Signaling Vascular Morphogenesis and Maintenance

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Blood vessels are constructed by two processes: vasculogenesis, whereby a primitive vascular network is established during embryogenesis from multipotential mesenchymal progenitors, and angiogenesis, in which preexisting vessels (both in embryo and adult) send out capillary sprouts to produce new vessels (1-3). Endothelial cells are centrally involved in each process: They migrate and proliferate and then assemble into tubes with tight cell-cell connections to contain the blood. Peri-endothelial support cells are recruited to encase the endothelial tubes, providing maintenance and modulatory functions to the vessels; such cells include pericytes for small capillaries, smooth muscle cells for larger vessels, and myocardial cells in the heart.

The establishment and remodeling of blood vessels is controlled by paracrine signals, many of which are protein ligands that bind and modulate the activity of transmembrane receptor tyrosine kinases (RTKs). This realization has emerged from the discovery and analysis of RTKs expressed on endothelial cells and of their ligands. Our basic view of RTK signaling has come from studies (performed largely in fibroblasts) of ligand-dependent autophosphorylation and activation of the branched Ras pathways. The results suggest that most RTKs are similarly coupled into the intracellular signal transduction cascade and are capable of inducing cell proliferation. However, the lessons from endothelial cells present a far more complicated picture. This complexity is highlighted by an article in this issue (4 page 55) of a ligand, called angiopoietin-2 (Ang2), that interferes with the kinase activity of an endothelial cell-selective RTK named Tie2. Remarkably, this inhibition of Tie2 kinase activity does not block endothelial cell proliferation and angiogenesis, but rather facilitates it.

Tie2 has two ligands, Ang1 and Ang2. Another major regulator of vasculogenesis and angiogenesis is vascular endothelial growth factor (VEGF, also called vascular permeability factor, VPF). VEGF signaling is itself mediated by two other endothelial cell-selective RTKs, called VEGF-R1 and VEGF-R2 (Flt1 and Flk1/KDR, respectively).

Mice carrying homozygous disruptions in the two highly homologous VEGF receptors die in mid-gestation of acute vascular defects, implicating each in vasculogenesis and developmental angiogenesis. However, the phenotypes are distinct--and instructive (see figure 1). VEGF-R2 knockout mice, which die by embryonic day 8.5 (E8.5), lack both endothelial cells and a developing hematopoietic system, implicating VEGF as a signal in the determination first of a hemato-angioblast progenitor and then of endothelial cells (5). In contrast, VEGF-R1 knockout mice, which

also die around E8.5, have normal hematopoietic progenitors and abundant endothelial cells, which migrate and proliferate but do not assemble into tubes and functional vessels (6). Thus, these highly homologous RTKs send distinctive signals in endothelial cells.

Tie2 knockout mice die somewhat later in embryogenesis (E9.5 to E10.5). The Tie2 null phenotype is distinct from that of the VEGF receptor knockouts and is also informative. Endothelial cells are present in normal numbers and are assembled into tubes, but the vessels are immature, lacking branching networks and proper organization into large and small vessels (7, 8). There is also an absence of the angiogenesis that vascularizes the neuroectoderm by capillary sprouting from the primitive vascular network (or plexus). Notably, the vessels that do form lack an intimate encapsulation by peri-endothelial support cells. In the heart, the endocardium and myocardium do not show tight association and structural complexity; rather, the endocardial cells have aberrant, rounded shapes, are only loosely attached to the surrounding basement membrane, and in many locations are disconnected from myocardial cells. Similar defects in vessel architecture are evident in other tissues. Thus, the Tie2 tyrosine kinase appears to control the capability of endothelial cells to recruit stromal cells to encase the endothelial tubes so as to stabilize the structure and modulate the function of blood vessels.

The fourth endothelial cell-selective RTK, Tie1, is remarkably similar in structure to Tie2 and appears to control another aspect of vessel integrity. Knockout mice lacking Tie1 die over a variable period, ranging from E14.5 to birth, of edema and hemorrhage, implicating the Tie1 signal in control of fluid exchange across capillaries and in hemodynamic stress resistance (8, 9).

New insights into the surprising concept that the Tie2 RTK is primarily coupled into a signal transduction circuit that elicits vessel maturation and maintains vessel integrity comes from functional analyses of the angiopoietins that bind to Tie2 and modulate its activity (4, 10, 11). The Tie2 ligands Ang1 and Ang2 are both ~75-kD secreted proteins with considerable sequence homology; each contains a coiled-coil and a fibrinogen-like domain. Both bind to the Tie2 receptor with similar affinity, and neither binds to the related receptor Tie1. Yet their effects on Tie2 are distinctive, as are their expression patterns in the mouse. Ang1 induces autophosphorylation of Tie2 in cultured endothelial cells. In marked contrast, Ang2, which binds with similar affinity, does not induce receptor phosphorylation. Rather, it can competitively inhibit Ang1--induced kinase activation of the Tie2 receptor. Thus Ang2 presents a negative signal to Tie2, a remarkable observation given its high homology to Ang1.

Moreover, this distinctive effect is apparently endothelial cell-specific. If a modified Tie2 is forcibly expressed in 3T3 fibroblasts, both Ang1 and Ang2 induce receptor phosphorylation and yet do not stimulate fibroblast proliferation. Similarly, Ang1-induced autophosphorylation of Tie2 does not affect endothelial cell growth in culture, consistent with the Tie2 knockout phenotype, which indicated that Tie2 is not required for endothelial cell proliferation during vasculogenesis. Functional studies in transgenic and gene-knockout mice support the notion that Ang1 signals Tie2 to recruit support cells, and that Ang2 inhibits this capability. Gene-knockout mice that lack Ang1 die with similar vascular defects to the Tie2 knockout mice (11). Transgenic mice overexpressing

the negative ligand Ang2 in endothelial cells also die during embryogenesis, again with similar vascular defects (4). Thus, overexpression of Ang2 phenocopies loss of Ang1 expression, consistent with their opposite activities. Collectively, the data argue that Ang1 is the major physiological ligand for Tie2's functional role in recruiting and sustaining peri-endothelial support cells, whereas Ang2 serves to block this function, thereby relaxing these intimate and critical associations.

Why then does Ang2 exist? No doubt the Ang2 gene-knockout phenotype will prove illuminating. Meanwhile, the prevailing evidence suggests that Ang2 allows vascular remodeling, and in particular angiogenesis, processes that may be restricted by the encapsulation with basement membrane and peri-endothelial support cells. The clues come from the expression patterns of Ang1 and Ang2.

Ang1 is widely expressed both in the embryo and in the adult (4, 10). Ang2 is also widely expressed in the embryo. However, its expression pattern in the adult (in a limited survey) is provocative: Ang2 is selectively expressed in ovary, uterus, and placenta, the three tissues subject to physiological angiogenesis (4). The possible association of Ang2 with adult angiogenesis was investigated during ovulation, which is marked by distinctive phases of vascular quiescence, angiogenesis, and vascular regression. Ang2 expression in these stages was compared to that of Ang1 and VEGF. In early follicles, the vasculature is quiescent, and Ang1 is expressed, with little or no VEGF or Ang2 expression. In late pre-ovulatory follicles and in the postovulatory corpus luteum, where angiogenesis is ongoing, both VEGF and Ang2 are up-regulated, while Ang1 expression persists. Notably, Ang2 expression appears punctate, or focal. Finally, in nonproductive follicles, which show vascular regression, Ang2 is expressed at uniformly high levels, provocatively in the absence of VEGF expression.

These patterns of expression, when considered in the context of the lessons from the various gene-knockout phenotypes (see Fig. 1), collectively suggest a model for control of vasculogenesis, vessel maturation and maintenance, angiogenesis, and regression, as illustrated in Fig. 2. The Ang1/Tie2 circuit appears to mediate vessel maturation from simple endothelial tubes into more elaborate vascular structures composed of several cell types, and the maintenance of those vessels via the cell-cell and cell-matrix associations that produce them. Consequently, Ang1 may also help preserve endothelial cell quiescence. Focal expression of Ang2 evidently blocks the Ang1/Tie2 signal, resulting in a loosening of this tight vascular structure and thereby exposing the endothelial cells to activating signals from angiogenesis inducers, including VEGF. If VEGF (or another angiogenesis inducer) is present, the endothelial cells become activated to migrate and proliferate, producing new capillary sprouts and in turn tubes. One can envision that the more uniform presence of Ang1 allows a shift in the local balance of Ang1/Ang2 back in favor of Ang1, to effect maturation and stabilization of the newly formed vessels. Thus, there appears to be a collaboration between VEGF, Ang2, and Ang1 to elicit angiogenesis. In contrast, vascular regression is associated with very high-level expression of Ang2 in the absence of the activating (survival) signal from VEGF, presumably overwhelming the Ang1 signal and thereby producing catastrophic detachment from matrix and support cells, most likely with consequent apoptosis.

The work by Maisonpierre et al. (4) introduces an inhibitory ligand, Ang2, for the Tie2 regulatory RTK that normally helps maintain vascular integrity. A model is emerging of a regulatory mechanism through which blood vessels are constructed, maintained, remodeled, and eliminated. Other factors are also involved, during embryonic vasculogenesis and angiogenesis, and for physiological and pathological angiogenesis in the adult. For example, gene-knockout mice have also implicated a G protein-coupled receptor in vascular regulation, because in the absence of Ga13, embryos die at E8.5 to E9.5, with defects in vascular assembly and angiogenesis (12). Moreover, a reciprocal paracrine signal has been revealed by gene-knockout mice that lack the genes for neuregulin, which is expressed in the endocardium of the heart, and the ErbB-2/3/4 RTKs, which are expressed in myocardium. Knockouts of this ligand or of its receptors produces defects in the developing heart analogous to those observed when Ang1 or Tie2 is missing (13-16). A similar role is suggested by gene-knockout mice for platelet-derived growth factor and its receptors in other tissue vasculature (17, 18). Furthermore, there is clear evidence in the adult for additional angiogenesis inducers and for an increasing number of angiogenesis inhibitors that act directly on endothelial cells (19-21). Thus, regulation of angiogenesis in ovulation and implantation, in wound healing, and in chronic pathological situations such as tumor progression will indeed be complex, but tractable using the power of animal models.

That complexity notwithstanding, the evidence is compelling that VEGF and the angiopoietins, and their cognate receptors, are critical components of the vascular regulatory machinery. It will be of particular interest to establish the possible contributions of Ang2 to tumor angiogenesis, whereby the quiescent vasculature (likely maintained in part by Ang1/Tie2) is activated to elicit and chronically effect the angiogenic phenotype that accompanies tumor growth and metastasis. Ang2 could well serve as an initial angiogenic signal, locally opening up the vessel structure to allow protease degradation of the basement membrane surrounding the endothelium and accessibility to that endothelium by angiogenesis inducers such as VEGF, thereby eliciting capillary sprouting and in turn new blood vessels that sustain a tumor as it expands.

Fig. 1. Lessons from gene-knockout mice

The endothelial cell-selective RTKs VEGF-R1, VEGF-R2, Tie1, and Tie2 have all been ablated in gene-knockout mice. Each RTK knockout produced embryonic lethality with vascular defects. However, their distinctive phenotypes indicate that each of these tyrosine kinases controls a specific, complementary function in endothelial cells that collectively can account for a significant part of endothelial cell morphogenesis into functional vessels. See (22) for receptor structures.

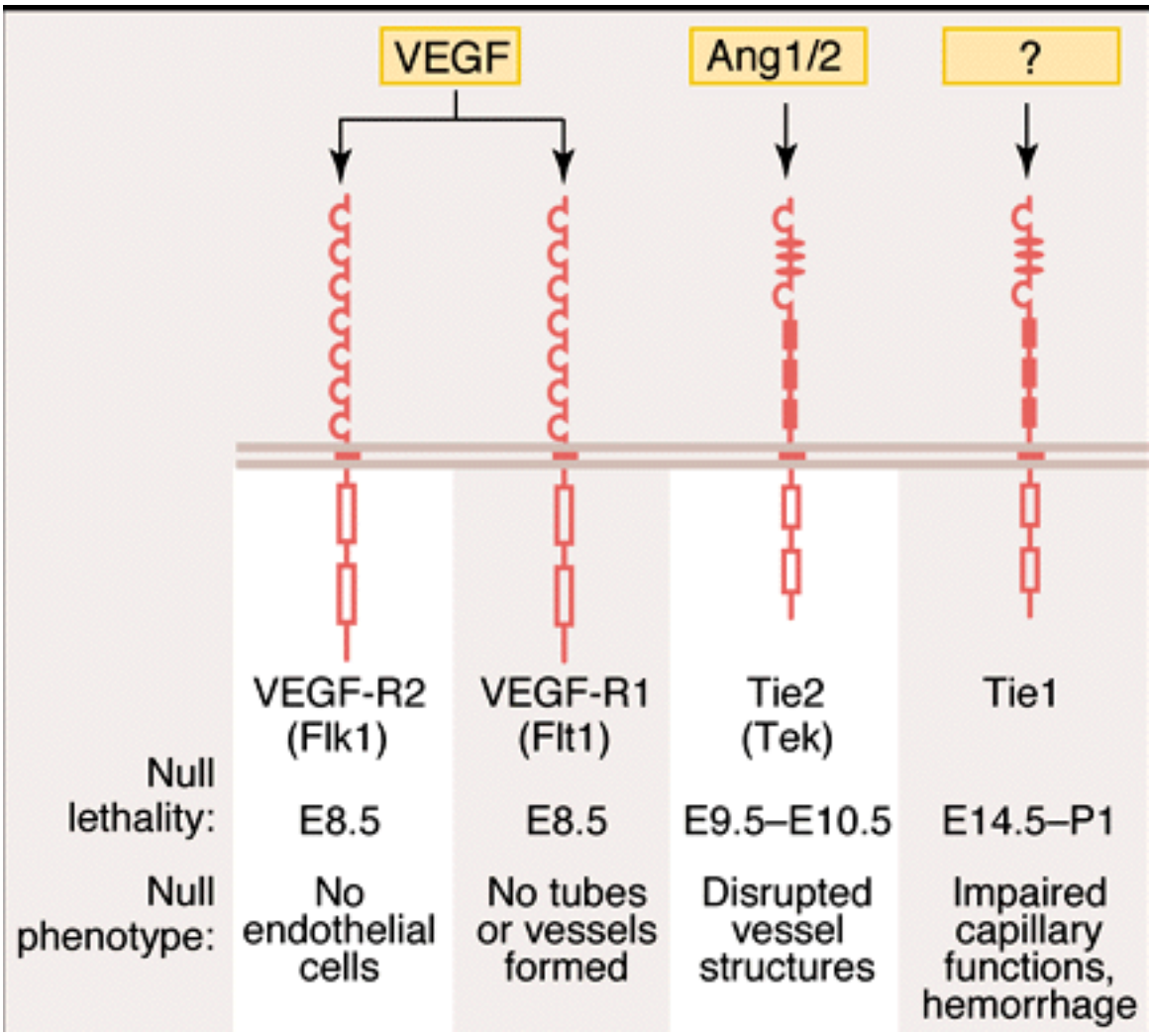

Fig. 2. Regulation of vascular morphogenesis, maintenance, and remodeling by RTKs and their ligands

A model for regulation of the vascular endothelium manifested by the prototypical angiogenesis factor VEGF and a new class of angiogenic regulators, Ang1 and Ang2. All three ligands bind to RTKs that have similar cytoplasmic signaling domains. Yet their downstream signals elicit distinctive cellular responses. Only VEGF binding to the VEGF-R2 sends a classical proliferative signal. When first activated in embryogenesis, this interaction induces the birth and proliferation of endothelial cells. In contrast, VEGF binding to VEGF-R1 elicits endothelial cell-cell interactions and capillary tube formation, a process that follows closely proliferation and migration of endothelial cells. Ang1 binding to the Tie2 RTK recruits and likely maintains association of peri-endothelial support cells (pericytes, smooth muscle cells, myocardiocytes), thus solidifying and stabilizing a newly formed blood vessel. The newly discovered Ang2, although highly homologous to Ang1, does not activate the Tie2 RTK; rather, it binds and blocks kinase activation in endothelial cells. The Ang2 negative signal causes vessel structures to become loosened, reducing endothelial cell contacts with matrix and disassociating peri-endothelial support cells. This loosening appears to render the endothelial cells more accessible and responsive toward the angiogenic inducer VEGF (and likely to other inducers). Finally, Ang2 is expressed at uniformly high levels in vascular regression in nonproductive ovarian follicles; the lack of VEGF coexpression suggests that loosening of cell-matrix interactions in the absence of a growth or survival signal elicits endothelial cell death, likely by apoptosis. No doubt additional factors play into these distinctive states, including the emerging class of angiogenesis inhibitors that directly block endothelial cell proliferation and migration.