Context-dependent functions of angiopoietin 2 are determined by the endothelial phosphatase VEPTP

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The angiopoietin (ANGPT)–TIE2/TEK signaling pathway is essential for blood and lymphatic vascular homeostasis. ANGPT1 is a potent TIE2 activator, whereas ANGPT2 functions as a context-dependent agonist/antagonist. In disease, ANGPT2-mediated inhibition of TIE2 in blood vessels is linked to vascular leak, inflammation, and metastasis. Using conditional knockout studies in mice, we show TIE2 is predominantly activated by ANGPT1 in the cardiovascular system and by ANGPT2 in the lymphatic vasculature. Mechanisms underlying opposing actions of ANGPT2 in blood vs. lymphatic endothelium are poorly understood. Here we show the endothelial-specific phosphatase VEPTP (vascular endothelial protein tyrosine phosphatase) determines TIE2 response to ANGPT2. VEPTP is absent from lymphatic endothelium in mouse in vivo, permitting ANGPT2/TIE2-mediated lymphangiogenesis. Inhibition of VEPTP converts ANGPT2 into a potent TIE2 activator in blood endothelium. Our data support a model whereby VEPTP functions as a rheostat to modulate ANGPT2 ligand effect on TIE2.

angiopoietin–TIE2 pathway | VEPTP | angiogenesis | lymphangiogenesis | tyrosine kinase

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The angiopoietin–TIE2 (tyrosine kinase with Ig and EGF homology domains, also known as TEK) receptor tyrosine kinase pathway regulates vascular homeostasis, maturation, and remodeling, and has been described as the “gatekeeper” of vascular quiescence (1–4). TIE2 phosphorylation enhances vascular stability by promoting endothelial cell survival, reducing responsiveness to inflammatory stimuli, and strengthening cellular junctions in mature vessels (5–10). The primary TIE2 agonist, angiopoietin 1 (ANGPT1), is secreted from perivascular cells, acting in a paracrine manner (7, 11). A second ligand, ANGPT2, has been described as a context-dependent agonist/antagonist, despite having similar receptor affinity as the agonistic ligand ANGPT1 (12–14). Unlike ANGPT1, ANGPT2 is secreted by endothelial cells and acts on the TIE2 receptor in an autocrine manner (15).

The ANGPT–TIE2 pathway has attracted attention due to strong associations and causal links with human diseases, including rare genetic disorders, such as hereditary vascular malformations and primary congenital glaucoma (16–18), as well as common diseases, such as sepsis, cancer, diabetes, and cardiovascular disease (7, 19, 20). Vigorous efforts have been made to understand and translate this pathway to the clinic. However, a major question remains: why does ANGPT2 display opposing context-dependent roles in different vascular beds (14)? In blood endothelial cells (BECs), ANGPT2 is described as an antagonist of ANGPT1-mediated TIE2 activation (1, 3, 12, 14), while the situation is reversed in lymphatic endothelial cells (LECs), where ANGPT2 serves as the primary TIE2 agonist (21).

In the blood endothelium, where TIE2 signaling plays an important role in vascular stability, elevated levels of circulating ANGPT2 in vascular diseases, such as sepsis, result in TIE2 inhibition, leading to increased capillary leakage and poor clinical outcomes (2, 19). Developmental mouse models provide further support for ANGPT2-mediated antagonism of ANGPT1–TIE2 activation in blood vessels (1, 3, 4). Deletion of either Angpt1 or Tie2 results in embryonic lethality at embryonic day (E) 10.5 due to severe defects in cardiovascular development (7–11). This phenotype is reproduced by endothelial overexpression of Angpt2, supporting an agonistic role for ANGPT2 in the blood endothelium (12).

In contrast to its antagonistic role in the blood vasculature, ANGPT2 functions as a TIE2 agonist in the lymphatic endothelium (1, 3, 21). Angpt2 knockout mice display lymphatic defects, including chylous ascites, as well as a sprouting defect in the retinal blood vascular capillaries (22–24). Intriguingly, only the lymphatic phenotypes were rescued by the obligate TIE2 agonist ANGPT1, supporting an agonistic role for ANGPT2 specific to the lymphatic endothelium (22–24).

Two recent papers have suggested that TIE2 is not required for lymphatic function in vivo, raising questions about the mechanism of ANGPT2-mediated lymphangiogenesis (25, 26). However, here we report that LEC-specific loss of TIE2 phenocopies the lymphatic defects observed in Angpt2 knockout mice, confirming that TIE2 is required for lymphatic development. Based on these data, outcomes are likely inherent to the regulation of lymphatic endothelial cell survival and function.

Significance

Reducing vascular leakage and stabilizing the endothelium through activation of the angiopoietin (ANGPT)–TIE2 receptor tyrosine kinase pathway is a promising therapeutic strategy for vascular diseases. ANGPT2 is one of two major ligands for the TIE2 receptor. Uniquely, ANGPT2 possesses an agonistic role in lymphatic endothelium, but acts as a competitive antagonist in blood endothelium. The molecular basis for the opposing actions of ANGPT2 in these two vascular beds is poorly understood. Here we demonstrate that the absence of VEPTP expression in the lymphatic endothelium confers an agonist function of ANGPT2 on TIE2 receptor, but VEPTP expression in blood endothelium abrogates its activity. Our findings provide mechanistic insights needed to advance therapeutic targeting of this pathway.

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we hypothesize that the context-dependent agonist/antagonist function of ANGPT2 and its opposing effects on TIE2 in different vascular beds (i.e., LEC vs. BEC) might be explained by differential expression of molecular components of the pathway, including negative regulators, such as the endothelial specific phosphatase, vascular endothelial protein tyrosine phosphatase (VEPTP) (27–30). To elucidate the molecular basis of opposing functions of ANGPT2 in LECs vs. BECs, we generated a series of gene-modified mouse lines and determined a critical cell-autonomous role for TIE2 signaling in lymphangiogenesis. We found that VEPTP is absent from LECs but abundant in BECs, and then used cell-biologic and proteomic-based approaches to explore the effect of VEPTP on ANGPT2–TIE2 activity. Our results show that VEPTP functions as a molecular “rheostat,” modulating receptor sensitivity to enable discrimination between ANGPT ligands, and provide a molecular mechanism to explain the opposing roles of ANGPT2 in blood and lymphatic vasculature.

Results

ANGPT2–TIE2 Signaling Is Essential for Embryonic Lymphangiogenesis. To identify the molecular basis of the differential functions of ANGPT2 in LECs and BECs, we characterized the role of ANGPT2–TIE2 signaling in lymphatic development, where ANGPT2 has a well-defined agonistic role (22–24). As expected, whole-body Tie2 deletion from conception using the Rosa26RTTA::tetO Cre bitransgenic system in mice harboring a Tie2 conditional by inversion (COIN) allele (Tie2Δ3.1Δ10.5) resulted in embryonic lethality between E9.5 and E10.5 (17, 18). However, embryos induced at E12.5 were found to survive until late gestation, allowing analysis of the lymphatic vasculature. At E16.5, all embryos were found alive. However, subcutaneous edema was observed in Tie2Δ3.1Δ10.5 knockout embryos, which was never observed in Tie2 wild-type or heterozygous controls (Fig. 1). This result was recapitulated in lymphatic-specific Tie2 knockout embryos generated using Prox1CreER (Tie2Δ3.1Δ10.5) mice, indicating that the edema was lymphatic in origin (Fig. 1B).

Compared with control littermates, immunostaining revealed a paucity of PROX1+ lymphatic vessels in the dorsal skin of Tie2Δ3.1Δ10.5 knockout mice at E14.5, confirming the importance of lymphatic-expressed TIE2 in lymphangiogenesis (Fig. 1C). Interestingly, unlike the phenotype observed in lymphatic-specific knockout embryos, dorsal skin lymphatics of whole-body knockout embryos were enlarged compared with littermate controls (Fig. S1A). This enlargement could be due to the combined deleterious effects of TIE2 deletion on BECs and LECs.

To determine the role for each ANGPT ligand in dermal lymphangiogenesis, we compared the phenotypes of either single or combined deletion of Angpt1 and Angpt2 genes with Tie2 conditional knockout mice. Unlike the marked edema of Tie2Δ3.1Δ10.5 embryos, single Angpt1 or Angpt2 whole-body knockout embryos induced at E12.5 had no apparent edema when dissected at E16.5 (Fig. 1D and Fig. S1B). The fact that loss of the TIE2 receptor led to a more severe phenotype than loss of either ligand alone suggested compensation or cooperative roles of the two ANGPT ligands. To test this possibility, we generated compound mutants lacking both Angpt1 and Angpt2 from E12.5 onward. This simultaneous loss of ANGPT1 and ANGPT2 expression (A1ΔA2) recapitulated the phenotype of Tie2Δ3.1Δ10.5 mice (Fig. 1D and Fig. S1B) and embryos exhibited marked edema.

ANGPT2–TIE2 Signaling Is Essential for Mesenteric Lymphatic Development. Angpt2 has a well-described role in the mesenteric and intestinal lymphatic vasculature. Because Tie2 whole-body or lymphatic knockout embryos induced at E12.5 were not viable, we tested whole-body deletion at a range of time points (22–24). Deletion at E13.5 or later resulted in viable mutant offspring. Tie2Δ3.1Δ10.5 knockout pups exhibited chylous ascites with severely disturbed lymphatic vessel morphology, indicating defects of mesenteric lymphatic function (Fig. S2). We then examined the role of each ANGPT ligand in this TIE2-mediated developmental process. Whole-body Angpt1 knockout pups induced at E13.5 had no apparent phenotype, but chylous ascites were observed in mice lacking Angpt2 alone or both Angpt1 and Angpt2 (Fig. S2B). Deletion of Tie2 after E15.5 did not result in overt chylous ascites (Fig. S2A), although a reduced number of lymphatic valves was observed in the mesentry (Fig. S3).

To better understand the role of ANGPT2 in mesenteric lymphatic development, we utilized a cell-type-specific approach to delete Angpt2 in endothelial cells (24). In contrast to the well-developed lymphatic vessels in control mice, endothelial deletion of Angpt2 using a lymphatic-expressed Lyve1-Cre (Angpt2ΔΔLyve1Cre) resulted in severely disturbed lymphatic vessel morphology with leakage of chyle, phenocopying the Tie2Δ3.1Δ10.5 knockout (Fig. S2D). Collectively, these results demonstrate a requirement for ANGPT2–TIE2 signaling in mesenteric lymphatic development.

A Regulatory Phosphatase for TIE2, VEPTP, Is Absent from Lymphatic Endothelium. TIE1 and VEPTP are both known to modulate activation status of TIE2 and are expressed in BECs (1–4). However, while TIE1 is expressed in LECs in vivo where it is required for lymphatic development (26, 31), VEPTP expression
VEPTP Abrogates ANGPT2 Agonistic Activity on TIE2. Given the striking difference in expression pattern of VEPTP in blood vs. lymphatic vasculature in mice in vivo and its negative regulatory role on TIE2, we reasoned that VEPTP might block ANGPT2 agonistic function in BECs. To test this hypothesis, we characterized the interaction of TIE2 and VEPTP in a heterologous cell model using HEK293 cells, where endogenous expression of both proteins was absent. In cotransfection experiments, VEPTP effectively reduced autophosphorylation of TIE2. Phosphorylation was restored by treatment with a small-molecule inhibitor of VEPTP, AKB-9785, or with recombinant human (rHu) ANGPT1 (Fig. 3 A and B). VEPTP also reduced the phosphorylation of a TIE2 gain-of-function mutant (R849W) identified in patients with hereditary venous malformations (16), confirming its high enzymatic activity (Fig. S5A). TIE2 and VEPTP form a stable complex when transfected in cells and they reciprocally regulate each other, as evidenced by the TIE2-dependent phosphorylation of catalytically inactive VEPTP (Fig. S5 B and C).

Receptor tyrosine kinases signal through trans- and autophosphorylation of tyrosine residues (34). To better understand the regulation of TIE2 by VEPTP and TIE1, we performed phosphoproteomic analysis of the full-length TIE2 and TIE1 receptors in cells. Overall, mass spectrometry detected peptide fragments covering all but Y1024 of the 19 intracellular tyrosine residues on TIE2 (Fig. 3C). Among the 18 tyrosine residues, 13 were phosphorylated at varying levels, including the C-terminal Y1102 and Y1108 that are known to recruit downstream signaling adaptors, such as p85 of PI3 kinase and DokR (35–37). Juxta-membrane Y816, which has been reported to recruit Shp2 and Grb14 for signaling, was also phosphorylated (35). Next, we tested how VEPTP coexpression modulates baseline TIE2 phosphorylation levels on individual tyrosine sites. We observed marked reduction of all phosphorylation in the presence of VEPTP, highlighting the broad impact of this phosphatase to overall TIE2 signal strength (Fig. 3C).

TIE1, a homolog of TIE2, is an orphan receptor tyrosine kinase with no known ligand (1, 3). TIE1 has been shown to interact with TIE2 and its importance both in lymphangiogenesis
and angiogenesis has been well-characterized (26, 31, 38, 39). Recently, extracellular cleavage of TIE1 has been shown to play a role in ANGPT2-mediated TIE2 antagonism in BECs during inflammation (40). Our heterologous expression system showed that TIE1 maintained low levels of autophosphorylation compared with TIE2 (Fig. S6A). Phosphoproteomic analysis of TIE1, either with or without TIE2, revealed that TIE1 phosphorylation is induced by TIE2 coexpression (Fig. S6B). Conversely, when a TIE2 kinase dead mutant protein was coexpressed with wild-type TIE1, the kinase-dead TIE2 became robustly phosphorylated (Fig. S6C), indicating reciprocal cross-talk between these kinases.

Ligand-induced TIE2 activation was studied using stable cell-lines expressing either TIE2-FLAG alone or together with VEPTP-GFP in HEK293 cells (Fig. S7A). Treatment with rHuANGPT1 and rHuANGPT2 increased TIE2 phosphorylation in TIE2-expressing cells in a dose-dependent fashion (Fig. 3 D and E). However, in the presence of VEPTP, only rHuANGPT1 was found to activate TIE2, while treatment with rHuANGPT2 had no effect (Fig. 3 D and E).

**VEPTP Inhibition Restores ANGPT2 Agonistic Activity on TIE2.** In a variety of disease conditions, circulating ANGPT2 levels increase, leading to elevated ANGPT2:ANGPT1 ratios (2, 7, 19, 41). We wondered if VEPTP inhibition might enable ANGPT2 to become a TIE2 agonist in BECs as seen in LECs. VEPTP inhibition alone or in combination with rHuANGPT2 treatment increased TIE2 phosphorylation in TIE2/VEPTP-expressing stable cells, whereas rHuANGPT2 alone did not (Figs. 3E and 4D). To determine if ANGPT2 enhances TIE2 signaling above the effect of VEPTP inhibition alone, we tested the downstream signaling activity in response to either ANGPT2 or VEPTP inhibition alone or in combination in primary and transformed endothelial cell-lines. Human umbilical vein endothelial cells (HUVECs) and EA.hy926 cells express abundant TIE2 and VEPTP.TIE1, the kinase-dead TIE2 became robustly phosphorylated (Fig. S8A). Collectively, our results have demonstrated that VEPTP inhibition in conjunction with ANGPT2 stimulation activates TIE2 signaling and drives translocation of TIE2 to the cell junctions in BECs, mimicking the actions of ANGPT1.

**Discussion**

The ANGPT–TIE pathway is comprised of two receptor tyrosine kinases (TIE1 and TIE2), three ligands (ANGPT1, -2, -3/4), and one phosphatase (VEPTP), which serves as a negative regulator of TIE2 activation (1–4). While previous knockout mouse studies have demonstrated a role for ANGPT2 in lymphatic development, to our knowledge this report of a requirement for TIE2 signaling in lymphatic development, demonstrating ANGPT2 activation of TIE2 occurs in LECs in vivo, is unique.

Angiogenesis and lymphangiogenesis share several fundamental signaling cascades, including growth factor/receptor tyrosine kinase pathways needed to establish and remodel the vascular plexus (1–4). However, while many growth factors have similar effects on LECs and BECs (4), ANGPT2 has opposing effects on TIE2 signaling in cultured BECs vs. LECs (1, 3, 4, 12, 21). Many studies have been performed to identify the molecular basis of the context-dependent functions of ANGPT2 (14), as mediated increases in pAKT signaling are reported to cause forkhead box O1 (FOXO1) phosphorylation, leading to its nuclear exclusion (40, 41). This finding was reproduced in our model, where rHuANGPT1 induced nuclear FOXO1 protein to translocate to the cytoplasm, while rHuANGPT2 did not (Fig. 4D). However, consistent with our pAKT findings, treatment with VEPTP inhibitor alone or VEPTP inhibitor in combination with rHuANGPT2 also markedly reduced nuclear accumulation of FOXO1 (Fig. 4D).

ANGPT1 reinforces vascular junctions and stabilizes blood vasculature by initiating TIE2 phosphorylation and activating downstream signaling networks, but also by physically bridging TIE2 receptors between juxtaposed cells (42, 43). Immunocytochemical analyses revealed TIE2-FLAG protein localized at cellular junctions following stimulation with either rHuANGPT1 or rHuANGPT2, but not VEPTP inhibitor, as previously reported (28, 42). This ANGPT2-induced cellular junctional localization was not affected by VEPTP inhibition (Fig. S7E). Collectively, our results demonstrate that VEPTP inhibition in conjunction with ANGPT2 stimulation activates TIE2 signaling and drives translocation of TIE2 to the cell junctions in BECs, mimicking the actions of ANGPT1.
limited mechanistic understanding of this ligand remains an obstacle to therapeutic development targeting the ANGPT–TIE2 pathway. Outside of the lymphatic endothelium, researchers have identified several conditions in which ANGPT2 can act as an agonist, including (i) high ANGPT2 concentration in vitro (44), (ii) in stressed endothelial cells where FOXO1 nuclear accumulation is reduced (45), and (iii) the presence of functional TIE1 protein in cells where TIE2 is expressed (40). Additionally, ANGPT2 exerts proangiogenic/vascular-destabilizing signals through integrin-mediated pathways (46, 47). We propose a simple new model, where TIE2 receptor sensitivity in BECs and LECs is established by the presence or absence of VEPTP (Fig. 5).

Recent studies have reported crystal structure analysis of TIE2 and provide evidence that the differences in oligomerization, but not the receptor-binding domains of ANGPT ligands, are a major determinant of their potency (39, 48). Higher oligomerization status is required to cluster the TIE2 receptors in cis and elicit downstream signaling cascades due to the relatively large “physical” distance between them, as characterized by the wide angular conformational structure on cell membrane (39, 48). Lower oligomerization status is sufficient to interact with TIE2 receptors across endothelial cellular junctions in trans between juxtaposed cells (39, 48). Although ANGPT2 can form high-order oligomers, it is primarily observed as a dimer (49). In contrast, ANGPT1 is expressed mostly in high-order oligomers through intermolecular disulfide bridges, giving ANGPT1 a stronger TIE2 clustering ability (49). Furthermore, chimeric fusion protein analyses showed both ANGPT1 and ANGPT2 receptor-binding domains have similar TIE2 activating functions when artificially multimerized, emphasizing the importance of oligomerization status (50). Consistent with the model proposed by Leppänen et al. (39), we show that ANGPT2 can bridge the TIE2 receptor in trans, as demonstrated by movement of TIE2 to interendothelial junctions upon ligand exposure, but cannot activate downstream signaling in HUVECs that require clustering TIE2 receptors in cis. We posit that the low availability of higher-order ANGPT2 oligomers necessitates a highly responsive cellular status, such as that provided by the absence of VEPTP, to efficiently activate TIE2 signaling.

The ability of phosphatases to set response thresholds for external signals has been described for signaling through the T cell antigen receptor (TCR), where phosphatases set the threshold for discrimination between self/weak antigen and strong agonist (51). For example, PTPN22 limits the downstream signal from TCR stimulated with a weak agonist, but allows full activation by strong antigens (52). In a similar manner, we propose that VEPTP limits ANGPT2-mediated TIE2 phosphorylation and downstream signaling by setting a high threshold, but allows full activation by strong agonist ANGPT2. The divergent expression pattern of ANGPT2 from an antagonist to an agonistic ligand (Fig. 5). In summary, we have shown that LECs lack VEPTP, conferring a TIE2 agonistic function on ANGPT2. Conversely, BECs express high levels of VEPTP, which raises the activation threshold of TIE2 and prevents activation by the weak agonist ANGPT2. The data support a model in which VEPTP serves as a molecular rheostat for TIE2 receptor sensitivity and confers a cell-type–specific function on ANGPT2. VEPTP inhibition is an attractive therapeutic target to promote vascular health through direct activation of the TIE2 receptor and conversion of ANGPT2 from an antagonist to an agonistic ligand (Fig. 5).

Materials and Methods

Animals. The mouse lines used for our study have been previously described (7, 17, 18, 24, 30, 56–58). Whole-body timed deletion of target genes was achieved by using a bigenic Rosa26rtTA; tetOCre system, as previously described (7, 17, 18). The transgenic mouse lines were maintained on a mixed background due to the large number of transgenes required. However, littermate controls were used for all phenotype analyses. Full details of mouse analysis are described in SI Materials and Methods.

Cell Culture Experiments and Phospho-Proteomics. The cells were cultured with standard methods and stimulated with rhuANGPT1 (R&D Systems), rhuANGPT2 (R&D Systems), and AKB-9785 (a VEPTP inhibitor) (54) for 30 min at 37 °C unless otherwise mentioned. For phospho-mapping analysis, HEK293 cells were transfected with plasmid vectors for expressing TIE2–FLAG, TIE1–FLAG, and VEPTP–GFP, either alone or in combination. The proteins in corresponding SDS gel pieces were digested with trypsin and chymotrypsin, and digested peptides were analyzed with LC-MS/MS. Abundance of phosphorylation sites were semiquantitatively compared across the samples using spectral counting (number of peptide spectrum matching or PSM) (59). Full details of analysis are described in SI Materials and Methods.

Statistics and Reproducibility. Results are expressed as means ± SEM. Statistical analysis was carried out using two-tailed Student’s t test or one-way ANOVA followed by Tukey–Kramer’s test or Dunnett’s correction for
multiple comparisons using GraphPad Prism software. A P value less than 0.05 was considered as statistically significant.

**Study Approval.** All animal experiments were approved by the Animal Care Committee of Mount Sinai Hospital, University of Toronto, and the Institutional Animal Care and Use Committee of the Center for Comparative Medicine at Northwestern University, Chicago.

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