growth.

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