Angiopoietins

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Abstract The formation of new blood vessels plays an important role during the development and progression of a disease. In recent years, there has been a tremendous effort to uncover the molecular mechanisms that drive blood vessel growth in adult tissues. Angiopoietins belong to a family of growth factors that are critically involved in blood vessel formation during developmental and pathological angiogenesis. The importance of Angiopoietin signaling has been recognized in transgenic mouse models as the genetic ablation of Ang-1, and its primary receptor Tie2 has led to early embryonic lethality. Interesting and unusual for a family of ligands, Ang-2 has been identified as an antagonist of Ang-1 in endothelial cells as evidenced by a similar embryonic phenotype when Ang-2 was overexpressed in transgenic mice. In this review, we focus on the functional consequences of autocrine Angiopoietin signaling in endothelial cells.

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2.1 Introduction

Angiogenesis involves the complex signaling between multiple angiogenic growth factors, and requires the coordinated interaction between endothelial and adjacent cells. Vascular endothelial growth factor (VEGF) possesses a dominant role in mediating endothelial cell sprouting, migration, and network formation as indicated by the early lethality of VEGF-deficient mice (Carmeliet et al. 1996; Ferrara et al. 1996; for review see Ferrara et al. 2003; Conway et al. 2001; Risau 1997). The Angiopoietin (Ang) family has primary roles in the latter stages of vascular development and in the adult vasculature, where it controls vessel remodeling and stabilization. Ang-1 has the capability to stimulate Tie2 receptor activation while Ang-2 has been identified as an antagonizing ligand (Suri et al. 1998; Maisonpierre et al. 1997). Ang-2 overexpression in transgenic mice led to embryonic death with a phenotype similar to Ang-1 or Tie2 deletion (Maisonpierre et al. 1997). Thus, genetic evidence suggests that signaling through Tie2 appears to depend on the balance between Ang1 and Ang2. In the quiescent vasculature in adults, Ang1 provides a basal signal to maintain the integrity of the endothelial cells (Brindle et al. 2006). In contrast, Ang-2 induced by VEGF or hypoxia suppresses these effects

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and leads to vessel destabilization. Consequently, effects mediated by Ang-2 allow vessel growth or regression, depending on the presence of additional growth factors (Hanahan 1997).

2.2 Importance of the Angiopoietin/Tie System During Developmental Angiogenesis

Ang-1 and Ang-2 (Davis et al. 1996; Maisonpierre et al. 1997) are best characterized among the Angiopoietin family. Additional members are designated as Ang-3 and Ang-4, and represent diverging counterparts in mice and humans (Valenzuela et al. 1999). Ang-1 and Ang-2 are ligands for the receptor *t*yrosine kinase with *i*mmunoglobulin and *e*pidermal growth factor homology domains 2 (Tie2; Maisonpierre et al. 1997; Davis et al. 1996; Sato et al. 1995; Dumont et al. 1995) with predominant expression in endothelial cells. They share approximately 60% of aminoacid identity (Maisonpierre et al. 1997). Ang-1 has initially been discovered as the primary Tie2 ligand (Davis et al. 1996). Although Ang-1 and Ang-2 act as antagonizing molecules, they bind to Tie2 with similar affinities. They share the same binding domains of the Tie2 receptor, including the first Ig-like loop and the epidermal growth factor-like repeats (Barton et al. 2006; Fiedler et al. 2003). The two highly related members of the Tie receptor tyrosine kinase family, Tie1 and Tie2, display unique extracellular domains: epidermal growth factor repeats, immunoglobulin-like domains, fibronectin-type III repeats, and a separated tyrosine kinase domain in the cytoplasmic region (Dumont et al. 1993; Sato et al. 1993; Schnurch and Risau 1993; Partanen et al. 1992). Although Tie2 is the best established receptor for Ang-1, there are emerging data showing that the ligand may also signal through the related tyrosine kinase Tie1 (Saharinen et al. 2005).

Engagement of Tie2 by Ang-1 is responsible for receptor phosphorylation and the induction of survival signals in endothelial cells (Jones et al. 1999; Papapetropoulos et al. 2000). There is additional evidence that Ang-1 plays an active role in vessels sprouting as Ang-1 overexpression in mice increased vessel density and branching (Suri et al. 1998). Ang-1-mediated endothelial cell sprouting and migration has also been proven in in vitro models (Audero et al. 2004; Hayes et al. 1999; Koblizek et al. 1998). Consistent with these findings, interactions between endothelial and pericytes/smooth muscle cells are stabilized only in the presence of Ang-1, and decreased association of endothelial cells with support cells is evident in Ang-1 mutant mice (Suri et al. 1996). In the adult vasculature, Ang-1 binding to Tie2 is constitutive and essential to maintain endothelium in the quiescent state (Wong et al. 1997; Saharinen et al. 2008; Fukuhara et al. 2008). By contrast, opposing functions have been described for Ang-2. Binding of Tie2 by Ang-2 antagonizes receptor phosphorylation in transgenic animals (Maisonpierre et al. 1997; Reiss et al. 2007), thereby disrupting contacts between endothelial- and periendothelial support cells. Ang-2 also disrupts endothelial monolayer interaction with smooth muscle cells in culture (Scharpfenecker et al. 2005). This process is fundamental for the initiation of vessel sprouting or regression.

Evidence for the importance of the Angiopoietin/Tie2 system for the vascular development is derived from genetic experiments following the ablation of Ang-1 or Tie2 in transgenic mice (Suri et al. 1996; Sato et al. 1995; Dumont et al. 1995). A summary of genetic mouse models available of Angiopoietins and Tie receptors are displayed in Table 2.1. Embryos lacking Tie2 receptor tyrosine kinase or Ang-1 ligand display aberrant vascular development and die around embryonic day E11 as a consequence of insufficient remodeling of the primary capillary plexus. Analysis of the vasculature of mice deficient for Tie2 or Ang-1 has indicated abnormal interactions between endothelial cells and peri-endothelial support cells (Suri et al. 1996; Sato et al. 1995; Dumont et al. 1995). Contrary to these findings, mice with targeted expression of Ang-1 in the skin exhibit larger and

Table 2.1 Transgenic mice resulting from Angiopoietin/Tie deletion and overexpression

Ang-1
Ang-1$^{-/-}$
Lethal at E11–12.5, defective vessel remodeling, enlarged vessels, and poor endothelial cell interaction with perivascular cells, complementary to Tie2$^{-/-}$ phenotype (Suri et al. 1996)
Ang-1 overexpression
Overexpression in skin increases number, size and branching of vessels (hypervascularization), vessel sealing, anti-inflammatory (Suri et al. 1998; Thurston et al. 1999)
Ang-2
Ang-2$^{-/-}$
Lethal at postnatal day 14 (depending on genetic background), normal embryonic vascular development, defects in postnatal angiogenic remodeling (disturbed hyaloid vessel regression), and defects in the lymphatic vasculature (disorganization/hypoplasia in dermal and intestinal lymphatics) (Gale et al. 2002)
Ang-2 overexpression
Ang-2 overexpression in the vasculature, lethal at E9.5–10.5. complementary to Ang-1 and Tie2 mutant phenotypes but more severe, rounded endothelial cells, poor interaction with matrix, endocardial defects (Maisonpierre et al. 1997)
Inducible Ang-2 overexpression in endothelial cells
Tie1 promoter driven, Tet-inducible expression of Ang-2 in endothelial cells, embryonic lethality during gestation, defective collateral artery growth, and smooth muscle cell coverage during pathological angiogenesis (limb ischemia) (Reiss et al. 2007)
Tie receptors
Tie1$^{-/-}$
Die embryonic day >13.5 (E13.5), vessel hemorrhage, edema, rupture, endocardial defects (Sato et al. 1995; Puri et al. 1995; Puri et al. 1999)
Tie2$^{-/-}$
Lethal E9.5–10.5, complementary phenotype to Ang-1$^{-/-}$, defective vessel remodeling, dilated vessels, decreased branching, rounded endothelial cells lacking pericytes, hemorrhage, vessel rupture (Dumont et al. 1994; Sato et al. 1995)
Tie1 and Tie2$^{-/-}$
Similar to Tie2$^{-/-}$ but more severe. Tie1$^{-/-}$embryos sensitive to Tie2 gene dosage, Tie1$^{-/-}$/Tie2$^{-/-}$endothelial cells absent from capillaries of adult chimeric wildtype/double knockout mice (Puri et al. 1999)
Tie1$^{-/-}$/Tie2$^{-/-}$cells have reduced capacity to contribute to hematopoiesis in the adult, but not in the fetus (Puri and Bernstein 2003)

more numerous branched vessels that are resistant to vascular leakage induced by permeability factors, such as VEGF (Suri et al. 1998). These findings support the present concept that the Angiopoietin/Tie2 system plays an important role in the interaction between endothelial and mural cells. Angiogenic remodeling of the mature vasculature requires a progressive disengagement of endothelial cells from the surrounding support cells, and this destabilization can result in vessels sprouting or regression. The distinct expression pattern of Ang-2 at sites of active vascular remodeling (Maisonpierre et al. 1997) and in highly vascularized tumors (Holash et al. 1999; Stratmann et al. 1998) has implicated Ang-2 in the blockade of the Ang-1 stabilizing function to facilitate angiogenesis. In addition, transgenic overexpression in embryonic endothelial cells

resulted in a similar phenotype as the deletion of the Tie2 gene, supporting the view that Ang-2 is an antagonistic ligand (Maisonpierre et al. 1997). However, genetic ablation of Ang-2 in mice resulted in a less severe phenotype, which is compatible with life, as such providing evidence that Ang-2 is not redundant with Ang-1 (Gale et al. 2002). Ang-2 is selectively upregulated in tumor vessels before the onset of VEGF in adjacent tumor cells, and can synergize with VEGF to enhance neovascularization. This indicated that Ang-2 might be antagonist in particular environments, such as in postnatal remodeling or pathological angiogenesis (Gale et al. 2002; Holash et al. 1999).

2.3 Angiopoietins and Tumor-Associated Angiogenesis

The essential role of angiogenesis for the expansion of solid tumors is demonstrated by the observation that avascular tumors are not able to grow beyond a certain size unless they acquire new blood vessels for the supply of nutrients and oxygen (Folkman 1971; Hanahan and Folkman 1996; Yancopoulos et al. 2000). Co-option of existing vessels from the neighboring tissue thereby displays one possible mechanism to promote tumor growth (Holash et al. 1999). In addition, tumor cells provide endothelium-specific growth factors such as VEGF and Angiopoietins for the recruitment of new blood vessel.

During development, Tie2 expression is present on virtually all endothelial cells (Dumont et al. 1995; Sato et al. 1995). In addition, Tie2 expression is increased during physiological and pathological angiogenesis in the adult. However, endothelial cells of the vasculature remain quiescent during adult life. Numerous studies have demonstrated altered expression patterns for Angiopoietin ligands and corresponding Tie receptors in a variety of tumors. This clearly indicated important roles for Angiopoietin/Tie signaling beyond development in experimental models of tumor growth (Reiss et al. 2005; Tait and Jones 2004). Tumor vessels are known to have abnormal phenotypes that include changes in the architecture and assembly of the vessel wall (Morikawa et al. 2002; Ward and Dumont 2002). These vessel abnormalities are likely the cause for increased vascular permeability within the tumor. With respect to potential targeted interventions of angiogenesis in tumors, it is required to decipher the mechanisms that promote or inhibit the vessel growth. Regarding the current knowledge of Angiopoietin biology during tumor angiogenesis, results are controversial and include pro- and antiangiogenic functions for both, Ang-1 and Ang-2. In detail, overexpression of Ang-1 in experimental tumors induced stabilization by the recruitment of pericytes and smooth muscle cells to recently formed vessels (for review see (Reiss et al. 2005; Tait and Jones 2004)). Consequently, reduced tumor growth or tumor stasis has been reported by a number of research laboratories in experimental tumors, such as colon-, lung-, mammary- and squamous cell carcinoma (Stoeltzing et al. 2003; Hawighorst et al. 2002; Tian et al. 2002; Stoeltzing et al. 2002; Ahmad et al. 2001; Yu and Stamenkovic 2001; Hayes et al. 2000). However, findings derived from certain tumor types, including our own results, indicate proangiogenic functions when overexpressing Ang-1 (Shim et al. 2002; Machein et al. 2004). These controversial findings may be related to differences in the presence of growth factors within the tumor types investigated. Although effector functions of Ang-1 on the outcome of tumor growth are not completely resolved, an improved vessel architecture in the presence of Ang-1 is typically observed. This is mainly exerted by a higher degree of pericyte coverage. Ang-2 in contrast, is necessary to initiate vessel sprouting and is associated with pericyte loss of the host tumor vasculature (Reiss et al. 2009; Cao et al. 2007; Machein et al. 2004; Zhang et al. 2003; Hu et al. 2003; Ahmad et al. 2001; Yu and

Stamenkovic 2001; Etoh et al. 2001; Tanaka et al. 1999). This is achieved through the destabilizing actions on the previously quiescent vasculature. At present, findings that have been reported for the role of Ang-2 during tumor progression are not well understood. However, what can be concluded from the literature with regard to Ang-2 functions in tumors is a shift in the balance of Ang-1 and Ang-2 in favor of Ang-2. Consequently, instability of the host vasculature and aberrant, nonfunctional vessels were often observed (Reiss et al. 2009; Reiss et al. 2005). Furthermore, Lewis lung carcinoma, mammary carcinoma, gastric and brain tumors overexpressing Ang-2 display increased frequencies of metastatic dissemination and are highly invasive (Hu et al. 2003; Yu and Stamenkovic 2001; Etoh et al. 2001). In summary, evidence from the literature implies that vessel destabilizing defects of Ang-2 might be caused by the disengagement of pericytes from the tumor vessels, and the defective cellular linings caused by openings between endothelial cells might to some extent explain increased permeabilities within tumor vessels (Hashizume et al. 2000). Ang-2-mediated functions during tumor angiogenesis are illustrated in Fig. 2.1.

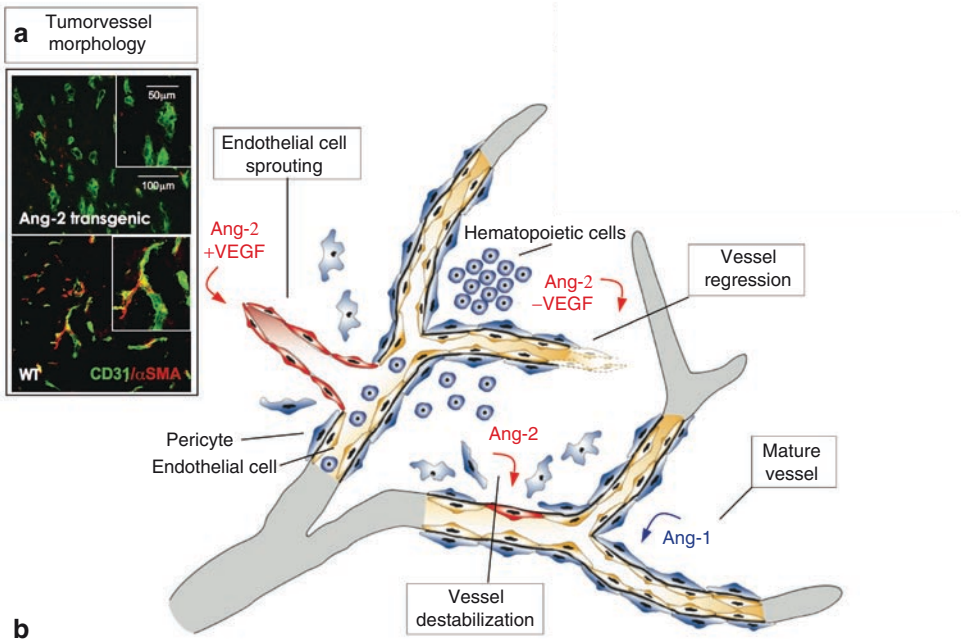

Fig. 2.1 Influence of the Angiopoietin/Tie system on the formation of new blood vessels in tumors. Inducible Ang-2 expression in the vasculature of transgenic animals (adapted from Reiss et al. 2007) leads to increased vascular densities (*green*: αCD31 immunohistochemistry) in subcutaneous Lewis lung tumors, indicative for excessive vessel sprouting (**a**). Furthermore, reduced in red pericyte coverage (**a**, indicated by αSMA labeling) in red is prominent within the tumor vasculature (insets: higher magnification). A schematic drawing of Angiopoietin/Tie mediated functions in tumors is illustrated in (**b**). Ang-1 contributes to the stabilization and maturation of new blood vessels in tumors. In concert with VEGF, Ang-2 destabilizes the vasculature and leads to vessel sprouting or regression (modified after (Reiss et al. 2005)) Ang-2 additionally might be able to promote the recruitment of hematopoietic cells during tumor progression or other pathological conditions as Ang-2 deficient mice display delayed inflammatory cell recruitment (Fiedler et al., 2006)

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Ang-2 is highly regulated at the transcriptional level (Hegen et al. 2004) and induced in endothelial cells in areas of active angiogenesis (Holash et al. 1999; Stratmann et al. 1998) such as in tumors, making it an attractive target for therapeutic intervention. Moreover, Ang-2 has been associated with poor prognosis and lymph-node metastasis in human tumors pointing towards a need for therapeutic intervention (Ochiumi et al. 2004; Hu et al. 2003; Sfiligoi et al. 2003; Etoh et al. 2001). Pharmacological inhibition of Angiopoietin functions by sequestration with soluble Tie2 (Siemeister et al. 1999; Lin et al. 1998; Lin et al. 1997) or by the usage of dominant-negative Tie2 mutants has earlier been shown to have a negative impact on tumor growth and progression. Furthermore, neutralization of Ang-2-Tie2 interactions (Oliner et al. 2004) or overexpression of Ang-2 (Cao et al. 2007) inhibited tumor angiogenesis and tumor growth in mice. Whether targeted intervention of Ang-2 will be applicable in human tumors as well remains to be elucidated in the future.

In spite of the intense research on Angiopoietin functions during physiological angiogenesis (Suri et al. 1998; Maisonpierre et al. 1997) and tumor angiogenesis (Holash et al. 1999; Stratmann et al. 1998), the biological actions of Angiopoietins during tumor progression have not been fully ascertained. Clearly, molecular mechanisms for a more precise understanding of Angiopoietin/Tie-mediated effector functions that may lead to increased vessel integrity or drive vascular remodeling/regression are largely missing. In detail, it is well established that tumor vessels display highly permeable vessels, but only few studies focused on the cellular basis of tumor vessel permeability (McDonald et al. 1999; Morikawa et al. 2002; Hashizume et al. 2000). For instance, it is largely unknown how Ang-1 prevents and Ang-2 increases vessel permeability, although they both seem to interfere with cell–cell interactions and junctional proteins (e.g., stabilize or destabilize EC junctions in vitro) (Gamble et al. 2000; Scharpfenecker et al. 2005). Recently, two reports provided some insight in the molecular mechanism of Ang-1-induced Tie2 signaling in regulating endothelial cell quiescence vs. angiogenic activation (Saharinen et al. 2008; Fukuhara et al. 2008). Using an in vitro system, the authors elegantly showed that Ang1-activated Tie2 assembles novel signaling complexes leading to preferential activation of different downstream signal transduction proteins in the presence vs. absence of cell–cell contacts.

In our own studies, we analyzed the cellular consequences of Angiopoietin expression on tumor vessel morphology in two mouse mammary carcinoma models which naturally displayed distinct Ang/Tie2 expression profiles and generated mammary carcinomas to express Ang-1 and Ang-2 (Reiss et al. 2009). Analysis of Angiopoietin-overexpressing mammary xenografts at the ultrastructural level strongly supported the hypothesis that Ang-1/Tie2 signaling is essential for proper vessel organization, and suggested that Ang-2 is mainly responsible for the induction of disrupted endothelial cells (Reiss et al. 2009). Furthermore, our findings supported the hypothesis that Ang-2 can trigger important signals that are decisive for a switch of vascular phenotypes within tumors. Current results also imply that disruption of cell–cell contacts between endothelial cells might be inversely regulated by Ang-1 and Ang-2. For instance, it has been shown that VEGF-mediated disruption of cell–cell interactions is attributed to the dissociation of β-catenin from VE-cadherin (Wang et al. 2004). Interestingly, this effect can be opposed by Ang-1 as it specifically counteracts the ability of VEGF to induce the phosphorylation-dependent redistribution of VE-cadherin, thereby rescueing the endothelial barrier function (Gavard et al. 2008). Our own observations in tumors of Ang-2 transgenic animals (unpublished data);

(Reiss et al. 2007) suggest that high serum levels of Ang-2 are mainly responsible for improper vessel function. Future studies will help to unravel participating cellular elements during pathological angiogenesis more precisely.

2.4 Therapeutic Implications

Clearly, the effects of Angiopoietins in vivo suggest that manipulation of this ligand could have therapeutical potential. Pharmacological inhibition of Angiopoietin functions by sequestration with soluble Tie2 (Siemeister et al. 1999; Lin et al. 1998; Lin et al. 1997), or by the usage of dominant-negative Tie2 mutants has previously been shown to have a negative impact on tumor growth and progression. Until now, novel inhibition strategies for cancer treatment are at the preclinical level in murine angiogenesis models. Possible manipulation includes neutralization of Ang-2-Tie2 interactions (Oliner et al. 2004) or overexpression of Ang-2 (Cao et al. 2007), which inhibited tumor angiogenesis and tumor growth in mice. Whether targeted intervention of Ang-2 will be favorable in human tumors needs to be determined in the future. However, interfering with Ang-2 will shift the relative level of Ang1 and Ang-2. In case of Ang-2 inhibition, increased levels of Ang-1 will be beneficial for vessel perfusion and permeability and might lead to increased angiogenesis. Thus, Angiopoietin dosage is critical for the net outcome on angiogenesis inhibition and has to be taken into account for possible therapeutic interventions. Interestingly, VEGFR2 blockage can temporarily normalize tumor vessel structure (increased pericyte coverage) and lead to vascular normalization via expression of Ang-1 (Winkler et al. 2004). As a consequence, transient stabilization of vessels and improved oxygen delivery to hypoxic zones is achieved following VEGF neutralization which may facilitate drug delivery into tumors. The delivery of drugs utilizing the Angiopoietin/Tie system as a vehicle has recently been reported by De Palma et al. (De Palma et al. 2008). In this study, the authors exploited the tumor-homing ability of proangiogenic Tie2-expressing monocytes to deliver IFN-α to tumors which inhibited tumor growth and metastasis.

The complex interplay between complementary and yet conflicting roles of both Angiopoietins during tumor angiogenesis has impeded the development of drugs interfering with this angiogenic pathway. Collectively, a better understanding of the molecular mechanisms of Ang-1 and Ang-2 signaling during pathological angiogenesis may set the stage for novel therapies targeting this pathway.

2.5 Conclusions

Angiopoietins (Ang-1 and Ang-2) and their Tie receptors have wide-ranging effects on tumor malignancy that includes angiogenesis, vascular stabilization and permeability, and the recruitment of inflammatory cells. These multifaceted pathways present a valuable opportunity in developing novel inhibition strategies for cancer treatment. Ang-1 is not significantly upregulated in the majority of tumors. In contrast, Ang-2 is highly induced in the tumor vasculature, even prior to the induction of VEGF. As such, a shift in the Ang-1:Ang-2 balance in advantage of Ang-2 is the consequence. Therefore, it is evident that Ang-2 dosage is critical in shaping the outcome of angiogenesis. However, the regulatory role of Ang-1 and Ang-2 in tumor angiogenesis remains controversial, and the complex interplay between complementary yet conflicting

roles of both the Angiopoietins during adult angiogenesis need to be addressed more precisely, for example, by using Ang-2 transgenic animals. Further studies are needed to discern how Angiopoietins cooperate with other molecules and to develop new strategies for therapy targeting the Ang/Tie pathway.

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