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Angiopoietins: a link between angiogenesis and inflammation

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The angiopoietin (Ang)–Tie ligand–receptor system has a key regulatory role in regulating vascular integrity and quiescence. Besides its role in angiogenesis, it is an important regulator in numerous diseases including inflammation. Ang-1-mediated Tie2 activation is required to maintain the quiescent resting state of the endothelium. Agonistic Ang-1 functions are antagonized by Ang-2, which is believed to inhibit Ang-1–Tie2 signaling. Ang-2 destabilizes the quiescent endothelium and primes it to respond to exogenous stimuli, thereby facilitating the activities of inflammatory (tumor necrosis factor and interleukin-1) and angiogenic (vascular endothelial growth factor) cytokines. Intriguingly, Ang-2 is expressed weakly by the resting endothelium but becomes strongly upregulated following endothelial activation. Moreover, endothelial cells store Ang-2 in Weibel–Palade bodies from where it can be made available quickly following stimulation, suggesting a role of Ang-2 in controlling rapid vascular adaptive processes. This suggests that Ang-2 is the dynamic regulator of the Ang–Tie2 axis, thereby functioning as a built-in switch controlling the transition of the resting quiescent endothelium towards the activated responsive endo thelium.

Introduction

The vascular endothelium lines the inside of all blood vessels, forming a non-thrombogenic surface that controls the entry and exit of plasma and white blood cells to and from the bloodstream. It is one of the largest internal surfaces of the body and can be considered conceptually as a systemically disseminated organ. The quiescent endothelium has turnover rates of months to years, and proliferates only following angiogenic activation [1]. The molecular mechanisms controlling the quiescent endothelial-cell phenotype are poorly understood. Nevertheless, the loss of quiescence is a common feature of conditions such as inflammation, atherosclerosis, restenosis, angiogenesis and various types of vasculopathies, and might be a pathogenic mechanism linking different diseases that are associated with endothelial-cell activation. Recent research indicates that vascular morphogenic molecules also have crucial roles in controlling vascular homeostatic functions of the quiescent endothelium. Among these, the Tie2 ligand angiopoietin (Ang)-2 has a pivotal role in controlling the responsiveness of the endothelium to exogenous cytokines.

Role of the vascular endothelium as a systemically distributed organ system

Blood vessels provide the growing embryo with nutrients and oxygen [2]. The formation of the blood vascular system begins with the assembly of embryonic progenitor cells to produce a primitive vascular plexus in a process known as vasculogenesis [3]. Following the formation of this primary vascular plexus, the vascular network expands by sprouting, remodeling and regression (pruning) in a process known as angiogenesis. Vasculogenesis and angiogenesis are downregulated in the healthy adult and are – except for the organs of the female reproductive system – almost exclusively associated with pathology when angiogenesis is induced by microenvironmental factors (e.g. hypoxia or inflammation) [1]. Pathologic processes associated with, or induced by, angiogenesis include diseases as diverse as cancer, psoriasis, macular degeneration, diabetic retinopathy, thrombosis, and inflammatory disorders including arthritis and atherosclerosis, but also obesity, diabetes, asthma, infections and endometriosis. Moreover, insufficient angiogenesis is characteristic of ischemic heart disease and pre-eclampsia [2]. This impressively illustrates the broad array of diseases that are associated with the activated endothelial-cell phenotype. Intriguingly, most of these diseases are restricted to specific vascular beds and organs; for example, thrombosis occurs primarily in arterial blood vessels. Atherosclerosis arises preferentially in the arterial system. Leukocyte adhesion occurs preferentially in postcapillary venules [4]. Tumor cells metastasize site-specifically to particular organs, and endothelial cell-surface molecules are believed to be causally involved in this process [5]. Thus, a specific set of adhesion molecules is specifically expressed by, or presented to, the vascular bed following activation of the endothelium, for example, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 at inflammation sites, and tumor-endothelium markers (TEMs) at tumor vessels. Likewise, even circulating cytokines and chemokines function locally on specific vascular beds. However, the mechanisms controlling vascular-bed-specific activation programs and subsequent adhesion-molecule expression are unknown.

The quiescent vascular endothelium forms a tight barrier that controls the passage of plasma and cells from

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Available online 12 October 2006.

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the bloodstream to the underlying tissues [4]. This barrier consists of a single layer of endothelial cells covering the lumenal side of blood vessels. Ablumenally, endothelial cells are anchored to an organ- and caliber-specific basement membrane [1]. Mural cells (pericytes and smooth muscle cells) ensheath the endothelial layer. They penetrate the basement membrane and make direct contacts with endothelial cells. The barrier functions of the endothelium are achieved through tight cell–cell contacts between the endothelial cells that form junctions [6]. Moreover, direct contact and cell–cell communication of endothelial cells with mural cells and components of the extracellular matrix also control the quiescent endothelial phenotype [7]. Endothelial cells adhere to each other through junctional transmembrane proteins that are linked to specific intracellular structural and signaling complexes [6]. Junctional proteins controlling endothelial barrier functions include vascular endothelial (VE)-cadherin and N-cadherin at adherence junctions, and occludin, claudins and junctional adhesion molecules (JAMs) at tight junctions [8–10]. Interestingly, the organization of endothelial junctions varies in different vascular beds – for example, the brain vasculature is extremely tight to restrict permeability and to form the blood–brain barrier. Consequently, the brain vasculature contains numerous tight junctions [11]. By contrast, the number of tight junctions in postcapillary venules is low, enabling sensitivity to permeability-inducing agents and the paracellular transmigration of adherent leukocytes [12]. Thus, different vascular beds are differentially susceptible to exogenous stimuli. Innate endothelial lineage and vascular-bed-specific properties could be responsible for these differences. Alternatively, microenvironmental cues might control a specific regional endothelial-cell phenotype in a more dynamic fashion.

The endothelial layer can undergo a transition from the resting anti-adhesive state to the active adhesive state (Box 1) [13]. Activation of the endothelium results in the expression of adhesion molecules. This leads to, for example, leukocyte adhesion and transmigration or thrombus formation (Box 1). Thus, endothelium activation is not only prerequisite for initiating angiogenesis but also for the initiating inflammation and, concomitantly, inflammation-associated diseases. In fact, there is increasing evidence that angiogenesis-regulating receptor tyrosine kinases are also crucially involved in controlling endothelial-cell responses during inflammation. Of these, the Ang–Tie system is emerging as a key regulator of vascular maintenance and quiescent endothelial-cell homeostasis in which the antagonistic ligand Ang-2 functions as an autocrine switch of vascular responsiveness to exogenous stimuli.

Box 1. Activation of the endothelium – the role of Weibel–Palade bodies

Endothelial activation is controlled by multiple processes and factors, including physical damage, hypoxia and altered shear-stress, in addition to bacterial and viral infections. These stimuli trigger an inflammatory response program that is mediated by soluble factors including thrombin, histamine, endotoxin, oxidized lipoproteins, prostaglandins, leukotrienes, interleukins, TNF and VEGF. The activated endothelium facilitates immune-cell recruitment, thrombus formation and local fluid accumulation owing to the changes in endothelial-cell adhesiveness and permeability [4,66,83]. The activation of endothelial cells involves rapidly acting, presynthesized and stored molecules, in addition to a subsequently slower transcriptionally regulated response program. Presynthesized molecules are stored in endothelial-specific storage granules, known as Weibel–Palade bodies (WPBs) [84]. The primary constituent of WPBs is von-Willebrand factor (vWF). In addition to a processed multimeric form of vWF, WPBs also store P-selectin, CD63, IL-8, endothelin-1, tissue plasminogen activator (t-PA) and Ang-2 [84]. Interestingly, all of these molecules are involved in controlling rapid endothelial responses, including hemostasis, inflammation, hemodynamic adaptation, fibrinolysis and permeability [66,84]. The molecules are released from WPBs within seconds to minutes in response to multiple secretagogues, including thrombin, histamine, serotonin, superoxides and sphingosine-1-phosphate [85–87]. These secretagogues are potent inducers of inflammation, coagulation, angiogenesis and other endothelial responses, suggesting that the release of WPBs functions as an initial step in the transition from the quiescent, resting endothelium to the activated, responsive endothelium. Endothelium activation is associated with the loosening of interendothelial junctional complexes and the surface presentation of different adhesion molecules [6,13,66]. For example, inflammatory activation triggers a molecular cascade of events that results in leukocyte recruitment and transmigration. The process starts with the rolling and tethering of leukocytes to the activated endothelium, which is mediated by the interaction of endothelial-cell and leukocyte selectins with their corresponding counter-receptors. The surface presentation of WPB-stored P-selectin is one of the first steps in the cascade of events that lead to leukocyte recruitment. P-selectin- (and, later, E-selectin)-mediated leukocyte rolling is followed by firm adhesion. Firm adhesion is controlled by members of the immunoglobulin superfamily of adhesion molecules, including ICAM-1 and VCAM-1, which are expressed on the lumenal side of inflammatory cytokine-activated endothelial cells. Firm and irreversible adhesion of leukocytes to the endothelium is followed by transendothelial migration and extravasation into the underlying tissues [13,88].

Regulation of vascular maintenance and homeostasis through the Ang–Tie system

The Ang–Tie ligand–receptor system consists of two receptor tyrosine kinases, Tie1 and Tie2, and four corresponding ligands, Ang-1, Ang-2, Ang-3 and Ang-4 [14]. The Tie receptors are almost exclusively expressed by endothelial cells and hematopoietic stem cells [15–20]. Tie2 expression could also be detected on a subset of tumor-associated monocytes and eosinophils [21,22]. Tie1 and Tie2 share a similar overall structure consisting of an extracellular domain with 33% similarity and an intracellular tyrosine kinase domain with 76% similarity [19]. The angiopoietins were originally identified as ligands for Tie2 [23–25]. Ang-1 and Ang-2 are the best-characterized ligands, and were the first to be identified [23,24]. Ang-4 and the mouse ortholog of Ang-4, Ang-3, were identified later [25]. Surprisingly, Ang-3 and Ang-4 function both as species-specific agonists and antagonists of Tie2 [26]. Intriguingly, no specific ligand has been identified for Tie1. However, at high concentrations, Ang-1 binds to Tie-1 through integrins [27,28].

Ang-1 is constitutively expressed by many different cell types: Ang-1 expression is found in pericytes, smooth muscle cells, fibroblasts and some tumor cells [23,29,30]. This is in contrast to the expression of Ang-2, which is almost exclusively expressed by endothelial cells themselves, and is also detectable in Kaposi's sarcoma cells and in Müller cells in the retina [29–34]. Ang-2 mRNA is almost undetectable in the quiescent vasculature; however, it is induced dramatically at sites of endothelial-cell activation. Ang-2 expression

is induced by various cytokines, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2, and by microenvironmental factors (e.g. hypoxia) [29,35,36].

Both Ang-1 and Ang-2 bind to the same site in the extracellular domain of Tie2 with similar affinities [24,37]. The binding of Ang-1 to Tie2 mediates rapid receptor autophosphorylation that promotes endothelial-cell migration and survival. By contrast, Ang-2 binding to Tie2 does not elicit rapid Tie2 autophosphorylation, suggesting that Ang-2 functions as an antagonist ligand of Tie2. This concept is also supported by the phenotypes of genetically manipulated mice. Ang-1- and Tie2-deficient mice have similar phenotypes. Both die in midgestation owing to severe vascular remodeling defects causing perturbed vascular integrity. The phenotypic similarity of Ang-1- and Tie2-deficient mice strongly suggests that Ang-1 is the nonredundant, agonistic ligand of Tie2 [38,39]. Conversely, systemic embryonic Ang-2 overexpression results in embryonic lethality. The phenotype of these Ang-2-transgenic mice largely phenocopies Ang-1- and Tie2-deficient mice [24]. In contrast to the lethal embryonic phenotype of Ang-2-transgenic mice, Ang-2-deficient mice develop normally. These mice seem phenotypically normal at birth but die within 14 days as a consequence of chylous ascites (on a C129 genetic background) or develop normally throughout adulthood (on a C57/Bl6 genetic background) [32,40]. Together, these findings suggest that Ang-2 is dispensable for proper embryonic development. However, strong systemic Ang-2 elevation is potentially dangerous, as evidenced by the embryonic lethal phenotype of Ang-2-transgenic mice. The genetic data have also solidly established Ang-2 as the functional antagonist of the constitutively functioning Ang-1–Tie2 axis. The role of Ang-1–Tie2 signaling as an important vascular maintenance factor is also supported by the observation that constitutive low-level Tie2 phosphorylation can be detected in the adult in different vascular beds [41].

Ang-1-mediated Tie2 phosphorylation signals primarily through the protein kinase B (PKB)–Akt pathway that transduces survival signals (Figure 1) [42–45]. Akt signaling leads to inactivation of the forkhead transcription factor FKHR-1, which, in turn, is a potent inducer of Ang-2 expression and also prevents Ang-2 secretion [46,47]. Thus, Ang-1-mediated PKB–Akt signaling directly inhibits endothelial-cell apoptosis and prevents activation of the endothelium by inhibiting Ang-2 expression and secretion. Tie2 activation also results in the recruitment of A20-binding inhibitor of nuclear factor (NF)-κB (ABIN)-2, which inhibits the NF-κB pathway [48,49]. This protects endothelial cells from apoptosis and inhibits inflammatory responses (Figure 1) [48,50]. Thus, constitutive Tie2 phosphorylation and signaling involves several signaling pathways that are collectively anti-apoptotic and maintain the quiescent state of the resting endothelium.

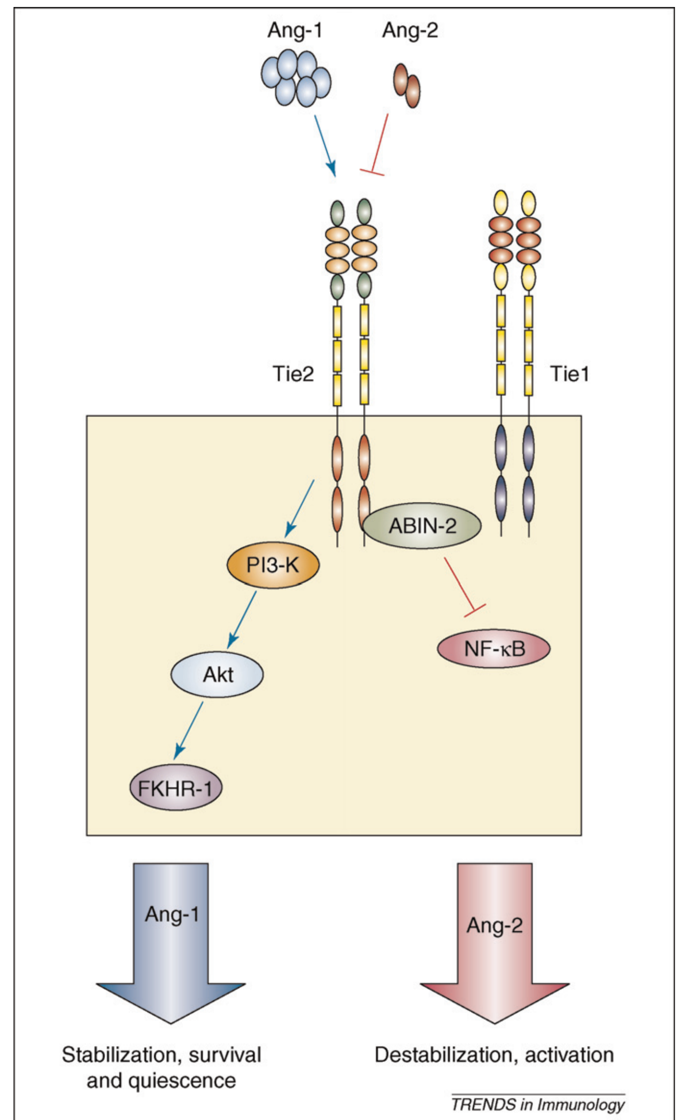

Figure 1. Angiopoietin and Ang-2 signaling in regulating the quiescent and activated endothelial-cell phenotype. The Tie2 ligand Ang-1 binds to Tie2 and induces its autophosphorylation. Ang-1-mediated PI 3-kinase activation results in the phosphorylation and activation of Akt. Akt signaling controls the quiescent endothelial-cell phenotype and promotes endothelial-cell (yellow box) survival. Moreover, Akt phosphorylates and inactivates FKHR-1. Consequently, endothelial Ang-2 expression is inhibited. Phosphorylated Tie2 also interacts with ABIN-2 and prevents NF-κB signaling, thereby suppressing the expression of inflammation-associated molecules. Ang-2 release from endothelial storage pools (WPBs) and binding to Tie2 interferes negatively with Ang-1-mediated Tie2 signaling and results in destabilization, thereby rendering the endothelium responsive to stimulation by inflammatory and angiogenic cytokines. Abbreviation: PI3K, PI 3-kinase.

Autocrine regulation of vascular homeostasis and responsiveness through Ang-2

Constitutive Ang-1 expression and low-level Tie2 phosphorylation in the adult vasculature suggest that Ang-1-mediated Tie2 signaling functions as the default pathway to control vascular quiescence. Ang-1 exerts a protective effect on the endothelium and limits its ability to be activated by exogenous cytokines. Ang-1 seals the vasculature: it is anti-inflammatory, protects against cardiac allograft arteriosclerosis and radiation-induced endothelial-cell damage, and promotes wound healing [48,50–55]. Furthermore, Ang-1 can inhibit VEGF-induced blood-vessel formation and adhesion-molecule expression [56,57], indicating that Ang-1-mediated Tie2 signaling controls vascular homeostasis and endothelial activation.

Proper vascular homeostasis is tightly controlled by balanced Tie2 signaling. Ang-1 overexpression induces blood-vessel and lymphatic angiogenesis in particular

experimental settings [56,58,59]. Moreover, Tie2 overexpression in the skin causes a psoriasis-like phenotype [60]. The most compelling genetic evidence for a strict Tie2-activity dosage concept is the observation that an activating Tie2 mutation causes venous malformations that are composed of dilated endothelial channels [61]. The role of Ang-2 in this scenario is not well understood. Genetic manipulation experiments in mice and cell-culture experiments suggest that Ang-2 antagonizes Ang-1-mediated Tie2 functions (Figure 2) [24,38,39,62]. However, although Ang-2 is well-established functionally as an antagonist of Ang-1–Tie2 signaling, direct evidence for an inhibitory effect of Ang-2 on Tie2 phosphorylation is lacking.

Ang-2 expression is tightly controlled. Ang-2 mRNA is almost absent in the quiescent resting vasculature and dramatically upregulated in tumor blood vessels [29]. Ang-2 expression is regulated by several different endotheliotropic cytokines [e.g. FGF-2, VEGF and tumor necrosis factor (TNF)] and environmental cues (hypoxia, high glucose levels and superoxides) [31,34,35,63,64]. Ang-2 protein is stored in endothelial-cell Weibel–Palade bodies (WPBs) and, thus, is readily available following endothelial stimulation with WPB secretagogues such as phorbol 12-myristate-13-acetate (PMA), thrombin and histamine [32,65]. The release of Ang-2 results in rapid destabilization of the endothelium, suggesting that Ang-2 functions as an autocrine negative regulator of the quiescent resting endothelium [62,66]. Moreover, Ang-2 triggers an inflammatory response by activating the endothelium and inducing permeability [67,68]. This was further supported by inflammation experiments in Ang-2-deficient mice [32]. These mice cannot elicit an acute inflammatory response following intraperitoneal injection of thioglycolate or *Staphylococcus aureus*. Detailed mechanistic analyses revealed that Ang-2-deficient mice have an impaired ability to express cytokine-inducible adhesion molecules on their lumenal cell surface after inflammatory activation. Ang-2 does not affect endothelial-cell adhesion-molecule expression directly. Instead, it primes the quiescent endothelium to control the responsiveness to inflammatory cytokines [32,66]. These findings support a model that implies that a balanced Ang-1:Ang-2 ratio determines the functional status of the vasculature (Figure 2). According to this model, vascular quiescence is maintained by constitutive Ang-1–Tie2 signaling, with an Ang-1:Ang-2 ratio in favor of Ang-1 owing to downregulated Ang-2 production. Following endothelial-cell activation, WBP-stored Ang-2 is released rapidly and Ang-2 is transcriptionally upregulated strongly in endothelial cells, locally shifting the Ang-1:Ang-2 ratio in favor of Ang-2. Consequently, Ang-2-mediated negative interference with constitutive Ang-1–Tie2 signaling destabilizes the endothelium and primes it to acquire responsiveness to other cytokines. The endothelium switches back to the quiescent state in the absence of an additional stimulus. The presence of other exogenous stimuli, such as TNF or VEGF, induces an inflammatory and angiogenic response, and, additionally, induces Ang-2 overexpression (Figure 2). Alternatively, Ang-2 upregulation in the absence of other exogenous stimuli might result in vascular destabilization and subsequent vessel regression, for example as observed

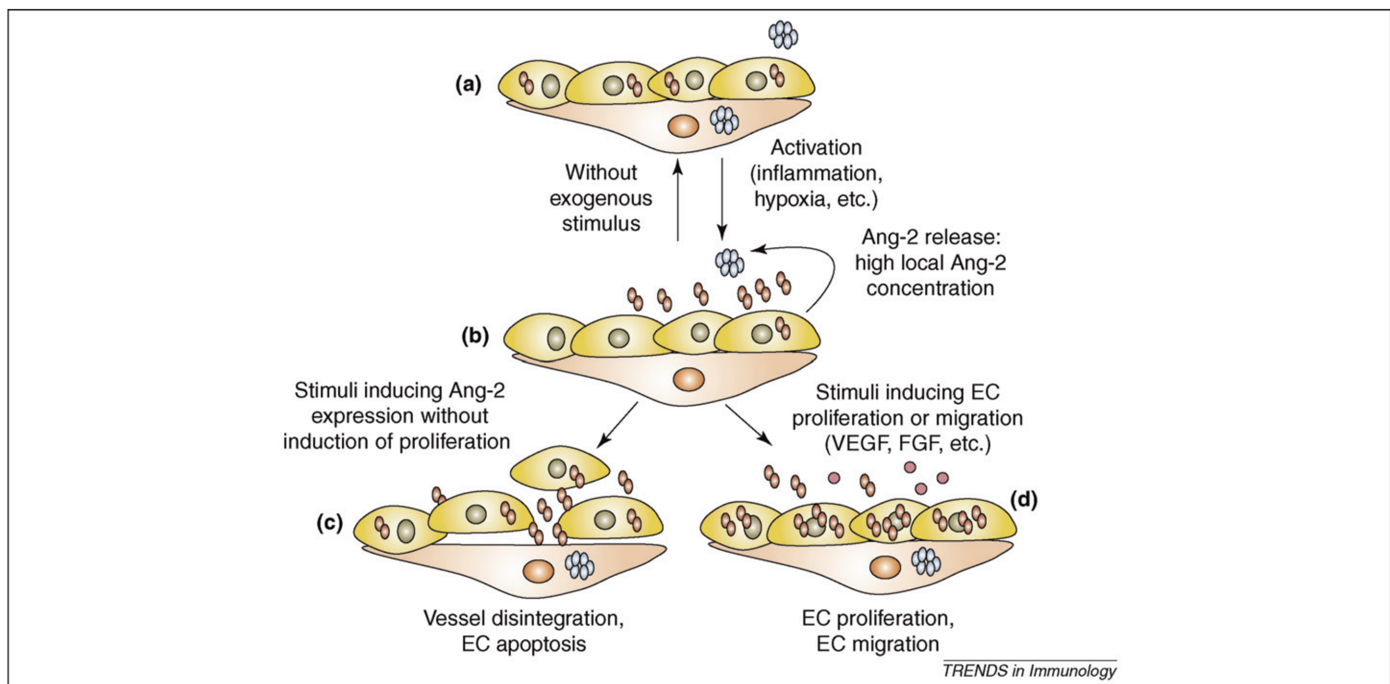

Figure 2. Proposed model of Ang–Tie interactions in regulating **(a)** vascular quiescence, **(b)** vascular responsiveness, **(c)** vascular regression and **(d)** angiogenesis. (a) The quiescent phenotype of the endothelium is maintained by constitutive Tie2 activation mediated by the binding of oligomeric Ang-1 (blue), which is secreted by smooth muscle cells and pericytes (orange). Quiescent endothelial cells (green) store dimeric Ang-2 (red). (b) The activation of endothelial cells results in Ang-2 liberation, which interferes with constitutive Ang-1–Tie-2 signaling and yields endothelial cells responsive to the activities of other cytokines (e.g. inflammatory or angiogenic). (c) Continued exposure of endothelial cells to Ang-2 in the absence of other cytokines (pink) results in endothelial apoptosis and subsequent vessel regression. (d) Conversely, the continued induction of Ang-2 protein expression primes endothelial cells towards angiogenic stimuli. Transcriptional upregulation of Ang-2 in endothelial cells is an early event of the angiogenic cascade. Abbreviation: EC, endothelial cell.

in the regressing ovarian corpus luteum during luteolysis (Figure 2). A shift in the ratio from Ang-2 to Ang-1 controls vascular responsiveness and homeostasis locally. Systemic effects of Ang-2 are unlikely. Massive systemic transgenic overexpression results in embryonic lethality [24]. Physiologically, however, circulating Ang-1 concentrations in healthy adult humans exceed systemic Ang-2 levels, and might, thereby, counteract the potentially deleterious effects of systemically released Ang-2.

Ang-2 functions are context-dependent. Ang-2 facilitates angiogenesis if it functions in concert with VEGF, and it leads to vessel regression in the absence of VEGF [69]. In fact, endothelial cells in contact with smooth muscle cells require Ang-2 to enable them to respond to VEGF stimulation in a cellular model of sprouting angiogenesis [70]. Similarly, Ang-2 modulates the sensitivity of retinal vessels to VEGF, providing *in vivo* evidence for a VEGF-priming role of Ang-2 [71,72]. The underlying molecular mechanisms by which Ang-2 sensitizes endothelial responsiveness to exogenous cytokines are presently poorly understood. Ang-2-mediated negative interference with constitutive Ang-1–Tie2 signaling might alter the integrity of interendothelial cell contacts and junctional complexes, and, thereby, affect the cell-surface expression of different growth factor receptors, including receptors for inflammatory and angiogenic cytokines. Alternatively, it is also conceivable that Ang-2 signals by itself through Ang-1–Tie2-independent mechanisms. Surprisingly little is known about the effects of Ang-2 on Tie2 signaling. There is evidence that Ang-2 functions as a Tie2 agonist in a concentration- and spatiotemporal-dependent manner in certain experimental settings. For example, it has been shown that the long-term sustained stimulation of endothelial cells with Ang-2 results in Akt signaling, and promotes endothelial-cell survival, sprouting and migration [70,73–75]. Furthermore, Ang-2 overexpression *in vivo* promotes wound healing and protects against cardiac allograft vasculopathy [55,76]. Moreover, there is accumulating evidence that Ang-2 functions vary in an organ-specific and vascular-bed-specific manner: for example, experiments in which the gene encoding Ang-1 was knocked into the Ang-2 locus showed that Ang-2 functions as an agonist in lymphatic system development and as an antagonist for the development of the hyaloid vessel in the eye [40,77,78]. Thus, Ang-2 might also have agonistic effects on certain vascular beds. However, these findings are based mostly on cellular assays using high amounts of recombinant Ang-2 and systemic overexpression of Ang-2 *in vivo*. Nevertheless, transgenic mouse experiments are clearly showing that Ang-2 functions antagonistically on the Ang–Tie system by interfering with the Ang-1–Tie2 axis, which induces blood-vessel regression and primes the vascular bed towards stimulation by angiogenic and inflammatory cytokines [24,32]. Clearly, further studies will be needed to understand fully the agonistic versus antagonistic functions of Ang-2, that is, (i) to unravel the molecular mechanisms by which Ang-2 modulates rapid vascular homeostatic functions and responsiveness towards different cytokines, and (ii) to elucidate the effects of Ang-2 on chronic vascular disease, most notably, atherosclerosis, arthritis and tumor growth.

Concluding remarks and future perspectives

Changes in the integrity and quiescent state of the vascular endothelium are directly or indirectly involved in many human diseases. The Ang–Tie system functions as a key regulator of vascular quiescence. Ang-2 is the dynamic player of the system, controlling the quiescence of the endothelium as an autocrine built-in switch of endothelial cells. These concepts are highly compatible with the phenotypes of genetically manipulated mice (i.e. Ang-1 = Tie2 agonist; Ang-2 = Tie2 antagonist). Much needs to be learned about the molecular mechanisms of angiopoietin-ligand interactions with the Tie2 receptor and the co-receptor function of Tie1 to understand rationally under what conditions of long-term stimulation Ang-2 can function as an agonist of Tie2 signaling, which induces Tie2 phosphorylation. The detailed molecular analysis of the mechanisms underlying angiopoietin function should have major therapeutic implications. Ang-1 has potent anti-inflammatory potential in several animal models [49,51–55]. However, Ang-1 therapies might have crucial side effects. In addition to inducing angiogenesis and vascular remodeling, Ang-1 also promotes pulmonary hypertension [58,59,79–81]. Likewise, given that Ang-1 functions constitutively and Ang-2 is dynamically regulated, it is likely that Ang-2-manipulatory therapies will be preferred. Ang-2-neutralizing reagents have been developed as potential anti-angiogenic tumor drugs [82]. Given that Ang-2 is dispensable for embryonic vascular development [40], it has yet be seen what the prospect of Ang-2-neutralizing tumor therapies will be. Nevertheless, Ang-2-neutralizing therapies could prove effective in acute settings to interfere with disease processes associated with rapid vascular activation. Conceptually, regulating vascular homeostatic maintenance function in the adult using angiogenic cytokines marks an interesting paradigm shift in the field of angiogenesis research. It has raised awareness of the potential side effects of prolonged anti-angiogenic treatments in tumor patients. In turn, it might also broaden the scope of angio-manipulatory drugs beyond angiogenesis to include non-neoplastic indications, including inflammation or atherosclerosis.

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