

Roles of main pro- and anti-angiogenic factors in tumor angiogenesis

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Abstract

Tumor growth without size restriction depends on vascular supply. The ability of tumor to induce new blood-vessel formation has been a major focus of cancer research over the past decade. It is now known that members of the vascular endothelial growth factor and angiopoietin families, mainly secreted by tumor cells, induce tumor angiogenesis, whereas other endogenous angiogenic inhibitors, including thrombospondin-1 and angiostatin, keep tumor in dormancy. Experimental and clinical evidence has suggested that the process of tumor metastasis depends on angiogenesis or lymphangiogenesis. This article summarizes the recent research progress for some basic pro- or anti-angiogenic factors in tumor angiogenesis.

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INTRODUCTION

In the early time of the 20th century, Lewis observed that the vascular architecture depended on the tumor type, and proposed that the tumor environment determined the growth and morphological characteristics of tumor vessels. Hitherto it is the cognition that tumor vascularization is a vital process for the progression of all solid tumors from a small, localized focus to a large tumor with the capability to metastasize^[1,2]. This progression begins with the sprouting or intussusception from pre-existing host vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, also contribute to tumor angiogenesis^[3,4].

Contrarily, adult normal tissue vasculature is quiescent because of the balance between angiogenic promoters and inhibitors. The early development of tumor is dormant because of the same reason. Break of the balance results in physiological angiogenesis such as wound healing and pathological angiogenesis such as tumor angiogenesis.

Tumor-induced angiogenesis is thought to depend on the production of pro-angiogenic growth factors by the tumor cells, which affect the existing vessels^[5]. This turns on the tumor "angiogenic switch", and then promotes tumor growth and metastases.

ANGIOGENIC FACTORS

Vascular endothelial growth factors and vascular endothelial growth factor receptors

In 1983, Senger and his colleagues discovered a protein that induced vascular leakage and named it as tumor vascular permeability factor (VPF)^[6]. Subsequently Ferrara and Connolly showed that VPF and vascular endothelial growth factor (VEGF) were the same molecule^[7,8]. In 1992, Breier and Risau found that the expression of *VEGF* mRNA temporally and spatially correlated with blood-vessel growth in the developing mouse embryo. This supported the idea that VEGF was an angiogenic factor *in vivo*^[9]. Later Veikkola and Alitalo also demonstrated that inactivation of one of the two VEGF alleles in the mouse embryos led to severe defects in vascular formation, ultimately resulted in embryonic lethality, whereas increased expression of VEGF seemed to be essential for tumor growth beyond size restriction^[10]. *In-situ* hybridization studies have identified that the expression of *VEGF* mRNA is increased in renal^[11], ovarian^[12], gastric carcinomas^[13], and particularly the highly vascular and aggressive glioblastoma^[14]. VEGF is considered as a prognostic molecular marker in hepatocellular carcinoma^[15]. *VEGF* gene is localized on the short arm of chromosome 6^[16], with eight exons and seven introns^[17]. Predominant isoforms of *VEGF* are *VEGF*₁₈₉, *VEGF*₁₆₅, *VEGF*₁₂₁, and *VEGF*₂₀₅. They are generated by alternative mRNA splicing and proteolytic processing. *VEGF*₁₂₁ differs from all the other higher molecular weight species by the absence of the heparin-binding domain encoded by exon 7 that can bind to neuropilin-1 (NP-1)^[18,19]. Therefore, VEGF₁₂₁-diphtheria toxin (DT385) conjugation treatment completely prevents tumor angiogenesis *in vivo* without toxicity to NP-1^[20]. VEGF is a major inducer of angiogenesis and structurally resembles (placenta growth factor), VEGF-B, VEGF-C, VEGF-D and the Orf virus derived VEGF (also called VEGF-E)^[21-26].

Interestingly, VEGF is highly expressed in most ischemic areas of the tumor, indicating that VEGF expression might be induced by hypoxia. In hypoxic tissue the hypoxia inducible factor-1 (HIF-1) has been proven to play a central role in inducing the transcription of genes that are involved in glycolysis and angiogenesis, including VEGF^[27]. Subsequent studies have shown that the mechanisms of hypoxic regulation of VEGF and erythropoietin are similar^[28]. *VEGF* gene contains two hypoxia-regulated element (HRE) in the 5' and 3' regions (Figure 1). HIF-1 recognizes and binds HRE^[29,30]. Hypoxia also induces an RNA-binding protein, HuR, which can bind to the 3' untranslated region (UTR) of *VEGF* mRNA and increase the mRNA stability^[31]. Oncogenes including members of the *ras* and *erbB* families also up-regulate the expression of VEGF^[32,33]. The products of *ras* gene activate the HIF-1 α and promote the stability of the HIF-1 α via Raf/MEK/MAPK and PI3K/PDK-1/AKT pathways^[34]. The activated AKT increases the phosphorylation of GSK3 β on Ser9, and then enhances HIF-1 α stabilization, since unphosphorylated GSK3 β inhibits HIF-1 α accumulation^[35] (Figure 1). Recent studies have indicated that the chronic myelogenous leukemia (CML)-associated oncogene *BCR/ABL* induces VEGF expression in growth factor-dependent Ba/F3 cells by promoting the expression of HIF-1^[36]. However, the product of the von

Hippel-Lindau tumor suppressor gene (vHL) inhibits the expression of VEGF^[37,38]. The ubiquitin ligase E3 complex containing the vHL tumor suppressor protein increases the HIF-1 α degradation^[39]. In addition to vHL, other tumor suppressor proteins such as p53 also inhibit the transcriptional activity of HIF-1 by binding to the HIF-1/P300 complex and then promoting Mdm2-mediated ubiquitination and proteasomal degradation of the HIF-1 α , which ultimately results in the decrease of VEGF expression^[40,41]. These findings indicate that HIF-1 is critical for the regulation of VEGF expression. VEGF expression is also regulated by estradiol in human breast cancer cells^[42]. Similarly, 17 beta-estradiol also enhances VEGF expression via the regulation of adenylate cyclase in differentiated THP-1 cells^[43].

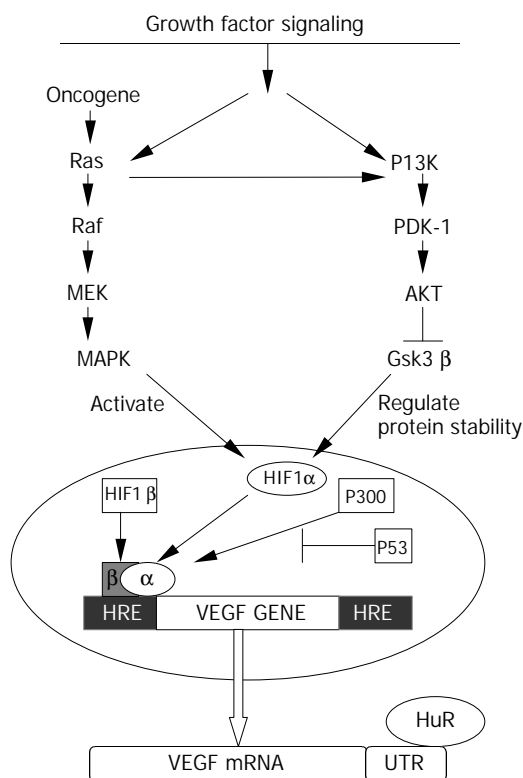


Figure 1 The regulation of the VEGF expression. HIF1 plays central roles in this regulation.

VEGFs induce angiogenesis through the binding to their relevant receptors that are mainly expressed in endothelial cells. The first VEGF receptor was identified to be Flt1 (FMS-like-tyrosine kinase), also known as VEGFR1^[44]. Subsequently, the other highly specific VEGF-binding receptors (KDR, Flk1, or VEGFR2) were discovered^[45,46]. Scientists investigated the function of VEGFR1 and VEGFR2 in endothelial cells via Flt1 or Flk1 mutant endothelial cells or an embryonic model. They counted the number of mitotic endothelial cells in embryo to confirm the effect of proliferation of endothelial cells. In VEGFR1 knockout mice, the reason causing early embryonic lethality was not the absence of vascularisation but the disorganized embryonic and extra-embryonic vasculature^[47]. This suggests that Flt1 signal pathways mainly regulate normal endothelial cell-cell or cell-matrix interactions during the process of vascular development. The ability of VEGFR1 to bind to VEGF is 10 times stronger than VEGFR2, but the activity of tyrosine kinase of VEGFR1 is weaker than that of VEGFR2. A study demonstrated that Flt1 null mutation resulted in early embryonic death, but Flt1 lacking tyrosine kinase domain homozygous mice developed normal vessels and survive^[48]. These observations indicate that Flt1 without

tyrosine kinase domain is sufficient to promote embryonic normal angiogenesis. The effects of VEGFR2 in endothelial cells, such as cell survival and proliferation, are not induced by treatment with VEGFR1 specific ligands or in the VEGFR1 overexpressing cells lacking VEGFR2^[49,50]. These data suggest that VEGFR2 plays the central role in VEGF signal transduction in endothelial cells. VEGFR1 is likely to be the antagonist of VEGFR2, acting as a negative regulator of angiogenesis via sequestering VEGF and thus blocking the VEGF signal transduction mediated by VEGFR2. Recent studies have confirmed this conclusion. The *Flt1* gene encodes not only the full-length Flt-1, but also a soluble short form of Flt-1, designated sFlt1. In placenta, sFlt-1 is abundant in the trophoblast layer during pregnancy, suggesting that it is a negative regulator to excess angiogenesis at the fetomaternal border in mammals^[51]. Subsequently Kearney confirmed that Flt-1 normally modulated vascular growth by controlling the rate of endothelial cell division *in vitro* and *in vivo*^[52]. The downstream signaling events of VEGF signal transduction pathway via VEGFR2 includes receptor dimerization, transphosphorylation of intracellular VEGFR2 tyrosine residues (Tyr951/996 and Tyr1054/1059)^[53], the activation of protein kinase C, and then the activation of the MEK/MAPK pathway to promote endothelial cell proliferation (Figure 2). However, Meyer's data showed that phosphorylation of tyrosine 1212 of VEGFR2 was also necessary for VEGFR2-mediated angiogenesis^[54]. In addition to VEGF, scientists have found that there are other molecules that activate VEGFR2 or enhance the expression of VEGFR2. Recent studies have indicated that tumor necrosis factor (TNF) has anti-tumor activity by down-regulating Flk-1 expression in tumor endothelial cells^[55]. Moreover, in cardiac capillary endothelial cells, Bradykinin (BK) also stimulates angiogenesis via the activation of the B2-type receptor, which leads to the tyrosine phosphorylation and dimerization of Flk-1 as does VEGF itself^[56]. This implies a novel mechanism by which a G protein-coupled receptor activates a receptor tyrosine kinase to generate biological response. In addition to the stimulation of endothelial cell migration and proliferation, VEGF also elevates the survival capacity of endothelial cells *in vitro*. The underlying mechanism is that VEGF activates VEGFR2, which further activates phosphatidylinositol 3-kinase (PI3-K) and Akt^[57]. VEGF also prolongs the survival of human dermal microvascular endothelial cells (HDMECs) *in vitro* by inducing expression of the anti-apoptotic protein Bcl-2^[58].

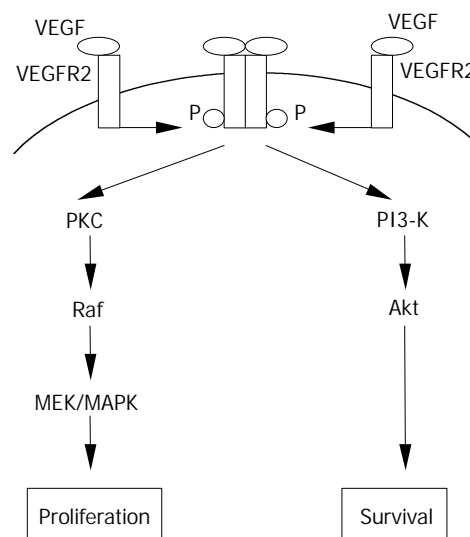


Figure 2 The intracellular downstream signaling of VEGF signal transduction via VEGFR2.

Interestingly, new data have revealed that NP-1, originally found to control the neuronal axon guidance, also enhances the mitogenic effects of VEGFR2 signaling in endothelial cells^[59]. So far, the potential role of NP-1 in pathologic angiogenesis remains unknown. Some experiments have indicated that VEGF up-regulates NP-1 via VEGFR2-dependent pathway. Endothelial proliferation stimulated by VEGF₁₆₅ is inhibited significantly by antibody neutralization of NP-1. In a murine model of VEGF-dependent angioproliferative retinopathy, intense NP1 mRNA expression was observed in the newly formed vessels^[60]. In breast carcinoma cells neuropilin expression also enhances VEGF-inducing cells survival signal^[61]. Furthermore, in rat uterus the neuropilins (NP-1 and NP-2) might participate in hormonally regulated changes occurring throughout the female reproductive cycle^[62].

The other factor VEGF-C and its receptor (VEGFR3) are essential to the lymphangiogenesis and play a direct role in the metastatic dissemination of tumor cells. It is known that VEGFR3 is mainly expressed in lymphatic endothelium during development^[63,64]. Later in embryonic development, VEGFR3 is expressed in lymphatic cells, and further induces lymphangiogenesis^[63,64]. However, in early embryonic development without lymphatic vessels, the embryos lacking VEGFR3 gene usually die due to cardiovascular failure^[65]. These data indicate that VEGFR3 is necessary in the formation of the primary cardiovascular network. In addition, VEGFR3 is also expressed in the tumor endothelial cells, which helps tumor neovascularization^[66].

Angiopoietins and Tie receptors

The second family of growth factors that specifically act on the vascular endothelium has been identified, namely the angiopoietins^[67,68]. To date, there are four members of this family, mainly including Ang-1 and Ang-2, which both only bind to the Tie-2 receptor and control the blood vessel stabilization signals^[67,68]. The results of early experiments showed that the mouse embryonic development lacking Ang-1 or Tie-2 died in the later stage of angiogenesis because the remodeling and stabilization of the primitive vasculature induced by VEGF was severely perturbed^[69-71]. Similarly, the increased Ang-1 expression in human cervical cancer HeLa cells by transgene promotes the growth of human cervical cancers in mice via promoting tumor angiogenesis^[72]. In addition to in the active angiogenesis tissue, Ang-1 seems to be widely expressed in the normal adult tissues^[73], indicating

that Ang-1 may act on the stabilization of existing vessels. The underlying mechanism is thought to modulate the interaction between endothelial cells and surrounding support cells, such as smooth muscle cells^[71,74]. Ang-2 was cloned in 1997. It is thought to be a natural antagonist for the Ang-1/Tie-2 interaction now. The transgenic overexpression of Ang-2 in embryo results in early embryonic lethality, similar to the phenomenon in embryonic development without Ang-1 or Tie-2^[68].

The relation of VEGF and angiopoietins in angiogenesis has become a research hotspot (Figure 3). In the onset of cerebral ischemia, the increase of VEGF mRNA goes with the decrease of Ang-1 mRNA. Later increased expression of VEGF/VEGFRs and Ang-1/Tie2 is detected^[75]. This data show that Ang-1 works at the later stage of angiogenesis. Other experiments directly demonstrate the above mentioned role of VEGF and Ang-1. The transgenic overexpression of VEGF in the skin of mice induces the formation of numerous leaky blood vessels. In contrast, overexpression of Ang-1 leads to an enlargement of dermal vessels with less leaking, instead of an increase in the number of vessels. If both VEGF and Ang-1 are overexpressed, the size and number of skin vessels are both increased. Furthermore, these vessels are not leaky. Remarkably, it is found that Ang-1 also reduces VEGF-stimulated inflammation by suppressing expression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)^[76] and E-selectin^[77]. The expression of Ang-2 is not broader than that of Ang-1. Holash *et al* demonstrated that endothelial cells apoptosis occurred prior to the wide spread loss of tumor cells in the necrotic tumor core^[78]. This implies that there is a mechanism to induce this process. Ang-2 promotes endothelial cell death and vessel regression if the activity of VEGF is inhibited. By contrast, Ang-2 promotes a rapid increase in capillary diameter and stimulates sprouting of new blood vessels in the presence of endogenous VEGF *in vivo*^[79]. These results indicate that the pre-effect of existing blood vessels by Ang-2 is in favor of the step of VEGF-inducing angiogenesis. Thereby, in the regulation of angiogenesis, VEGF can convert the role of Ang-2 from an anti- angiogenic factor to a pro-angiogenic factor. Other data further confirm that Ang-2 expression precedes that of VEGF in the early stage of tumor angiogenesis^[80].

Altogether, VEGF/VEGFRs and angiopoietins/Ties co-regulate the tumor vessels regression, growth and maturation.

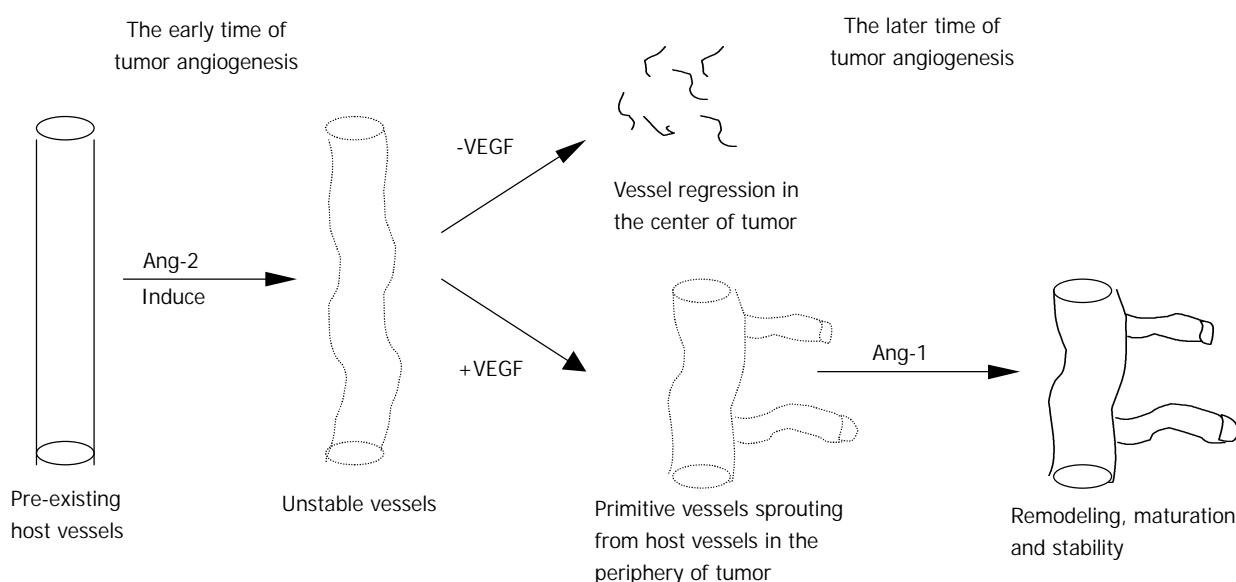


Figure 3 The interaction relation between VEGF and angiopoietins in the development of tumor angiogenesis.

Expression of Ang-2 without VEGF induces vessels regression in core of a necrotic tumor. Then, hypoxia induces tumor cells to express VEGF. The angiogenic properties of VEGF may be easier to act on the vessels, which are destabilized by Ang-2. Newly formed tumor vessels are abnormal, including highly disorganization, poor differentiation, tortuousness and excessive branch, because continuous expression of Ang-2 in newly tumor vessels inhibits the maturation and stabilization signals via Ang-1. In the later development of tumor angiogenesis, the increasing expression of Ang-1 promotes the maturation of neovascularization. In fact, hyper-vascular hepatomas with aberrant vasculature show high levels of Ang-2 expression in the endothelium.

Other angiogenic factors

The mechanism of tumor angiogenesis is complicated. It involves many factors and some different signal pathways. Therefore, if only one signal molecule (for example VEGF) is blocked, tumor may switch to another molecule (for example bFGF) for the induction of angiogenesis. The basic fibroblast growth factor (bFGF, also known as FGF-2) isolated from a chondrosarcoma was identified to be the first pro-angiogenic factor^[81]. bFGF is the main member of FGF family which is a structurally homologous protein family, including at least 20 members. FGF has a high affinity to heparin sulfate proteoglycans (HSPGs), which locates on the surface of most cells and within the extracellular matrix^[82].

Four receptor tyrosine kinases (known as FGFR-1,-2,-3,-4) can interact with FGF. The FGF/FGFRs signal pathway may play a crucial role in angiogenesis. The FGFR-1 mutant mouse embryos exhibited abnormal embryonic and extra-embryonic vascularization, suggesting that FGF/FGFR-1 plays a role in the development and maintenance of a matured vascular network in the embryo^[83]. The mechanisms for VEGF- and FGF-mediated angiogenesis are not the same. In astrocytoma, bFGF and VEGF expression levels positively correlated with tumor growth and angiogenesis. However, bFGF and VEGF expression levels were significantly different in various grades of astrocytoma^[84]. In the culture of embryonic stem cell-derived vascular precursors *in vitro*, bFGF and VEGF both exhibited effects on the survival of angioblasts. However, VEGF induced the formation of primitive endothelial tubes, whereas bFGF did not^[85]. This finding suggests that VEGF but not bFGF plays a major role in angiogenesis. The mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 suppresses FGF-induced angiogenesis, indicating that the Ras-MEK-MAPK pathway is important for the biological effect of FGF^[86]. Subsequently, Cross *et al* found that the Shb adaptor protein binding to Tyr766 in the FGFR-1 promoted FRS2 phosphorylation, and further regulated the Ras-MEK-MAPK pathway^[87]. FGFR-1-mediated intracellular signal cascades include the Ras pathway, Src family tyrosine kinases, phosphoinositide 3-kinase (PI3K) and the PLC pathway^[88]. Some members of the FGF family (such as FGF-3) promote oncogenesis, and are identified as proto-oncogenes^[89].

ANTI-ANGIOGENIC FACTORS

In humans or vertebrates, normal vascularization is quiescent, suggesting that *in vivo* humans or vertebrates should have anti-angiogenic factors against angiogenic factors. Recently, experiments *in vitro* or *in vivo* confirm the presence of endogenous anti-angiogenic factors.

The primary anti-angiogenic factors include thrombospondins (TSP), angiostatin and endostatin. Now the TSP family includes five members, known as TSP-1, -2, -3, -4 and TSP-5/COMP. They play multiple functions via binding to matrix proteins, plasma proteins and cytokines^[90-93]. Some

reports have shown that TSP-1 and TSP-2 have anti-angiogenic properties. TSP-1 inhibits angiogenesis via a direct way that suppresses migration and induces apoptosis of endothelial cells or via an indirect way that inhibits the mobilization of pro-angiogenic factors and blocks their access to co-receptors on the endothelial cell surface^[94]. TSP-2 has the similar functions^[95]. The signal pathway of TSP includes TSP/CD36 receptor, cytoplasmic tyrosine kinase p59^{lyn}, caspase-3-like proteases and p38 mitogen-activated protein kinase (MAPK), and results in apoptosis of endothelial cells^[94,96,97]. It has been proven that TSP is an anti-angiogenic factor or acts as prognostic predictor in pancreatic ductal carcinoma^[98], bladder cancer^[99], non-small cell lung cancer^[100,101], breast cancer^[102], cervical cancer^[103] and colon cancer^[104]. TSP expression is regulated by several factors. Hypoxia increases TSP-1 expression in endothelial cells by promoting the post-transcriptional stabilization of the *TSP-1* mRNA^[105]. This property does not match with its anti-angiogenic property. Contrarily, *in vitro* hypoxia suppresses TSP-1 expression and induces VEGF expression in rodent cells, regardless of the p53 genotype^[106]. In cultured fibroblasts, loss of the wild-type allele of the p53 results in reduced expression of TSP-1. Transfection assays have revealed that p53 stimulates the expression of the endogenous *TSP-1* gene and positively regulate *TSP-1* gene promoter sequences^[107,108]. A novel p53-inducible gene, named *BAI1*, was found in 1997^[109]. *BAI1* contains five TSP-type 1 repeats, and significantly suppresses angiogenesis. Some oncogenes have been also found to regulate TSP1 expression, including *c-jun*^[110] and *H-ras*^[106]. These data indicate that oncogenes or loss of tumor suppressor genes also regulate TSP expression to turn on the angiogenic switch and promote tumor growth.

A number of experimental studies have demonstrated that a primary tumor inhibits a secondary distant tumor^[111,112], indicating that there are some inhibitors from the primary tumor to inhibit the growth of metastases. Endostatin is a circulatory inhibitor, and identified to be an anti-angiogenic factor^[113]. It is a 20 kD C-terminal fragment of collagen XVIII. Treatment with endostatin by transgene inhibits angiogenesis and liver tumor growth^[114]. In other similar diseases such as rheumatoid arthritis, treatment with endostatin showed beneficial effects^[115]. Interestingly, endostatin immunostaining was stronger in area accompanied with blood vessel maturation. Endostatin expression was significantly prominent in vessels of tumor marginal zone where angiogenesis was highly active. These observations suggest that endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes in angiogenic active region in tumors^[116]. Intracellular signaling mechanisms by endostatin remain poorly understood. Jiang *et al* found that endostatin increases intracellular Ca²⁺ concentration approximately three-fold, indicating that endostatin induces intracellular Ca²⁺ signal^[117]. Endostatin also modulates the Wnt signaling by regulating β -catenin stability via a novel glycogen synthase kinase 3(GSK3)-independent mechanism^[118].

Angiostatin is another circulatory anti-angiogenic factor. It was isolated from a lung carcinoma cell line and is particularly effective in suppressing metastatic growth when the primary tumor is removed^[119]. Angiostatin is a 38 kD fragment of plasminogen. Treatment with angiostatin inhibits angiogenesis in a murine breast cancer model^[120]. Now it is found that angiostatin plays a crucial role in inflammation via directly inhibiting neutrophil migration and neutrophil-mediated angiogenesis^[121]. Recent studies have shown that angiostatin has structural similarities to hepatocyte growth factor (HGF), which induces proliferation and migration of both endothelial cells and smooth muscle cells via its cell surface receptor, c-met. Therefore, the disruption of HGF/c-met

signaling is a novel mechanism for the anti-angiogenic effect of angiostatin^[122]. Experiments by Mauceri *et al* indicated that TNF- α enhances the production of angiostatin in tumor cells by inducing the activity of the plasminogen activator and the release of MMP-9 by tumor cells^[123].

CONCLUSIONS

Tumor angiogenesis plays crucial roles in tumor growth and metastases. Tumor angiogenesis is a complex process involving interactions of many molecules and several different or similar signal pathways. Despite the rapid research progress in the field, there are many mysteries regarding the molecular mechanism of tumor angiogenesis. Once we fully understand the precise functions of these pro- and anti-angiogenic factors in tumor angiogenesis, clinical application of those novel research results for tumor therapy would become practical.

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