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Vascular endothelial growth factor (VEGF) — part 1: in physiology and pathophysiology

Naczyniowo-śródbłonkowy czynnik wzrostu (VEGF) — część 1: w fizjologii i patologii

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Abstract

Angiogenesis is an important component of many physiological processes, such as the female sexual cycle, placenta formation, the processes of growth and differentiation of tissues, and reparative processes including wound healing, fracture repair, and liver regeneration. The formation of new blood vessels during angiogenesis and vasculogenesis allows the growth and functioning of multicellular organisms. Pathological angiogenesis most commonly occurs in ischaemic, inflammatory and neoplastic diseases. Conditions in the pathogenesis of which angiogenesis plays an important role are sometimes labelled angiogenic diseases. To date, a number of pro-and anti-angiogenic factors have been defined. VEGF is the only specific mitogen for endothelial cells. It stimulates their growth and inhibits apoptosis, increases vascular permeability in many tissues, promotes vasculogenesis and angiogenesis. VEGF signalling activity in relation to the cell is dependent on having its specific membrane receptors (Flt-1, KDR, Flt-4). Angiogenesis plays a protective role in ischaemic heart disease and myocardial infarction. Angiogenesis extends life for patients after a stroke. Most of the facts about physiological angiogenesis are derived from studies into liver regeneration as a result of an acute injury or partial hepatectomy. Pathological hepatic angiogenesis occurs in the course of inflammation, fibrosis, hypoxia, and during tumourogenesis. There is interesting data relating to liver steatosis and obesity. **(Pol J Endocrinol 2011; 62 (5): 444–455)**

Key words: *VEGF, angiogenesis, physiology, pathophysiology, KDR, Flt-1, liver, cancer, neoplasm, growth factor*

Streszczenie

Angiogeneza stanowi ważny element wielu procesów fizjologicznych, takich jak cykl płciowy kobiety, tworzenie łożyska, procesy wzrostu i różnicowania się tkanek, procesy reparacyjne (w tym gojenie ran, złamań, regeneracja wątroby). Dzięki powstawaniu nowych naczyń krwionośnych w procesach waskulogenezy i angiogenezy możliwe są wzrost i funkcjonowanie organizmów wielokomórkowych. Angiogeneza patologiczna występuje najczęściej w chorobach niedokrwiennych, zapalnych i nowotworowych. Choroby, w patogenezie których angiogeneza odgrywa istotną rolę obejmowane są czasem wspólnym mianem chorób angiogennych. Dotychczas zdefiniowano wiele czynników pro- i antyangiogennych. Czynnik VEGF jest jedynym swoistym mitogenem dla komórek endotelialnych. Stymuluje ich wzrost i hamuje apoptozę, zwiększa przepuszczalność naczyń w wielu tkankach, promuje waskulogenezę i angiogenezę. Aktywność sygnalizacyjna VEGF w stosunku do komórki jest zależna od posiadania przez nią swoistych receptorów błonowych (Flt-1, KDR, Flt-4). Angiogeneza odgrywa protekcyjną rolę w chorobie niedokrwiennej serca i zawale serca. Angiogeneza przedłuża życie chorym po udarze mózgu. Większość wiadomości o fizjologicznej angiogenezie pochodzi z badań nad regeneracją wątroby będącą wynikiem jej ostrego uszkodzenia lub częściowej hepatektomii. Patologiczna angiogeneza wątrobowa występuje w przebiegu zapalenia, włóknienia, niedotlenienia, podczas tumorogenezy. Interesujące dane dotyczą stłuszczenia wątroby i otyłości. **(Endokrynol Pol 2011; 62 (5): 444–455)**

Słowa kluczowe: *VEGF, angiogeneza, fizjologia, patofizjologia, KDR, Flt-1, wątroba, rak, nowotwór, czynnik wzrostu*

Angiogenesis in physiology and pathophysiology

Angiogenesis and vasculogenesis are the terms specifying the formation of new blood vessels. Vasculogenesis is the process of formation of blood vessels that occurs during organogenesis [1, 2]. The development of blood vessels during the embryo stage takes its origin from mesenchymal cells called angioblasts. Angiogenesis is a process that occurs during the post embryo stage when the formation of new blood vessels originates from existing blood vessels [2, 3]. In the process of angiogenesis, vascular capillaries are usually formed. The formation of new blood vessels during angiogenesis and vasculogenesis allows the growth and functioning of multicellular organisms [4]. Angiogenesis is an important component of many physiological processes [5, 6], such as the female sexual cycle (cyclic renewal

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of the endometrium [1, 5, 7], corpus luteum formation [7–10]), placenta formation [2], the processes of growth and differentiation of tissues [4, 11], reparative processes (including wound healing, fractures, liver regeneration) [1, 5, 8, 12, 13]. Pathological angiogenesis (often called neoangiogenesis) most commonly occurs in ischaemic, inflammatory and neoplastic diseases [4, 11, 14–20]. Conditions in the pathogenesis of which angiogenesis plays an important role are sometimes called angiogenic diseases.

Disease processes which have been shown to increase vascularity include: 1) vascular disease — atherosclerosis (pathological vascularisation [21] and growth of atherosclerotic plaque [8, 22]), haemangioma [23], acute myocardial infarction [16]; 2) diseases of the skin and mucosae — pyogenic granuloma, Kaposi's sarcoma, scar keloids, allergic oedema, cancer, excessive hair growth (hirsutism); 3) ovarian disease — endometriosis, ovarian hyperstimulation, cancer, follicular cysts, functional vaginal bleeding; 4) within the muscle — muscle overload; 5) within the adipose tissue — obesity [4]; 6) bone and joint diseases — rheumatoid arthritis [5, 8, 11], synovitis, bones and cartilage destruction, osteomyelitis, osteophyte formation, neoplasms; 7) liver disease — inflammatory and infectious processes (hepatitis), cancer [12, 18, 20]; 8) kidney disease — infectious and inflammatory processes (glomerulonephritis), cancer [12], diabetic nephropathy [24]; 9) respiratory diseases — infectious and inflammatory processes, bronchial asthma, nasal polyps, cancer [12]; 10) brain disease — leukomalacia, cancer [4]; 11) eye disorders — retinopathy occurring in premature infants [4, 25], diabetic retinopathy [1, 4, 8, 11, 22, 26, 27], uveitis and other ocular illnesses; 12) endocrine glands illness — acromegaly [14, 17], autoimmune thyroid disease, goitre, neoplasms [4, 15, 19, 20, 28]; 13) transplantation of organs — the liver, pancreas, kidneys, lungs; 14) lymphatic vascular disease — metastases, lymphoproliferative illness; 15) haematologic illness — AIDS (Kaposi's sarcoma), blood cancers [4]; 16) disease processes in which increased vascularisation occurs in addition to strong vascular permeability — ascites [29], peritoneal fibrosis in dialysis patients, metastasis formation [4].

Disease processes which have been shown to reduce vascularity include: 1) skin and mucosae disorders — ulcers (including stomach and intestines), impaired wound healing; 2) gynaecological diseases — placental insufficiency; 3) within the muscle — ischaemic heart disease, arms and legs; 4) bone disorders — sterile bone necrosis, impaired healing of fractures; 5) hypertension (pulmonary and systemic); 6) diseases of the brain, nerves — vascular dementia [4], Alzheimer's disease [4, 25], leukoencephalopathy [4].

Disease processes leading to abnormal vascular remodelling include: 1) vascular disease — vascular malformations [4]; 2) skin diseases — psoriasis (skin vessels enlarge and become crooked) [8]; 3) gynaecological diseases — preeclampsia; 4) pulmonary hypertension [4]; 5) diabetes mellitus [4, 30].

To date, a number of pro- and anti-angiogenic factors have been defined.

Activators of angiogenesis include: 1) VEGF — induces and enhances angiogenesis/vasculogenesis, vascular permeability, induces and enhances endothelial cells (EC) proliferation, migration and adhesion of leukocytes; 2) receptors for VEGF — integrate and transmit stimuli; 3) nitric oxide (NO) — dilates blood vessels; 4) integrins: $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ [receptors for extracellular matrix (ECM) proteins and macromolecules] — mediate the sprouting blood vessels, are mobilised during EC migration, are involved in intercellular communication (as ECM receptors); 5) transforming growth factor beta 1 (TGFβ1) and its receptors — promotes maturation of blood vessels, deposition of ECM components, induces EC proliferation, differentiation of mesenchymal cells to pericytes; 6) growth factors: acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), TGFα [4, 7, 20, 31–33], insulin-like growth factor I (IGF-I) [4, 14] — induce proliferation of EC and at various stages stimulate angiogenesis; 7) platelet-derived growth factor (PDGF) and its receptors — recruit smooth muscle cells [4, 31, 32, 33]; 8) plasminogen activators (uPA — urokinase plasminogen activator), matrix-metalloproteinases (MMPs), heparinases, chymases, tryptase, cathepsin — rebuild ECM, release and activate growth factors [4, 31, 32, 33]; 9) angiopoietin-1 (Ang-1) — prevents apoptosis of EC, promotes vascular sprouting and branching, stabilises vessels and intercellular contacts, inhibits vascular permeability; Tie-2 — receptor for Ang-1 and Ang-2 (the soluble form of sTie-2 is their natural inhibitor); angiopoietin-2 (Ang-2) — in the presence of VEGF facilitates vascular sprouting [4, 16, 24, 29, 31–37]; 10) plasminogen activator inhibitor 1 (PAI-1) — stabilises new blood vessels (protects against degradation of the provisional ECM located around the new blood vessels) [4, 31–33]; 11) angiotensin II — induces the VEGF [38]; 12) monocyte chemotactic protein 1 (MCP-1); 13) chemokines; 14) hypoxia-inducible factor 1 alpha (HIF-1α) — induces angiogenesis [4, 31–33] and 15) IL-8 [31, 32], IL-1, epidermal growth factor (EGF), prostaglandin (PGE 1, PGE 2, PGF), erythropoietin, histamine, bradykinin, fibrin, heparin [39], tumour necrosis factor alpha (TNFα) [19].

Angiogenesis inhibitors include: 1) soluble Flt-1 (sFlt-1) and NRP-1 (sNRP-1) — binding VEGF and

thereby reducing its biological activity; 2) Ang-2 — an antagonist of Ang-1, increases vascular permeability, destabilises the vessel, causes the death of EC; 3) thrombospondin-1 and -2 (TSP-1, -2) — inhibits the migration, growth, adhesion of endothelial cells, and shortens their lifespan (inhibits EC proliferation), inhibits the formation of vessel lumen; 4) angiostatin (an internal fragment of plasminogen) — inhibits angiogenesis, tumour growth; 5) endostatin (collagen XVIII fragment) — inhibits the migration and shortens the lifespan of endothelial cells, increases apoptosis of tumour cells; 6) vasostatin — inhibits the proliferation of EC; 7) platelet factor-4 — inhibits the binding of VEGF and bFGF, EC proliferation; 8) tissue inhibitor of metalloproteinases (TIMPs) — inhibit the degradation of ECM, EC proliferation, pathological angiogenesis; 9) interferon alpha (IFNα), IFNβ, IFN, inteleukin 4 (IL-4), IL-12, IL-18 — inhibit EC proliferation and migration, inhibit bFGF; 10) VE-cadherin (vascular endothelial cadherin), platelet endothelial cell adhesion molecule-1 (PECAM-1) — adhesion molecules (including intercellular adhesion), increase the tightness of vessels; 11) claudins, occludin, junctional adhesion molecules (JAM) -1, -2, -3 — molecules "tightening" vascular connections, intercellular adhesion, increases vascular integrity; 12) connexins — molecules facilitate intercellular communication; 13) αvβ3 and αvβ5 integrins — shorten lifespan of EC, which is mediated by VEGF and KDR; 14) antithrombin III, LIF (leukemia inhibitory factor) — inhibit the proliferation of EC; 15) an excess of Ang-1 — over-sealing blood vessels makes them harder to sprout; 16) PRL — inhibits VEGF and bFGF; 17) osteopontin — interferes with the transmission of the integrin stimuli [4, 16, 24, 29, 32, 33, 35, 40]; 18) somatostatin [31, 41]; and 19) angiostatic steroids (medroxyprogesterone, 2-metoxyoestradiol) [39].

Angiogenesis occurs as a result of interaction between proangiogenic factors and their inhibitors, and between them and the ECM [2, 32]. Both physiological and pathological angiogenesis are the result of pro-angiogenic factors having an advantage over inhibitors of this process [2, 42, 43]. In tissues in which there is angiogenesis, ECM remodelling is influenced by proteolysis and neosynthesis of its components, which creates conditions for the migration of EC. EC proliferate, differentiate and form new blood vessels. Angiogenesis is thus a multi-stage process involving the endothelium and ECM [6, 32]. It begins with a vasodilation, loss of connections between endothelium cells and vascular leakage, thence it becomes extravasation of plasma adhesion molecules (laminin, fibronectin, fibrin), which together with the components of the ECM will create a scaffold for EC migrating toward the angiogenic stimulus [33, 34, 44, 45]. VEGF binding to its receptors in EC induces NO production in them. This in turn is a major mediator of VEGF-induced vasodilatation and increasing blood flow *in vivo*, that precede angiogenesis [46, 47]. The main factor responsible for vascular permeability is VEGF. VEGF and NO need to overcome the forces maintaining vascular integrity. VE-cadherin, PECAM-1, claudins, occludin, JAM-1, -2, -3, and connexins are involved in the formation and maintenance of vascular integrity. Whereas VEGF causes a loss of interactions between the above-mentioned substances [33, 34, 44, 45], Ang-1 stabilises them [33, 34].

EC proliferate in the course of angiogenesis in response to factors secreted by EC themselves or by neighbouring cells (e.g. leukocytes, hepatocytes, Kupffer cells). VEGF stimulates the migration and proliferation of EC in arteries, veins and capillaries *in vitro* and *in vivo* [48]. Other growth factors such as aFGF, bFGF, HGF, TGFα, TGFβ1, and placenta growth factor (PIGF) also exhibit stimulatory action on EC proliferation such as the number of cytokines, lipid mediators, hormones, and neuropeptides, while angiostatin, endostatin, IFNβ, antithrombin III, LIF, and platelet factor-4 inhibit the proliferation of EC [33, 34, 49]. EC proliferation and migration are possible due to prior degradation of vascular basement membrane (composed primarily of collagen IV and laminin) and ECM (composed primarily of collagen I, elastin) surrounding vessels that occur through specialised proteinases such as uPA, PAI-1, MMPs, TIMPs, heparinase, chymase, tryptase and cathepsins [33]. Expression of collagenases is induced by e.g. VEGF [50]. In addition, ECM proteolysis leads to the release of factors rooted in the ECM, which also promotes proliferation and migration of EC. Insufficient ECM proteolysis makes EC migration difficult or impossible, whereas excessive ECM degradation destabilises a supportive structure (scaffold), after which EC migrate. Both of these situations result in inhibition of angiogenesis. Under conditions of excessive proteolysis and subsequent degradation of the scaffold, there is no supportive structure for migrating EC, and there for they form cysts instead of tubular structures [33]. The degradation of vascular basement membrane and ECM surrounding the vessels leads to the formation of intercellular spaces that will constitute the lumen of the new vessel.

The beginnings of new vascular capillaries are called vascular sprouts. They consist of proliferating and migrating EC and vascular tubes, in which the lumen of vessel is created. The endothelium produces collagen types I and III, which is the the beginning of the basal membrane. Newly created vascular matura-

tion covers the formation of the basement membrane next to the recruitment of pericytes, myocytes and fibroblasts [33]. The processes of forming the lumen, its diameter and length are precisely regulated by VEGF, integrin $\alpha v\beta 3$, $\alpha v\beta 5$ [51], Ang-1 [33]. Integrins are involved in EC migration through the regulation of EC attaching to ECM. It appears that integrins $\alpha v\beta 3$, $\alpha v\beta 5$, which have long been recognised as proangiogenic factors, may also inhibit angiogenesis by decreasing VEGF- and Flt-1-dependent survival of EC [51] and may inhibit angiogenesis induced by bFGF. TSP-1 is involved in the inhibition of light vessels formation, while Ang-1 secreted by pericytes stabilises newly formed vessels by binding to the receptor Tie-2 [33, 37] (its excess, however, makes the vessels more tight and hinders their sprouting [35]). Ang-2 may have dual, opposing effects — it acts as an antagonist of Ang-1, destabilises vessels and causes EC death, leading to regression of vascularisation [33], whereas in the presence of VEGF it promotes a rapid increase in capillary diameter, and facilitates vascular sprouting [35]. TGFβ1 promotes vascular maturation by stimulating ECM deposition and induction of mesenchymal cells differentiation into pericytes [33]. Pericytes, located on the external side of a capillary, inhibit EC proliferation and in the final stage of capillary vascular formation determine the size of newly formed vessels. The process of vessel formation finalises the appearance of collagen IV and V, which in turn inhibit the mitotic divisions of EC. The persistence of newly formed blood vessels depends on maintaining the ability to survive of EC; factors responsible for this include VEGF and Ang-1. On the other hand, angiogenesis inhibitors induce EC apoptosis [4, 37]. New endothelium capillaries usually have a wide connection, numerous channels and endothelial fenestration [39].

Starting the process of angiogenesis is usually dependent on several factors. These include metabolic stress (e.g. hypoxia, low pH, hypoglycaemia) [4, 16, 32], mechanical stress (e.g. pressure triggering cell proliferation), the immune/inflammatory response (e.g. immune/tissue infiltrating inflammatory cells), mutations (e.g. activation of oncogenes or deletion in tumour growth suppressor genes that control production of angiogenesis regulators) [4], and hormonal changes (e.g. increased levels of oestrogen, IGF-I, TSH) and VEGF, which play a role in all the situations in which angiogenesis occurs [32].

A crucial substance controlling the process of angiogenesis is HIF-1 [52], which provides information about hypoxia. Mammalian cells that require for their survival oxygen and nutrients are located within 100–200 microns of blood vessels. This is also the maximum distance for oxygen diffusion. The growth and functioning of multicellular organisms are therefore possible due to the formation of new blood vessels [4]. HIF-1 is a heterodimer constantly containing a β subunit (HIF-1β) and regulated by a concentration of oxygen and growth factors α subunits (HIF-1α and HIF-2α). In hypoxic conditions, HIF-1 dimer binds to the VEGF promoter HRE fragment (hypoxia response element), leading to VEGF transcription [53]. This implies that hypoxia, by inducing the synthesis of HIF-1 [52], leads to increased transcriptional activity of VEGF mRNA [52, 54] and thus VEGF production [4, 25, 54, 55]. VEGF as a physiological regulator of angiogenesis in this way controls the oxygenation of tissues [56]. Hypoxia is a potent stimulator of VEGF both *in vitro* and *in vivo* [57]. HIF-1 also induces NO, PDGF, Ang-2, and contributes to apoptosis of hypoxic cells [4]. In hypoxic conditions, VEGF transcriptional activity is regulated by cytokines and growth factors such as IL-1, IL-6, TNFα, TGFβ, TGFα, bFGF, HGF, EGF, PDGF, IGF-I, IGF-II, and keratinocyte growth factor (KGF) [19, 58, 59, 60] which have generally stimulatory effects on VEGF [19, 56, 61]. VEGF arising from hypoxia stimulates the receptors Flt-1 and inhibits KDR [56]. Expression of Flt-1 is greater in vessels of hypoxic tissue [62, 63]. Moreover, under the influence of hypoxia, mast cells located outside the vessels secrete histamine — a strong vasodilator, and macrophages synthesise TGFβ1, TNFα, IL-1, IL-6, IFNα, PDGF. In hypoxia, the amount of tissue factor (TF) in the EC, monocytes/macrophages and muscle cells is also increased [42, 43, 52]. In turn, the TF increases the expression of VEGF and FGF5 [43]. VEGF is produced by most inflammatory cells [60]. It can be assumed that the congestion of the tissues that is characteristic of inflammation is the result not only of vasodilation, but, after a while, also angiogenesis. Among the mechanical factors that trigger angiogenesis, an important role is played by increased vessel wall tension caused by the increased pressure prevailing in the artery (e.g. hypertension), increased blood flow in the capillary vascular (e.g. hyperthyroidism), and interaction between elements of the blood and endothelial cells (e.g. in polycythemia vera) [64]. Factors that stimulate the secretion of VEGF in smooth muscle cells also include endothelin-1, steroid hormones and heavy metals (Co +2, Cd +2, Ni +2, Mn +2) [56].

Most of the mechanisms of angiogenesis are common to the various organs. The differences concern, among others, the existence of different types of vessels e.g. in the liver — large vessels such as the portal vein, central vein and hepatic arterioles which are characterised by a continuity of EC, and hepatic sinusoids which are characterised by fenestrations.

Vascular endothelial growth factor (VEGF) and its receptors

VEGF, previously known as VPR (permeability factor), was first described and identified by Senger et al. in 1983 [65]. The genetic information for VEGF is located on the short arm of chromosome 6 (6p21.3) [66, 67]. VEGF has a mass of 45 kDa [48], and belongs to a family of platelet derived growth factors. Thus far, several forms of VEGF have been distinguished — A, B, C, D, [2], and E [68]). The biological significance of the different forms of VEGF is not yet definitively known. Of the above, VEGF A, known in the literature (and in this paper) as VEGF [69], is the commonest. VEGF is a homodimeric glycoprotein occurring in several basic isomeric forms containing respectively 121, 145, 165, 189 and 206 amino acids. Clinically significantly, the best-established isoform is $VEGF_{165}$. It is believed that both in normal and transformed cells, gene product 165 is the ommonest [2, 70], and has the highest biological activity [2, 67, 70, 71]. $VEGF_{165}$ and $VEGF_{121}$ have mitogenic activity and increase vascular permeability, while $VEGF_{189}$ and $VEGF_{206}$ are likely only to increase vascular permeability [72].

VEGF is the only specific mitogen for EC (in the rest only 0.01% of the EC undergoes mitotic division [4]). It stimulates their growth and inhibits apoptosis, increases vascular permeability in many tissues, promotes vasculogenesis and angiogenesis [58, 59, 73]. VEGF mRNA and protein are localised in many normal tissues including lung, heart, kidney, liver, brain, uterus, ovaries, pituitary, adrenal, skin, gastric mucosa [7, 20, 58, 59]. Northern blotting methods and in situ hybridisation have detected the largest number of VEGF transcripts in alveolar epithelial cells, renal glomeruli, adrenal cortex and cardiac muscle cells [9]. VEGF is produced by the EC (e.g. pulmonary alveoli, kidney, intestines), fibroblasts, smooth muscle cells [10], and the majority of inflammatory cells (macrophages, lymphocytes, neutrophils and eosinophils). Megakaryocytes demonstrate VEGF expression which can be stored in their α granularities and released during the activation [74]. The physiological role of platelets may rely on limiting the angiogenic activity of circulating VEGF to sites where coagulation occurs, such as wound healing [75]. *In vitro* studies have shown that VEGF produced through thyreocytes as a result of excitation located in the specific receptors for TSH, stimulates Flt-1 in EC thyroid gland, causing their proliferation and increasing vascularity of the gland [66]. VEGF B has been located mainly in muscle (including cardiac muscle), and the lack of it in the EC [25]. VEGF C and VEGF D have been detected in vascular smooth muscle cells. The presence of VEGF C has been demonstrated in α cells of the islands of Langerhans, PRL-secreting, adrenal medulla and neuroendocrine cells of gastrointestinal tract, whereas VEGF D has been demonstrated in the adrenal cortex and various neuroendocrine cells [76].

VEGF signalling activity in relation to the cell is dependent on having its specific membrane receptors Flt-1 (fms-like tyrosine kinase-1, VEGFR1), KDR (Flk-1, fetal liver kinase-1, VEGFR2) [67, 70], Flt-4 (VEGFR3) [70, 76]. All three receptors are characterised by the presence of seven extracellular domains [71, 77] and an intracellular cytoplasmic domain with tyrosine kinase activity [78]. The second and third extracellular domains are responsible for ligand binding, whereas domains 4–7 are responsible for receptors dimerisation [71, 77]. VEGF binding to the extracellular part of the receptors induces their dimerisation [79] and activation of tyrosine kinase [78, 79], in consequence of which there is receptor autophosphorylation and subsequent signal transduction. This leads to activation of phospholipase Cγ (PCγ), which catalyses decomposition of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The resulting second messengers cause an increase in intracellular calcium levels by activating protein kinase C (PKC). Through the RAF kinase, activation of MAP-kinases occurs and further regulates the VEGF-dependent gene expression [67]. Further receptors are neuropilina-1 (NRP-1) [70, 76] and soluble forms of receptor Flt-1 (sFlt-1, sVEGFR1) [25] and KDR (sKDR, sVEGFR2). Flt-1 and KDR are present primarily in the EC [6, 11, 25, 62, 80–82]. Flt-1 is also located in trophoblasts, macrophages [73], and monocytes [25]. KDR is also located in retinal cells, haematopoietic cells, megakaryocytes [83], and haemangioblasts [81]. Flt-4 is present in the endothelium of lymphatic vessels (such as chest tube) [70, 76, 84] and in the endothelium of capillaries with fenestration in the bone marrow, spleen, hepatic sinusoids, kidney glomerulus, and endocrine glands [76]. VEGF acts on the EC particularly through Flt-1 and KDR [6, 11, 25, 62, 80–82]. VEGF B acts through Flt-1 [25]. VEGF C and VEGF D operate through KDR and Flt-4 [76]. Flt-4 preferentially binds VEGF C, therefore stimulates lymphangiogenesis [59, 68, 70, 76, 84]. NRP-1 specifically binds $VEGF_{165}$ and enhances its binding to Flt-1, which indicates that it is a co-receptor [70]. sFlt-1 and sKDR are strong VEGF antagonists. The soluble receptors, by combining VEGF molecules, are suppressing their action on specific membrane receptors. They can be current in the specific tissue as well as circulate within blood. They participate in this manner in angiogenic homeostasis of the organism.

There are differences in biological activity between Flt-1 and KDR. Both Flt-1 and KDR bind to VEGF with high affinity [85, 86], but for Flt-1 this is higher [79, 87]. However, the tyrosine kinase activity of Flt-1 is usually about one tenth that of KDR [79, 88, 89] resulting

in a more potent biological effect after the stimulation of KDR [83]. In this way, stimulation of Flt-1 receptor, albeit with a higher affinity for VEGF, shows a weak, or not capable of being defined, response, which may suggest that Flt-1, like sFlt-1, negatively regulates (inhibits) VEGF action and angiogenesis [77]. Flt-1 expression in EC is low, which makes it difficult to detect, whereas KDR expression is high quality and easy to detect [79, 87]. It has been demonstrated that while KDR is responsible for VEGF signal transduction, Flt-1 regulates the signal [6]. Certainly, Flt-1 is involved in vasculogenesis [25]. The role of Flt-1 in angiogenesis is less clear. Abolishing Flt-1 and KDR expression in mice made development of blood vessels and of the foetus impossible, leading to death, but an isolated deficiency of each of these genes gave a distinct angiogenic phenotype [79]. KDR-knockedout mouse foetuses died due to EC differentiation and vessel formation and lack of the precursors of haematopoietic cells [90]. Mouse foetuses with a lack of Flt-1 also died, but had very expressly differentiated EC and very large malformed vessels [91]. These observations thus confirm the activity of Flt-1 in regulating angiogenesis. Foetal mice lacking Flt-4 died before the lymphatic system was developed [32], while the vessels of homozygous mice with Flt-1, deprived of the intracellular domain, were nearly correct [92]. This indicates that in early embryogenesis, the extracellular domain of Flt-1 is sufficient to protect the animals against the development of lethal anomalies [91]. This implies that lethality in mice lacking Flt-1 would not be the result of an inability to signal VEGF, but rather of the lack of negative regulation of growth and differentiation of haemangioblasts [77, 91]. Flt-1 activation by VEGF induces monocyte chemotaxis, and increases expression of urokinase, PAI-1 and MMPs in smooth muscle cells [93]. Most likely, Flt-1 activation by VEGF acquires meaning only in pathological angiogenesis [11], because Flt-1 and KDR have different biological activities in different pathological conditions [3].

VEGF and angiogenesis in pathological processes

Angiogenesis plays a protective role in ischaemic heart disease and myocardial infarction [16, 39], and creating a new network of blood vessels in the ischaemic myocardium improves its function [4, 26]. It has been shown that the amount of VEGF in the myocardium increases significantly 24 hours after hypoxia and lowers to baseline values 24 hours after the restoration of myocardium oxygenation [57]. Endothelial cells of a properly blood-supplied cardiac muscle possess a reduced number of Flt-1, and its increase is observed in conditions of hypoxia [64]. Commonly occurring polymorphisms of KDR (SNP604, SNP1192, SNP1719) coexist with the risk for coronary heart disease [94]. VEGF also has the ability to expand the coronary vessels. This results in the production and release of NO by the endothelium. Growth factors released in areas of ischaemia affect the opening of the already existing vascular anastomoses. Chronic myocardial ischaemia of a different aetiology(for example due to iron deficiency anaemia) leads to expansion of the coronary vessels, opening of the vascular connections, the proliferation of capillaries and the elongation of existing capillaries [64]. In addition to hypoxia, mechanical factors may cause vascular remodelling. Hypertension (including portal) leads to hypertrophy and proliferation of arterial smooth muscle, and thus to change their structure. Angiogenesis can lead to haemorrhagic rupture of atherosclerotic plaque [4].

Hypoxia exerts a significant effect on VEGF secretion in lung diseases: animal experiments have revealed that acute and chronic hypoxia increases VEGF secretion and the number of KDR in the lung tissue. VEGF enhances pulmonary vessels permeability [95]. An increased expression of VEGF and Flt-1 in alveolar macrophages and granulosa cells of sarcoidosis, and elevated VEGF levels in the blood, have been found in patients with extrapulmonary and disseminated sarcoidosis [2].

Angiogenesis extends life after a brain stroke [4, 26]. The development of angiogenesis in the CNS is dependent on the area of the brain ischaemia. In rats after 130 days of hypoxia the amount of vessels in 1 mm^3 increases in the cortex, hippocampus and corpus striatum, while in the cerebellum and medulla oblongata it increases only slightly and the amount of VEGF is increased in ischaemic areas of the brain [96]. In cerebral oedema, increased VEGF secretion and Flt-1 expression are apparent already in the first hours of hypoxia. VEGF expression is most intense two days after, and Flt-1 expression three days after, the beginning of hypoxia [97]. On the other hand, angiogenesis occurring in the eyeball is the commonest cause of blindness in diabetic retinopathy and retinal ischaemia. Hypoxia-induced angiogenesis can also cause blindness in premature neonates [4, 26]. VEGF increases CTGF mRNA levels (connective tissue growth factor) in retinal vessels via the Flt-1 and KDR [98]. Excessive deposition of ECM in tissues additionally reduces the supply of oxygen, causing hypoxia in diabetes, and Alzheimer's disease in premature neonates treated with oxygen under high pressure [4, 25].

KDR deficiency leads to disorders of haematopoiesis [81]. In women with endometriosis in whom angiogenesis is responsible for disease progression, elevated VEGF levels in peritoneal fluid have been found [39]. Angiogenesis and increased VEGF expression occur

in arthropathy. For rheumatoid arthritis, their cause is both hypoxia and cytokines involved in the inflammation process [33, 99]. It has been shown that VEGF expression in the epithelial cells of skin tissue is closely related to the healing process [100]. Angiogenesis also participates in the excessive accumulation of body fat in the obese. It is known that adipose tissue has a rich vascularisation, and preadipocytes migrate to places of neovascularisation. Mediators of angiogenesis in adipose tissue are insulin-induced VEGF, bFGF and leptin (antiangiogenic therapy has been studied in the treatment of obesity) [4].

VEGF and angiogenesis in hepatology and gastroenterology

Most facts about physiological angiogenesis are derived from studies on liver regeneration being a result of acute injury or partial hepatectomy [13, 101, 102]. Physiological hepatic angiogenesis occurs during regeneration of this organ, and is based on the formation of new functionally efficient hepatic sinusoids, whereas pathological hepatic angiogenesis, for example during fibrosis, is characterised by the capillarisation of hepatic sinusoids [12, 33]. In patients with fulminant hepatitis during the recovery period, a profound regeneration of this organ has been found to coexist with a distinct increase in serum VEGF concentrations, in contrast to patients who did not achieve clinical improvement [103]. On the other hand, in animals after partial hepatectomy, VEGF promotes the proliferation of hepatocytes and the reconstruction of hepatic sinusoids by EC [104].

Liver regeneration is a multi-stage process. In the first stage, hepatocytes in response to the action of growth factors [13, 102] move from G_0 to G_1 phase of the cell cycle (6–8 hours). This process is controlled by cytokines (especially TNFα and IL-6) and transcription factors. Following this process, the expression of genes involved in cell cycle of hepatocytes occurs. Initiation of replication hepatocytes requires the presence of HGF and TGFα, but over time this process becomes autonomous, which means that the presence of growth factors is no longer needed. In rats, two peaks of hepatocytes DNA synthesis are observed: one after 24 hours and another after 36–48 hours. Termination of the hepatocytes replication cycle occurs under the influence of several factors (including TGFβ1) which are potent inhibitors of hepatocytes DNA synthesis [13, 105]. In the early phase of liver regeneration, proliferating hepatocytes show hypoxia-induced VEGF expression which initiates a process aiming at achieving the proper flow of blood through the liver. Hepatocytes then proliferate to the greatest extent around the portal vein (the area around the portal), which rarely is accompanied by reconstruction of the hepatic sinusoids. Then, as a result of angiogenesis, hepatocytes begin to proliferate in conditions of sufficient blood supply, something that is already accompanied by liver hepatic sinusoids reconstruction. Growth factors that stimulate it directly are therefore essential to the proliferation of hepatocytes, such as HGF and TGFα (in the early phase), and, indirectly, VEGF (in the late stage of regeneration) [104]. Their activity is proportional to the amount of liver tissue removed [102]. It has been shown that the appearance of VEGF mRNA expression in hepatocytes and nonparenchymal cells of mouse liver precedes hepatocyte proliferation after partial hepatectomy [106]. In rats, hepatic expression of VEGF and its receptors increases in the early period after such surgery, and coexists with the proliferation of endothelium [107].

VEGF promotes the repair of damaged tissues. In freshly isolated healthy rat liver cells, the presence of VEGF mRNA evaluated by Northern blot analysis has been found in hepatocytes, as has the presence of Flt-1 mRNA and KDR mRNA in nonparenchymal cells (including the SEC) [108, 109]. In turn, in the liver after partial hepatectomy, VEGF mRNA expression has been demonstrated in both hepatocytes and in nonparenchymal cells [12] (mainly in the SEC, Kupffer cells, macrophages, and hepatic stellate cells (HSC), ECM [108, 110]). VEGF mRNA expression increases in hepatocytes around the portal area within a short time (48–72 hours) after surgery [12, 104, 108–110] (after 24 hours by [111]) and then progressively decreases [110]. Expression of Flt-1 mRNA and KDR mRNA in the sinusoidal endothelial cells (SEC) has been found to be increased 72–120 hours after partial hepatectomy [110] (72–168 hours by [108, 109]). Simultaneously, the SEC proliferative activity around the portal area was transcendent 48–72 hours after partial hepatectomy and coexisted with increased proliferative activity of hepatocytes of this area. Convergence between VEGF-expressing hepatocytes and the location of proliferating SEC has been demonstrated. It was therefore concluded that, secreted by the proliferating hepatocytes, VEGF promotes proliferation of SEC in the paracrine manner, and the reconstruction of the hepatic sinusoids by the SEC in regenerating liver begins exactly in the area around the portal, which is also where proliferation of the hepatocytes starts [104, 110]. In rats, the share of VEGF in angiogenesis associated with liver regeneration has also been demonstrated through the simultaneous execution of partial hepatectomy and administration of VEGF-neutralising antibodies (anti-VEGF) which led to the suppression of proliferation of hepatocytes and SEC (Ki-67 index was assessed) 48–96 hours after surgery. It has also

been demonstrated that, concurrent with partial hepatectomy, the application of exogenous VEGF in turn exacerbates the proliferation of the abovementioned cells 48 hours after surgery [104]. It has been shown that injection of VEGF promotes the proliferation of hepatocytes and is involved in liver regeneration [112]. After liver transplantation, VEGF promotes the simultaneous formation of new blood vessels and new liver tissue [113]. The administration of adenoviruses with VEGF (mitogen SEC) and HGF (hepatocyte mitogen) to a cirrhotic rat liver before a 70% hepatectomy led to a marked increase in the amount of hepatic mRNA VEGF, HGF plasma concentrations, proliferation of hepatocytes and SEC 24–48 hours after surgery, and thus liver regeneration was enhanced [114]. *In vitro* VEGF DNA synthesis was increased after 24 hours once EGF or HGF had been added to culture hepatocytes [108, 109]. Delivery of VEGF *in vivo* increased the liver weight of mice but did not stimulate *in vitro* proliferation of hepatocytes in the absence of the SEC because of the lack of HGF produced by the SEC and its paracrine action. *In vivo*, selective activation of Flt-1 stimulated the proliferation of hepatocytes and reduced toxic liver injury in mice. This was based on the observation that Flt-1 agonists can be used to treat certain liver diseases [115]. The increasing expression of VEGF in regenerating the liver is involved (through the Flt-1 and KDR) in SEC proliferation, leading to the reconstruction of the hepatic sinusoids [108, 109].

The role of VEGF in liver regeneration, however, is broader than a simple stimulation of cell proliferation [13]. Selective activation of Flt-1 means that the SEC produces a number of antiapoptotic factors which may protect parenchymal cells from damage and initiate regeneration [115]. The action exerted by VEGF is not limited to the SEC and hepatocytes. Under the influence of hypoxia, the expression of VEGF and Flt-1 can appear in activated HSC and lead to their proliferation [116]. *In vivo* studies conducted in rats under conditions of hypoxia showed an increase in the amount of VEGF and Flt-1 in hepatocytes and an unchanged number of KDR and Flt-4 [117]. The expression of Flt-1 was found in the endothelium of hepatic arterioles and in SEC and HSC, and it was highest 72 hours — 12 days after partial hepatectomy [107, 118, 119]. KDR expression was found in the endothelium of large vessels of the liver and in SEC, and it was highest 72 hours — 12 days after surgery [119]. Increased expression of Ang-1 has been demonstrated in the period 72–96 hours, and of Ang-2 72–168 hours, after partial hepatectomy. Ang-2 in the presence of VEGF intensifies the early angiogenesis during liver regeneration, and in the absence of VEGF inhibits the growth of blood vessels in the later stages [107]. During liver regeneration (from 48 hours after surgery), Tie-2 expression in the SEC appears in the endothelium of large vessels of the liver, and Tie-1 expression in EC appears in the non- vascularised areas of the organ [119]. Most receptors for angiogenic factors localised in the liver EC are found at a low level of expression in the quiescent state, but this level is clearly higher during liver regeneration. Increasing the expression of hypoxia-dependent genes, which occurs at a later stage of liver regeneration, is a response to hypoxia deepening in newly established, non-vascularised areas of hepatocytes [33]. The physiological mechanism of adaptation to hypoxia in human cells is the activation of transcription regulators. One of these, HIF-1, increases the expression of genes involved in the processes of glycolysis, erythropoiesis and angiogenesis [120]. Blood flow through the liver was lowest 36 hours after partial (70%) hepatectomy, and expression of HIF-1α mRNA was highest in the period 24–120 hours after surgery. In turn, the expression of VEGF and Flt-1 was highest 120 hours and 12 hours respectively after this procedure. This implies that the expression of Flt-1 and HIF-1α, that precedes VEGF expression, may participate in the reconstruction of the hepatic sinusoids [121]. Growth factors and cytokines (such as EGF, TGFβ, TGFα, PDGF, IGF-I, KGF, IL-1β, IL-6 [71, 100] as well as hormones (TSH, ACTH, steroids, angiotensin II) [53, 120, 122], are involved in increasing transcriptional mRNA activity and VEGF secretion under conditions of hypoxia, depending on the type of tissue in which these substances act.

Pathological hepatic angiogenesis occurs in the course of inflammation, fibrosis, hypoxia, and during tumourogenesis [18, 20, 33]. Chronically persistent and excessive angiogenesis is a hallmark of inflammatory diseases of multiple organs, including the liver. Monocytes, macrophages, platelets, mast cells, and leukocytes next to inflammatory mediators release the angiogenic factors including VEGF, TGFβ1, bFGF, HGF, TNFα, PDGF, IGF-I, Ang-1, MCP-1 [4], EGF, TGFα [64] and others. Many of these act on the damaged cells ("wound" cells) leading to the subsequent release of these angiogenic factors. Intrahepatic inflammatory cells leave the circulatory system vessels and accumulate in areas of inflammatory infiltration and liver injury. The mechanisms responsible for leukocyte migration to sites of inflammation include, inter alia, interactions between proteins present on the surface of leukocytes and on the surface of the endothelium. Adhesion molecules include intercellular adhesion molecule 1 (ICAM-1), integrins, and selectins. Increased permeability and vasodilatation in the course of inflammation help these factors to reach and penetrate the surroundings. In the inflam-

matory process, angiogenesis is being triggered among others by hypoxia in a mechanism requiring the participation of pro-inflammatory cytokines and growth factors [4]. Hypoxia and pro-inflammatory cytokines induce HIF-1α and VEGF production [33]. Cytokines and angiogenic molecules produced by immune cells can modulate cellular expression of adhesion molecules and other superficial substances on the cells of the endothelium or inflammably changed tissue. For example, VEGF and TNFα increase, while TGFβ1 and bFGF reduce, the amount of adhesion molecules [4]. In HBV and HCV (hepatitis B and C viruses), infected patients produce, under the influence of VEGF, NO in the liver which leads to vasodilatation. VEGF participates in increasing vascular permeability [33]. During HCV infection, capillaries are formed by EC in inflamed changed areas [123]. An increase of EC proliferation, both in viral and non-infectious liver diseases (including cirrhosis stage) have been documented [124, 125]. VEGF, which as a factor increasing vascular permeability is 50,000 times more potent than histamine [65], may enhance the release of acute phase proteins from cells and vessels and thus play a key role in acute inflammation [59]. KDR blockage reduces vascular permeability in a cirrhotic rat liver [29]. In chronic hepatitis and liver cirrhosis, the primary lesions, excessive fibrosis, and nodules formation are similar regardless of the aetiological agent (viruses, alcohol, parasites) [59]. Fibrosis of liver tissue occurring during chronic inflammatory processes at a certain point leads to increasing resistance in blood vessels and obstructing blood flow, reducing the oxygenation of tissues, which, although stimulating angiogenesis, may, in advanced disease, be ineffective [123]. Advanced liver fibrosis and cirrhosis, by reducing the amount of liver cells and the creation of portal-venous and arterio-venous connections, lead to impairment of organ perfusion. Brodsky et al. [126] showed that the amount of blood vessels and the simultaneous VEGF expression in the cirrhotic liver are very diverse. Namely, VEGF expression in the regenerative nodules is smaller than in the surrounding tissues and in the healthy liver tissue, whereas in liver tissue modified by fibrosis, that surrounds regenerative nodules, VEGF expression is higher compared to the nodule tissues and the healthy liver tissue. Low VEGF blood concentrations in patients with liver cirrhosis [59, 127, 128] probably reflect both the degree of portal hypertension and the organ dysfunction [59, 127] and may be associated with a low degree of hepatocytes regeneration, because hepatocytes in the cirrhotic liver are in conditions of reduced blood flow (as well as chronic ischaemic kidneys). In less advanced forms of hepatitis, the reduction in the oxygen concentration strongly stimulates VEGF, with the result that induces mitoses in the SEC, hepatocytes, EC of intrahepatic arterial and venous vessels. In this context, VEGF is an agent acting against liver cirrhosis, but the destruction of liver cells by the disease process and the rebuilding of the liver do not lead to an expected increase in VEGF. However, the scientific community is far from agreed as to whether angiogenesis and VEGF play a positive or a negative role in chronic hepatitis and liver cirrhosis [59].

There is interesting data relating to liver steatosis. Up to 20% of 163 patients with sleep apnoea syndrome had transaminase activity elevations; in more than half of them this co-existed with steatosis, and in some of them with fibrosis. Patients with sleep apnoea syndrome are a risk group for the development of liver steatosis. This confirms that logically one of the mechanisms of liver injury may be its hypoxia. Although changes in hepatic expression of VEGF have not been observed in patients with sleep apnoea syndrome, nevertheless the above facts and the attempt to use VEGF as a marker of hepatic hypoxia are interesting [129]. VEGF as a marker of hepatic ischaemia has been used in other studies [130].

The degree of VEGF expression in epithelial cells of stomach tissues is closely associated with the process of healing ulcers [131]. Patients with ulcerative colitis and Crohn's disease had higher VEGF levels compared to healthy subjects and to patients with no inflammatory bowel disease. Higher values of VEGF are present in patients with hereditary haemorrhagic telangiectasia [2]. In liver haemangiomas, high expression of VEGF and KDR has been seen [23].

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