Review paper

VEGF and tumor angiogenesis

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Abstract

Angiogenesis is a critical process in tumor growth and development. VEGF is the most specific stimulator of vascular endothelial cell proliferation. VEGF is known to be involved in the growth and development of cancer and is expressed early in the progression of each tumor. High levels of VEGF may also predict poor response to conventional systemic therapy. Two of the novel and most promising targets in treatment of many types of cancer are receptor for epidermal growth factor (EGFR) and VEGF. The last one is the predominant regulator for this pathological process. The most promising drugs are Bevacizumab and Cetuximab. Other growth factors as PDGF, TGF, HGF, and bFGF which are potent endothelial cell mitogens, are relatively nonselective for endothelium and can also stimulate divisions in any other cell types. Thus, in tissues VEGF is most important for activation of angiogenesis process. It is produced in response to different cellular and environmental stimuli.. VEGF has been shown to facilitate survival of existing vessels, contribute to vascular abnormalities that may inhibit effective delivery of antitumor compounds, and stimulate new blood and lymphatic vessels growth. Expression of VEGF also correlates with invasiveness of many types of cancer cells, vascular density in different tumors, appearance of metastasis, tumor recurrence, and poor prognosis for patients including early death.

The objective of this review is to summarize data on various members of VEGF family and on their role in tumor angiogenesis.

Key words: *angiogenesis, VEGF, tumor growth, anti-angiogenic drugs, VEGF receptors.*

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Introduction

Near 40 years after the articulation of hypothesis concerning treating solid tumors by inhibiting process of its new blood vessels formation, dependent on the fact, that tumors are unable to grow beyond 1-3 millimeters of diameter unless they have a blood supply, it is now known that angiogenesis and probably also lymphangiogenesis are an underlying processes in the pathogenesis and invasion of neoplasms. Process of angiogenesis usually occurs in normal physiological conditions, but may appear also in the pathology. Ultimately, metastases to local lymph nodes via the lymphatic vessels are a common step in the spread of solid tumors [1, 2].

Capillaries, very small and thin blood vessels, extend into all tissues of the body. They are responsible for supplying nutrients and carrying off waste products. Under physiological conditions, capillaries do not grow and do not spread in the body, because the endothelial cells which build these narrow blood and lymphatic vessels almost do not divide. But in certain circumstances diseased or injured cells can produce and release angiogenic growth factors. That proteins diffuse into the nearby tissues and bind to specific receptors located on the endothelial cells of nearest blood vessel.

When growth factors bind to their receptors, signals are sent from cell's surface to the nucleus and endothelial cells become activated. Then, they can produce and secrete new cytokines and proteolytic enzymes, that disrupt their intercellular connections, the vascular basement membrane and, consequently, migrate to the new places, where give the rise to a new blood vessel. New capillaries develop from preexisting ones.

The increase in length of a new capillaries is due to the proliferation of endothelial cells. Specialized molecules called adhesion molecules, or integrins (αvß3 and αvß5) help

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new blood vessel sprout forward. This stage is followed by the maturation of a newly developed blood vessel. Additional enzymes like matrix metalloproteinases (MMP) are produced to dissolve the tissue in front of the sprouting vessel to help accommodate it. For example, it has been shown that neutrophil-derived MMP-9 is able to release biologically active VEGF(165) from the ECM of colon cancer cells by the cleavage of heparan sulphates [3, 4].

Angiogenic factors and its receptors

It is widely known that particular stages of angiogenesis are regulated by endogenic factors present in a patient's tumor and serum and they include cytokines (hormone-like peptides and low-molecule albumins affecting the functions and interactions of cells) and growth stimulating factors. They are mainly concerned with activation of different enzymes (proteases, collagenases, gelatinases, heparinases) responsible for degradation of the extracellular matrix ad proteins of the basal membrane, and generation of the proliferation and migration of the endothelial cells. Heparinase activity is strongly implicated in tumor angiogenesis and metastasis attributed to remodeling of the sub-epithelial and sub-endothelial basement membranes.

PDGF, EGF, and VEGF are factors that enable the mobilization of human mesenchymal stem cells (MSC) from the bone marrow into the blood stream and their recruitment to and retention in the tumor. The monoclonal antibodies like Glivec, Erbitux and Avastin are the specific inhibitors, which can interfere with migration of these cells [5-7].

One of the triggers of new blood vessels formation is the secretion of VEGF/VPF family factors (vascular endothelial growth factor/vascular permeability factor), which consists of six now known members. VEGFs are the multifunctional cytokines that exerts many effects on vascular endothelium what in special circumstances can contribute to angiogenic response. These action include the changes in cell morphology and cytoskeleton accompanied by stimulation of endothelial cell migration and proliferation. They are able to promote survival, induce and enhance invasion of endothelial cells by their interactions with tyrosine kinase receptors. Vascular Endothelial Growth Factor (VEGF) is considered to be one of the most important regulators of angiogenesis and a new key target in anti-cancer treatment. Various clinical trials have validated the clinical importance of anti-VEGF or anti-VEGF receptor (VEGFR) therapy. Currently the humanized monoclonal antibody bevacizumab (blocks VEGF-A), and the tyrosine kinase inhibitors sunitinib and sorafenib (inhibit VEGFRs) are approved for patients with various malignancies and several others are expected in the coming years [1, 5, 8].

There are 5 known, structurally related, but encoded by separate genes – VEGFs and PlGF. They differ in number of amino acids in their chains. As was written, all they have varied expression in different tissues, as well as they play various biological functions. The VEGF gene is located on the short arm of chromosome 6 and is differentially spliced to yield several different isoforms, the three most prominent of which encode polypeptides of 189, 165, and 121 amino acids in human cells (corresponding murine proteins are one amino acid shorter).

Genes for all isoforms of VEGF contain exon 1 to 5 and 8, and they differ in having various combinations of other exons.

The two primary isoforms of VEGF-A ($VEGF_{121}$ and $VEGF_{165}$) have varied expression during angiogenesis in different tissues, as well as varied biological functions, and the next two ($VEGF_{189}$ and $VEGF_{206}$), probably play a role in lymphangiogenesis. There are other variants, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{183}$ – which are distinguished by their ability to binding heparin or heparin sulfate, what may be important mechanism of interaction with drugs like heparin and low molecular weight heparins [1, 3, 9]

The several VEGF isoforms have distinct physical properties. VEGF 121 is freely soluble and does not bind heparin. By contrast, isoforms 165 and 189 have increasing basic charge and bind heparin with increasing affinity; in fact, VEGF 165 was originally purified on the basis of its affinity for heparin. Heparin, heparan sulfate, and heparinase all displace the larger VEGF isoforms from proteoglycan-binding sites; plasmin cleavage also activates these isoforms by freeing them from cells or matrix. Despite these physical differences, the several VEGF isoforms apparently have identical biologic activities when free in solution. Angiogenesis is initiated only at a stage when cells express metalloproteases that liberate VEGF from matrix. Therefore, in this and likely other instances, proteases are required to trigger so called " angiogenic switch".

First and most distinctive biologic activity of VEGF-A is the increase of microvascular permeability. It results in leakage of plasma proteins, including fibrinogen and other clotting proteins. The clotting system is rapidly activated by means of the tissue factor pathways and results in deposition of extravascular fibrin in tumor stroma. Vascular endothelial growth factor A (VEGF-A), the founding member of the vascular permeability factor (VPF/VEGF) family of proteins, is probably the most important angiogenic cytokine with critical role in tumor angiogenesis. There are a lot of mechanisms by which VEGF-A induces angiogenesis, and it has multiple functions in this process. Other way, there are several mechanisms, by which VEGF-silenced tumors might stimulate angiogenesis. For example, tumors may induce surrounding stromal cells to secrete VEGF. In addition, not only the cancer cells but also inflammatory cells (macrophages, monocytes and other) that infiltrate the tumor and the extracellular matrix can be a source of angiogenesis factors. Also various tumor cell lines produce VPF (dimeric heparine-binding glycoprotein) which increase the proliferative ability of vascular endothelial cells in vitro, because it is a highly specific mitogen for this type of cells.

VEGF-A is over expressed not only by invasive cancer cells, but also by at least some premalignant lesions (eg, precursor lesions of breast, cervix, and colon cancers); furthermore, expression levels increased in parallel with malignant progression. Thus, measurements of circulating levels of VEGF-A may have value in estimating prognosis, and VEGF-A and its receptors are potential targets for therapy. Recognized as the single most important angiogenic cytokine, VEGF-A has a central role in tumor biology and will likely have an important role in future approaches designed to evaluate patient prognosis. It may also become an important target for cancer therapy.

At a molecular level, VEGF-A reprograms endothelial cell gene expression, leading to increased expression of a number of different proteins, including the procoagulant tissue factor, proteins associated with the fibrinolytic pathway (urokinase, tissue-type plasminogen activator, type 1 plasminogen activator inhibitor, urokinase receptor), matrix metalloproteases, the GLUT-1 glucose transporter, nitric oxide synthase, numerous mitogens, and a number of antiapoptotic factors (eg, bcl-2, A1, survivin, XIAP). VEGF-A also serves as an endothelial cell survival factor, protecting endothelial cells against apoptosis.

According to above, we can say, that VEGF-A is one of the most important secreted glycoprotein, responsible for stimulating angiogenesis during development of embryo, wound healing on menstruation cycle, as well as during tumor formation, growth and metastasis. VEGF also functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels.

Besides of VEGF-A, there are other described members in the family.

VEGF-B, which probably plays a role in coronary artery development (is present in heart tissues in high concentration), and also in cancer angiogenesis, because of ability to endothelial cell function regulation. It is a heparin binding growth factor, which is also highly abundant in skeletal muscle and pancreas. High levels of VEGF-B, like also VEGF-C, are usually associated with lymph node metastasis of colorectal cancer.

VEGF-C, VEGF-D, that are essential for development of lymphatic system and can also increase vascular permeability as well as induce angiogenesis. VEGF-C binds to VEGFR-3/Flt-4, expressed on lymphatic endothelium, and induces tyrosine fosforylation of VEGFR-2 and VGFR-3, that are implicated in lymphangiogenesis. Both VEGF-C and VEGF-D are mitogenic for lymphatic endothelial cells and promotes via VEGFR-3 survival of this cells. VEGF-D, structurally related to VEGF-C, is an endothelial cell mitogen and interacts with VEGRFR-2 and VEGFR-3. Especially, it activates VEGFR-2. In addition, VEGF-D (also known as c-fos-induced growth factor, FIGF) activates VEGFR-3/Flt-4 receptor.

Other members of VEGF family are VEGF-E not to well known, and the last one - PIGF (placenta growth factor) was discovered in placenta, so it is not highly expressed in normal adult tissues except placenta. It is thought to have an accessory role in pathologic angiogenesis, serving as a factor which increase the activity of VEGF-A.

Biologically VEGF-A, PIGF and VEGF-B are secreted forms, while VEGF-C and VEGF-D are synthesized as proteins that require post-translational processing, and appear to be bound to the cell surface.

VEGFs are expressed by the vast majority of tumors, often at elevated levels and throughout the tumor life cycle. Thus, tumor cells can induce new blood vessels formation by producing VEGF, that in turn can nourish the tumor cells. This is the self perpetuating paracrine loop, because as the tumor develops, it may begin to activate another angiogenic pathways. As these secondary pathways emerge, VEGF continues to be over-expressed and remains one of the critical mediators of angiogenesis. Therapeutic disruption of this loop, may give the possibility of successful treating of cancer. Numerous growth factors, cytokines, and lipid mediators up regulate VEGF expression in different cells, including epidermal growth factor, transforming growth factor alpha (TGF-α), FGF-2, TGF-ß, keratinocyte growth factor, tumor necrosis factor, interleukin-1 and interleukin-6, insulin-like growth factor 1, hepatocyte growth factor, and prostaglandins E1 and E2 [1, 3, 10-15].

Signal transduction by vascular endothelial growth factors (VEGFs) through their receptors, which are the tyrosine kinases, follows the consensus scheme for receptor tyrosine kinases. Thus, binding of ligand induces dimerization of receptor and activation of the tyrosine kinase enzyme through transphosphorylation between receptor molecules. This leads to initiation of intracellular signal transduction through the proper pathways. Certain signal transduction pathways are shared with most, if not all, tyrosine kinase receptors, whereas some may be unique (e.g., transduced only by VEGF receptors). Indications that such unique signaling pathways may be discerned only when VEGF receptors are expressed in their proper context (i.e., in endothelial cells of microcapillary origin) [14, 16-19].

VEGF receptors were identified on endothelial cells. Different VEGFs bind to different receptors. There are known five types of tyrosine kinase receptors for VEGF – VEGF-R1(flt-1), VEGF-R2(KDR/flk-1), VEGF-R3(Flt-4), neuropilin-1 (NRP-1), and neuropilin-2 (NRP-2). The 3 major transmembranous tyrosine kinase receptors for VEGF: (Flt-1), (flk-1/KDR) and Flt-4 bind to all secreted VEGF-isoforms. This binding initiates a cascade of signaling events that induce autophosphorylation of tyrosine kinase receptors, and signal transduction begins.

On the endothelium of blood vessels are found VEGF-R1 and VEGF-R2, whereas VEGF-R3 is found on the endothelium of lymphatic vessels. Also, both VEGFR-1 (Flt-1) and VEGFR-2 (flk-1/KDR) are over expressed in the vasculature of tumors that express VEGF-A. VEGF-R1 is also expressed on other cells like monocytes, macropha-

ges, pericytes, vascular smooth muscles and colorectal tumor cells. It may have an independent role in stimulating cell motility, and is a transmembrane protein which binds VEGF-A, VEGF-B and PlGF. Soluble form of this receptor (sVEGF-R1) can act as inhibitor of VEGF. While affinity of VEGF-A to VEGF-R1 is 10× higher than to VEGF-R2, its tyrosine kinase activity is 10× weaker.

VEGF-R2 binds VEGF-A, VEGF-C, and VEGF-D. It is responsible for mediating microvascular permeability and it was demonstrated that it is the primary mediator of VEGF signaling. VEGF-R2 is expressed on endothelial cells and also on hematopoetic steem cells, vascular smooth muscle cells, megacariocytes and retinal progenitor cells. It is also present on some tumor cells (breast, neuronal and gastrointestinal cancer cells).

VEGF-R3 is expressed mainly on embryonic endothelial cells, and during development its expression decreases and become restricted in physiological conditions in adult tissue to the lymphatic endothelial cells only. It binds only mature forms of VEGF-C.

Neuropilin-1 (NRP-1), a non-kinase receptor, originally identified as receptor for family of neuronal mediators on neuronal cells, although it is known, that endothelial cells can also express it. NRP-1 acts as an isoform specific receptor for $VEGF_{165}$. Previously, neuropilin had been shown to mediate of neurons growth but later it was found on cancer endothelium and tumor cells (breast, lung, pancreatic, gastric, and colon cancer cells). It has been recently found to potentiate VEGF-A's binding to VEGFR-2, but because Neuropilin is less selectively expressed on vascular endothelium than VEGFR-1 and VEGFR-2, its role in tumor angiogenesis is unclear and still under investigation. Neuropilin-2 (NRP-2) is a receptor for placental growth factor (PlGF), but it can bind also $VEGF_{165}$ [2, 17, 19, 20].

There are different agents such as antibodies, tyrosine kinase inhibitors, VEGF-toxin conjugates, small molecule VEGF receptors antagonists, and aptamers using in research works and clinical trials to stop angiogenesis and cancer nowadays.

The role of hypoxia

It is known that vascular endothelial cell growth factor (VEGF) as well as hypoxia plays important role in tumor angiogenesis. It is because of fact that VEGF gene expression is up-regulated in tumors under hypoxic conditions.

We know from pathology that when the tumors increase in their size, in the central part of the tumor develops hypoxic area and central necrosis. During tumor growth, hypoxic areas usually develop and VEGF expression is then increased.

Also in many tumor cells from different tissues, numerous hypoxia-related genes are significantly up-regulated. One of them is hypoxia-inducible factor 1 (HIF-1). Hypoxia-inducible factor 1, is a protein transcription factor. It is composed of two subunits (α and ß), and is primarily responsible for transcriptional regulation of VEGF under hypoxic conditions. It is important to know, that HIF-1α is very rapidly degraded under normooxygenic conditions. But during hypoxia HIF-1α is stabilized and together with HIF-1ß binds to the VEGF-promoter and activates gene expression. Hypoxia leads to significant increases in VEGF expression mostly through HIF-1α-mediated increase in VEGF transcription.

It was shown, that green tea extract and (-)-epigallocatechin-3-gallate inhibit hypoxia- and serum-induced HIF-1-alpha protein accumulation and VEGF expression in human cervical carcinoma and hepatoma cells. Also epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. Moreover some polyphenols (e.g. green tea catechins) inhibit VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule [12, 15, 21, 22].

Lymphangiogenesis

The lymphatic system collects fluids that leak from tissues and blood vessels and returns them to the circulation. Lymphatic vessels have been implicated in various diseases including lymphoedema and systemic sclerosis, and lymphatic vessels also provide a conduit by which tumor cells can metastasize. Arja Kaipainen, M.D., Ph.D., in Folkman's lab has found that some angiogenesis inhibitors also inhibit lymphangiogenesis, and might become possible treatments for tumor metastasis and other diseases involving lymph-vessel abnormalities.

As well VEGF-D promotes angiogenesis and lymphangiogenesis in tumors it was shown, that lymphatic vessels can be formed in solid tumors and that tumor-induced lymphangiogenesis then can contribute to metastatic spread.

It was proved on animal tumor model that binding of VEGF-D to its receptor induces formation of new lymphatic vessels. Also expression of VEGF-D in tumor cells leads to spread of the tumor to lymph nodes. Lymphatic spread induced by VEGF-D could be blocked with an antibody specific for VEGF-D. Furthermore, VEGF-D promotes tumor angiogenesis and growth. The dissemination of malignant cells from the primary tumor to local tissue or to distant organs via the lymphatic or blood vessels is characteristic for cancer progression.

The molecular mechanisms underlying the spread of cancer by the lymphatic vessels are probably concerned with vascular endothelial growth factor VEGF-D, which is a ligand for the VEGFR-3/Flt-4 receptor expressed on lymphatic endothelium in adult tissues. Connection between both is responsible for the ability to induce formation of lymphatic vessels (lymphangiogenesis), similarly to the process of blood vessel formation, which is based on VEGF A, and VEGF B reactions with their receptors [1, 3, 6].

Despite the large diffusion and rapid development of anti-VEGF therapy in clinical practice it is very difficult, at present, to identify validated and useful biomarkers to monitor the efficacy of these compounds and to appropriately select patients most likely to benefit from such treatments. Several agents that target vascular endothelial growth factor, are administered either as single agents or in combination with chemotherapy. They have been shown to give the benefits to patients with advanced-stage malignancies. The therapeutic benefit associated with such therapy is complex, and probably involves multiple mechanisms. A better understanding of these mechanisms will lead to future advances in the use of different anti-angiogenic agents in the clinic.

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