Tie2 is tied at the cell-cell contacts and to extracellular matrix by Angiopoietin-1

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Abbreviations: Angptl, angiopoietin-like family; FAK, focal adhesion kinase; ISV, intersomitic vessel; KLF2, Krüppel-like factor 2; VE-cadherin, vascular endothelial cadherin; VE-PTP, vascular endothelial protein tyrosine phosphatase; VSMC, vascular smooth muscle cells

Abstract

Angiopoietin-1 (Ang1) binds to and activates Tie2 receptor tyrosine kinase. Ang1-Tie2 signal has been proposed to exhibit two opposite roles in the controlling blood vessels. One is vascular stabilization and the other is vascular angiogenesis. There has been no answer to the question as to how Tie2 induces two opposite responses to the same ligand. Our group and Dr. Alitalo's group have demonstrated that trans-associated Tie2 at cell-cell contacts and extracellular matrix (ECM)-anchored Tie2 play distinct roles in the endothelial cells. The complex formation depends on the presence or absence of cell-cell adhesion. Here, we review how Ang1-Tie2 signal regulates vascular maintenance and angiogenesis. We further point to the unanswered questions that must be clarified to extend our knowledge of vascular biology and to progress basic knowledge to the treatment of the diseases in which Ang1-Tie2-mediated signal is central.

Keywords: angiopoietin-1; angiostatic proteins; cell movement; cell proliferation; extracellular signal-regulated MAP kinases; neovascularization, pathologic; neovascularization, physiologic; proto-oncogene proteins c-akt; receptor, TIE-2

Introduction

Blood vessels are developed in embryogenesis

and reorganized in ischemia and cancer to deliver oxygen and nutrients to the tissues. Vascular formation during embryogenesis requires both vasculogenesis and angiogenesis, whereas neovascular formation in adult requires angiogenesis (Adams and Alitalo, 2007). Therefore, in the adult vasculature, blood vessels are maintained by the balance between vascular stabilization and vascular angiogenesis. Vascular stabilization depends on the coordination of cell-cell adhesion and cell survival. Vascular angiogenesis depends on the de-adhesion of pre-existing cell-cell adhesion and migration/proliferation of endothelial cells.

The inter-endothelial cell adhesion and the adhesion between endothelial cells and mural cells are regulated by cell-cell adhesion molecules, and thereby contribute to cell survival. Vascular endothelial cadherin (VE-cadherin) and N-cadherin elicit adhesive functions in inter-endothelial cell junctions and endothelial-mural cell junctions, respectively. In addition to the adhesion molecules, endothelial cell-specific growth factors and their receptor-mediated signals play central roles in endothelial cell survival. Receptor tyrosine kinases including vascular endothelial growth factor receptor (VEGF-R), Eph, and Tie family are required for vascular formation (Adams and Alitalo, 2007).

Among endothelial cell-specific receptors, Tie2 receptor-mediated signaling exhibits a vascular protective action. Tie2 constitutes the Tie family and dimerizes with Tie1 and functions as a receptor for angiopoietin-1 (Ang1) (Saharinen *et al.*, 2005). The Ang family consists of Ang1, Ang2, Ang3, and Ang4 (Valenzuela Koh *et al.*, 1999; Koh *et al.*, 2002; Lee *et al.*, 2004). Of them, Ang1 and Ang2 are well characterized (Gale *et al.*, 2002; Fiedler *et al.*, 2006a; Fiedler and Augustin, 2006). Ang1 binds to and activates Tie2, while Ang2 has similar affinity to Tie2 to compete with the binding of Ang1 to Tie2. Therefore, Ang2 is thought to inhibit Ang1-mediated vascular protective action. It is also reported that Ang1-Tie2 is essential for developmental angiogenesis, ischemia-induced angiogenesis, and tumor angiogenesis. Hence, the Ang1-Tie2 signal plays central roles in two opposing vascular regulation: vascular quiescence and angiogenesis (Figure 1). However, it is not known how Ang1-Tie2 signal regulates two paradoxical actions. Here, we focus on Ang1-Tie2 signaling and do not refer to the details of other Ang family

Figure 1. Schematic illustration of two opposite functions mediated by angiopoietin-1 (Ang1)-Tie2 signaling. Ang1-Tie2 regulates both angiostasis and angiogenesis. In the mature vessels, vascular endothelial cells are supported by pericytes or vascular smooth muscle cells (VSMCs) that release Ang1. In clear contrast, it is reported that Ang1-Tie2 signal is required for developmental and pathological angiogenesis in which inter-endothelial cell adhesions are disrupted by vascular endothelial growth factor (VEGF).

and Tie1. Ang and Tie are elegantly reviewed elsewhere (Brindle *et al.*, 2006; Fiedler and Augustin, 2006). We and Alitalo's group have recently demonstrated the different signals mediated by Tie2 in the presence or absence of endothelial cell-cell contacts (Fukuhara *et al.*, 2008; Saharinen *et al.*, 2008). One is trans-associated Tie2 by Ang1 and the other is extracellular matrix-anchored Tie2 by Ang1. In this review, we further suggest that these distinct signals may play specific roles in vascular quiescence and angiogenesis.

Ang1-mediated vascular stabilization

Ang1 induces endothelial cell survival, inhibits vascular leakage, and reduces vascular inflammation. All these effects of Ang1 on vascular endothelial cells lead to preservation of vasculature from vascular damage. Ang1 activates phosphatidylinositol 3-kinase and subsequently induces phosphorylation of AKT, which plays essential roles in cell survival (Kim *et al.*, 2000; Papapetropoulos *et al.*, 2000). Pro-apoptotic activities of the AKT substrates are inhibited by the phosphorylation by AKT, resulting in cell survival (Manning and Cantley, 2007; Duronio, 2008). Ang1 reduces vascular leakage by strengthening PECAM-1 and VE-cadherin-regulated inter-endothelial adhesion (Gamble *et al.*, 2000). *In vivo*, Ang1 antagonizes VEGF-induced vascular permeability (Thurston *et al.*, 2000). Moreover, Ang1 protects endothelial cells from VEGF-induced inflammation by inhibiting the expression of ICAM-1 and VCAM-1 on endothelial cells, which are required for the attachment of inflammatory cell infiltration into the vasculature

(Kim *et al.*, 2001b). Accumulating evidence indicate that the inter-endothelial cell-cell adhesion and Ang1-Tie2 signaling co-operatively regulate endothelial cell survival and vascular integrity to maintain the established vasculature. Ang1 is produced from mural cells that support the endothelial cells. Thus, both inter-endothelial cell adhesions and endothelial-mural cell adhesions are important for maintenance of vascular integrity. By contrast, it is of note that Ang2 produced in endothelial cells and stored in Weibel-Palade bodies are released to induce inflammation, resulting in destabilization of endothelial cell adhesion (Fiedler *et al.*, 2004, 2006b; Scharpfenecker *et al.*, 2005). Thus, stabilization and destabilization may be balanced by the counteraction on Ang1 and Ang2.

Ang1-Tie2 in angiogenesis

In clear contrast, during angiogenesis cell-cell adhesions must be disassembled to accelerate the branching and sprouting of new vessels. Therefore, the vascular reorganization process must overcome stabilization. Ang1-Tie2 signal is required for developmental angiogenesis during embryogenesis (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996). In adult vascular angiogenesis, the activation of Tie2 has been reported in tumor vessels and remodeling vessels, suggesting the involvement of Ang1-Tie2 signal in adult angiogenesis (Wong *et al.*, 1997; Peters *et al.*, 2004).

Angiogenesis requires vascular endothelial cell proliferation and migration. Although the proliferation of vascular endothelial cells has been controversial (Witzenbichler *et al.*, 1998; Kanda *et al.*, 2005), ERK activation, the hallmark of proliferation, by Ang1 has been reported (Kim *et al.*, 2002; Harfouche *et al.*, 2003). It is still unclear how Ang1-Tie2 signal induces ERK activation. Tie2-mediated endothelial cell migration involves Dok-R. Dok-R is an adaptor molecule, which contains multi-phosphotyrosines and signals multiple downstream molecules including Nck (Master *et al.*, 2001; Jones *et al.*, 2003). Cell migration is also controlled by Rho family GTPases, Rac and Rho. The activation of Rac1 by Ang1 is suggested to be involved in endothelial cell migration (Cascone *et al.*, 2003).

The distinct roles of trans-associated Tie2 and extracellular matrix-anchored Tie2

We and Dr. Alitalo's group have recently clarified how Tie2 responds to Ang1 and activates distinct

downstream signaling in the presence or absence of cell-cell contacts. First, we both noticed that the localization of Tie2 engaged with Ang1 depends on the presence or absence of cell-cell contacts. Tie2 is recruited to the cell-cell borders in the presence of cell-cell contacts, whereas it is localized to cell-ECM contacts in the absence of cell-cell contacts. In the cell-cell contacts, Ang1 induces trans-association of Tie2, thereby locating Tie2 at the borders. Ang1 is capable of binding to ECM, resulting in anchoring of Tie2 to cell-ECM attachments.

We examined the activation of AKT and ERK, which are hallmarks of cell survival and proliferation/migration, respectively (Eliceiri *et al.*, 1998; Manning and Cantley, 2007). AKT is preferentially activated by Ang1 in the presence of cell-cell contacts. Consistently, Alitalo's group clearly showed Ang1-induced eNOS activation that is a substrate of AKT. In clear contrast, ERK is activated in the absence of cell-cell contacts. We delineated the signaling by which ERK is preferentially activated. Ang1 tied Tie2 to ECM, thereby accelerating the attachment of the cells to ECM. In turn, the assembly of initial cell-ECM adhesions, focal complexes, is promoted. Focal adhesions implicate focal adhesion kinase (FAK) in the activation of ERK, as evidenced by the fact that depletion of FAK resulted in the decreased ERK activation by Ang1. Alitalo's group noticed that in migrating endothelial cells lacking the cell-cell adhesions, Dok-R is phosphorylated by Ang1 and colocalized with Tie2 at the cell-ECM in the rear of the motile cells, suggesting the involvement of Dok-R in endothelial cell migration upon Ang1 stimulation.

Ang1 may not induce sprouting or branching from the pre-existing vessels, because pre-existing vessels are stabilized by Ang1. However, once cell-cell adhesion is disrupted by VEGF, Ang1 accelerates angiogenesis cooperatively with VEGF (Asahara *et al.*, 1998). This potential angiogenic activity of Ang1 is confirmed in rabbit ischemic hind limb model (Chae *et al.*, 2000). It is of note that Ang1 counteracts VEGF in the presence of cell-cell adhesion to inhibit VEGF-induced inflammation and VEGF-induced permeability (Gamble *et al.*, 2000; Kim *et al.*, 2001a). (We discuss this point later in this manuscript.)

How does Ang1-Tie2 signal regulate vascular integrity?

Alitalo's group have demonstrated that Ang1 reduces vascular endothelial cell permeability in association with vascular endothelial protein tyrosine phosphatase (VE-PTP). VE-PTP enhances VE-

cadherin-mediated cell-cell adhesion and reduces vascular permeability (Nawroth *et al.*, 2002). The important role of VE-PTP for vascular development is confirmed by knockout mice of VE-PTP (Baumer *et al.*, 2006). In addition, it has been reported that VE-PTP interacts with Tie2 (Fachinger *et al.*, 1999). Therefore, the same authors investigated the involvement of VE-PTP in the reduction of permeability mediated by Ang1-Tie2 and showed enhanced colocalization of Tie2 with VE-PTP.

We identified a panel of genes upregulated by Ang1-Tie2 signal in the presence of cell-cell contacts using microarray analyses. Among them, we further focused on a transcription factor, Krüppel-like factor 2 (KLF2). KLF2 belongs to a zinc-finger family of transcription factor (Lomberg and Urrutia, 2005; Atkins and Jain, 2007) and exhibits vascular protective roles (SenBanerjee *et al.*, 2004; Bhattacharya *et al.*, 2005). Therefore, we hypothesized that Ang1-Tie2-dependent stabilization of vasculature is mediated by KLF2. First, we examined the involvement of MEF2 transcription factor in Ang1-induced KLF2 expression, because MEF2C is shown to regulate KLF2 expression in shear stress-induced KLF2 expression (Parmar *et al.*, 2006). Second, we tested whether MEF2-dependent KLF2 expression was mediated by phosphatidylinositol-3 kinase/AKT. Finally, we investigated whether the inhibitory effect of Ang1 on VEGF-mediated inflammation was dependent on KLF2. Collectively, we delineate a role for the Tie2-PI3K/AKT-MEF2-KLF2 signaling activated by Ang1 in anti-inflammatory action (Sako *et al.*, 2008). We summarize the molecular mechanism how Ang1 maintains vascular quiescence and Ang1 accelerates vascular angiogenesis in the presence or absence of cell-cell adhesions, respectively (Figure 2).

The unsolved important questions in Angiopoietin-Tie signaling

There are still unanswered questions to fully understand Ang-Tie signaling, although Tie2 and Ang1 have been extensively studied. Is Tie1 a real receptor of Ang1? If so, what is the difference between Tie1 and Tie2? We first need to clearly analyze when and where Tie1 and Tie2 are expressed in the vasculature of the whole body. Development of anti-Tie1-specific antibody and anti-Tie2-antibody that can be used for immunohistochemistry will help us to scrutinize the expression of these two important receptors (Brown *et al.*, 2000). The simultaneous examination of Tie1 mRNA and Tie2 mRNA by *in situ* hybridization may also help to investigate the functional difference bet-

Figure 2. Distinct roles of trans-associated Tie2 for vascular quiescence and extracellular matrix (ECM)-anchored Tie2 for angiogenesis. Trans-associated-Tie2 at the cell-cell contacts preferentially activated AKT and upregulates Krüppel-like factor 2 (KLF2) transcription factor, resulting in cell survival and resistance to inflammation by VEGF. On the other hand, ECM-anchored Tie2 induces ERK activation preferable for cell migration and proliferation once cell-cell adhesions are disrupted by VEGF or other signaling. Therefore, Tie2-expressing cells that dissociate from surrounding endothelial cells contribute to angiogenesis.

ween two receptors for angiogenesis.

Does Ang2 really antagonize Ang1 for the activation of Tie2? Forced expression of Ang2 disrupts the embryonic angiogenesis (Maisonpierre *et al.*, 1997). It is very important to clarify when Ang2 is released from vascular endothelial cells and which signal induces Ang2 release, as several signaling cascades that regulate the expression and release of Ang2 have been identified (Jang *et al.*, 2008; Simon *et al.*, 2008; Xue *et al.*, 2008). What is the physiological role for Ang2? It has been suggested that Ang2 induces inflammation to remodel the vasculature (Audoy-Remus *et al.*, 2008; Tressel *et al.*, 2008). Tie2 family receptors were originally identified in hematopoietic cells (Batard *et al.*, 1996; Hashiyama *et al.*, 1996). It is necessary to underpin this Ang2-mediated inflammation to extend the involvement of Ang family molecules in angiogenesis. Especially, it would be interesting to examine whether Tie2 family receptors are expressed in M1 macrophages and if they are activated by Ang2 released from vascular endothelial cells (Gordon, 2003).

There are still uncharacterized angiopoietins, Ang3 and Ang4 (Valenzuela *et al.*, 1999; Xu *et al.*, 2004). Besides these, angiopoietin-like family (Angptl) molecules have been recently shown to be involved in angiogenesis (Hato *et al.*, 2008). The receptors for Angptls are not yet isolated, to date. Thus, to thoroughly understand Ang- and Angptl-regulated angiogenesis, the ligand-receptor coupling should be molecularly defined. We here summarize what should be clarified in the future

Figure 3. Unsolved questions in Ang-Tie signaling. The role of Ang1-Ang4 as well as angiopoietin-like factors (Angptl) and their specific receptors must be identified to clearly understand Ang-Tie signaling. Additionally, we need to know when and where Ang-Tie family members are expressed and activated during angiogenesis (e.g. during inter-somitic vessel (ISV) formation after sprouting from dorsal aorta (DA)). Furthermore, it would be interesting to examine the involvement of M1 macrophages in inflammatory angiogenesis. To test this possibility, it should be clarified whether Tie receptors are expressed in M1 macrophages and whether Ang2 activates M1 macrophages.

(Figure 3). Once we can fully characterize Angs and Tie family receptors, we can optimize the treatment for anti-angiogenesis or pro-angiogenesis.

Conclusion

We overviewed the Ang1-Tie2-regulated signals for angiostasis and angiogenesis. The diversity of downstream signaling activated by Tie2 depends on the presence or absence of cell-cell contacts. Alitalo's group and ourselves have now demonstrated where and how Ang1-Tie2 complexes are formed in the vascular endothelial cells to determine the intracellular signaling. Finally, we further refer to the points that are unsolved but require answering in order to extend our knowledge to clinical translation in the treatment of patients who are suffering from excessive angiogenesis in tumors, ischemia in coronary artery diseases (Carmeliet, 2003, 2005), and leaky vessels in lymphedema (Lohela *et al.*, 2003; Karpanen and Makinen, 2006).

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