Angiogenesis as targeted treatment

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Angiogenesis: health and disease

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introduction

The formation of new blood vessels is a fundamental process that occurs during embryonic and post-natal development but also in a number of pathologies ranging from cancer to chronic inflammatory disease. Blood vessels can be generated by either angiogenesis or vasculogenesis.

Angiogenesis is implicated in embryonic, post-natal and pathological vascular development. On the contrary, vasculogenesis was thought to be only restricted to embryonic vascular development. However, results from many laboratories indicate that vasculogenesis is not only involved in post-natal vascular development but also occurs in many pathologies such as cancer. For example, 40% of the endothelial cells in a tumor were found to derive from endothelial progenitor cells that originate in the bone marrow [1].

Angiogenesis is now a major focus of research. A number of basic mechanisms of angiogenesis have been deciphered and several targets for therapeutic intervention identified. Key players of blood vessel formation, such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and angiopoietins, have been identified and joined by many others such as ephrin, netrin, Notch/Delta, semaphorin, and roundabout/slitfamilies of proteins [2].

The study of lymphatic vessel development (lymphangiogenesis) has emerged in recent years and has become a subfield of angiogenesis research attracting many investigators. A number of lymphangiogenesis factors have been identified such as VEGF-C and VEGF-D [3]. Lymphangiogenesis plays an important role in cancer metastasis or lymphedema and may lead to new venues for the development of therapeutic strategies.

Furthermore, an important link between angiogenesis and neurobiology has also emerged. Angiogenesis factors such as VEGF also act on neurons and may regulate neurogenesis, neuron survival, axonal growth and complex processes in the brain such as spatial or associative learning [4]. Conversely, neuronal regulators such as guidance molecules or their receptors are expressed in the vasculature and participate in the modulation of the angiogenic phenotype [5].

Angiogenesis research has let to an intense pharmaceutical development. For example, bevacizumab (Avastin, Genetech-Roche) the first drug derived from angiogenesis research is now in clinical use in the treatment of colon cancer. Many other molecules are expected to come in the forthcoming years.

In this article, we present a short overview of our current understanding of angiogenesis and describe some recent advances in the field. For more extensive information the reader may refer to a series of excellent review articles published recently on the subject [6].

basic mechanisms of vascular development

the angiogenic switch

Vasoformation is influenced by molecular regulations in both healthy and pathological tissue. This process is dependent on paracrine angiogenesis signals that induce proliferation and migration of vascular cells and their assembly into functional vessels. Vessel stabilization and remodeling are very important events that occur at a later stage. In microvessels, two major cell types, endothelial cells and pericytes, participate in these processes. In larger vessels, smooth muscle cells are involved instead of pericytes. A number of soluble factors, receptors, and extracellular matrix molecules play a role in vascular morphogenesis. Expression of these factors is under the control of a molecular switch inside the cells [7].

Hypoxia is a driving force for angiogenesis in tumors or ischemic tissue [8]. Hypoxia regulates angiogenesis via an increase in hypoxia-inducible transcription factor-1α (HIF-1α) that initiates a program of survival and adaptive gene expression. In angiogenesis, the major factor regulated through the HIF-1α system is vascular endothelial growth factor (VEGF). In the presence of oxygen, the enzyme prolyl 4-hydroxylase (PHD) binds molecular oxygen and hydroxylates proline residues in HIF-1α. Hydroxylated HIF-1α associates with the von Hippel-Lindau (VHL) gene product, passes to the proteasome, and is rapidly degraded. Under hypoxic conditions, hydroxylation is inhibited and HIF-1α levels are stabilized.

There are three sequence-related PHDs. Probably only one of these forms, PHD2, which resides in the cytoplasm, is involved in HIF-1α regulation during angiogenesis. PHD2 is also transcriptionally induced by HIF-1α in a low-oxygen environment. This provides an autoregulatory feedback loop. In vascular cells, another form of HIF, HIF-2, is responsible for regulating gene expression. For example, an important receptor that binds VEGF, VEGFR2, is up-regulated through a HIF-2α-dependent mechanism.

Tissue ischemia is not only dependent on hypoxia but also on nutrient deprivation such as hypoglycemia. We have obtained recent data that indicate that hypoglycemia induces VEGF expression but a pathway independent of HIF (Drogat et al., in preparation).
The molecular angiogenesis switch is not only dependent on hypoxia or hypoglycemia, but also on oncogenic transformation, which occurs for example in tumor cells, or on autocrine growth factor loops [9]. For example, activation of the ras gene product induces the expression of VEGF, a stimulator of angiogenesis, and down-regulates inhibitors such as thrombospondins in tumor cells. Similar effects are also observed when autocrine growth factor loops are present in tumor cells.

**key players in the angiogenic process**

Among the most important regulators of angiogenesis are the VEGFs (Table 1). This family is composed of VEGF-A, -B, -C, and -D and the related placental growth factors (PLGFs) [10]. VEGFs are essential in embryonic and postnatal vascular development. They also play an important role in ischemia-driven or tumor angiogenesis. In fact, one of the VEGF prototypes, VEGF-A, is a permissive factor for multistage carcinogenesis, as evidenced in the Rip-Tag mouse model.

VEGFs bind three types of tyrosine-kinase receptors: VEGFR1 (flt1), VEGFR2 (flk1 or KDR), and VEGFR3 (flt3). VEGF-A binds VEGFR2 and VEGFR1. In contrast, VEGF-B binds only VEGFR1. VEGF-C and VEGF-D both preferentially bind VEGFR3 but also interact with VEGFR2. Finally, PLGFs bind only VEGFR1 [10, 11].

VEGFR2 is the critical receptor for angiogenesis in blood vessels and for vascular permeability [10, 11]. VEGFR1 may synergize with VEGFR2 in postnatal and pathological angiogenesis. VEGFR1 is also found on hematopoietic cells that accumulate at angiogenic sites. However, only VEGFR2 seems to be necessary for embryonic vascular development. Nevertheless, both receptors are needed for repair-associated, tumor, or retinal neo-angiogenesis in the adult [12].

VEGFR3/flt4 is required for the growth and the maintenance of lymphatic vessels (see below) and is also mutated in primary human lymph node edema (Milroy’s disease), which illustrates the importance of this receptor in the lymphatic tissue [3].

**Table 1. Angiogenesis and lymphangiogenesis factors**

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<thead>
<tr>
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<th>Angiogenesis</th>
<th>Lymph-angiogenesis</th>
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<tbody>
<tr>
<td>Ang1</td>
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<tr>
<td>Ang2</td>
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<td>Ephrin B2</td>
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<td>FGF1 FGF2</td>
<td>+ (EC, pericytes, SMC)</td>
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<td>HGF</td>
<td>+</td>
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<td>IGF-1, 2</td>
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<td>PLGFs</td>
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<tr>
<td>PDGFBB</td>
<td>+ (pericytes)</td>
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<td>VEGF-A</td>
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<td>VEGF-D</td>
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Ang, angiopoietin; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, Insulin-like growth factor; PLGFs, placental growth factors; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

Targeted inactivation of VEGFR3 in mice results in abnormal lymphatic vessel growth.

VEGF prototypes also use co-receptors for binding to target cells. Among the co-receptors, neuropilins seem to be the most important. For instance, neuropillin-1 is a co-receptor of VEGFR2. This interaction is critical for VEGF-A binding to VEGFR2. VEGF-A is the main VEGF form that regulates angiogenesis in blood vessels, whereas lymphangiogenesis is primarily dependent on VEGF-C. Several splice variants of VEGF-A (VEGF-165, VEGF-121, and VEGF-189) have been identified that exhibit variable affinities for heparan sulfates [10]. This interaction is important for VEGF’s biodisponibility. VEGF expression is regulated by a number of factors, including hypoxia, oxidative stress, reactive oxygen species, tumor suppressor genes or growth factors, or cytokines that activate the MAP kinase pathway.

A number of other factors act not only on vascular endothelial cells but also on other vascular cells, such as pericytes. Among these factors are fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), angiopoietins, developmental gene products such as Notch/Delta, and molecules classically involved in axonal guidance [2, 13]. Particularly noteworthy among the latter are netrins. Netrins are axonal guidance molecules with repellent function and also provide guidance cues for blood vessels [14]. It has been demonstrated that netrins also provide repellent cues to growing blood vessels in vitro and in vivo.

The major system involved in vessel stabilization is the angiopoietin system. Angiopoietin-1 (Ang-1) is thought to stabilize vessels by rendering them less sensitive to VEGF [15]. The stabilizing effect of Ang-1 is disrupted by Ang-2 binding to the Ang-1 receptor Tie-2. Ang-1 stimulates endothelial cell migration by activating tie-2 and inducing the recruitment of Dok- R, NcK, and Pack. Ang-1, but not Ang-2, has been shown to be in the extracellular matrix. Ang-1 induces PDGF, which is released and stimulates migration of pericytes, smooth muscle cells, and other accessory cells and, thus, favors mural cell coverage. Ang-1 is able to trigger vessel remodeling, as demonstrated in the retina [16].

Angiogenesis is also under the control of proteolytic enzymes and inhibitors, including the plasminogen activator system and matrix metalloproteinase (MMP) and their inhibitors. The basic principle of the activity of these enzymes is that they must be present at a critical concentration at the cell surface to promote invasion of vascular tubes. Furthermore, inhibitors such as tissue inhibitor of metalloproteinase-2 of MMPs may be required to localize the proteolytic activity at the cell surface, thus promoting activation of MMPs. This may account for the paradoxical stimulatory effects observed for these inhibitors in some circumstances. It has been recently reported that MT1-MMP is critical for sprouting angiogenesis in vitro but not MMP2 or MMP9 [17], which is in contradiction with results published earlier [18]. This observation has been only made in an in vitro model of angiogenesis and should be confirmed in vivo before general conclusion can be drawn.

Cell adhesion molecule receptors such as integrin αVIβ3 (and, to a lesser extent, α1β5) also play a critical role in angiogenesis. For example, the integrin αVIβ3 is highly expressed in proliferating endothelial cells, and both a monoclonal antibody...
to vVβ3 and a low-molecular-weight antagonist have been shown to inhibit angiogenesis in *in vivo* models [19]. This indicates that integrin vVβ3 has a promoting role in angiogenesis and may constitute a potential interesting therapeutic target. However, observations of vVβ3-knockout mice have challenged this view [20]. In particular, it has been reported that mice lacking β3 integrin show enhanced pathological angiogenesis.

The reason for these apparent differences is not clear. Cheresh and collaborators attempted to explain the differences using the concept of ligated and unligated integrins [21]. Unligated integrins provide dead signals to endothelial cells that are blocked by ligation. Knocking-out integrins affects both ligated and unligated integrins and may artificially increase the invasiveness of endothelial cells by increasing endothelial cell survival, assuming that more unligated than ligated integrins are present.

**Lymphangiogenesis: a new field in angiogenesis research**

Lymphangiogenesis research has undergone a fast development in the last five years when critical players such as VEGF-C and D and the VEGFR3 receptor tyrosine kinase were identified and their role unraveled [3, 22, 23]. The repertoire of lymphangiogenic factors has been recently increased when it became apparent that other growth factor molecules or angiogenic factors are also regulating lymphangiogenesis. For example, it has been recognized that fibroblast growth factors such as FGF-1 or FGF-2 inducing lymphangiogenesis by both direct and indirect mechanisms [24–26]. FGFs can bind lymphatic endothelial cells and induce lymphatic cell proliferation and migration. Prox1, the master regulator of lymphatic cell differentiation induces the expression of FGFFR3 thus sensitizing the lymphatic vessel to FGF. In addition to this direct mechanism [24, 25], FGFs may regulate VEGF-C and D expression and thus exert an indirect action on the lymphatic vasculature. VEGF-A, angiopoietin-1, Insuline-like growth factors or hepatocyte growth factors have also recently joined the list of lymphangiogenic factors [27–30].

Lymphatic vessels are genetically very different from blood vessels as evidenced by comparative gene profiling studies [3, 31–32] (Table 2).

**Angiogenesis in pathology and therapy**

Angiogenesis is a driving forces for a number of pathologies, such as cancer, ocular neovascular disease, ischemic disease, and chronic inflammatory diseases.

**Tumor angiogenesis and lymphangiogenesis**

Cancer cells express a complex molecular repertoire that critically impacts the surrounding vascular stroma. It is clearly recognized that tumor cells produce both negative and positive regulators of vasoformation, and that the net effect on the vasculature is the outcome of the balance between these two types of regulators. That the growth of tumors is dependent on the vasculature was recognized by Algire in 1945 and later formulated as a paradigm by Judah Folkman [33, 34]. In general, tumors less than 2 mm³ are avascular and grow slowly. Tumor cells are then activated intracellularly by a mechanism called angiogenic switch (see above) and then start to favor the positive regulators over the negative regulators. First insight into the molecular mechanisms of the angiogenic switch was gained through the analysis of tumor progression in the RIP-Tag mouse model [35].

For many years, tumor vascularization was explained solely by the ingrowth of new vessels into the tumor from preexisting ones. However, in recent years, additional mechanisms have been recognized. These include angioblasts recruitment, cooption, vasculogenic mimicry and mosaic vessels [36]. These different mechanisms may exist concomitantly in the same tumor or may be selectively involved in a specific tumor type or host environment. For example, uveal melanoma cells develop partially by vasculogenic mimicry in the eye and by sprouting angiogenesis when implanted subcutaneously [37].

Although different mechanisms of tumor vascularization were characterized, little is known about their relative implications in tumor development rendering difficult for specifically targeting them for anti-tumor therapy. The host-tumor interaction in a given tissue has also to be considered. For example, tumor cells inactivated for the VEGF-A inductor HIF1α and injected subcutaneously develop small and poorly vascularized tumors. No reduction in tumor size and in vascularization were observed when the same cells where injected in the mouse brain [38].

Most of the anti-vascular therapy in tumors is anti-angiogenic aimed to block the function of specific growth factor or receptor. For example, targeting VEGF-A is the most powerful strategy used by many groups to inhibit tumor angiogenesis. Many drugs or antibodies acting against the growth factor are in clinical trials and are listed in http://www.cancer.gov/clinicaltrials/developments/anti-angio-table. The most

<table>
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<td>PDGFR-β</td>
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<td>Prox1</td>
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<tr>
<td>Podoplanin</td>
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<tr>
<td>PV1 or FELS</td>
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<td>Sox18</td>
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<tr>
<td>VEGFR3</td>
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<td>VEGFR2</td>
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<td>vWF</td>
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ACE, angiostatin-converting enzyme; FELS, fenestrated endothelial-linked structure protein; FGFR, fibroblast growth factor receptor, FGFR; Lyve-1, lymphatic vessel hyaluronan receptor; NR2P, neuropilin2; PDGFR-β, platelet-derived growth factor receptor-β; prox1, prosper1; PV1, plasmalemma vesicle1; VEGFR3, vascular endothelial growth factor receptor-3; VWF, von Willebrand factor.
promising agent is an anti-VEGF-A humanized antibody, bevacizumab (Avastin, Genentech). Phase III clinical trial showed an increased in survival of patient with colon, kidney and lung cancer treated with bevacizumab in conjunction with chemotherapy. The anti-angiogenic agent has been recently approved by the American Food and Drug Administration for the treatment of colorectal cancer [39].

However, not all tumors are dependent on VEGF-A for being vascularized and other players are also involved providing additional targets for therapy. Thus, a better knowledge of the tumor vasculature is important for widening the therapeutic spectrum. Moreover, since in angiogenesis vessels are at least partially stabilized by pericytes and may become refractory to VEGF-A inhibitors, molecules that interfere with endothelial cell/pericyte interactions become critically important. A step forward into this direction has been made by Bergers and colleagues who demonstrated increased therapeutic efficacy in a transgenic mouse tumor model when inhibitors (such as SU 6668) are used that both inhibit VEGFR2/FGFR present on endothelial cells and PDGFR-alpha present on pericytes [40] and [41].

Angiogenic inhibitors are increasingly used in combination with chemotherapy. This had lead to promising results in experimental mouse models and in humans [42]. This may be surprising because chemotherapy may no more reach successfully the tumor tissue when vessels are inhibited. One explanation for the benefit of this combination is that anti-angiogenic therapy normalize the vasculature by shutting-down non-functional blood vessels. Tumor cells are genetically instable and often become refractory to chemotherapy. Because endothelial cells are different from tumor cells, it was postulated that they cannot become refractory to anti-angiogenic therapy. However, recently it was shown that endothelial cells isolated from tumors, grew independently of the presence of endothelial growth factors, suggesting that tumor endothelial cells can acquire some characteristics that make them less sensitive to anti-angiogenic therapy [43].

Another issue to consider is the relative dependency of tumor cells on the vasculature, which may vary during tumor development and in response to anti-angiogenic therapy. For example, by inhibiting tumor vessels it is possible to select tumor cells with high proliferating capacity that are able to grow under hypoxia independently of the presence of tumor vessels [44]. Furthermore, Casanovas et al. have recently reported that tumor cells can acquire resistance to a single anti-angiogenic drug such as anti-VEGF receptor molecules by upregulating another repertoire of angiogenic growth factors, in their case FGFs [45].

An alternative approach to anti-angiogenesis is vascular targeting. In this approach, inhibition of the function of is irrelevant. Vascular targeting agents aim to localize specifically in tumor blood vessels and to induce destruction or occlusion of neovascularization. Examples of the validity of this approach are studies related to the L19 antibody, which recognizes the EDB domain of fibronectin specifically expressed in the tumor vasculatures. L19 fused to cytokines (TNF-α, IL-2, IL-12) is able to induce regression of established tumors in experimental mouse tumor models [46].

In addition to blood vessels, lymphatic vessels are also required for dissemination of tumor cells. Factors critically involved in the development of lymphatics, such as VEGF-C and FGFs, may also play a role in lymphangiogenesis in tumors. Blocking lymphangiogenesis in a highly metastatic human lung cancer cell line by inhibiting VEGF-C suppresses lymph node metastasis [47]. Furthermore, crossing RIP-Tag mice with mice expressing VEGF-C under the control of the insulin promoter yields bigenic mice that develop pancreatic tumors with metastatic spread through the lymphatic system [48]. Most important, peritumoral and intratumoral lymphangiogenesis is associated with poor survival in melanoma and cervical carcinoma patients [49].

Ocular neovascular disease
Ocular neovascular disease is another pathological condition in which abnormal angiogenesis plays a leading role. Diseases include diabetic retinopathy and age-related macular dystrophy (AMD). VEGF seems to be one of the principal factors in the pathophysiology of these diseases. In the case of AMD, the fas/fas ligand system may also have a critical role [51]. Indeed, fas/fas ligands are expressed in the retinal pigmented epithelium and the choroid vessels. Knockout mice

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**Figure 1.** Mechanisms of the angiogenic switch.

**Figure 2.** Anti-angiogenesis strategies.
for fas/fas ligand exhibit aberrant ocular neovascularization resembling AMD.

Antagonizing VEGF may be a valuable strategy to treat AMD[52]. Indeed Pegaptanib sodium injection (Macugen®, Eyetech Pharmaceuticals, Pfizer, New York, NY, USA) is now proposed for the treatment of the neovascular form of age-related macular degeneration (AMD) characterized by the growth of abnormal blood vessels into the macula. Pegaptanib is aptamer that is able to bind to the 165 amino acid isoform of VEGF-A but does not bind VEGFRs.

angiogenesis and tissue ischemia

Angiogenesis is implicated in ischemic disease in two ways. First, capillary growth within the walls of large arteries may contribute to the establishment of a proliferative lesion and invasion into the intima. It has been reported that inhibition of plaque neovascularization reduces the accumulation of macrophages and progression to advanced atherosclerotic lesions. Second, the growth of the collateral circulation that limits the extent of the ischemic lesion is also controlled by angiogenesis factors such as VEGF or FGFs. Third, administration of hematopoietic precursor or endothelial precursor cells is beneficial in patients with ischemic cardiovascular disease. This has offered novel therapeutic opportunities to salvage the ischemic area and to improve morbidity and mortality in patients with coronary or peripheral artery disease [53, 54].

chronic inflammatory disease

Chronic inflammatory disorders such as chronic polyarthritis are also angiogenesis-dependent. In the early phase, this disease is characterized by a proliferative lesion of synoviocytes in the synovia. The neovessels in rheumatoid joints are dysfunctional contributing to a hypoxic environment [55].

Within the inflamed synovia, the number and quality of microvessels are also altered. VEGF and integrin αvβ3 seem to play an essential role since blocking their activity in animal models results in disease improvement. Recent data suggest that tumor necrosis factor-α blockade may modify angiogenesis in rheumatoid arthritis by acting possibly on integrin function in endothelial cells [56].

conclusion

Angiogenesis research is a fast growing field that now holds the promise to generate useful therapeutic molecules and strategies for the treatment of many diseases. Bevacizumab, the first drug developed out of angiogenesis research, is a clear example that validates anti-angiogenesis-based therapies in oncology. After successful clinical trials, this molecule has now entered into the clinic. Vascular targeting approaches are also being developed and will offer new therapeutic opportunities in the near future. However, the angiogenic process is rather complex and multiple players and scenarios are at work. Thus, there is still a long way to go to fully understand its mechanisms and to translate the angiogenesis paradigm into efficient therapies.

references


