Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Tie1 is required for lymphatic valve and collecting vessel development

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ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
10 December 2014
Accepted 17 December 2014

Keywords:
Tie1
Lymphatic valve
Lymphovenous valve
Lymphatic vessel remodeling
Conditional knockout
Prox1

ABSTRACT

Tie1 is a receptor tyrosine kinase with broad expression in embryonic endothelium. Reduction of Tie1 levels in mouse embryos with a hypomorphic Tie1 allele resulted in abnormal lymphatic patterning and architecture, decreased lymphatic draining efficiency, and ultimately, embryonic demise. Here we report that Tie1 is present uniformly throughout the lymphatics and from late embryonic/early postnatal stages, becomes more restricted to lymphatic valve regions. To investigate later events of lymphatic development, we employed Cre-*loxP* recombination utilizing a floxed *Tie1* allele and an *Nfatc1Cre* line, to provide *loxP* excision predominantly in lymphatic endothelium and developing valves. Interestingly, unlike the early prenatal defects previously described by ubiquitous endothelial deletion, excision of *Tie1* with *Nfatc1Cre* resulted in abnormal lymphatic defects in postnatal mice and was characterized by agenesis of lymphatic valves and a deficiency of collecting lymphatic vessels. Attenuation of Tie1 signaling in lymphatic endothelium prevented initiation of lymphatic valve specification by Prox1 high expression lymphatic endothelial cells that is associated with the onset of turbulent flow in the lymphatic circulation. Our findings reveal a fundamental role for Tie1 signaling during lymphatic vessel remodeling and valve morphogenesis and implicate it as a candidate gene involved in primary lymphedema.

Introduction

The lymphatic vasculature is essential for the maintenance of normal tissue fluid homeostasis and for support of the immune response. It consists of a complex network of capillaries and collecting vessels. The lymphatic capillaries have discontinuous basement membrane and loose intercellular junctions, lack of pericyte coverage, and are therefore highly permeable to large macromolecules. In contrast, larger collecting lymphatic vessels are surrounded by a substantial basement membrane and by smooth muscle cells (SMC)/mural cells, which help to pump the lymph forward while the luminal valves in these vessels prevent lymph backflow (Oliver and Detmar, 2002). These structural features allow efficient fluid uptake of protein-rich lymph from tissue interstitium by capillaries and transport of lymph back to the blood vascular system by collecting vessels (Tammela and Alitalo, 2010). Lymphatic vessel hypoplasia or defective lymphatic valves can impair the ability of the lymphatic vasculature to collect and transport fluids and lead to lymphedema (Alitalo, 2011; Schulte-Merker et al., 2011).

The mammalian lymphatic system has been shown to originate from embryonic veins. Lymphatic vessel development starts at embryonic day (E) 9.5 in mice. After formation of the primary lymph sacs, primitive lymphatic capillary networks assemble by a process of centrifugal sprouting from the lymph sacs, and then subsequently these networks combine and remodel into a hierarchal network of capillaries and collecting lymphatic vessels (Koltowska et al., 2013; Tammela and Alitalo, 2010). Maturation of collecting vessels is accompanied by the downregulation of lymphatic marker molecules such as Lyve1, the acquisition of partial smooth muscle cell coverage, and the formation of intraluminal valves (Bazigou et al., 2009; Sabine et al., 2012).

Formation of intraluminal lymphatic valves is one of the hallmarks of the collecting lymphatic vessel characteristic of later stages of lymphatic development. Lymphatic valve formation in mice is initiated during late embryonic development (around E16.5). It has recently been shown that mechanical stimulus of turbulent hemodynamics caused by the onset of lymph flow establishes the location, often at branch points of lymphatic vessels, where valves develop by upregulating the expressions of Prox1 and Foxc2 (Sabine et al., 2012). Foxc2 cooperates with Prox1 to control Connexin37 (Cx37) expression and to activate Nfatc1/Calcineurin signaling (Sabine et al., 2012). Other molecular regulators such as EphrinB2 (Makinen et al., 2005), integrin 9 and its extracellular matrix (ECM) ligand FN-EIIIA (Bazigou et al., 2009), EMILIN1/α9β1 Integrin

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http://dx.doi.org/10.1016/j.ydbio.2014.12.021
0012-1606/

(Danussi et al., 2013), Connexin37 and Connexin43 (Kanady et al., 2011), Bmp9 (Levet et al., 2013), and Semaphorin3a and their receptors Neuropilin1/plexin A1 (Bouvrée et al., 2012; Jurisic et al., 2012) have been reported to be required for either lymphatic valve formation, elongation of lymphatic valve leaflets, or valve maturation. Despite this knowledge, the signaling mechanisms regulating lymphatic remodeling and maturation, and early stages of lymphatic-valve development are still not well understood. Importantly, although FOXC2 is induced by oscillatory shear stress in vitro (Sabine et al., 2012), surprisingly Prox1 is not. Thus further analysis of lymphatic endothelial cell mediators of altered hemodynamic shear stress should provide important insights on the molecular mechanisms required for the initiation of lymphatic valve formation.

Tie1 is an orphan endothelial receptor tyrosine kinase sharing a high degree of homology with Tie2, the receptor for the angiopoietins (Peters et al., 2004; Yancopoulos et al., 2000). It is expressed throughout both the blood and lymphatic vasculature endothelium from early embryonic stages to the adult (Partanen et al., 1992; Dumont et al., 1995; Qu et al., 2010) and has previously been shown to be involved in the regulation of growth and integrity of lymphatic capillaries (D'Amico et al., 2010; Qu et al., 2010). Accordingly, *Tie1*-deficient embryos have abnormal lymphatic patterning and architecture, leading to a lymphedema associated with inefficient lymph drainage and increased leakage and embryonic demise. In addition, our laboratory has recently shown that Tie 1 is at least partially responsible for mechanotransduction of turbulent flow required for initiation and maintenance of atherosclerotic plaque formation at the branch points of the systemic vasculature in the adult animal (Woo et al., 2011). Here, we report that Tie1 is expressed in developing collecting lymphatics and becomes progressively enriched at lymphatic valves. We subsequently demonstrate that Tie1 is a crucial regulator of lymphatic remodeling and valve morphogenesis by conditional deletion of Tie1 predominantly in the lymphatic valvular endothelium. Early post-natal animals exhibit major lymphatic defects, including a failure to remodel their primary lymphatic capillary plexus into a hierarchical vessel network with a definitive collecting vessels, and lack of luminal valve formation. Furthermore, we demonstrate that Tie1 is necessary for assembling the critical patterned high expression of Prox1 and Foxc2 in prospective valve-forming cells, which is required for the initiation of valve morphogenesis. This suggests that as previously demonstrated in the adult systemic circulation, Tie1 may serve as a component of the mechanotransduction machinery required to mediate the altered hemodynamics essential for lymphatic valve ontogeny.

Materials and methods

Mouse models

Generation of *Tie1*$^{+/lz}$ (Puri et al., 1995) , *Tie1*$^{fl/fl}$ (Qu et al., 2010), and *Nfatc1Cre* mice (Wu et al., 2012) has been described previously. R26R reporter (*R26*^{fslz}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All strains were maintained on a mixed 129 and C57/BL6 background. The animals were handled in accordance with institutional guidelines with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine.

Antibodies and whole-mount immunohistochemistry

Mesenteries, hearts, diaphragm, head and back skin (from embryos), and ventral skin (from neonates) were dissected, fixed in paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C and then washed with PBS. Whole-mount immunostaining was performed as described previously (Qu et al., 2010). Briefly, the samples were washed 3 × 10 min with PBST (0.1% Tween-20 in PBS) following by 2 washes with PBTD (1% dimethyl sulfoxide in PBST), blocked for minimum of 2 h with blocking solution (10% serum in PBTD) and then incubated with primary antibodies diluted in blocking solution overnight at 4 °C. At day 2, the samples were washed for at least 4 h with PBTD and then incubated with secondary antibodies diluted in blocking solution overnight at 4 °C. At day 3, the samples were washed for 2–3 h with PBTD. The following primary antibodies were used: rat anti-mouse CD31 (Pharmingen, monoclonal MEC13.3), goat anti-mouse Vegfr3 (R&D Systems, AF743), rabbit anti-Lyve1 (Abcam, ab14917), and Cy3 conjugated anti-α-Smooth Muscle Actin (SMA) (Sigma, C-6198), mouse anti-human Nfatc1 (Santa Cruz Biotechnology, clone 7A6), rabbit anti-Prox1 (Abcam, ab37128), goat anti-human Prox1 (R&D Systems, AF2727), rat anti-mouse CD105 (endoglin) (eBioscience, 14–1051), goat anti-mouse integrin-α9 (R&D Systems, AF3827), rabbit anti-human laminin α5 (Ringelmann et al., 1999). For fluorescence staining, Alexa Fluor 488, 594, and 647 fluorochrome-conjugated secondary antibodies (Invitrogen) were used for signal detection. Images were acquired with a Leica TCS SP2 confocal system (Leica Microsystems). To visualize anti-Vegfr3 staining with light microscopy, biotinylated rabbit anti-goat IgG (Vector Laboratories, BA-5000) secondary antibodies were used in horseradish peroxidase stainings with the Vectastain kit (Vector Elite PK-6100) and DAB kit (Vector Laboratories, SK-4100).

Staining for β-galactosidase activity

Whole-mount X-gal staining was performed as previously described (Qu et al., 2010). Briefly, whole embryos or intestine with mesenteries were harvested in PBS, fixed in 4% PFA in PBS, and then washed three times for 15 min with a detergent rinse (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40, in PBS). The staining was developed in staining solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and X-gal 1 mg/ml, in PBS) at 37 °C overnight. After staining, the samples were used for histological analysis or imaged using a Leica M205 FA stereomicroscope and a Leica DFC310 FX digital camera (Leica Microsystems).

Quantitative RT-PCR

RNA from mesenteries of *Tie1* conditional knockout (*Tie1*-cko) and their control littermates at E18.5 and P1 was isolated using TRIZOL Reagent (Invitrogen) with additional DNase treatment (Promega). cDNA was then generated using the SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative PCR was performed on the CFX96 Touch™ thermal cycler (Bio-Rad) using iQ™ SYBR Green Supermix (Bio-Rad). All assays were repeated at least twice, and all samples were run in triplicate. Analysis of gene expression was carried out using the comparative Ct (ΔΔCt) method as described by the manufacturer. Relative quantification of gene expression was normalized to 18S mRNA expression level. Sequences of the PCR primers used are listed in Supplementary Table 1.

Quantification of lymphatic valves

Mesenteric valves were identified as areas of strongly positive Prox1 staining and counted from duodenum to ileum. The number of valves at each developmental stage was quantified using four mesenteric vessels in 4 mice/genotype.

Please cite this article as: Qu, X., et al., Tie1 is required for lymphatic valve and collecting vessel development. Dev. Biol. (2015), http://dx.doi.org/10.1016/j.ydbio.2014.12.021