CCBE1 Is Essential for Mammalian Lymphatic Vascular Development and Enhances the Lymphangiogenic Effect of Vascular Endothelial Growth Factor-C In Vivo

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Rationale: Collagen- and calcium-binding EGF domains 1 (CCBE1) has been associated with Hennekam syndrome, in which patients have lymphedema, lymphangiectasias, and other cardiovascular anomalies. Insight into the molecular role of CCBE1 is completely lacking, and mouse models for the disease do not exist.

Objective: CCBE1 deficient mice were generated to understand the function of CCBE1 in cardiovascular development, and CCBE1 recombinant protein was used in both in vivo and in vitro settings to gain insight into the molecular function of CCBE1.

Methods and Results: Phenotypic analysis of murine Ccbe1 mutant embryos showed a complete lack of definitive lymphatic structures, even though Prox1⁺ lymphatic endothelial cells get specified within the cardinal vein. Mutant mice die prenatally. Proximity ligation assays indicate that vascular endothelial growth factor receptor 3 activation appears unaltered in mutants. Human CCBE1 protein binds to components of the extracellular matrix in vitro, and CCBE1 protein strongly enhances vascular endothelial growth factor-C–mediated lymphangiogenesis in a cornal micropocket assay.

Conclusions: Our data identify CCBE1 as a factor critically required for budding and migration of Prox-1⁺ lymphatic endothelial cells from the cardinal vein. CCBE1 probably exerts these effects through binding to components of the extracellular matrix. CCBE1 has little lymphangiogenic effect on its own but dramatically enhances the lymphangiogenic effect of vascular endothelial growth factor-C in vivo. Thus, our data suggest CCBE1 to be essential but not sufficient for lymphangiogenesis. (Circ Res. 2011;109:486-491.)

Key Words: Ccbe1 lymphangiogenesis Hennekam syndrome VEGF-C mouse model

The vascular system consists of blood and lymphatic vessels, both of which are lined by endothelial cells (ECs). Lymphatic vessels play a key role in fluid homeostasis and are central to transport of macromolecules, immune system function, and intestinal lipid absorption. Mice lacking lymphatic vessels due to loss-of-function mutations in vascular endothelial growth factor (VEGF)-C or Prospero homeobox 1 (Prox1) die in utero displaying severe edema. In humans, functional defects of lymphatic vessels result in a number of clinical conditions, such as lymphedema. Lymphatic vessels are also implicated in metastatic dissemination, therefore playing an important role in tumor progression.

Recently, we identified the collagen- and calcium-binding EGF domains 1 (ccbe1) gene to be required for embryonic lymphangiogenesis in zebrafish. Furthermore, collagen- and calcium-binding EGF domains 1 (CCBE1) was found to be mutated in a cohort of patients with Hennekam syndrome, a rare disease that presents with lymphedema, lymphangiectasias, and other pathological features. However, it has remained unclear in which stage of lymphatic development CCBE1 is required. Also, whether CCBE1 is an instructive or permissive factor has not been resolved.

Methods

Ccbe1 Mouse Allele

LacZ mice were generated by homologous recombination in embryonic stem cells with a lacZ cassette, replacing the first and second coding exons of Ccbe1 (Ccbe1floxed). Purification of CCBE1collagen-Fc Protein and Mouse Corneal Micropocket Assay

Recombinant human CCBE1collagen-Fc (NP_597716) contains amino acid residues 1 to 191 of CCBE1 fused with the Fc domain of
human IgG1. Protein pellets (inert hydron, 0.2 mm×0.2 mm) containing 500 ng CCBE1\textsuperscript{collagen-Fc} (Genentech), 250 ng VEGF-C (Genentech), or both were inserted to the base of the corneal micropocket, approximately 1 mm from the limbus. After 10 days, corneas were harvested and processed as described.\textsuperscript{10} An expanded Methods section is available in the Online Supplement.

**Results**

To unravel CCBE1 function during murine lymphatic development, we targeted the murine Ccbe1 locus by generating a lacZ allele (Ccbe1\textsuperscript{lacZ}) (Figure 1A). During embryonic development, cells expressing Ccbe1 were found in multiple locations such as the brain, the mesothelium of the heart, and in close proximity to nascent blood and lymphatic vascular structures, with weak lacZ expression in some of these regions (Figure 1B and Online Figure I). In Ccbe1\textsuperscript{−/−} mice, patterning and density of blood vessels exhibited no gross abnormalities (Figure 1C). From E13.5 on, however, Ccbe1\textsuperscript{−/−} mice accumulated fluid and developed edema (Figure 1D, arrows). All mutant mice died prenatally. Further analysis revealed that Ccbe1\textsuperscript{−/−} embryos lack all lymphatic vessels such as those found in skin (Figure 1E, arrowheads). Thus, in mice, CCBE1 appears to be critically important for lymphangiogenesis but not for angiogenesis.

Because early markers for lymphatic fate are missing in zebrafish, it has remained unclear whether lymphatic fates are

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### Figure 1. Ccbe1 mutant mice develop edema and lack lymphatic vessels.

A. Knockout strategy for Ccbe1\textsuperscript{lacZ}.

B. Transverse sections of Ccbe1\textsuperscript{−/−} lacZ embryo at E10.5 and E12.5; expression (red arrows) can be detected near vascular structures cardinal vein (cv) and lymph sacs (ls).

C. Pecam-1 staining of a Ccbe1\textsuperscript{−/−} lacZ embryo revealing no abnormalities in blood vascular patterning.

D. Edema formation at E14.5.

E. Ccbe1\textsuperscript{−/−} skin that lacks lymphatic vessels at E14.5; Lyve-1 (green)/Pecam-1 (red).

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### Non-standard Abbreviations and Acronyms

- CCBE1: collagen- and calcium-binding EGF domains 1
- ECM: extracellular matrix
- LEC: lymphatic endothelial cell
- Lyve-1: lymphatic vessel endothelial hyaluronan receptor 1
- Pecam-1: platelet endothelial cell adhesion molecule 1
- Prox-1: Prospero homeobox 1
- VEGF-C: vascular endothelial growth factor C
- Vegfr-3: vascular endothelial growth factor receptor 3
established in the absence of ccbe1. Lyve-1 and Prox-1, early markers for LEC fate specification, were detected in E10.5 Ccbe1+/H11002 embryos (Figure 2A), suggesting that LEC differentiation is unaffected in the absence of CCBE1. Normally, differentiated LECs bud and migrate away from the anterior cardinal veins between E10.5 and E12.0 and form lymph sacs between E11.5 and E13.5, which subsequently give rise to most of the lymphatic vasculature. We observed no lymph sacs, and only a few Prox-1 cells and Lyve-1 LECs were present in the wall of the cardinal vein in Ccbe1+/H11002 embryos, with even fewer LECs outside the cardinal vein (Figure 2B, arrowhead), indicating an overall reduction in LECs at these stages and a primary defect before the formation of lymph sacs (Figure 2C and 2D). These findings indicate that CCBE1 is essential for budding and/or migration of LECs from veins.

Migration of LECs has previously been shown to be dependent on VEGF-C/Vegfr-3 signaling. We analyzed LECs for Vegfr-3 tyrosine phosphorylation (pTYR/Vegfr-3) in situ, using a proximity ligation assay. We quantified both the number of Lyve-1 cells and the number of pTYR/Vegfr-3 sites per Lyve-1 cell at E10.5 and E12.5 and confirmed a reduction in the number of Lyve-1 cells in Ccbe1+/H11002 embryos (Online Figure II). However, at critical stages of LEC migration, levels of phosphorylated Vegfr-3 per Lyve-1 cell were unaltered, indicating that Vegfr-3 activation is not affected by the absence of CCBE1 and suggesting that CCBE1 may function independently of Vegfr-3 phosphorylation (Online Figure II).

Because Vegfr-3 phosphorylation appeared normal in Ccbe1+/− LECs and as suggested by the CCBE1 domain structure, we considered whether CCBE1 might be part of the extracellular matrix (ECM). Because we were unable to produce full-length CCBE1, and because previous and new genetic evidence (Online Figure III) suggests that the majority of inactivating mutations is present in the EGF domains, we generated a truncated protein (CCBE1collagen-Fc) consisting of the EGF and calcium-binding EGF domains of CCBE1 fused to an Fc domain, but lacking the collagen repeat domain (Online Figure IV). CCBE1collagen-Fc appeared to bind to collagen IV deposited by cultured HUVECs. Binding particularly occurred in cell-free areas (Online Figure IV). We hence removed the cells before incubating the remaining ECM with either recombinant CCBE1collagen-Fc, or human Fc-containing proteins Alk1-Fc or anti-Nrp2 antibody as negative controls. Collagen IV (Figure 3A, arrows) but not laminin (data not shown) colocalized with CCBE1collagen-Fc. Control proteins anti-Nrp2 (Figure 3A, lower panels) and Alk1-Fc (data not shown) did not show binding to ECM components. Because it is possible that the colocalization of CCBE1collagen-Fc with ECM components is indirect (Colla-
gen IV and CCBE1 might both bind a third, independent component of the ECM), we used a binary system where plates coated with a single ECM protein were incubated with CCBE1\(^{\Delta\text{collagen}}\)-Fc protein. In three independent experiments we found reproducible and weak interaction of CCBE1\(^{\Delta\text{collagen}}\)-Fc with Collagen I, Collagen IV and Collagen V (Figure 3B), with Collagen IV binding being variable. To fibronectin and laminin, CCBE1\(^{\Delta\text{collagen}}\)-Fc did not show binding. Strongest binding, however, was reproducibly observed with vitronectin in a dose-dependent manner (Figure 3B).

Our analysis of \(\text{Ccbe1}^{--}\) mice demonstrates that CCBE1 is important for LEC budding, migration, and sprouting. We hence tested whether exogenous CCBE1 has lymphangiogenic activity on its own or whether it may modify the lymphangiogenic activity of VEGF-C in vivo. CCBE1\(^{\Delta\text{collagen}}\)-Fc protein was used in a corneal micropocket assay and its effect compared with the effects of VEGF-C, a known inducer of lymphangiogenesis in the avascular cornea of mice. A single administration of CCBE1\(^{\Delta\text{collagen}}\)-Fc weakly but significantly induced growth of lymphatic vessels from the limbus region underneath the implanted pellets, whereas VEGF-C elicited a stronger response (Figure 4A and 4C). Strikingly, the lymphangiogenic response was strongly increased when CCBE1\(^{\Delta\text{collagen}}\)-Fc was administered together with VEGF-C, yielding a greater effect than previously observed with any other protein (Figure 4A and 4C). Of note, lymphatic vessels induced by the combined activity of CCBE1 and VEGF-C show a dramatically increased number of filopodial extensions in comparison to vessels induced by VEGF-C alone (Figure 4B and 4D).

Because the application of CCBE1 alone causes a weak response of lymphatic vessel outgrowth, this might be taken as an argument that CCBE1 is a molecule facilitating LEC migration. However, it has been shown that the inflection of a wound in the cornea attracts macrophages, which are known to produce high levels of VEGF-C.\(^17\) Hence, it is conceivable that applying CCBE1 in this context is sufficient to elicit a
lymphangiogenic response, during which low levels of macrophage-produced VEGF-C are sufficient to stimulate LECs in the presence of exogenous CCBE1 in the environment. Further stimulation with exogenous VEGF-C (Figure 4A and 4C) will then cause a considerably stronger response in this assay for adult lymphangiogenesis.

**Discussion**

We have shown that Ccbe1 is required for murine embryonic lymphangiogenesis, independent of Vegfr-3 phosphorylation. Given the embryonic lethal Ccbe1−/− phenotype, mutations in CCBE1 in human Hennekam syndrome patients are likely to represent severe hypomorphic but not complete loss-of-function
situations. Supporting the functional importance of the EGF domains, a truncated form of CCBE1 containing these domains is sufficient to induce a strong lymphangiogenic response in conjunction with VEGF-C in an in vivo assay.

Because CCBE1 is not required to alter the Vegfr-3 activation status, and because the effects of CCBE1 are less dramatic in the corneal micro pocket assay than the effects of VEGF-C, we favor a model in which CCBE1 is not an instructive factor but rather acts as an essentially required permissive factor. As such, CCBE1 is part of, or binds to, the ECM and modulates the effect of VEGF-C on lymphangiogenesis.

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Disclosures
M.C., J.K., R.T., and J.A.E. are employees of Genentech.

References

Novelty and Significance
What Is Known?
• Collagen and calcium-binding EGF domains 1 (ccbe1f) is required for the development of lymphatic vessels during zebrafish embryonic development.
• CCBE1 has been implicated with the human Hennekam syndrome, in which lymphatic and cardiovascular function is impaired.

What New Information Does This Article Contribute?
• We have generated a mouse model to study the expression and function of Ccbe1 during mammalian development.
• Mice that lack Ccbe1 acquire severe edema and die prenatally.
• Lymphatic endothelial cells are specified and present in Ccbe1 mutants, but these cells fail to migrate away from the cardinal vein and consequently lymphatic vessels do not develop.
• Mutant lymphatic endothelial cells display apparently normal vascular endothelial growth factor receptor (Vegfr)-3 phosphorylation.

• Human recombinant CCBE1 protein binds to specific components of the extracellular matrix.
• Human recombinant CCBE1 protein enhances VEGF-C driven lymphatic response in a corneal micropocket assay.

CCBE1 has been implicated in the human Hennekam syndrome, but a mouse model and insights into CCBE1 function are lacking. We show that CCBE1-deficient mice acquire severe edema, lack lymphatic vessels, and die prenatally. CCBE1 recombinant protein binds to components of the extracellular matrix and enhances the lymphangiogenic effect of VEGF-C in an in vivo assay. Our work demonstrates CCBE1 to be an essential factor for embryonic lymphangiogenesis and suggests CCBE1 modulation as a possible strategy for stimulating lymphatic vessel growth and improving clinical outcome of lymphatic-related diseases.
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Online Supplemental Material

Online Figure I

Expression of Ccbe1lacZ and overall blood vasculature patterning of Ccbe1 deficient mice.
(A) β-galactosidase staining (blue) of an E11.0 Ccbe1+/− lacZ embryo showing expression near heart (black arrows) and brain (green arrow) (B) β-galactosidase staining (blue) and H&E (pink) showing expression in mesothelium of the heart. (C) Lyve-1 (green) /Pecam-1 (red) staining of E14.5 Ccbe1+/− skin which lack lymphatic vessels but retain Lyve-1+ macrophages. (D) Quantification of Pecam-1+ vessel coverage of the skin at 10 different regions.

Online Figure II

Vegr-3 tyrosine phosphorylation is unaltered in Ccbe1 deficient embryos.
Vegfr-3 and phosphorylation of Tyrosine (pTYR) were detected by in situ proximity ligation assays at E10.5 (A) and E12.5 (B) in Lyve-1+ cells. At E10.5 Lyve-1+ cells in both sibling and mutant are still located within the cardinal vein, while at E12.5 Lyve-1+ cells are found in the lymph sacs of sibling embryos (C). While fewer Lyve-1+ cells were present in Ccbe1+/− embryos at E12.5 compared to E10.5, normalizing per Lyve-1+ cell revealed that the total amount of phosphorylated pTYR/Vegfr-3 per Lyve-1+ cell is unaltered in Ccbe1+/− embryos at E10.5 and E12.5 in comparison to sibling embryos (D). cv: cardinal vein, jls: jugular lymph sac.

Online Figure III

Generation and binding of CCBE1Δcollagen-Fc protein.
(A) Schematic representation of wildtype and recombinant CCBE1Δcollagen-Fc protein. (B) CCBE1Δcollagen-Fc (green) binds to ECM component Collagen IV (red), predominantly in cell-free regions (lower panel).

Online Figure IV

Overview of mutations in human Hennekam Syndrome and zebrafish ccbe1 mutants.
(A) Schematic presentation indicating the currently known human CCBE1 and zebrafish ccbe1 alleles. Note clustering of mutations in the N-terminal half of the protein, containing the EGF binding domains. ccbe1del81-125 and ccbe1Y109N are novel zebrafish ccbe1 alleles. (B) Zebrafish trunk vasculature at 5dpf
visualized by double transgenic labeling of arteries and veins. Note the existence of some venous intersegmental vessels (stars) in ccbe1Y109N.

Supplement Material Methods

Mice
Mice were maintained at the Hubrecht Institute and Genentech, and experiments were performed according to national rules and regulations. LacZ mice were generated by homologous recombination in embryonic SC with a lacZ cassette, which replaces the first and second coding exons of Ccbe1 (Ccbe1lacZ).

Detection of β−galactosidase activity
Freshly obtained samples were fixed in a 2% paraformaldehyde/0.2% glutaraldehyde/PBS solution at RT for 30min. Samples were then washed 2 times with rinse buffer (2 mM MgCl2/0.1% NP40/PBS) and stained for 24 h in a solution consisting of 1 mg/ml X-gal, 5 mM ferrothiocyanide, 5 mM ferrithiocyanide in rinse buffer. Paraffin embedded samples were sectioned at 4 to 6 μm.

Immunohistochemistry
Whole mount tissues were fixed in 4% PFA and blocked prior to antibody staining. 6-25 μm paraffin or cryo-sections were blocked with 0.1%BSA, 5% milk or 10% goat or donkey serum, depending on type of tissue. Antibodies were incubated for 1hr to 24hrs, depending on the Ab. Samples were stained with Alexa 488, 568, 647 conjugated secondary antibodies (1:500; Molecular Probes) for 1 hr. Images were captured using Zeiss Axiophoto Fluorescence microscope, Leica SPE or Leica SP5 confocal microscopes. Antibodies used are Lyve-1 (R&D Systems), Vegfr-3 (R&D Systems), Pecam-1 (BD and Chemicon), Prox1 (Chemicon).

Proximity ligation assay
Proximity ligation assay was performed using the Duolink II Detection Kit (Olink Bioscience) and following primary antibodies: goat anti-Vegfr-3 (R&D Systems), mouse anti-phospho-tyrosine 4G10 Platinum (Millipore) and rabbit anti-Lyve1 (Abcam).

Purification of the CCBE1Δcollagen-Fc fusion protein
Recombinant human CCBE1ΔCollagen (NP_597716), residues (1-191) was expressed as a fusion with the Fc domain of human IgG1 from a transient transfection vector under control of the CMV promoter in Chinese Hamster Ovary (CHO) cells. Expression media containing human CCBE1-Fc was conditioned with 1mM Sodium Azide, and 0.5mM Phenylmethylsulfonyl Fluoride (pH pH 7.0), loaded over Protein A resin (ProSep-A, Millipore), washed and subsequently eluted with 50mM Sodium Citrate, pH 3.0. pH adjusted fractions (pH 5.0) were further purified by cation exchange chromatography (SP Sepharose FF, GE Healthcare) and eluted by a 0-1.0M NaCl elution gradient. Proteins were concentrated (10000 MWCO Amicron Ultra, Millipore) and dialyzed (10000 MWCO dialysis cassette, Pierce) into 25mM Tris/0.15M NaCl/2mM CaCl₂, pH7.5. Final protein samples were sterile filtered and characterized by SDS-PAGE, O.D. 280, endotoxin assay, laser light scattering, and mass spectrometry.

Mouse corneal micro-pocket assay
Adult (8-10 weeks old) CD1 mice were anesthetized using isoflurane. A 1-2 mm initial cut was made on the cornea surface near the center using a #15 surgical blade. A micropocket directed toward the limbus is created by blunt dissection using a modified microspatula (ASSI 80017). Growth factor pellets are inserted (inert hydron, 0.2mmx0.2mm) to the base of the pocket, approximately 1 mm from the limbus. Growth factors used were: CCBE1ΔCollagen-Fc (Genentech) at 500ng/pellet, VEGF-C (Genentech) at 250ng/pellet, or in combination. After 10 days, corneas were harvested and stained immunohistochemically.

ECM binding studies
HUVECs were plated onto 4-well chamber slides and allowed to reach confluence and deposit ECM for 3 days. Medium was removed and wells washed with 0,5% sodium deoxycholate to remove cells. Cells were briefly fixed in 4% PFA and then blocked in 1% BSA in PBS for 1 hr CCBE1ΔCollagen-Fc or anti-Nrp2 antibodies at 10ug/ml were incubated overnight. Wells were washed and IHC was performed. Collagen I, IV, V, fibronectin, laminin, vitronectin or BSA was coated on ELISA plates overnight. Plates were washed with PBS and blocked in 1% BSA for 3 hours. Increasing concentrations of CCBE1ΔCollagen-Fc, anti-Nrp2 antibodies, or Alk1-Fc were incubated overnight. Plates were washed in 0.05% tween-20/PBS and then incubated for 1 hour with anti-human-HRP 1:5000 in 1%BSA/0.05%tween-20/PBS. Plates were washed in PBS containing 0.05% tween-20 and then incubated in TMB in the dark for 20 minutes. 2N sulfuric acid was added to stop the reaction and plates read at 450nm (n=3 per condition).
Supplement Figure Online Figure II

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