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Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis

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Summary

This article discusses the role of transcription factors in vascular endothelial growth factor (VEGF) gene expression. Angiogenesis is a complex and multilevel process of new capillary formation on the basis of already existing blood vessels. Physiologically, it is a very strictly regulated process, which results in a balance between stimulatory (angiogenic) and inhibitory (angiostatic) factors to control the correct development of blood vessels. There are many very well characterized angiogenic and angiostatic factors that can modulate VEGF expression. Some of them (e.g. HIF-1, AP-1, and Sp-1) are transcription factors, proteins that bind to the VEGF promoter to initiate and activate the transcription of a gene directly. Others, like nitric oxide or cytokines, are agents that stimulate the transcription factors through different cellular signaling pathways. There are also oncogenes (V-SRC, bcl-2) and tumor suppressor genes (VHL), the mutations of which lead indirectly to increased transcription of the VEGF gene.

key words: **transcription factors • vascular endothelial growth factor (VEGF) • angiogenesis**

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BACKGROUND

Vascular endothelial growth factor (VEGF) is one of the most important agents to stimulate angiogenesis. The loss of even a single VEGF allele results in embryonic death, which indicates its irreplaceable role in the development and differentiation of the vascular system [1]. The human VEGF gene has been assigned to chromosome 6p21-p12 and is organized into eight exons, separated by seven introns. It is a homodimeric, heparin-binding glycoprotein occurring in at least four isoforms of 121, 165, 189, and 201 amino acids, due to the alternative splicing of the single gene.

Trascription factors are proteins that initiate and activate the transcription of a gene by the polymerase RNA II and III. These are molecules that can recognize and bind to specific sites of DNA sequences. The promoter contains all the DNA sequences crucial to the initiation of transcription. These sequences are divided into basic promoter sequences (where the process of initiating complex folding takes place) and upstream promoter sequences, to which activators (transcription factors) bind. The transcriptional regulation of the VEGF gene seems to play the pre-eminent role in the control of VEGF expression, and several response elements within the VEGF promoter region have been characterized in vitro. Analysis of the human VEGF gene promoter (of full-length 1.5 kb) revealed a single major transcriptional start site (nucleotides –749 to –720 and –714 to –685) 1038 bp upstream from the ATG initiation codon. A key region of the VEGF promoter, located at approximately –930 from the transcription start, is a target for a number of signals. This ~50-bp region is responsive for hypoxia, oncoproteins, and growth factor activators, primarily *via* activation of HIF-1. Expression from this region is repressed under non-stimulated normoxic condition by the p53 and von Hippel Lindau (VHL) tumor suppressor, which function by regulation of stability of the HIF-1α.

Agents that influence transcription factors induce angiogenesis through different intracellular signaling pathways, and mutations of oncogenes and tumor suppressor genes lead indirectly to increased transcription of the VEGF gene. A list of these factors is shown in Table 1.

I. TRANSCRIPTION FACTORS INDUCING VEGF GENE EXPRESSION

Hypoxia-inducible factor HIF-1α

Hypoxia-inducible factor-1 (HIF-1α) is the best characterized transcription regulator of the vascular endothelial growth factor gene. It is one of the first genes upregulated by hypoxia in both *in vitro* and *in vivo* models of ischemia [2]. In its active form it is a heterodimer composed of two subunits (HIF-1α and HIF-1β), both of which are members of the bHLH-PAS (basic Helix-Loop-Helix-PerArnt-Sim) family of proteins [3–5]. HIF-1(is an 826 amino-acid protein that functions as a transacting transcriptional activator of vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), lactate dehydrogenase, erythropoietin (Epo), and glycolytic enzymes [6–8]. Expression of the gene for HIF-1α is highly sensitive to the onset of cellular hypoxic conditions, making it one of the earliest effectors of the response to ischemia [9]. HIF-1α is a high-affinity protein that binds to HIF-1α in the cytosol and transports it into the nucleus, where HIF-1α may exert its trans-acting effect [10]. HIF-1α is a constitutively expressed nuclear translocator protein, not sensitive to hypoxia, that forms heterodimers with HIF-1α as well as with other nuclear proteins. HIF-1 DNA-binding activity, HIF-1α and HIF-1α protein levels depend on cellular oxygen concentrations, with a maximal response at an O_2 concentration of 0.5%, which occurs in hypoxic and ischemic cells [11].

Increased steady-state levels of HIF-1α mRNA are detected in myocardial specimens with pathological evidence of acute ischemia (within the first 48 hours) or early infarction (within the first 24 hours). VEGF transcripts are seen in specimens with evidence of acute ischemia or evolving infarction (within 24 to 120) [4]. Expression of VEGF persists for a longer time than that of HIF-1α. The response of HIF-1(to ischemia occurs early and is transient, whereas the VEGF response is of longer duration and necessary for the preservation of the myocardium and limitation of hypoxic cellular destruction. The presence of HIF-1α mRNA and subsequently the presence of VEGF mRNA in the heart tissue of patients with infarction provide evidence that HIF-1α contributes to the limitation of infarct size by promoting angiogenesis and vascular remodeling by increasing the levels of VEGF mRNA. Therefore HIF-1α mRNA increase can be an early molecular marker of myocardial ischemia and infarction [4].

Hypoxia-inducible factor HIF-2α

Hypoxia inducible factors HIF-1α and HIF-2α are two closely related protein complexes that activate transcription of target genes in response to hypoxia [12]. HIF-2α (also called HRF, HLF and EPAS1) is highly expressed by vascular endothelial cells and activates the transcription of endothelial cell-specific receptor tyrosine kinases (tie-2) and of vascular endothelial growth factor receptor (flk-1/VEGFR2), regulating in this way the development of blood vessels [13]. During embryonic development, the expression of HIF-2α is highly increased in endothelial cells and is closely correlated with that of VEGF, whereas the expression of HIF-1α remains apparently unchanged at a much lower level. The high expression level of HIF-2α mRNA in the oxygen delivery system of developing embryos and adult organs suggests that in a normoxic state, HIF-2α regulates gene expression of VEGF and is involved in the development of blood vessels [14]. There is an inverse correlation between HIF-1α and HIF-2α induction and cell survival under hypoxic conditions in tumors. Cells with reduced induction of HIF-1α or HIF-2α show high basal levels of VEGF and improved survival under hypoxia [12]. Thus, despite the fact that HIF proteins are necessary for optimal tumor growth and angiogenesis *in vivo*, overexpression of these molecules seems to be detrimental to tumor growth.

Table 1. Factors that affect VEGF gene expression.

I	Transcription factors	HIF-1α/Arnt, HIF-2α/HLF/HRF, Ap-1, Ap-2, HAF, Sp-1, Sp-3, NFχB, STAT proteins, Smad, p300/CBP, estrogens, progestins
II	Agents influencing transcription factors	IL-1β, Il-6, PDGF, TGF-α/β, TNF-α, EGF, heavy metals (Cd, Co, Ni, Pb), cAMP, Ca^{2+}, PGE_2, PGI, NO, insulin IGF_1, IGF_2, HGF, retinoic acid, Ox-LDLs, angiotensin II, trombine, LIF, steroid hormones, endothelin-1, UV-Ai UV-B radiation, dexamethasone, kardiothropin, organomercurial compound (mersalyl)
III	Genes	ERBB 2, von Hippel-Lindau, oncoprotein E1A, GPCR receptor, Cbfa1/Runx2, VHL, V-SRC, bcl-2

HIF-2α also induces the expression of VEGF in osteoblasts in response to hypoxia [15]. VEGF produced by osteoblasts functions as a proangiogenic factor during normal skeletal development and in fracture repair. Osteogenesis is intimately associated with angiogenesis. VEGF mRNA is highly expressed during fracture healing and promotes angiogenesis during skeletal development and following fracture. Exposure of osteoblasts to 1% O_2 for 24 h results in a four-fold increase in VEGF mRNA, which is preceded by a rise in the level of the HIF-2α protein [15]. In all cell types studied to date, both HIF-1α and HIF-2α are similarly induced by hypoxia and both subunits stimulate the VEGF promoter. It is notable, therefore, that transcriptional regulation of VEGF in response to hypoxia in osteoblasts occurs through HIF-2α but not the HIF-1α subunit of HIF. These data indicate the existence of cell-type specific mechanisms in the regulation of HIF-α subunits by hypoxia. Transcription factors of the bHLH-PAS protein family are also important regulators of neurogenesis, and mouse HRF is expressed most prominently in brain capillary endothelial cells [16].

Hypoxia response elements (HREs)

Transcription of hypoxia-inducible genes is regulated by hypoxia response elements (HREs) located in either the promoter or enhancer regions [17]. Analysis of these elements revealed the presence of one or more binding sites for HIF-1. As already mentioned, there are many genes that can be induced by hypoxia, such as those of VEGF, erythropoietin and glycolytic enzymes. Mutational analysis of the VEGF gene promoter revealed that an HIF-1 binding site (HBS) and its downstream HIF-1 ancillary sequence (HAS) within the HREs are required as cis-elements for transcriptional activation of VEGF by either hypoxia or nitric oxide (NO). This finding suggests a major role of HIF-1 protein in transcriptional activation of the VEGF gene. Analysis of the VEGF promoter also revealed that deletion of the HREs completely abolishes VEGF induction by NO and hypoxia. Further analysis of HREs showed that not only the HBS, but also its downstream HAS, is essential for induction by these stimuli and that the AP-1 site is required for its optimal response [5].

The core sequences of HBS and HAS form an imperfect inverted repeat with a spacing of 8 nt. This observation suggests that HAF, a protein that binds to the HAS, might be identical or similar to HIF-1. Although mutations that increase stability of an inverted repeat enhance the reporter activity, changes in spacing between half-sites abolish the activity. A strict requirement of HAS and the precise spacing between the two motifs indicate that HAF interacts with the HIF-1 heterodimer as a transcriptional factor in NO- and hypoxia-induced VEGF expression. If the HAF of VEGF is unique and distinct from other genes, inhibition of HAF function would be able to suppress VEGF induction without affecting the expressions of other hypoxia-inducible genes [17].

Transcription factor NF-κB

Transcription factor NF-κB is present in many types of cells and contributes to the expression of many genes, such as those for cytokine and cytokine receptors, growth factors, stress proteins, and adhesion and immunoregulatory molecules. NF-κB factor is present in the cellular cytosol and consists of structurally related proteins that belong to the Rel family. Each member of this family has a Rel-homology domain (RHD) as well as domains responsible for DNA binding, dimerization and translocation to the nucleus within the RDH [18]. In cells that are not stimulated, NF-κB remains in the cytosol combined with inhibitory proteins (IκB). A strong NF-κB activator is reoxygenation. These findings seem to explain the induction of many inflammatory genes subjected to reperfusion as a result of ischemia [19]. The NF-κB is also involved in the upregulation of VEGF and its activity in breast cancer cell lines (MDA-MB-231) is associated with the high expression of VEGF mRNA [20]. What is more, advanced glycation end products (AGE) which occur during diabetes increase the transcriptional activity of NF-κB and therefore upregulate mRNA levels of VEGF [21]. Cervistatin (a hydroxymethylglutaryl CoA reductase inhibitor) completely abolishes this process, and might therefore be a promising therapy for patients with proliferative diabetic retinopathy [21].

Transcription factor AP-1

Transcription factor AP-1 (activator protein-1) binds to TRE (TPA response element), which is located in the promoter region of many genes responsible for the proliferation and tumor progression of cells. Hypoxia highly induces the binding ability of AP-1 to DNA as well as the transcriptional activation of genes [22,23]. The whole complex of factors (HIF-1, AP-1) contributes to activation and expression of the VEGF gene in hypoxic conditions. However, transcription factor AP-1 is not necessary in the induction of expression of the VEGF gene [24]. The functional role of AP-1 was investigated in hypoxia-

induced expression of VEGF by using dexamethasone as an inhibitor of AP-1 activity. Platelet-derived growth factor (PDGF) causes an increase in VEGF mRNA expression, which is strongly suppressed in the presence of dexamethasone, whereas hypoxia-induced VEGF expression is not inhibited by dexamethasone. It was also proved that intracellular calcium (Ca^{2+}) is required for the expression of hypoxia-inducible genes [25]. However, in contrast to hypoxia, the elevation of intracellular Ca^{2+} neither induces the HIF-1α protein nor stimulates HIF-1-dependent transcription. On the contrary, it increases levels of c-Jun protein, causing its phosphorylation. During hypoxia an increase in intracellular Ca^{2+} activates a HIF-1-independent signaling pathway that involves AP-1-dependent transcription, and the cooperation between the HIF-1 and AP-1 pathways allows fine regulation of gene expression during hypoxia.

Transcription factor Sp1

The transcription factor Sp1 plays a significant role in the constitutive and induced expression of a variety of genes and therefore contributes to the processes of tumorigenesis or the promotion of VEGF gene transcription by interacting directly and specifically with the protein kinase C zeta (PKC zeta) isoform [26]. The study of human pancreatic cancer cells revealed an elevated steady-state level of VEGF mRNA due to enhanced VEGF gene transcription and increased constitutive VEGF promoter activity that was preceded by the activation of transcription factor Sp1 [27]. Therefore it is clear that a constitutive Sp1 activation is essential for the differential overexpression of VEGF, which in turn plays an important role in angiogenesis and the progression of cancer. The expression of Sp1 also precedes the increased synthesis of bFGF, PDGF and VEGF during the healing of duodenal ulcers [28]. It was shown that Sp1 contributes to accelerated healing without any changes in HCl secretion. In the same way, genetic therapy using the VEGF and angiopoietin genes contributes to a similar healing of gastric ulcers [29].

Estrogens

Estrogens are hormones and transcription factors which increase the expression of vascular endothelial growth factor mRNA by binding to the specific estrogen response elements in the VEGF gene [30]. This regulatory effect is rapid, beginning within 1 hour after hormone treatment, dose dependent, and blocked by the pure antiestrogen ICI 182, 780.

The induction of the transcript is blocked by inhibitors of RNA, but not of protein synthesis. The estrogen response elements were identified in the VEGF gene, and the present findings indicate that estrogens regulate VEGF expression at the transcriptional level *via* the classical nuclear receptor pathway. Progestins also induce VEGF expression in the uterus, although the effect is less marked and slower in onset than estrogenic effects [30]. The effect of progestins is blocked by mifepristone (RU-486), suggesting that it is also mediated by a classical nuclear receptor pathway.

II. Agents that influence transcription factors

Nitric oxide (NO)

Nitric oxide is an intracellular and intercellular signaling molecule generated in eukariotic cells from L-arginine by a reaction catalyzed by NO synthase (NOS). A wide range of biological effects are attributed to this molecule. Some of these are linked to its intracellular second messenger nature, while others result from its paracrine actions, mediated by the activation of the guanylate cyclase. Analysis results indicate that VEGF gene transcription is activated by NO as well as by hypoxia *via* the HIF-1 binding site and an adjacent ancillary sequence within the HRE of this gene [5]. This response to NO is mediated, at least in part, by activation of the HIF-1 complex independent of the guanylate cyclase pathway. It was proved that both hypoxia and NO induce VEGF gene transcription in human glioblastoma and hepatoma cells as well as in the brain after subarachnoid hemorrhage [31]. The transcription factor HIF-1 plays a central role in hypoxic induction of the VEGF gene by binding to its target DNA sequence. NO-induced VEGF expression is also, at least in part, mediated by activation and subsequent binding of HIF-1. Therefore, NO and hypoxia may share common features in the pathways of VEGF induction.

The role of NO in angiogenesis is controversial. On one hand it was proved that nitric oxide donors inhibit angiogenesis in the chick chorioallantoic membrane, the growth and metastatic properties of the Lewis lung tumor in mice, and VEGF expression in the arterial wall in response to balloon angioplasty as well as in lung during acute and chronic hypoxia [11]. The nitric oxide donor sodium nitroprusside (SNP) suppresses hypoxia-induced VEGF expression as well as HIF-1 binding activity in a dose- and time-dependent manner. Therefore nitric oxide decreases the VEGF transcription induced by hypoxia as well as by conditions other than hypoxia *in vivo*. L-penicylamine (SNAP) downregulates VEGF expression by inhibiting PKC-induced AP-1 binding activity in smooth muscle cells. Nitric oxide also inhibits proliferation and migration of endothelial cells and is a negative regulator of VEGF receptors in angiogenesis. On the other hand, however, NO increases the angiogenic activity. The increased nitric oxide synthase (NOS) activity correlates positively with the increase of vascular density and tumor growth [32]. Colon tumor cells transfected with a NOS-encoding gene grow faster and are more vascularized than the parent cell lines *in vivo*. Both exogenous and endogenous NO increase angiogensis *in vivo* and the proliferation and migration of endothelial cells *in vitro*. Endothelial nitric oxide synthase (eNOS), unlike the inducible one (iNOS), plays a predominant role in VEGF-induced angiogenesis as well as vascular permeability, and the lack of eNOS contributes to the reduced angiogenic response to VEGF observed in eNOS-deficient mice [33]. Thus, selective modulation of eNOS activity could be a promising strategy for altering angiogenesis *in vivo*.

These contradictory data indicate that NO exerts inhibitory as well as stimulatory effects on VEGF gene

transcription and angiogenesis, depending on the redox state of the cellular environment and the types of cells in which assays are performed [5]. Nitric oxide is highly redox-sensitive, and this may explain its contradictory effects on HIF-1 activation in different cell types. Given the importance of HIF-1 in the genomic responses to hypoxic cells, analysis results indicate that there is a direct link between NO and the adaptation of normal and neoplastic cells and tissues to low oxygen tension. This helps to explain why NO donors can exert such diverse beneficial therapeutic actions, for example in cardiovascular diseases and in ischemic brains. On the other hand, the very strong correlation between NO production and tumor angiogenesis makes long-term treatments with pharmacological agents that potentiate NO very hazardous.

Interleukin 1β (Il-1β)

Interleukin 1β is a multipotent cytokine participating in a variety of cardiovascular diseases. Treatment of neonatal rat cardiac myocytes with Il-1(increases the levels of VEGF mRNA in a time- and concentration-dependent manner [34]. Analyses of site-specific mutations revealed that Il-1β activates VEGF promoter activity through two G+C-rich sequences located at –73 and –62. It was also shown that Sp1 and Sp3 proteins specifically bind to the G+C-rich sequences and the half-life of VEGF mRNA is significantly increased in cells treated with IL-1β. These results indicate that Il-1β induces VEGF gene expression at both the transcriptional and posttranscriptional levels and that IL-1β evokes p38 MAPK and JNK signaling, which in turn stimulate the transcription of the VEGF gene through Sp1-binding sites [34].

Transforming growth factor α (TGF-α)

Transforming growth factor α also induces the transcription of the VEGF gene [35]. TGF-α strongly induces VEGF synthesis in keratinocytes, and TGF-α-mediated VEGF expression plays a significant role in the initiation and maintenance of increased vascular hyperpermeability and hyperproliferation in skin. A G+C-rich TGF-α-responsive region between –88 bp and –65 bp of the VEGF promoter has been identified and is responsible for constitutive and TGF-α-inducible transcriptional activation. This region binds Sp1-dependent protein complexes constitutively and an additional TGF-α-inducible complex that is distinct from Sp1 protein [35]. Both transcription factors, Ap-2 and Egr-1, are components of this complex, and Ap-2 protein is functionally important in TGF-α-induced VEGF gene expression.

Transforming growth factor β (TGF-β)

Hypoxia and TGF-β cooperate in the induction of VEGF gene expression [36]. Signaling by TGF-β family members is mediated by Smad proteins that regulate gene transcription through functional cooperation and association with other DNA-binding proteins. This cooperation has been mapped on the VEGF promoter within the region from –1006 to –954, which contains functional DNA-binding sequences for HIF-1 and Smads. Optimal HIF-1α-dependent induction of the VEGF promoter is obtained in the presence of Smad 3, suggesting an interaction between these proteins. THG-α and hypoxia synergize in the regulation of VEGF gene expression at the transcriptional level.

Tumor necrosis factor α (TNF-α)

Tumor necrosis factor α increases cellular HIF-1α protein content but not HIF-1α mRNA [37]. The expression of HIF-1α as well as VEGF was examined in cells subjected to acute inflammation due to injury, and TNF-α exerted a significant regulatory effect on both iNOS and VEGF.

Lead (Pb)

Analyses of DNA expression indicate the existence of Pb-sensitive genes, and one of the most sensitive is the VEGF gene [38]. Lead induces VEGF mRNA 3-fold and VEGF protein approximately 2-fold after incubation with lead acetate. It also activates the transcriptional factor AP-1 and increases AP-1-dependent luciferase expression. Lead induces VEGF expression *via* a PKC/AP-1-dependent and HIF-1-independent signaling pathway [38].

Prostaglandins (PGs)

Prostaglandins increase the expression of the VEGF gene through the specific signaling pathway that is mediated by two distinct receptors: prostaglandin E-specific receptor (PGE) and peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a member of the nuclear receptor superfamily of ligand-dependent transcription factors. Prostaglandins especially strongly induce VEGF production by activated macrophages through specific PGE receptor and PPARγ-mediated processes and may thereby promote tumor growth [39].

Insulin

Both insulin and hypoxia are factors that induce HIF-1α/ARNT transcription complex and thereby stimulate VEGF gene expression [40]. Apart from insulin, insulin-like growth factors (IGF-1 and IGF-2) also possess the same ability and may therefore lead to increased secretion of growth factors by HIF-1α overexpression [41], which takes place in tumor cells as well as in diabetic retinopathy [42].

Oxidized LDLs

Vascular endothelial growth factor has a significant meaning in the development of atherosclerosis as it promotes macrophage migration, and its remarkably increased expression takes place in activated macrophages, endothelial cells, and smooth muscle cells within coronary atherosclerotic lesions [5,43]. Oxidized low-density lipoproteins (LDLs) are also abundantly present in atherosclerotic arterial walls. Recent analyses have revealed that peroxisome proliferator-activated receptor γ (PPARγ) is expressed not

only in adipocytes, but also in monocytes/macrophages, human acute monocytic leukemia cells, and human coronary artery endothelial cells (HCAECs). In all of these cells, Ox-LDLs upregulate VEGF secretion [43].

Retinoic acid (RA)

A significant increase in VEGF expression in human bronchioloalveolar carcinoma cells is induced by all trans-retinoic acid (at-RA) [44]. A series of site-directed mutation analyses indicate that the G+C-rich sequence located at -81 and -52 is required for at-RA and retinoic acid receptor alpha-mediated induction of the VEGF promoter. Major constituents of the nuclear factors binding to G+C-rich sequences are Sp1 and Sp3. An increase in transcription of the VEGF promoter by at-RA is mediated through the Sp1 site, and both new protein synthesis and tyrosine kinase activation are necessary for this induction.

Ultraviolet-A and -B radiation (UV-A and UV-B)

UV-A and UV-B radiation induce gene expression of epidermal keratinocytes by distinct molecular mechanisms. A keratinocyte-derived vascular endothelial growth factor not only provides the major cutaneous angiogenic activity, but may also augment the malignant phenotype of tumor cells and therefore contribute to photocarcinogenesis. Ultraviolet-A strongly and rapidly induced vascular endothelial growth factor mRNA expression in a fashion comparable to that seen with transforming growth factor alpha (TGF-() through a consensus element for activator protein-2 transcription factor (AP-2) [45]. In human glioblastoma cells, which are very malignant and show resistance to radiation therapy, induction of the VEGF gene leads to angiogenesis and brain edema. Several analyses also revealed that the promoter activity of the VEGF gene is enhanced by ionizing radiation, and also that the ERK1/2 pathway is involved in the up-regulation of VEGF expression ionizing radiation mediated by AP-1, which may lead to further neovascularization and proliferation of glioblastoma cells resistant to radiation therapy [46].

Angiotensin II

Angiotensin II (AII) induces VEGF mRNA production [47]. The induction is augmented by cycloheximide and blocked by actinomycin D. Losartan, an AT1 receptor antagonist, abolishes the induction of VEGF mRNA by AII, whereas PD 123319, an AT2 receptor antagonist, has no effect on VEGF mRNA induction. Angiotensin II stimulates largely the expression of two VEGF gene isoforms, $VEGF_{120}$ and $VEGF_{164}$. Analyses of the VEGF promoter activity show that it is increased 2.2-fold upon AII stimulation and reveals an enhanced binding of transcription factors AP-1 and NF-κB. The upregulation of VEGF by AII may play a significant role in AII-induced hyperpermeability, which may modulate its impact on blood vessels.

Organomercurial compound (Mersalyl)

Expression of hypoxia-inducible factor 1 (HIF-1) can also be induced by exposure of cells to divalent metals, such as $CoCl_2$, or iron chelators, such as desferrioxamine (DFO). The organomercurial compound mersalyl induces the expression of VEGF and enolase 1 mRNA as well as HIF-1 activity [48]. Expression of reporter genes containing hypoxia response elements (HREs) from the erythropoietin (Epo) and VEGF genes was also induced by mersalyl treatment. Several analyses indicate that mersalyl induces the expression of HIF-1 and a subset of hypoxia-inducible genes by a mechanism involving the insulin-like growth factor-1 (IGF-1) receptor and mitogen-activated protein kinase (MAP) activity, which is distinct from the mechanisms of induction by hypoxia, $CoCl_2$, or DFO.

Leukemia inhibitory factor (LIF)

Expression of the VEGF gene is stimulated by LIF and by cardiotrophin-1 [49]. Activation of glycoprotein (gp) 130 transduces hypertrophic and cytoprotective signals in cardiac myocytes. These signals through gp130 increase the expression of VEGF in cardiac myocytes *via* the signal transducer and activator of transcription (STAT) 3 pathway. After activation of gp130 with LIF or cardiotrophin-1, expression of VEGF mRNA rapidly increases. A STAT family protein functions as a regulator of angiogenic growth factors, and gp130/STAT signaling in cardiac myocytes may control vessel growth during cardiac remodeling.

Adrenergic regulation

Brown adipose tissue (BAT) produces high levels of vascular endothelial growth factor mRNA in response to exposure to cold, which may contribute to angiogenesis associated with cold-induced hyperplasia of tissue. Preadipocyte stimulation by norepinephrine increases the level of VEGF mRNA and has a significant meaning in cold-induced angiogenesis [50].

III. GENES THAT INFLUENCE VEGF GENE TRANSCRIPTION

Vascular endothelial growth factor transcription plays a significant role in tumor angiogenesis; its expression in the case of cancer inversely correlates with patient survival, and vascular density is an important prognostic factor in cancer [51,52]. Development of new blood vessels (angiogenesis) is a very strictly regulated physiological process that is controlled by specific genetic alternations in oncogenes and tumor suppressor gene expression as well as by the physiological microenvironment that surrounds the tumor. VEGF expression can be induced by exposure of tumor cells to hypoxia or growth factors, but in both cases this expression is mediated by hypoxia-inducible factor 1 (HIF-1). This activates the transcription of genes whose products are required for critical aspects of tumor progression, including angiogenesis (PAI-1 and VEGF), iron homeostasis (transferin and transferin receptor), and metabolic adaptation (glucose transporters and glycolitic enzymes), as well as several factors that affect tumor cell survival or proliferation (endothelin 1, inducible nitric oxide synthase, and insulin-like growth factor 2) [32].

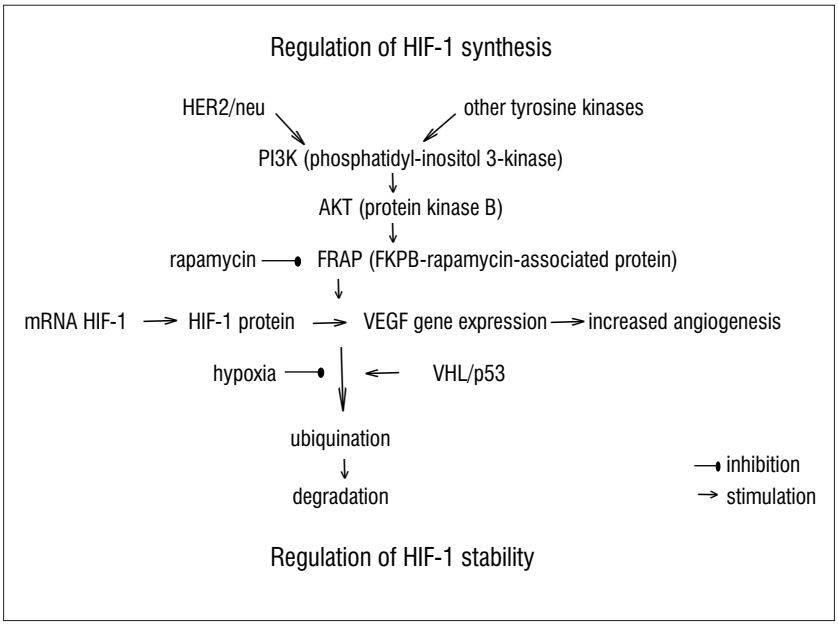

Figure 1. Regulation of HIF-1 synthesis and stability.

RA

The ERBB2 gene

Among the genetic alternations identified in human breast cancer, one of the most important is the increased activity of the HER2 receptor tyrosine kinase encoded by the ERBB2 gene on chromosome 17q21, which occurs in approximately one-third of breast tumors and is associated with increased tumor grade, chemotherapy resistance, and decreased rates of patient survival [32]. Treatment of breast cancer cells with a neutralizing antibody against HER2 results in both a dose-dependent inhibition of VEGF expression and tumor regression. The overexpression of HER2 is also associated with increased AKT (also known as protein kinase B) activity and, like heregulin stimulation, induces VEGF mRNA and protein expression in cancer cells [32]. Hypoxia and mutations in the tumor suppressor genes *VHL* and *p53* induce HIF-1 activity by inhibiting the ubiquitination and proteasomal degradation of HIF-1. HER2 signaling, in contrast, induces HIF-1α protein synthesis rather than inhibits its degradation (Figure 1) [32].

HER2 signaling provides increased resistance against apoptosis (induced by adverse conditions in the tumor microenvironment or chemotherapy) mediated by the PI3K-AKT pathway. HER2 signaling in nonhypoxic cells induces transcriptional activation of the VEGF gene by HIF-1 that is dependent upon PI3K, AKT and FRAP activity. Most surprising is the finding that activation of the PI3K-AKT-FRAP pathway by heregulin stimulation of MCF-7 human breast cancer cells does not affect HIF-1α stability, but instead dramatically increases the rate of HIF-1α protein synthesis. Previous studies have demonstrated that hypoxia and loss of p53 or VHL activity affect HIF-1α protein stability *via* altered ubiquitination. Whereas hypoxia increases both the stability of HIF-1α protein and its specific transcriptional activity, heregulin-HER2 signaling induces HIF-1α protein synthesis such that the combination of HER2 overexpression and hypoxia has a synergistic effect on VEGF gene expression. Data from cyclohexamide-addition experiments suggest that activation of the PI3K-AKT-FRAP pathway by other receptor and nonreceptor tyrosine kinases, including EGRF and VSRC, also induces HIF-1α protein synthesis. The PI3k-AKT-FRAP pathway may also be activated by physiological stimulation of normal cells, such as the induction of HIF-1α expression in vascular smooth muscle cells exposed to angiotensin II, platelet-derived growth factor BB (PDGF), or thrombin [53].

Recent studies indicate that a consequence of dysregulated expression of multiple tumor suppressor proteins and signal transduction pathways is an increase in HIF-1 transcriptional activity that occurs *via* three different molecular mechanisms:
– loss of p53 or VHL increases HIF-1α protein expression by interfering with its ubiquitination and proteasomal degradation [54];
– RAF/MEK/extracellular signal-regulated kinase signaling stimulates transcription of HIF-1-dependent target genes, but does not increase HIF-1α expression, suggesting a direct effect on transactivation [55];
– PI3K-AKT-FRAP signaling increases the rate of HIF-1α synthesis [32].

HER2 overexpression does not activate HIF-1-dependent gene transcription in isolation but rather in combination with other tumor-specific genetic and physiological alternations.

The von Hippel-Lindau tumor suppressor gene

The von Hippel-Lindau tumor suppressor gene has a critical role in the pathogenesis of clear-cell renal cell carcinoma (RCC), as VHL mutations have been found in both von Hippel-Lindau disease-associated and sporadic RCCs. The VHL gene product inhibits VEGF promoter

activity in a dose-dependent manner up to 10-fold [56]. The VHL-responsive element of the VEGF promoter is a G+C-rich 144bp region that specifically binds to the transcription factor Sp1. The VHL and Sp1 are part of the same complex and directly interact with each other [54]. Hemangioblastoma is the most frequent manifestation of the hereditary von Hippel-Lindau disease. It is a highly vascularized tumor of the central nervous system that upregulates the expression of the VEGF gene [57]. The overexpression of VEGF mRNA in stromal cells is highly correlated with elevated expression levels of HRF/HIF-2α mRNA, which suggests a role of HRF in VEGF-dependent vascular growth in hemangioblastomas and provides a link between transcriptional activation of the VEGF gene and loss of function of the VHL gene product.

V-SRC gene

Cells expressing the V-SRC oncogene have increased expression of HIF-1, VEGF and ENO1 under both hypoxic and normoxic conditions [58]. Expression of V-SRC is associated with increased transcription of reporter genes containing cis-acting hypoxia-response elements (HRE) from the VEGF and ENO1 genes, and this transcriptional activation requires an intact HIF-1 binding site. Tumor growth correlates with HIF-1 expression, which suggests the HIF-1 involvement in tumor progression, and the V-SRC oncogene induces HIF-1 expression in order to provide a mechanism for hypoxic adaptation by tumor cells.

E1A oncoprotein

Human adenovirus E1A oncoprotein activates or represses transcription from a variety of viral and cellular promoters by several complex mechanisms. It is essential for induction of cell cycle progression, cell immortalization, and neoplastic transformation [59]. E1A inhibits p53-mediated transactivation as well as represses the HIV-1 gene expression [60,61]. Hypoxic conditions lead to the formation of a DNA binding complex containing both HIF-1α and p300/CBP that are homologous transcription adapters targeted by the E1A oncoprotein [62]. They participate in numerous biological processes, including cell cycle arrest, differentiation, and transcription activation. Hypoxia-induced transcription from the Epo and VEGF promoter is specifically enhanced by ectopic p300 and inhibited by E1A binding to p300/CBP. Paradoxically, p300/ CBP is active in both transformation suppression and tumor development.

A constitutively active G protein-coupled receptor (GPCR)

A constitutively active G protein-coupled receptor (GPCR) encoded by the Kaposi's sarcoma-associated herpes virus (KSHV)/human herpes virus 8 is oncogenic and stimulates angiogenesis by increasing the secretion of VEGF, which is a key angiogenic stimulator and a critical mitogen for the development of Kaposi's sarcoma. The KSHV GPCR enhances the expression of VEGF by stimulating the activity of the transcription factor hypoxia-inducible factor (HIF-1α), which activates transcription from a hypoxia response element (HRE) within the 5'-flanking region of the VEGF promoter [63]. Moreover, specific inhibitors of the p38 (SKF86002) and MAPK (PD98059) pathways are associated with HIF-1α and are able to inhibit the activation of its activity as well as the VEGF expression and secretion in cells overexpressing this receptor.

Cbfa1/Runx2 gene

The Cbfa1 gene is a major regulator of bone development. It is essential for osteoblast and chondrocyte differentiation [64]. Cbfa1 belongs to a small family of transcription factors that show strong homology to the Drosophila gene *runt*. Mutations in Cbfa1 are responsible for the skeletal malformation syndrome cleidocranial dysplasia (CCD), which is characterized by multiple skeletal abnormalities including persistently open fontanelles, absent/hypoplastic clavicles, supernumerary teeth, short stature, and multiple other minor changes in skeletal growth and development [64,65]. Tissue-specific loss of VEGF expression is a result of lack or targeting of the Cbfa1/Runx2 gene. During endochondral bone formation, invasion of blood vessels into cartilage is associated with upregulation of VEGF in hypertrophic chondrocytes and increased expression of VEGF receptors in the perichondrium. The overexpression of Cbfa1 in fibroblasts induces an increase in their VEGF mRNA level and protein production by stimulating VEGF transcription, which proves that Cbfa1 is a necessary component of a tissue-specific genetic program that regulates VEGF during endochondral bone formation [66].

BCL gene

The bcl-2 oncogene contributes to apoptosis inhibition induced by a variety of stimuli, including hypoxia. Prostate carcinoma cells overexpressing bcl-2 oncogene, in both hypoxic and normoxic conditions, express significantly high levels of VEGF, which lead to the progression of tumor growth [67]. The impact of bcl-2 gene on VEGF expression suggests the existence of a factor (or factors) that is constitutively expressed in normoxic cells but is synthesized only temporarily in other cells in hypoxic conditions. The bcl-2 overexpression is associated with the development of androgen-independent prostate carcinomas as well as with increased resistance of such tumors to chemotherapy. One new therapeutic approach to treat prostate tumors is to inhibit their expression of bcl-2, which would restore the sensitivity of such tumors to apoptosis as well as limit their progression abilities by inhibition of angiogenesis, because the bcl-2 oncogene may contribute to tumor progression not only by the inhibition of apoptosis, but also by the induction of angiogenesis [67].

Gene expression is most effectively controlled by transcription factors, proteins that bind to cis-acting elements of DNA and guide the binding of polymerase II to start the transcription of specific mRNA. Such a strict and specific VEGF gene expression regulation has a basic and significant meaning in the maintenance of the physiologi-

cal balance between angiogenesis stimulation and suppression. However, uncontrolled angiogenesis is implicated in many serious dysfunctions and has a direct connection with disorders, such as proliferative retinopathy, age-related macular degeneration, psoriasis, and rheumatoid arthritis, and is crucial in the development of tumors. One of the new and promising therapies is the so-called therapeutic angiogenesis, which could be a rescue therapy for many patients affected by coronary or critical limb ischemia. Most effective in VEGF gene expression regulation seems to be gene therapy, because of the use of transcription factors in the process of angiogenesis modulation. Recent studies have demonstrated that zinc-finger protein (ZFP) transcription factors are able to stimulate therapeutic angiogenesis and VEGF expression. Specifically engineered transcription factors can regulate an endogenous gene expression *in vivo* and evoke potentially therapeutic effects in the whole organism [68].

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