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Absence of venous valves in mice lacking Connexin37

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ABSTRACT

Venous valves play a crucial role in blood circulation, promoting the one-way movement of blood from superficial and deep veins towards the heart. By preventing retrograde flow, venous valves spare capillaries and venules from being subjected to damaging elevations in pressure, especially during skeletal muscle contraction. Pathologically, valvular incompetence or absence of valves are common features of venous disorders such as chronic venous insufficiency and varicose veins. The underlying causes of these conditions are not well understood, but congenital venous valve aplasia or agenesis may play a role in some cases. Despite progress in the study of cardiac and lymphatic valve morphogenesis, the molecular mechanisms controlling the development and maintenance of venous valves remain poorly understood. Here, we show that in valved veins of the mouse, three gap junction proteins (Connexins, Cxs), Cx37, Cx43, and Cx47, are expressed exclusively in the valves in a highly polarized fashion, with Cx43 on the upstream side of the valve leaflet and Cx37 on the downstream side. Surprisingly, Cx43 expression is strongly induced in the non-valve venous endothelium in superficial veins following wounding of the overlying skin. Moreover, we show that in Cx37-deficient mice, venous valves are entirely absent. Thus, Cx37, a protein involved in cell–cell communication, is one of only a few proteins identified so far as critical for the development or maintenance of venous valves. Because Cxs are necessary for the development of valves in lymphatic vessels as well, our results support the notion of common molecular pathways controlling valve development in veins and lymphatic vessels.

Introduction

The first detailed description of venous valves occurring throughout the body was given by the great anatomist and surgeon Hieronymus Fabricius over 400 years ago (Caggiati et al., 2004). The morphological features of venous valves are well suited for their role as gatekeepers of unidirectional flow. In the lower leg, for example, the shape and mechanical properties of the valve cusps promote the unidirectional movement of blood, propelled largely by the contraction of skeletal muscles surrounding the veins, from the superficial veins to the deep veins of the leg and on towards the heart. Normal valve function is also needed to prevent a sudden rise in capillary and venule pressure during skeletal muscle contraction. In addition, the properties of venous valves allow them to act as flow modulators (Lurie et al., 2003).

Valvular incompetence, either congenital or acquired following scarring, fibrosis, or injury, is associated with prevalent venous disorders including chronic venous insufficiency, where there is typically sustained venous hypertension, and varicose veins, characterized by enlarged and tortuous superficial veins (Lim and Davies, 2009; Meissner et al., 2007; Oklu et al., 2012; Raffetto and Khalil, 2008). The mechanism(s) underlying the development of these disorders remain incompletely understood and are likely to be multi-factorial. Primary valve failure or even absence of valves may play an important role in venous disease in some cases, and recent genetic studies on families with inherited lymphatic and venous disorders support this notion. For example, mutations in the gene encoding the Foxc2 transcription factor have been found to cause lymphedema-distichiasis syndrome, where, along with lymphatic vessel dysfunction and probable valve defects, saphenous vein reflux is consistently observed in affected family members (Fang et al., 2000; Mellor et al., 2007; Ng et al., 2005).

In comparison to cardiac and lymphatic valves, the development of venous valves is poorly understood at the molecular and cellular level and has only recently been studied since Kampmeier's seminal histological description of the basic morphogenic events (Kampmeier and La Fleur Birch, 1927). Srinivasan and Oliver have shown that two functional copies of the *Prox1* transcription factor gene are required by the venous endothelial cells that form lymphovenous valves and venous valves (Srinivasan and Oliver, 2011). In addition, Bazigou et al. demonstrated the requirement of ephrin-B2 and integrin-α9 for normal development and maintenance of venous valves in mice (Bazigou et al., 2011). Significantly,

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all three of these proteins are also important for lymphatic valve development (Bazigou et al., 2009; Mäkinen et al., 2005; Sabine et al., 2012). A better understanding of the pathways controlling the development and maintenance of valves would facilitate development of novel molecular therapeutics targeting venous and lymphatic diseases.

Although a few studies have documented connexin (Cx) proteins in veins that lack valves (Chang et al., 2010; Inai and Shibata, 2009; Shiojiri et al., 2006), the expression and function of Cxs in venous valves has not yet been examined. Comprising a family of 21 members in humans, Cxs assemble into gap junction intercellular channels that allow for the direct diffusional transfer of small molecules, including active signaling agents, between adjacent cells (Goodenough and Paul, 2009). The gap junction channels themselves show selectivity and can be dynamically regulated in response to growth factors and other physiological signals (Ek-Vitorin and Burt, in press; Laird, 2006; Solan and Lampe, 2009). Cxs can also contribute to extracellular signaling by forming hemichannels, which are undocked channels that function as release (and possibly uptake) sites in the plasma membrane (Stout et al., 2004). In addition, Cx interactions with other proteins within the cell contribute to signaling pathways that may be unrelated to channel function (Dbouk et al., 2009; Jiang and Gu, 2005; Laird, 2010).

We have shown that specific deficiencies of Cx37 and Cx43 alone or in combination result in defective valve formation in lymphatic collecting vessels (Kanady et al., 2011). Moreover, evidence from Foxc2-/- embryos and siRNA knockdown studies with cultured lymphatic endothelial cells suggests that the Cx37 gene (*Gja4*) is a downstream target, possibly a direct target, of regulation by Foxc2 (Kanady et al., 2011; Sabine et al., 2012). Given the molecular similarities so far between lymphatic and venous valves and the observation that Foxc2 is also highly expressed at venous valves (Bazigou et al., 2011; Norrmén et al., 2011), we hypothesized that Cx37 (and perhaps Cx43 and Cx47) might also be turned on and play an important role during venous valve development.

In this study, we show for the first time that in valved veins, Cx37, Cx43, and Cx47 are expressed exclusively in the valves and that Cx expression is strikingly polarized between the upstream and downstream sides of the valve leaflets. Furthermore, we demonstrate the absence of venous valves in mice lacking Cx37, thus supporting emerging ideas about common molecular pathways controlling valve development in both veins and lymphatic vessels. Lastly, we present data on the effects of locally disrupting blood flow or wounding of the overlying skin on Cx expression in large superficial veins such as the saphenous vein.

Materials and methods

Mice

Cx37-/- (*Gja4*-/-) (Simon et al., 1997) mice were maintained on a C57BL/6 background and genotyped by PCR using previously published methods (Kanady et al., 2011). 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 Cx32 (71–0600, Invitrogen), Cx37 (Simon et al., 2006), Cx40 (Gabriels and Paul, 1998), Cx43 (C6219, Sigma), Cx43 (71–0700, Invitrogen), Cx47 (364700, Invitrogen), Prox1 (11–002, AngioBio), Prox1 (ab11941, Abcam), von Willebrand Factor, vWF (A0082, Dako); mouse antibodies to Cx43 (Cx43 IF1, a gift from Paul Lampe); rat antibodies to CD31 (550274, BD Biosciences), CD144 (555289, BD Biosciences); goat antibodies to EphB4 (AF446, R&D Systems), Foxc2 (ab5060, Abcam), VEGFR3 (AF743, R&D Systems). AffiniPure minimal cross reactivity secondary antibodies (conjugated to Alexa 488, Alex 555, Alexa 647, Cy3, Cy5, or Dylight 649) and unlabeled Fab fragments were from Jackson Immunoresearch. For some experiments, Cx37 antibody was directly conjugated to Alexa 555 using the Alexa Fluor 555 Protein Labeling Kit (A20174, Invitrogen) and Cx43 antibody (C6219, Sigma) was directly conjugated to Alexa 488 using the APEX Alexa Fluor 488 Antibody Labeling Kit (A10468, Invitrogen).

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, 1% donkey serum, 0.25% Triton X-100, and incubated with primary antibodies for 1.5 to 3 h at room temperature or overnight at 4 °C. Sections were washed with PBS containing 0.25% Triton X-100 and then incubated with secondary antibodies for 30–45 min. For experiments in which Alexa 555-Cx37 antibody or Alexa 488-Cx43 antibody (directly conjugated rabbit antibodies) were used for double labeling along with another unlabeled rabbit primary antibody, the unlabeled rabbit antibody was incubated on sections first, followed by the secondary antibody and a blocking step with 5% normal rabbit serum, before the directly conjugated antibody was applied. 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.

Saphenous artery/vein pair ligation

Mice were anesthetized with 1.5% isoflurane in O_2 during surgery and injected with buprenorphine (0.1 mg/kg) for analgesia prior to the operation. An incision was made in the left hindlimb skin and the saphenous artery–vein pair was exposed and then ligated at the knee, just above the sapheno-femoral junction, and again near the ankle, using 6–0 silk suture. The intervening saphenous artery–vein pair segment, with disrupted blood flow, was left in place and the incision was closed. For sham surgery controls, the procedure was the same except that the ligation was omitted or, in some experiments, only the surgical incision was done (for the same amount of time), without exposure of the blood vessels. 8, 24, or 72 h after surgery, the mice were sacrificed and the saphenous artery–vein pair was collected and frozen unfixed in Tissue-tek O.C.T. and sectioned longitudinally at 10 μm section thickness for immunostaining. In some experiments, the femoral and iliac artery–vein pairs were also collected.

Blood pressure measurements

Blood pressures were measured with a Hatteras Instruments multi channel blood pressure tail cuff analysis system (MC4000). Measurements were recorded on four days for each mouse with 15 measurements recorded in each individual run. 9 wild-type and 9 Cx37-/- mice were tested.

Results

In valved veins, Cx37, Cx43, and Cx47 are found exclusively at valves

Cryosections containing venous valves from mouse saphenous, brachial, iliac, or azygos veins were immunostained with antibodies