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Connexin37 and Connexin43 deficiencies in mice disrupt lymphatic valve development and result in lymphatic disorders including lymphedema and chylothorax

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ABSTRACT

Intraluminal valves are required for the proper function of lymphatic collecting vessels and large lymphatic trunks like the thoracic duct. Despite recent progress in the study of lymphvasculogenesis and lymphangiogenesis, the molecular mechanisms controlling the morphogenesis of lymphatic valves remain poorly understood. Here, we report that gap junction proteins, or connexins (Cxs), are required for lymphatic valvulogenesis. Cx37 and Cx43 are expressed early in mouse lymphatic development in the jugular lymph sacs, and later in development these Cxs become enriched and differentially expressed by lymphatic endothelial cells on the upstream and downstream sides of the valves. Specific deficiencies of Cx37 and Cx43 alone or in combination result in defective valve formation in lymphatic collecting vessels, lymphedema, and chylothorax. We also show that Cx37 regulates jugular lymph sac size and that both Cx37 and Cx43 are required for normal thoracic duct development, including valve formation. Another Cx family member, Cx47, whose human analog is mutated in some families with lymphedema, is also highly enriched in a subset of endothelial cells in lymphatic valves. Mechanistically, we present data from Foxc2−/− embryos suggesting that Cx37 may be a target of regulation by Foxc2, a transcription factor that is mutated in human lymphedema–distichiasis syndrome. These results show that at least three Cxs are expressed in the developing lymphatic vasculature and, when defective, are associated with clinically manifest lymphatic disorders in mice and man.

Introduction

Lymphatic (Ly) vessels are essential for tissue fluid balance, immune function, and the absorption and transport of dietary fat. Excess interstitial fluid is taken up by Ly capillaries and transported as lymph through valved collecting vessels, filtered by lymph nodes, and then carried through major Ly trunks which empty into the venous system. Valves are a crucial feature of the Ly vasculature because they ensure that lymph movement continues anterograde, propelled in part by Ly intrinsic contractions (Zawieja, 2009). Defects of the Ly system lead to a number of congenital and acquired disorders and syndromes including lymphedema, chylothorax, metabolic disorders, inflammation, and immune dysfunction. In addition, the Ly vasculature is a major route for tumor metastasis. Understanding the molecular mechanisms of lymphangiogenesis and lymphvasculogenesis both in normal contexts and during tumor growth will be important in efforts to develop novel molecular therapeutics (Jurisic and Detmar, 2009; Witte et al., 2001).

In recent years substantial progress has been made in identifying key genes and proteins involved in Ly development. Knockout and transgenic mouse models as well as other approaches have established the importance of key transcription factors (Prox1, Foxc2, Sox18, NFATc1, Coup-TFII, Net); signaling proteins (VEGF-C/D, Ang1 and Ang2, FIAF, EphrinB2, podoplanin, Syk, Akt/PKB (Zhou et al., 2010), PI3K); receptors (VEGFR-3, Np2); and cell-matrix interactions (integrin-α9, FnEIIIA, Emilin1) in Ly development (for reviews see (Mäkinen et al., 2007; Oliver and Srinivasan, 2010; Oliver and Srinivasan, 2008; Tammela and Alitalo, 2010)). In addition, several congenital Ly diseases have been linked to gene mutations, including Milroy disease (*VEGFR3*) (Ferrell et al., 1998), lymphedema–distichiasis syndrome (*FOXC2*) (Fang et al., 2000), hypotrichosis–lymphedema–telangiectasia (*SOX18*) (Irrthum et al., 2003), congenital chylothorax (*ITGA9*) (Ma et al., 2008), and generalized Ly dysplasia (*CCBE1*) (Connell et al., 2010). Despite these advances, the

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overall process of Ly development is still incompletely understood. The current view is that the process begins at E9.5-E10.5 in the mouse when a subgroup of committed Prox1-expressing endothelial cells in the anterior cardinal veins migrates laterally and form the Ly primordia (lymph sacs) (Oliver and Srinivasan, 2010). Primitive Ly 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 initial and collecting Ly vessels (Tammela and Alitalo, 2010). However, detailed knowledge about the signaling mechanisms that govern these processes, and in particular, those that control Ly valve morphogenesis is at an early stage.

One area that has not been investigated in any detail is the role of gap junction (GJ) proteins, or connexins (Cxs), in Ly vascular development and function. Cxs are a family of 21 proteins in humans which assemble into GJ channels, structures that allow for the direct transfer of small molecules between adjacent cells (Goodenough and Paul, 2009). GJ channels are dynamically regulated, rapidly at the single channel level, and on a slower timescale at the level of Cx synthesis, assembly, post-translational modification, and degradation (Laird, 2006; Solan and Lampe, 2009). Besides intercellular channels, Cxs can also form hemichannels (undocked channels) which act as release sites for extracellular signaling molecules (Stout et al., 2004). Some Cxs bind other proteins within the cell, contributing to signaling that may be unrelated to channel function (Dbouk et al., 2009; Jiang and Gu, 2005; Laird, 2010). It is well established that endothelial cells and smooth muscle cells of many blood vessels are coupled by Cx-comprised intercellular channels (Gabriels and Paul, 1998; Simon and McWhorter, 2003; Yeh et al., 1997). Furthermore, Cxs have been shown to be necessary for various aspects of blood vessel development, propagation of conducted arteriolar vasomotor responses, and for communicating antiinflammatory signals between blood vessel endothelial cells (Brisset et al., 2009; Figueroa and Duling, 2009; Simon and McWhorter, 2002; Walker et al., 2005). Regarding Ly vessels, it has been suggested that GJs could provide a pathway for conduction of spontaneously evoked contractions in Ly vessels (McHale and Meharg, 1992; Zawieja et al., 1993). To date, however, the argument for GJs in Ly vessels is based mainly on GJ inhibitor studies. Because the inhibitors used were not specific for GJ channels, the evidence for GJs in Ly vessels has not been conclusive. While recent microarray studies of human dermal Ly endothelial cells (LECs) have provided evidence of expression of Cx mRNA in cultured LECs, the expression and localization of Cx proteins in developing and mature Ly vessels, as well as their potential functions in these vessels, have not been investigated (Shin et al., 2008; Wick et al., 2007). In this study, we examine the expression of Cxs in both the developing and mature Ly vasculature and use Cx-deficient mice to investigate their functions in the Ly system.

Materials and methods

Mice and genotyping

Cx37−/− (*Gja4*−/−) (Simon et al., 1997), Cx40−/− (*Gja5*−/−) (Simon et al., 1998), Cx43−/− (*Gja1*−/−) (Reaume et al., 1995), and Foxc2−/− (Iida et al., 1997) mice have been described previously. Cx37−/− and Cx43+/− mice were interbred to generate mice deficient in both Cxs. Mice were maintained on a C57BL/6 background and genotyped by PCR using previously published protocols for Cx40−/− (Simon and McWhorter, 2002), Cx43−/− (Bobbie et al., 2010) and Foxc2−/− lines (Kriederman et al., 2003). Primers for Cx37−/− genotyping were: Primer 1: 5′-GATCTCTCGTGGGATCATTG-3′; Primer 2: 5′-TGCTAGACCAGGTCCAGGAAC-3′; and Primer 3: 5′-GTCCCTTCG-TGCCTTTATCTC-3′. Animal protocols were approved by the IACUC Committee at the University of Arizona (Tucson, AZ).

Antibodies

Primary antibodies used for immunostaining were as follows: rabbit antibodies to Cx37 (Simon et al., 2006), Cx37 (40–4200, Invitrogen), Cx40 (Gabriels and Paul, 1998), Cx43 (C6219, Sigma), Cx47 (36–4700, Invitrogen), LYVE-1 (ab14917, Abcam), NG2 condroitin sulfate (AB5320, Millipore), Prox1 (ab11941, Abcam); mouse antibodies to Cx26 (a gift from Paul Lampe), Cx43 (35–5000, Invitrogen), smooth muscle actin (C6198, Sigma), NFATc1 (sc-7294, Santa Cruz Biotechnology); rat antibodies to CD31 (MON1149, Cell Sciences), CD45 (550539, BD Biosciences), F4/80 (MF48000, Invitrogen), LYVE-1 (53–0443, eBioscience); goat antibodies to ephrinB2 (AF496, R&D Systems), Foxc2 (ab5060, Abcam), integrin-α9 (AF3827, R&D Systems), Prox1 (ab11941, Abcam), VEGFR-3 (AF743, R&D Systems); and hamster antibodies to CD3e (550275, BD Biosciences). AffiniPure minimal cross reactivity secondary antibodies (conjugated to Cy3, Cy5, or Dylight649) and unlabeled Fab fragments were from Jackson ImmunoResearch, AlexaFluor 488 goat anti-rat IgG from Invitrogen, and Vectastain Elite ABC kit (Rabbit IgG) from Vector Laboratories.

Section immunostaining

Tissues were frozen unfixed in Tissue-tek O.C.T. and sectioned at 10 μm. Sections were fixed in acetone at −20 °C for 10 min, blocked in a solution containing PBS, 4% fish skin gelatin, either 1% goat serum or 1% donkey serum, 0.25% Triton X-100, and incubated with primary antibodies for 2 h. Sections were washed with PBS containing 0.25% Triton X-100 and then incubated with secondary antibodies for 30 min. After washing, sections were mounted in Mowiol 40–88 (Aldrich) containing 1,4-diazobicyclo-(2,2,2)-octane and viewed with an Olympus BX51 fluorescence microscope fitted with a Photometrics CoolSnap ES2 camera or viewed with a Zeiss LSM 510 confocal microscope. Ly vessels were identified by staining with antibodies against Prox1, VEGFR-3, or LYVE-1.

Whole-mount immunostaining

Mesentery was fixed in 1% paraformaldehyde overnight at 4 °C, washed in PBS, permeabilized with PBS containing 0.3% Triton X-100, and then blocked overnight in PBS containing 3% goat serum and 0.3% Triton X-100. Primary antibodies diluted in PBS containing 0.3% Triton X-100 were applied to the tissue overnight at 4 °C. After washing, fluorescently labeled secondary antibodies were incubated overnight at 4 °C. Following final washes, the mesenteries were mounted on slides in Citifluor mountant (Electron Microscopy Sciences). Ear tissue was treated similarly except fixation was for 1 h at room temperature. For whole-mount Prox1 immunostaining of the TD and diaphragm muscle from embryos, the procedure was the same as with mesentery except Vectastain Elite ABC kit secondary and tertiary reagents and DAB substrate were used.

Lymphangiography with Evans blue dye

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and kept warm. Evans blue dye (EBD) (1% w/v) was injected intradermally into the hindpaws and a dissecting microscope was used to examine EBD transport. Evidence of abnormal dye reflux into hindlimb skin or mesenteric lymph nodes was noted if present. The thoracic cavity was then opened and the presence of EBD in the TD was noted along with any abnormal dye reflux into intercostal Ly vessels. In some mice, EBD was also serially injected into forepaws, snout, or ear to examine EBD movement in axillary, jugular, and ear regions, respectively.