Commentary

Is Angiopoietin-2 Necessary for the Initiation of Tumor Angiogenesis?

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Almost all functional cells are located within 30 μm of a blood capillary. Acute changes in blood flow are regulated in response to tissue needs by changes in the constriction level of blood vessels,¹ whereas long-term regulation of tissue perfusion is achieved by growth of new blood vessels or by vascular regression.² Physiological angiogenesis is limited to wound healing and changes in female reproductive organs during the menstrual cycle and pregnancy but blood vessels maintain their ability to grow and regress throughout life. Changes in the vasculature occur in association with many pathological processes, such as ocular neovascularization, inflammatory diseases, and cancer.³ The concept that solid tumor growth depends on angiogenesis is well established.⁴ Indeed, tumor size is restricted to a few cubic millimeters if it is not able to attract new blood vessels.

The primitive embryonic vasculature is laid down by vasculogenesis,⁵ which involves *in situ* differentiation of endothelial cells (ECs) from mesodermal precursors and their organization into a primary vascular plexus. In adults, all new blood vessels appear to be formed by angiogenesis, which is based on sprouting of blood vessels from existing ones or on intussusceptive growth involving *in situ* remodeling of the vessels by protruding interstitial tissue columns. In embryos, some developing organs including the brain and kidneys are vascularized by angiogenesis. The initiation of blood vessel growth involves focal reduction of intercellular interactions and interactions between the cells of the blood vessel and the surrounding extracellular matrix (ECM). This is associated with a loss of pericytes (PCs) and possibly of smooth muscle cells (SMCs) from the existing vessels.² Many angiogenic factors have been shown to be mitogenic and chemoattractive for ECs. The ECs have been shown to distort malleable substrata in a process called traction^{6,7} and the reorganized ECM may facilitate the formation of complex weblike EC structures. Formation of functional blood vessels requires remodeling of this EC meshwork. Initiation of blood flow enables adaptation to changing

blood and oxygen pressure conditions and further remodeling of the vascular network.⁸ The maturation of newly formed vessels involves the accumulation of a basal lamina and tightly associated PCs or SMCs on the abluminal side. Although many phases of vessel growth overlap, this classification shows that complex orchestration is required in order for angiogenesis to proceed.

Numerous substances can trigger the angiogenic process by causing a reprogramming of cells in the blood vessels⁹ and these responses are beginning to be elucidated. In this issue of *The American Journal of Pathology*, Stratmann et al¹⁰ report their novel finding that the angiopoietin-2 (Ang-2) signaling molecule is up-regulated in a spotlike fashion in the endothelium of growing blood vessels in glioblastoma. The authors also show that angiopoietin-1 (Ang-1), a related signaling molecule, is secreted from tumor cells and that Tie-2, a receptor for both Ang-1 and Ang-2, is up-regulated in the endothelium of vessels undergoing angiogenesis.

Endothelial-Specific Receptor Tyrosine Kinases

Thus far two growth factor receptor tyrosine kinase (RTK) subfamilies, which are mainly expressed in ECs have been found. They seem to control major aspects of blood vessel growth.^{11,12} The vascular endothelial growth factor (VEGF) receptor family consists of three members, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). At least five endogenous ligands (VEGF, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor or PlGF) bind to one or two of these receptors.¹³ VEGF and its major mitogenic receptor, VEGFR-2, are crucial for embryonic vasculogenesis and angiogenesis.¹⁴ VEGF has been shown to be up-regulated in various types of tumors and inhibition of its signaling shows promise as a way to control angiogenesis in cancer therapy.¹⁵ The fact that

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deletion of one allele of the VEGF gene causes embryonic death indicates the importance of VEGF regulation in vascular development.^{16,17} In addition to their crucial role in blood vessel growth, some members of these families are also involved in the growth of lymphatic vessels and the regulation of vascular permeability.

Another family of EC-specific RTKs consists of two members, Tie-1 (tyrosine kinase with immunoglobulin (Ig) and epidermal growth factor homology domains) and Tie-2 (also called Tek), which have been cloned from human, mouse, rat, and zebrafish sources.^{18–28} Tie-2 has at least three known ligands, Ang-1, Ang-2, and an as yet less characterized Ang-4/Ang-3 (Dr. George Yancopoulos, personal communication). Tie-1 is still an orphan receptor.

Structure and Expression Pattern of Tie-1, Tie-2, Ang-1, and Ang-2

The functions of the different extracellular domains of Tie-1 and Tie-2 have not been clarified. Immunoglobulin homology domains are found in many RTKs and they are typically involved in ligand binding and receptor dimerization. Epidermal growth factor and fibronectin type III homology domains are found in other situations to mediate protein-protein interactions involved in cell adhesion. Both Ang-1 and Ang-2 consist of an amino-terminal coiled-coil domain and a carboxy-terminal fibrinogen-like domain.^{29,30} The coiled-coil structure is commonly involved in multimeric intermolecular interactions.³¹ One out of nine cysteine residues present in Ang-1 is absent from Ang-2. This residue maps to a region between the coiled-coil and fibrinogen-like domains.³⁰ For technical reasons an altered form of Ang-1, denoted as Ang1*, has been used in many assays of Ang-1 activity. In Ang1*, the nonconserved cysteine residue has been mutated to the corresponding serine residue of Ang-2 and the first 77 residues of human Ang-1 have been replaced with the first 73 residues of Ang-2.³²

Ang-1 is known to form hexamers (Dr. George Yancopoulos, personal communication). Interestingly, tenascin-C, an extracellular protein with poorly characterized biological activities, shares sequence homology with the angiopoietins and has been shown to form hexamers.³³ Tenascin-C is a chemoattractant for SMCs and the part responsible for this activity was mapped to a 32-amino acid-long part of the fibrinogen-like domain,³⁴ a homologous sequence also present in the angiopoietins. An intriguing possibility is that Ang-1 and Ang-2 can form heteromers, which would allow a complex pattern of receptor regulation.

Tie-1 and Tie-2 are restricted mainly to ECs and their precursors^{23,35} but they are also expressed in hematopoietic progenitors and differentiating megakaryoblasts.^{18–21,24,28,36,37,66} Tie-1 is down-regulated after the fetal period in ECs of many organs, but enhanced expression is commonly found during neovascularization.³⁸ Increased expression was reported in metastatic melanomas,³⁹ breast cancers,⁴⁰ and malignant gliomas.⁴¹ Tie-1 mRNA and protein levels are also increased in ECs

of arteriovenous malformations,⁴² although they have not been analyzed in venous malformations caused by an activating point mutation of the Tie-2 tyrosine kinase domain,⁴³ in von Hippel-Lindau disease, or in the endothelium of hereditary hemorrhagic telangiectasia patients. Tie-2 is also expressed in the quiescent vasculature and up-regulated in angiogenic capillaries in breast cancer⁴⁴ and in gliomas.¹⁰ Interestingly, tyrosyl phosphorylation on Tie-2 can also be detected in the normal vasculature,⁴⁵ suggesting that Tie-2 signals have an active role in the maintenance of blood vessels.

Of the Tie-2 ligands, Ang-1 is expressed first in the developing heart, but later the expression pattern includes mesenchymal cells and SMCs surrounding blood vessels.²⁹ In midgestational mouse embryos, Ang-2 transcripts were seen in the SMC layer surrounding some vessels, such as the dorsal aorta, but the expression pattern was reported to be more punctate than for Ang-1.³⁰ Initially, Ang-2 expression in adults was thought to be restricted to sites of vascular remodeling, ie, the placenta, ovaries, and uterus.³⁰ More detailed analysis showed strong Ang-2 expression at the forefront of invading vessels in the developing corpus luteum. In aged corpus luteum with regressing vessels large amounts of Ang-2 mRNA were detected, possibly after the switch-off of VEGF expression. In addition, Ang-2 mRNA was observed in quiescent vessels and also in several EC lines *in vitro*.⁴⁶ Constitutively high Ang-2 expression was also seen in a cell line of Wilms' tumor origin³⁶ (JL, unpublished results).

Lessons from Phenotypes of Genetically Altered Mice

Embryos deficient in Tie-1 or Tie-2 have been generated independently by two groups^{47–49} and reanalysis has been carried out at an ultrastructural level.⁵⁰ Embryos homozygous for a disrupted Tie-1 allele died between embryonic day (E)13.5 and birth as a result of loss of vascular integrity, whereas the Tie-2-null mice died between E9.5 and E10.5.^{47,49} The ECs of Tie-1^{–/–} embryos seemed to be “electron light” because of numerous intracellular and transcellular holes.⁵⁰ Plasma and blood cells extravasated through the ECs due to altered internal structure of the ECs, resulting in edema and hemorrhage. The structure of heart endocardium was immature and abnormal. The vascular density was increased even though the total number of ECs was unaltered. Analysis of mice chimeric for Tie-1-null cells revealed that there is a continuous selection against ECs lacking Tie-1 and that this selection is especially strong in those parts of the vasculature formed by sprouting angiogenesis during the embryonic period.⁵¹

Tie-2^{–/–} mice failed to form extensive branches in cardiac vessels and they lacked capillary invasion to the neuroectoderm, which occurs by sprouting angiogenesis.⁴⁹ The endothelium of the dorsal aorta, which is laid down by vasculogenesis, was disorganized, and the endocardium was weakly associated with the myocardium.⁴⁷ Heart defects seen in the Tie-2-null embryos could

depend on decreased heparin-binding epidermal growth factor (HB-EGF) or neuregulin secretion by the ECs.^{70,79} The ECs seemed rounded, maybe because of defective contacts with mesenchymal cells.⁵⁰ The blood vessels also appeared to be more uniformly sized and the dead-end vessel structures were more frequent than normal. Interestingly, Dumont et al⁴⁷ reported that in Tie-2-null embryos the number of ECs was reduced. Because Tie-2 is required for embryonic viability, it is difficult to tell whether all of the observed phenotypes reflect primary functions of Tie-2. Interestingly, disruption of rasGAP, which is known to be a downstream signaling molecule for Tie-2,⁷⁸ produced phenotypes with many similarities to Tie-2-null mice.⁵² Recent results from crosses of Tie-1 and Tie-2 mutant mice suggest that Tie-1 and Tie-2 have largely overlapping functions in embryonic development (Mira Puri, Dr. Allan Bernstein, and Dr. Juha Partanen, personal communication).

Targeted disruption of the Ang-1 gene resulted in embryonic death at E12.5.⁵³ The defects were largely similar but somewhat milder than in the Tie-2-null embryos, consistent with the role of Ang-1 as the major activating ligand for Tie-2. The structure of the endocardium in Ang-1^{-/-} embryos was less intricately folded and there were defects in trabecular formation. The blood vessels were more uniformly sized and the number of large vessels was reduced. Interestingly, the already formed inter-somitic vessels seemed to regress with time. This could result from a failure to recruit periendothelial cells during the critical period of vascular development or from a role for Tie-2 in cell survival signaling. Most of the mice with a disrupted Ang-2 gene died perinatally or within a couple of days after birth, although some have survived into adulthood, whereas Ang-3-null mice seem to be viable (Dr. Tom Sato and Dr. George Yancopoulos, personal communication).

Overexpression of Ang-2 driven by the Tie-2 promoter produced a phenotype having many similarities to that of the Ang-1 and Tie-2 knockout mice, but defects in the vasculature were generally more severe. The transgenic embryos died at E9.5 to E10.5.³⁰ These effects of Ang-2 *in vivo*, together with Tie-2-receptor phosphorylation studies, support the idea that Ang-2 is an antagonist of Ang-1. However, another physiological role of Ang-2 may be masked by the abnormal spatiotemporal overexpression pattern. Overexpression of Ang-1 in mice under the K14 promoter, which targets the gene product to the basal layer of epidermis, yielded a hypervascular phenotype in which vessel branching was dramatically increased.⁵⁴ Strikingly, overexpression of Ang-2 under the same promoter caused embryonic death at E14 (Dr. George Yancopoulos, personal communication). The mild phenotype of Ang-2^{-/-} mice and the severe defects in mice overexpressing Ang-2 highlight the importance of controls of Ang-2 expression.

PCs and Vascular Remodeling

Characteristics of the phenotypes of embryos lacking angiopoietin-Tie-2 signaling components suggest that

the regulation of interactions between PCs and ECs may be an important function of Tie-2. PCs, SMCs, and cardiomyocytes, collectively called mural cells, surround ECs abluminally.⁵⁵ PCs are within the same basement membrane that surrounds nearby ECs in the microvasculature. The phenotypes of PCs and SMCs seem interchangeable; the latter encapsulate ECs around larger vessels and cardiomyocytes surround the endocardium. PCs and ECs form intimate contacts by interdigitations and gap junctions. The morphology and function of PCs depend on their location. Only PCs and ECs together form a functional entity that fulfills the physiological requirements of mature blood vessels.⁵⁵

PCs have been shown to restrict the proliferation of ECs in co-cultures.⁵⁶ This inhibition, requiring contacts between ECs and PCs, has been attributed to activation of transforming growth factor β from its latent complex by plasmin in the EC-PC intercellular space.^{57,58} PCs may also restrict EC proliferation by forming a physical barrier, and they have been shown to inhibit EC migration.⁵⁸ Heparin-binding epidermal growth factor and platelet-derived growth factor-BB are involved in the EC-to-PC signaling: ECs have been shown to stimulate PC migration and proliferation via platelet-derived growth factor-BB secretion during developmental angiogenesis^{59,60} (Dr. Christer Betsholtz, personal communication), and epidermal growth factor receptors may be up-regulated in the cytoplasmic interdigitations of PCs facing ECs.⁶¹

Initiation of vessel sprouting in adults is preceded by a local drop-off of PCs from the existing vessel that enables ECs to overcome growth inhibition and start proliferating and migrating. More discordant is the role of PCs in vascular sprouting. A body of published reports (for references, see 62) supports the theory that during angiogenesis, PC migration is delayed until after EC migration, but contrary data have also been published.^{62,63} The migration of PCs from existing vessels to the new branches has been shown to lag several days behind the formation of the endothelial plexus.⁸ The function of delayed PC migration may be to form a plasticity window during which hyperoxia-induced vascular pruning and fine-tuning of vessel capacity occur. Subpopulations of rat intimal/subintimal SMCs are also able to differentiate into PCs during angiogenesis *in vitro*.⁶⁴ Some PCs in angiogenesis may, however, be formed by *in situ* differentiation from mesenchymal precursors. Such PCs may precede ECs in angiogenic sprouting during development and may act as guidance cells for the ECs.⁶² The molecular characteristics of PCs have been shown to change during neovascularization.⁶⁵

Functions of Angiopoietin Signaling: Emerging Concepts

Although both Ang-1 and Ang-2 bind to Tie-2 with high affinity, only Ang-1 seems to cause receptor autophosphorylation in ECs.³⁰ In the ECs, Tie-2 phosphorylation can be induced by Ang-1, and this can be inhibited by an excess of Ang-2. In the hematopoietic BaF3 cell line expressing a transfected Tie-2 construct, both Ang-1 and

Table 1. The biological functions of the angiopoietin-Tie-2 system based on evidence from:

Gene disruption studies	Reference:
Endocardium-myocardium interactions	47, 50
EC-mesenchymal cell interactions	50
Sprouting angiogenesis	49
Vascular remodeling	49, 50
EC survival	47
Integrity of the endothelium	47
Transgenic studies	
Antagonistic role of Ang-2	30
Role of Ang-1 in vessel branching	Dr. George Yancopoulos, personal communication
<i>In vitro</i> studies	
Sprouting/migration of ECs	32, 46
Differentiation/shape of ECs	50
Plasminogen activation	Dr. Urban Deutsch, personal communication
Hematopoietic cell adhesion	66
<i>In vivo</i> models	
Stimulation of angiogenesis in the presence of VEGF	76
Vessel pattern formation	76
Role in pathological angiogenesis	10, 68, 69

Ang-2 induced Tie-2 phosphorylation, although the phosphorylation induced by Ang-1 was more prominent.⁶⁶ Tie-2 is constitutively phosphorylated when overexpressed in fibroblasts.^{30,67} However, a genetically modified Tie-2 with decreased basal level of receptor phosphorylation was autophosphorylated by both Ang-1 and Ang-2 in the fibroblasts.³⁰ The endogenous expression pattern of Tie-2 is restricted to ECs and their precursors and thus, at least in mature ECs, Ang-2 may serve as a physiological antagonist for Ang-1.

The observation by Stratmann et al that Tie-2 is up-regulated in the endothelium of growing blood vessels in glioblastomas is compatible with the active role suggested for Tie-2 in angiogenesis. (See Table 1.) Inhibition of Tie-2 signaling in tumor vessels using a recombinant soluble Tie-2 receptor or an adenoviral Tie-2 vector seems to prevent tumor growth in mice and rats.^{68,69} However, long-term therapeutic interventions based on the blocking of Tie-2 signaling could result in systemic side effects because Tie-2 is phosphorylated in quiescent vasculature.⁴⁵

During development, Ang-1 is expressed by mesenchymal cells surrounding blood vessels and this expression pattern may persist in adult tissues. Because of the paracrine expression pattern of Ang-1 and the observed defects in genetically altered mice, it has been suggested that Ang-1 contributes to stabilization of vessel structure.⁷⁰ The linkage of the Tie-2 gene to venous malformations characterized by a deficient SMC layer⁴³ emphasizes the importance of unperturbed Tie-2 signaling in mediating interactions between ECs and surrounding cells. Analogous to the possible role of Tie-2 signaling in mediating vessel-stabilizing EC-SMC interactions, it could also mediate EC-ECM interactions. During development, cells expressing Ang-1 are scattered through the brain parenchyma and Ang-1 may be expressed in glial cells or neuronal cells, but not in SMCs or PCs associated with blood vessels (Dr. Christer Betsholtz, personal communication). Stratmann et al now report that in glioblastomas, Ang-1 is up-regulated in the tumor cells. This expression pattern of Ang-1 agrees with the previous finding of Ang-1 mRNA in several tumor cell lines including C6 rat glioma cells.^{36,71}

In tumors, Ang-1 may stimulate blood vessel invasion synergistically with VEGF. Indeed, Ang1* and Ang-1 induce sprouting of ECs in a cordlike fashion from the

Figure 1. Hypothetical role of Ang-2 in tumor angiogenesis. Ang-1 expression in mesenchymal cells around vessels with Tie-2 receptor-positive ECs leads to constitutive Tie-2 activation and signaling (1). Increased production of VEGF and bFGF in tumors may contribute to the observed up-regulation of Ang-2 focally in the angiogenic EC sprouts. In addition, regional hypoxia may up-regulate Tie-2 and Ang-2 expression via the endothelial PAS domain protein 1 (EPAS1/HIF-2) transcription factor activation.⁷⁷ By inhibiting Ang-1-Tie-2 signaling, Ang-2 may disrupt the interactions between ECs and their microenvironment, including PCs and SMCs. This may sensitize the ECs to the mitogenic and chemotactic signals mediated by tumor-secreted angiogenic factors and release them from growth inhibition caused by PCs (2). When ECs are in an activated state, Ang-1 secreted by the tumor cells and Ang-2 produced by the endothelium may promote angiogenesis in concert with VEGF(3).

surface of microcarrier beads embedded in a fibrin gel.³² Ang1* also stimulated EC migration in fibronectin and gelatin-coated Boyden chamber, and this migration was inhibited by an excess of Ang-2. Ang-2 by itself had no effect on cell migration.⁴⁶ Interestingly, Ang-1 failed to induce tube formation in collagen matrices²⁹ (Ang-2 was not tested in this assay). Differences between migration and tube-formation assays are not yet clear at the molecular level. In the latter, junctional complexes mediating cell-cell interactions may have a more prominent role,⁷² whereas adhesive interactions between ECs and ECM may be more critical for migration. The rather uniform mesenchymal expression pattern of Ang-1 may be necessary for the vessel-stabilizing functions of Ang-1, whereas the increased expression of Ang-1 in tumor cells may generate an Ang-1 gradient in the ECM. This could cause polarization of the ECs and their pericellular interactions and enable cell migration.⁷³ These findings also call for the elucidation of the factors that increase the expression of Ang-1 in tumors.

The demonstration by Stratmann et al that Ang-2 is expressed in a subset of angiogenic vessels, namely in small vessels with few PCs, fits well with the suggested role of Ang-2 as an inhibitor of constitutive Ang-1 signaling, which may be important for the interactions between ECs and the surrounding cells.⁷⁴ However, if Ang-1 is not expressed in SMCs and PCs but instead in mesenchymal cells farther from the vessels, a role for angiopoietins in the reciprocal signaling between the ECs and PCs/SMCs seems less likely. Instead, Ang-2 may initially induce a weakening of EC interactions with their microenvironment, including the EC-PC interactions. This may convert ECs into a more active and immature phenotype, and enhanced expression of EC-derived Ang-2, in combination with tumor-derived Ang-1 and VEGF, may then act synergistically to stimulate angiogenesis (Figure 1). In cultured ECs Ang-2 mRNA is up-regulated by VEGF and bFGF (and, curiously, also by hypoxia), and down-regulated in response to transforming growth factor β 1, which is associated with vessel stabilization.⁷⁵ VEGF expressed by glioblastomas could provide at least one stimulus for Ang-2 expression.

After activating the ECs by inducing PC drop-off, Ang-2 may have further effects that differ from its effects on quiescent vasculature. Such altered responses of angiogenic activated ECs to cytokine signaling could be central to angiogenesis. For example, the expression of VEGFR-3 is normally restricted to the lymphatic endothelium in adults, but it can also be up-regulated in the tumor vessel endothelium (Dr. Reija Valtola and KA, unpublished results). Earlier investigations have shown that Ang-2 is expressed in the forefront vessels invading the corpus luteum.³⁰ Indeed, after destabilization of vessel structure Ang-2 may take another role in angiogenesis. Recently it was shown that Ang-1 and Ang-2 enhanced VEGF-induced angiogenesis *in vivo* in the corneal micro-pocket assay but neither Ang-1 nor Ang-2 alone stimulated angiogenesis.⁷⁶ Curiously, in this assay both Ang-1 and Ang-2 induced the recruitment of periendothelial cells, but the effect of Ang-1 was five times stronger than that of Ang-2. This indicates that in active angiogenic

ECs, Ang-2 is able to induce at least some level of Tie-2 signaling, although it may act very locally as an autocrine endothelial growth factor. Although its exact role remains enigmatic, the available evidence indicates that Ang-2 is important for the initiation of angiogenesis and for vascular sprouting.

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