**Teaching unit 9**

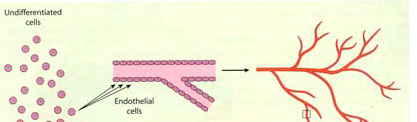
**TUMOR ANGIOGENESIS. MECHANISMS OF NEOANGIOGENESIS.**

**TUMOR BLOOD VESSELS. MEDIATORS OF ANGIOGENESIS**

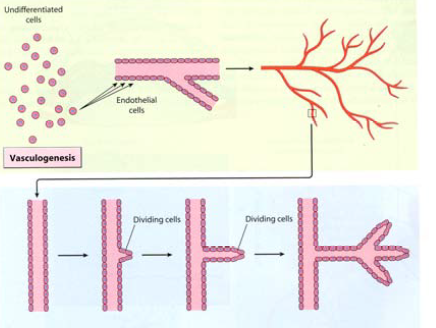
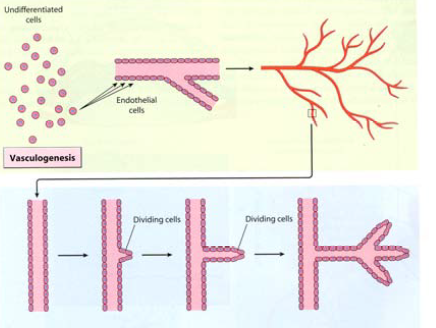
**INTRODUCTION**

**Microvascular network** consists of the smallest blood vessels: capillaries, venules and arterioles. The main functions of the microcirculation are the delivery of oxygen and nutrients to tissues and the removal of carbon dioxide (CO2) and other metabolic wastes from tissues. It also serves to regulate local blood flow and conducts blood–tissue exchange thereby affecting blood pressure and responses to inflammation which can include edema (swelling).

Even in the absence of changes in oxygen demand or other functional requirements, microvascular network structures are dynamic and likely undergo continuous changes. New blood vessels are formed by two basic processes: vasculogenesis and angiogenesis. **Vasculogenesis** means the *de novo* formation of initial vascular networks by the differentiation of endothelial progenitor cells (EPCs). At early stages of development blood vessels are formed from angioblasts, which differentiate into endothelial cells (Figure 1). **Angiogenesis** is the process of forming new blood vessels from existing blood vessels, whereby the endothelial cells of the pre-existing blood vessels proliferate and migrate, thus building an initial capillary tube subsequently expand and form new blood vessel structures (Figure 2).



**Picture 1.**Vasculogenesis



**Figure 2.** Angiogenesis

Angiogenesis is under the strict control of molecules that are produced in the body. Some of these molecules act as proangiogenic factors that stimulate angiogenesis processes, while other molecules are antiangiogenic factors and they inhibit angiogenesis.

Angiogenesis is important in a number of physiological processes. **Physiological angiogenesis** occurs during embryonic development, wound healing, during the formation of the *corpus luteum* and ovarian follicles, as well as during the growth of the uterine endometrium. Pathological (abnormal or uncontrolled) angiogenesis can be involved in the pathogenesis of various diseases, such as:

* diabetic retinopathy
* atherosclerosis
* psoriasis
* rheumatoid arthritis
* tumor.

In 1971, M. Judah Folkman proposed a hypothesis that angiogenesis is key players in the tumor progression. Due to limited oxygen and nutrient resources, solid tumors cannot grow more than any 2-3 millimeters in diameter without an angiogenesis. In addition, the increased interstitial pressure in the tumor inhibits the diffusion of metabolites and nutrients necessary for the multiplication and survival of malignant cells. This environment stimulates the malignant cells to induce the formation of new vessels from pre-existing blood vessels, which allows the tumor to be supplied with oxygen and nutrients. Folkman and colleagues hypothesize that by inhibiting the development of blood vessels in tumors, tumor dormancy can be prolonged and potentially improved the survival of patients with minimal toxicity. This hypothesis about the essential role of angiogenesis in tumor growth, as well as the potential therapeutic benefit of anti-angiogenic drugs, has been partially confirmed in some tumor types.

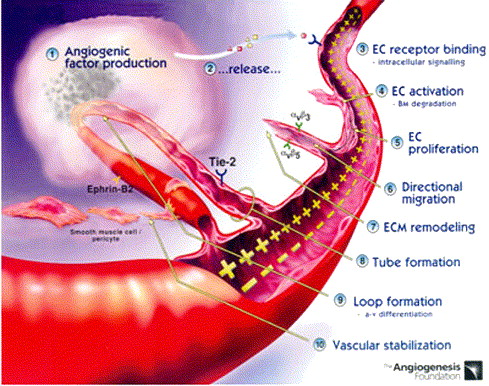
Tumor growth and establishment of metastases depend on the development of new blood vessels that will deliver oxygen, nutrients and growth factors. Tumor angiogenesis is a vital process resulting in the formation of new vessels from pre-existing blood vessels in the tumor tissue. The term “neoangiogenesis” is often applied to pathological angiogenesis in tumours. Tumor cells stimulate the creation of new blood vessels, and the mechanisms are the same as in physiological angiogenesis. This process plays an important role in tumor growth and metastasis. The new blood vessels supply the malignant cells with oxygen and nutrients and thus allow the tumor to grow and invade the surrounding structures. In addition, since these blood vessels are in direct contact with the tumor, they represent the entry point of malignant cells into the circulation, from where they spread in the body and thus enable the creation of new tumor foci (metastases).

**MECHANISMS OF NEOANGIOGENESIS**

**Basic steps of the tumor blood vessel development**

Angiogenesis is a genetically programmed and dynamic process that is locally activated by stimulating signals. The angiogenesis is a complex multi-step process, involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. This cascade angiogenic process comprised of several steps (Figure 3):

* production of angiogenesis factors and their binding to receptors on endothelial cells
* endothelial cell activation
* basement membrane and extracellular matrix breakdown by proteolytic enzymes
* endothelial cell proliferation and migration
* budding and growth of endothelial bands and formation of a capillary loop
* maturation and stabilization of the new blood vessels.



**Figure 3.**The process of angiogenesis

The first step in the formation of a capillary shoot from an pre-existing blood vessel is the local degradation of basement membrane enclosing the postcapillary venule. This leads to disruption of the postcapillary venule, which allows the endothelial cell migration towards neighboring tumor cells, in response to gradients of proangiogenic molecules. Local proteolytic degradation of the basement membrane and extracellular matrix is ​​a consequence of the action of various proangiogenic growth factors produced by tumor cells and/or reactive stroma cells. Under the influence of these factors, the synthesis and release of numerous proteolytic enzymes are induced, such as matrix metalloproteinases, cathepsins, and urokinase plasminogen activators.

The next step involves the direct movement/migration of endothelial cells toward an tumor cell-derived proangiogenic stimuli. Released proangiogenic factors bind to their receptors on endothelial cells, resulting in the activation of endothelial cells that acquire an angiogenic phenotype. These endothelial cells reorganize their cytoskeleton, change their phenotype, break adhesion, and then migrate through the basement membrane. This is simultaneously accompanied by the endothelial cell proliferation. Proliferation of endothelial cells resulting in the formation of a capillary bud that grows as a solid band from pre-existing blood vessels. This is followed by the formation of the lumen, the connection with another capillary tube, the formation of a capillary loop and ultimetllly the establishment of circulation. These unstable nascent capillaries are surrounded by a newly formed basement membrane. Mesenchymal cells are recruited into these capillaries, where they differentiate into pericytes (perivascular wall cells). A critical cellular component in this process is the specialized endothelial cells at the ends of newly formed capillaries, called "tip" cells, and they fuse with each other to form a new capillary network. Coordinated activation of pericytes and smooth muscle cells, together called mural cells, results in blood vessel maturation. Along with the mentioned **angiogenic sprouting process**, second mechanism known as **intussusceptive angiogenesis** also occurs, whereby the forming solid bands of endothelial cells create a barrier inside the lumen of an existing blood vessel. In this way, the existing blood vessel is divided into independent new blood vessels. This sequence of events is thought to be quite similar to the formation of new capillaries that occurs during embryonic development. However, the structure/morphology and function of many blood vessels in the tumor tissue can be extremely irregular, heterogeneous and functionally abnormal. The mechanism of angiogenesis can be organ- and/or tumor-specific. For example, it is hypothesized that in tumors such as melanoma, parts of the blood vessel wall may contain partially (mosaic blood vessels) or completely (vascular mimicry) malignant melanocytes. In addition, during angiogenesis, numerous and diverse molecular changes are detected on endothelial cells in tumor blood vessels.

**The role of pericytes in angiogenesis**

In addition to endothelial cells, pericytes are the next important components of blood vessels. They modulate the functions of endothelial cells and are critical for the development of the mature vascular network. Pericytes have long been known as supporting cells that are closely apposed to the outher surface of the entothelila tubes in normal tissue vasculature. They regulate vascular function including blood vessel diameter and thus blood flow, as well as vascular permeability. These cells also provide mechanical support and stability to the blood vessel wall and enable endothelial cell survival either by direct intercellular contact or by paracrine action.

Given that pericytes play an important role in the survival of endothelial cells, these cells have become an important therapeutic target in the inhibition of angiogenesis. In general, tumor blood vessels are immature and surrounded by sparse pericytes. However, in some tumors the blood vessels are surrounded by a dense layer of pericytes and such blood vessels are less sensitive to vascular endothelial growth factor receptor blockers. Pericytes are thought to mediate resistance to antiangiogenic therapy. Therefore, it is assumed that in order to more effectively inhibit tumor angiogenesis, in addition to endothelial cells, the therapeutic targets should also be pericytes.

**TUMOR BLOOD VESSELS**

Blood vessels in solid tumors exhibit persistent structural abnormalities that likely have a significant impact on tumor growth, progression, and tumor response to therapy.

Tumors achieve their own blood supply in several ways. In tumor-induced angiogenesis, which is similar to physiological angiogenesis, tumor cells release growth factors that stimulate the formation of new blood vessels from pre-existing capillaries. In addition, the tumor recruit circulating endothelial cell precursors from the bone marrow that than participate in the formation of tumor blood vessels (a process known as neovasculogenesis). In some tumors (eg, melanoma), malignant cells and macrophages show marked plasticity; they show the ability to dedifferentiate into cells endothelial-like cells and thus enable the formation of their own vascular network. This phenomenon is known as vascular mimicry.

Tumor blood vessels show various structural abnormalities. The walls of these blood vessels are comprised of tumor and endothelial cells (referred as mosaic vessels). At the periphery of the tumor in the blood vessels, pericytes are usually absent or rarely present, while the basement membrane is incomplete. The blood vessels of the tumor are dilated and also tortuous or twisted. Due to the incomplete basement membrane and the presence of fenestra (transcellular holes or large intracellular gaps between endothelial cells), tumor blood vessels are extremely permeable. Such aberrant blood vessels arise under the influence of dysregulated angiogenic signals in the tumor tissue, which is, among other things, the result of oncogene hyperactivation and loss of function of tumor-suppressor genes.

The mentioned structural abnormalities of blood vessels are variable within a solid tumor mass and heterogeneity is also reflected in the density of blood vessels. In certain tumor regions, dense blood vessels are present, while other regions are poorly vascularized. This may explain slow tumor growth in some regions and faster growth in others. Thus, blood flow in the tumor environment is also heterogeneous. In some areas of the tumor, the blood flow is slow, which is why those areas are deprived of oxygen and nutrients, resulting in severe hypoxia.

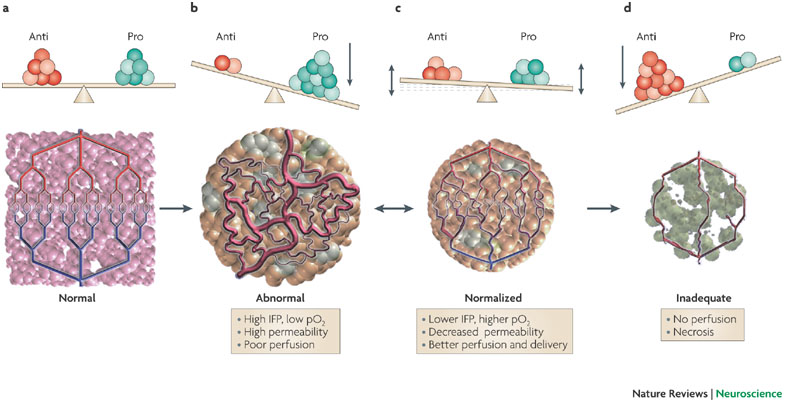
Increased permeability of tumor blood vessels leads to extravasation of plasma proteins, as well as fluid in the extracellular microenvironment within the tumor, resulting in an increase in interstitial pressure.

It is believed that this nature of tumor blood vessels, in addition to being necessary for progressive tumor growth and hematogenous metastases, also limits the effectiveness of therapy, especially chemotherapy, as well as the supply of oxygen necessary for the optimal effectiveness of radiotherapy.

Pronounced hypoxic regions in the tumor protect tumor cells from radiation therapy, the effectiveness of which depends on the generation of reactive oxygen species that kill these cells. In addition, these hypoxic regions select resistant malignant cells and directly stimulate angiogenic signals in the tumor microenvironment.

**MEDIATORS OF ANGIOGENESIS**

The beginning of the process of angiogenesis in a tumor is related to changes in the local balance between proangiogenic and antiangiogenic molecules (Figure 4). The malignant cells express an angiogenic phenotype resulting from an "angiogenic switch" (predominance of proangiogenic factors). In the first phase, activation of an oncogenes (e.g. HER-2) and/or mutation and inactivation of an anti-oncogene (e.g., p53) induce the expression of genes responsible for angiogenesis. In the second phase, malignant cells are additionally exposed to various stress factors in the tumor environment that stimulate angiogenesis. An angiogenic tumor phenotype is closely related to the increased proliferation of malignant cells, as well as the acquisition of invasive and metastatic potential.



**Figure 4.**Angiogenic tumor phenotype

Several different families of growth factors (proangiogenic factors) are known that stimulate tumor angiogenesis. Some factors, such as vascular endothelial growth factor (VEGF), exert their effect directly by binding to a receptor expressed on endothelial cells, especially on activated cells. There are other factors that act indirectly. In other words, these factors act either by stimulating the expression of the main factors of angiogenesis, or by activating cells at the site of angiogenesis that then enhance angiogenic processes. For example, indirect proangiogenic factors are TGF-β, TNF-α, proinflammatory cytokines (IL-8 and IL-6), granulocyte lineage-stimulating factors, chemokines such as stromal cell-derived factor-1 (SDF-1) as well as hormones (estrogen and testosterone). Platelet-Derived Growth Factor (PDGF) is also involved in angiogenesis, and its receptor is expressed on pericytes.

The primary angiogenic factors that act directly are the VEGF family and their receptors with tyrosine kinase activity, angiopoietins (angiopoietin-1 and -2) and their receptors (especially tie-2), as well as Notch signaling receptors (e.g., Notch-4) and their family ligands (e.g., Delta like ligand-4, DLL4). All these factors show a high, but not absolute degree of specificity for endothelial cells, particularly for activated endothelial cells. Eph receptor for ephrin-B2 is another type of receptor with tyrosine kinase activity that is involved in angiogenesis. Ephrin-B2 is a transmembrane ligand that regulates the function of endothelial tip cells involved in budding and branching of new capillaries. This transmembrane ligand also participates in the regulation of VEGF-R2 function.

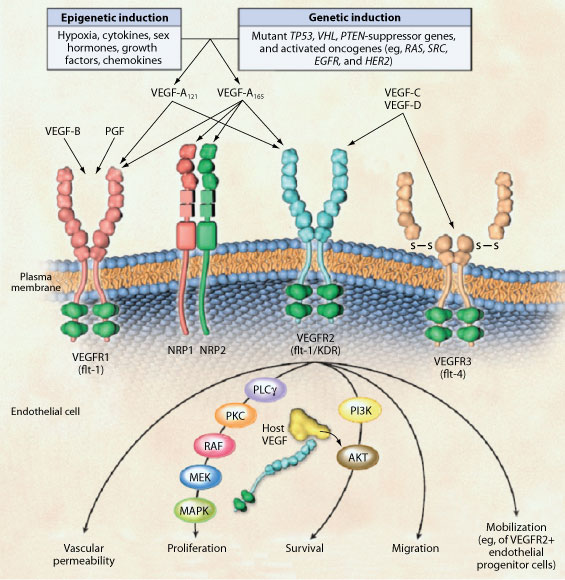
The different stages of angiogenesis are specific regulated by many molecules including: adhesive molecules (e.g., integrins, selectins and cadherins) that enable cell adhesion to components of the extracellular matrix (ECM), ECM components (such as collagen, fibronectin, laminin and proteoglycans) that are responsible for cell- ECM interactions, enzymes of the ECM (e.g., matrix metalloproteinases) which cause proteolysis of ECM components, cytokines (such as IL-1β, IL-8 and TNF) and proangiogenic growth factors (e.g., VEGF, bFGF, TGF-β, EGF and angiopoietin-1).

One of the most important and strongest stimulators of tumor angiogenesis are VEGF and bFGF. **VEGF** was first discovered as a vascular permeability factor because it increases microvascular permeability and endothelial fenestration. The effect of VEGF on vascular permeability is extremely strong (50,000 times stronger than histamine), which suggests that this factor plays a significant role in the tumor blood vessel permeability. Increased vascular permeability is thought to be due to the presence of intracellular fenestrae, reduced pericyte support (which represent another barrier to vascular permeability), and/or the presence of specialized endothelial organelles called vesiculovacuolar organelles. These transmembrane vacuoles form channels that allow the extravasation of fluids and proteins from blood vessels. VEGF is highly specific and is a potent mitogen for endothelial cells.

VEGF (also called VEGF-A) is a member of the VEGF family with approximately 40-80% homology. The VEGF family includes: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF is present in several isoforms, which are created by alternative splicing of VEGF gene. In humans, the most common VEGF isoforms are VEGF121, VEGF165, VEGF189 and VEGF206. VEGF121 is the shortest isoform that is present in the circulating form, while VEGF189 and VEGF206 are bound to the cell surface or are sequestered in the ECM in an inactive form and are activated by proteases. VEGF165 is also bound to the cell surface, but is also present in a circulating form. VEGF121 and VEGF165 isoforms are thought to be the main mediators of angiogenesis.

There are three types of receptors with tyrosine kinase activity to which VEGF binds: VEGFR-1, VEGFR-2 and VEGFR-3 (Figure 5). VEGFR-1 and VEGFR-2 are expressed on endothelial cells of blood vessels, while VEGFR-3 is expressed on endothelial cells of lymphatic vessels, and VEGF-C, which plays an important role in lymphangiogenesis, binds to this receptor.

VEGF121 and VEGF165 bind to VEGFR-1 and VEGFR-2. The main signaling receptor in angiogenesis is VEGFR-2, while VEGFR-1 signaling is weaker than that VEGFR-2. It is assumed that the soluble form of VEGFR-1 serves as a negative regulator in physiological angiogenesis. Neuropilin-1 and neuropilin-2 bind to the longer isoforms of VEGF. VEGF121, an shorter isoform, does not contain the neuropilin-binding domain. Neuropilin is thought to contribute to angiogenesis by serving as a coreceptor that promotes binding of VEGF-A to its receptor, VEGFR-2. Antibodies that target both VEGF and neuropilin have been shown to be more effective in treatment than antibodies that target only one of these proteins.



**Figure 5.** Members VEGF and their receptors

VEGF promotes tumor angiogenesis in several ways: 1) stimulates the endothelial cell proliferation; 2) induces the endothelial cell migration; 3) prevents apoptosis of endothelial cells; 4) mobilizes endothelial cell precursors from the bone marrow to the site of new blood vessel formation. In addition, VEGF increases vascular permeability by allowing the extravasation of plasma proteins, such as fibrin, which cross-link to form a fibrin gel in the tumor extracellular milieu that serves as a matrix for endothelial cell migration and new vessel formation. This growth factor also increases the expression of nitric oxide (Figure 5).

VEGF is produced by many types of cells, such as tumor cells, macrophages, vascular smooth muscle cells, and fibroblasts. VEGF is overexpressed in many human tumors compared with normal tissue. This increased expression of VEGF in tumor tissue is due to many factors (Figure 5). Of these factors, hypoxia is the most significant. Tissue hypoxia is an important stimulus for VEGF expression. Hypoxia stabilizes and increases the expression of the transcription factor, hypoxia-inducible factor-1α (HIF-1α). Lack of oxygen induces an increased intracellular level of the HIF-1α active form. HIF-1α stimulates the VEGF transcription, which is secreted, diffuses through the tissue, reaches endothelial cells and binds to specific receptors on their surface. In addition to hypoxia, a wide range of oncogenes (e.g., ras, src, HER-2), as well as mutation/inactivation or deletion of tumor-suppressor genes such as p53, PTEN, and VHL induce high VEGF expression.

Another important signaling pathway involved in the regulation of tumor angiogenesis is the angiopoietin/tie-2 signaling pathway. The angiopoietin family consists of numerous members including angiopoietin-1,-2-3 and -4. Angiopoietin-1 and angiopoietin-2 bind to tie-2 receptor. The role of angiopoietin-1 in angiogenesis is reflected in the fact that it promotes the maturation and stabilization of newly formed blood vessels by triggering the activation of the Akt/survivin signaling pathway. Conversely, angiopoietin-2 induces blood vessel destabilization, pericyte detachment, and budding of pre-existing blood vessels.

Basic fibroblast growth factor (bFGF) is also important in tumor angiogenesis. Together with VEGF, bFGF stimulates the formation of new blood vessels, and one of the functions of this growth factor is to induce the secretion of enzymes, MMPs, plasminogen activators, as well as collagenases, all of which are responsible for the ECM breakdown. Additionally, bFGF works by increasing the production of VEGF in vascular smooth muscle cells and also by increasing the expression of VEGF receptors on endothelial cells.

A close functional connection between two types of cells, endothelial cells and pericytes, is necessary for the maturation and maintenance of the vascular network. In mice lacking the gene for platelet-derived growth factor-B (PDGF-B) or its receptor PDGF-Rβ, a disorder in pericyte recruitment was registered, resulting in increased permeability and disorder in blood vessels organization, as well as increased apoptosis of endothelial cells. A population of bone marrow-derived precursors expressing the surface markers, C-Kit and Sca-1, was also identified. These cells are recruited to perivascular regions in the tumor where they differentiate into pericytes and thus participate in the stabilization of blood vessels. Thus, increased PDGF-B expression stimulates pericyte recruitment and blood vessel stabilization, while inhibition of the PDGF-B signaling pathway decreases pericyte recruitment and consequently increases endothelial cell apoptosis.

**The role of circulating bone marrow-derived cells and TAMs in angiogenesis**

Many types of cells derived from the bone marrow can be mobilized to the site of the formation of new blood vessels, where they amplify the angiogenesis processes. Hematopoietic CD45+ cells, such as monocytes or other myeloid cells, may be involved in angiogenesis processes. These cells express endothelial cell markers such as VE-cadherin, VEGFR-1, VEGFR-2 and tie-2. Neutrophils and macrophages also exert a pro-angiogenic effect.

Apart from these mentioned cells, non-hematopoietic CD45- cells are also involved in tumor angiogenesis. Circulating endothelial cell precursors are thought to be incorporated into the wall of growing blood vessels, where they then differentiate into endothelial cells.

In 1863. Virchow was the first to discover leukocytes in tumor tissue as well as tumor enviroment. Today, it is known that tumor-associated macrophages (TAMs) comprise large portion of the leukocyte infiltrate of many tumors (including primary tumors and metastases). TAMs affect tumor growth in two ways: cause either tumor regression via TAM-1 or tumor progression via TAM-2.

During progression, numerous regions of hypoxia are formed, and are characteristic of most solid tumors. Different factors (MSP-1, GM-CSF...) are secreted in these regions, which act chemotactically and attract macrophages to the tumor microenvironment. Macrophage infiltration accelerates tumor spread by inducing angiogenesis and invasion. Tumor-associated macrophages, M2 phenotype (TAM-2), can be an important source of VEGF and matrix metalloproteinases involved in the process of tumor angiogenesis.

**Endogenous inhibitors of tumor angiogenesis**

In addition to numerous molecular stimulators of angiogenesis, endogenous inhibitors of angiogenesis are also present. Thrombospondin-1 is an extracellular matrix glycoprotein that binds to the CD36 receptor, and functions as a potent endogenous inhibitor of angiogenesis. Tumor suppressor gene p53 has been shown to increase the expression of thrombospondin-1 in various malignancies. Thrombospondin-1 inhibits the endothelial cell proliferation and migration, and at the same time induces apoptosis of these cells.

Another endogenous inhibitor of angiogenesis is angiostatin. Angiostatin, a fragment of plasminogen, inhibits cell proliferation and induces endothelial cell apoptosis. Angiogenesis inhibitors are also type IV collagen fragments such as endostatin, tumstatin and canstatin. Recombinant endostatin has numerous antiangiogenic functions, such as the ability to interfere with the VEGF and bFGF signaling pathways, inhibit endothelial cell motility, arrest the cell cycle in endothelial cells, and cause their apoptosis. It achieves all the above effects by binding to integrins, including α5β1, αVβ3 and αVβ1. Tumastatin stimulates apoptosis and suppresses the proliferation of endothelial cells. Another endogenous inhibitor is a fragment of calreticulin also known as vasostatin. Vasohibin is a secreted protein produced by endothelial cells under the influence of VEGF.

Thus, the initiation of angiogenesis in tumors requires a major event: on the one hand induction and enhanced expression of one or more proangiogenic factors such as VEGF, and on the other hand reduced expression of one or more endogenous inhibitors such as thrombospondin-1.

**Antiangiogenic therapy**

Given that VEGF plays different roles in angiogenesis processes; regulates the development, function and morphology of new blood vessels, so this molecule is the main target in antiangiogenic therapy. For example, bevacizumab is a humanized monoclonal antibody that neutralizes VEGF. The effectiveness of this neutralizing antibody is increased if it is used together with chemotherapy. For example, such a combination is useful in the treatment of advanced breast cancer, colorectal cancer, as well as gastric and ovarian cancer. However, the findings of some preclinical studies indicate that when anti-angiogenic drugs are discontinued, this results in accelerated tumor growth, as well as increased tumor invasiveness and metastatic potential. One of the assumptions for this increased aggressiveness of malignant tumors is increased hypoxia in the tumor tissue due to the use of anti-angiogenic drugs, which results in increased expression of HIF-1α. This transcription factor then regulates numerous genes that contribute to malignant cell migration, invasion and metastasis.