Mouse phenotyping with near-infrared fluorescence lymphatic imaging

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Abstract: We demonstrate the ability to non-invasively and quantitatively image lymphatic architecture and contractile function using dynamic near-infrared (NIR) fluorescence imaging with injection of indocyanine green in normal and transgenic mice. Unlike normal mice, which showed well defined lymphatic drainage patterns and orthograde propagation of contraction waves, we observed tortuous and mispatterned lymphatic vessels and persistent retrograde lymph flow in mice with deficiency in Prox1, a transcription factor essential for lymphatic vascular development. NIR fluorescence imaging provides a method for quantifying lymphatic function for future studies designed to discern differences in lymphatic function in murine models of human lymphatic disease.

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1. Introduction

The lymphatic system plays an important yet poorly understood role in cancer metastasis, immune surveillance, lipid absorption, and fluid homeostasis. Impaired lymphatic function has been implicated in many pathophysiological conditions, including asthma, diabetes, edema, and obesity [1–3]. The potential impact of lymphatic dysfunction has spawned vigorous research programs in the study of molecular mechanisms of developmental and adult lymphangiogenesis, the process by which new lymph vessels are formed [1]. Yet, although the lymphatics represent an important component of the microcirculation system, there is little understanding of its architecture and function, due presumably to the lack of imaging techniques to non-invasively image lymphatic vessels.

In mouse models of human disease, lymphatic architecture and function has been examined using traditional imaging techniques with injection of Evans blue dye (EBD) or dextran bound with common fluorescent dyes that excite in the visible wavelength range [4–6]. All of these studies either employed an invasive, acute preparation that can impact observations can be directly made. In man, lymphatic function and architecture is currently imaged with lymphoscintigraphy, a nuclear imaging technique which lacks the temporal and spatial resolution required to assess dynamic, lymphatic contractile function.

Recently, we developed non-invasive, near-infrared (NIR) fluorescence imaging of the lymphatics with intradermal (i.d.) injection of indocyanine green (ICG) [7–9], a dye that associates with albumin and has been used clinically in retinopathy and hepatic studies on the basis of its dark green color [10,11]. Upon employing NIR excitation at 780 nm to collect fluorescence at 830 nm, tissue penetration is enhanced and background owing to autofluorescence is minimized [12]. Due to its association with albumin, we have found that ICG is preferentially and rapidly taken up by the initial lymphatics when administered intradermally. Using non-invasive dynamic NIR fluorescence imaging, we have reported for...
the first time, lymphatic contractile function in healthy nude mice [8] as well as the change in lymphatic architecture and contractile function in tumor-bearing mice with lymph node (LN) metastasis [9]. We have also used NIR fluorescence imaging of lymphatic trafficking in patients with breast cancer, head and neck cancer, and lymphedema [13–16]. Although hairless immunodeficient nude mice are useful for the study of fluorescence imaging of propulsive lymph flow because their lack of hair prevents interference with NIR fluorescence imaging, in vivo studies must be performed in immunocompetent mice to match the strain backgrounds of transgenic mice that are engineered to mimic human lymphovascular disorders. In this study, we show lymphatic contractile function in C57BL/6 mice, the background of most transgenic mice.

A number of genetic mutations have been associated with the congenital and hereditary human lymphovascular disorder of lymphedema, a broadly classified disease in which the lymphatics fail to transport excess fluid and macromolecules that have filtered from the blood vasculature causing irreversible edema and a sequela of inflammatory responses. There is no cure for lymphedema. While several transgenic mouse models of lymphatic disorders have been developed, non-invasive characterization of lymphatic contractile function has not been performed in any of these models. PROX1 is a gene that encodes for a transcription factor unique to the lymphatics and essential for developmental lymphangiogenesis [17]. Deletion of the PROX1 gene is embryonically lethal in mice, whereas heterozygous PROX1 (PROX1+/-) mice survive [3]. However, these mice are obese compared with their wild-type counterparts and indirect, invasive imaging with injection of EBD showed abnormal lymphatic drainage [3]. Yet to date lymphatic contractile function in PROX1+/- mice has not been assessed in vivo. Herein, we present NIR fluorescence imaging of the lymphatics of athymic nude, C57BL/6, and PROX1+/- mice. The objective of this study is to show that non-invasive, dynamic NIR fluorescence imaging is sensitive enough to phenotype transgenic animal models of lymphatic disorders. This work provides an example of transgenic mouse imaging to phenotype lymphovascular disorders that might be linked to hereditary lymphatic disorders in humans.

2. Materials and methods

2.1. Animals

Athymic nude and C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) and PROX1+/- mice (provided by Dr. Guillermo Oliver, St. Jude’s Hospital, Memphis, Tennessee) were maintained in a pathogen-free mouse colony at the Frensley Center of Imaging Research at Baylor College of Medicine and at the Brown Institute of Molecular Medicine, University of Texas Health Science Center at Houston (UTHSC-H), which are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Imaging studies of C57BL/6 mice were conducted at UTHSC-H. All animal experimentation was performed after approval by the Institutional Animal Care and Use Committee.

2.2. In vivo NIR lymphatic imaging

In order to prevent fur from interfering with the fluorescent signal, C57BL/6 and PROX1+/- mice were clipped and a depilatory agent (Nair, Church & Dwight Co., Inc., Princeton, NJ) was used to remove residual hair 24 hr before fluorescence imaging. At the time of imaging, animals were placed under isofluorane anesthesia and maintained at 37 °C. A volume of 10 to 40 μL of 645 μM of ICG (IC-Green, Akorn, Inc., Buffalo Grove, IL) or 10 μl of EBD (5mg/ml; Sigma, St. Louis, MO) reconstituted in USP standard 0.9% Sodium Chloride (Hospira, Inc., Lake Forest, IL) was injected intradermally into the base of each mouse tail with 31-gauge needles (BD Ultra-Fine™ II Short Needle, Becton and Dickinson Medical, Franklin, NJ). Fluorescence images were acquired immediately after injection and for up to 30 min following injection.

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For fluorescence imaging, a field of view (FOV) across the whole body of an animal was illuminated with 785 nm light from a laser diode (85 mA, 785 nm, model Sanyo DL7140-201, Newton, NJ) using a convex lens and diffuser to create a uniform excitation field. Filter sets used in this study included an interference filter (Image quality, model 830.0-2.0, Andover Corp., Salem, NH) to transmit fluorescent emission light and a holographic filter (model HNPF-785.0-2.0, Kaiser Optical Systems, Inc., Ann Arbor, MI) to reject reflected excitation light. The fluorescence images were captured by an electron-multiplying charge-coupled device (EMCCD) camera (PhotonMax 512B, Princeton Instruments, Tucson, AZ) with 100 or 200 ms integration time for dynamic imaging and 800 ms for static imaging. To acquire white-light images for registration purposes, the optical filters were removed and a low-power lamp illuminated the animal. Image acquisition was accomplished by V++ software (Digital Optics, Auckland, New Zealand). To achieve a greater magnification, a macro lens (Leica Z16 APO, Leica Microsystems, Inc., Bannockburn, IL) was placed on the EMCCD camera and holographic and bandpass filters were attached to the macro lens for fluorescence imaging.

At the end of the imaging session, intravital fluorescence imaging was performed. An arc-shaped midway incision was made in the abdominal skin from the inguinal to the axillary regions. The subcutaneous (s.c.) connective tissue was separated to free the skin flap without injuring the lymphatic vessels. The inguinal LN (ILN) and the axillary LN (ALN) were exposed from the inner side. After mice were placed in a supine position and the skin flap was carefully spread and fixed, intravital fluorescence imaging was conducted. For intravital color imaging, EBD was intradermally injected several millimeters distal to the site of the ICG injection. Color images were acquired using a stereomicroscope (MZ16 A, Leica Microsystems, Inc., Bannockburn, IL).

2.3. Data analysis

Matlab (The MathWorks, Inc., Natick, MA) and ImageJ (National Institutes of Health, Washington, DC) were used to analyze the fluorescence imaging data in the following manner. Sequential frames of fluorescence images affected by respiratory motion were excluded for the subsequent data analysis presented herein. Fixed regions of interest (ROIs) of equivalent areas were selected along the entire length of the fluorescent lymphatic vessels on fluorescence images as was similarly done for human lymphatic imaging [14,18]. The averaged fluorescent intensity within each selected ROI was plotted as a function of imaging time and length of the lymphatic vessels. Thus, a three-dimensional plot of fluorescent intensity as a function of time and distance was generated to assess the propagation velocity and the frequency of an ICG-laden lymph fluid “packet” propelled along the lymphatic vessels. From the time between disappearance of a lymph “packet” from a selected ROI and its appearance at another ROI a known distance away from the first, the propagation velocity was directly computed for each “packet.” By assessing the number of fluorescent pulses or “packets” arriving at a ROI, the contractile frequency was computed.

3. Results and discussion

Figure 1A shows an example of a fluorescent image frame from Media 1 in the ventrolateral view of a nude mouse displaying the lymphatic trafficking of ICG from the base of the tail, where ICG was intradermally injected, to the ILN, and subsequently to the ALN. The lymphatic vessels on the lower left ventral side and contralateral regions were also visualized. In order to determine lymphatic contractile function, a series of 66 ROIs were selected along the entire fluorescent collecting lymphatic vessel as shown in Fig. 1B.

Figure 1C shows a generated, three-dimensional (3-D) spatial-temporal map of fluorescent intensity as a function of time and length, showing coordinated propagation of 19 contraction waves. The frequency of propagating contractions averaged 6.24/min. We also observed lower frequency of contractions (1.33/min) in inguinal afferent lymphatic vessels and asynchronous propulsion of ICG between afferent (pre-nodal) and efferent (post-nodal) collecting lymphatic vessels. The different lymph pump activities between the afferent and
efferent lymphatic vessels in the LN were observed previously in isolated lymphatic vessels [19,20].

To quantify the apparent propagation velocity of contraction waves, two ROIs were selected over the lymphatic vessel. As an example, Fig. 1D shows fluorescent intensity as a function of time for 10 s for ROI 1 and ROI 66 (i.e., the first and the last ROI as shown in Fig. 1C, respectively). The transit time was determined by indentifying the multiple fluorescent intensity peaks in both ROIs and was found to average 6.78 ± 2 s during 19 propagating contractions. Since the distance between two ROIs was 1.8304 cm, the averaged apparent propagation velocity was calculated to be 2.96 ± 1.02 mm/s. Unlike the contractile frequency, there was no apparent difference in propagation velocities between afferent and efferent lymphatic vessels. Propulsive lymph velocities and contraction frequencies ranged from 2.11 ± 0.74 to 8.13 ± 2.96 mm/s and 1.35 to 6.32/min, respectively, in 6 nude mice.

In addition to the difference in lymphatic function between inguinal afferent and efferent lymphatic vessels, we also observed differences in lymphatic contractility along the lymphatic vessel.

As shown in Fig. 2B, an analysis of fluorescent intensity versus time and length along the entire efferent lymphatic vessel shows (i) four contraction waves starting propagations approximately at ROI 30 and (ii) the different patterns of lymphatic propulsion, which are likely mediated by the different lymphangions in which the ROI’s were selected. In order to elucidate the different modes of lymphatic contraction and relaxation, four ROIs (i.e. ROIs 5, 18, 30, and 66) were selected and fluorescent intensities within each ROI were plotted as a function of time as shown in Fig. 2C. Fluorescent intensity profiles in ROI 5 and ROI 18 illustrate no propulsive flow and a slightly pulsatile flow, respectively.
Fig. 2. A 3-D plot of fluorescent intensity as a function of time and distance (B) after selecting 66 ROIs along the lymphatic vessel (A) shows propagating contraction waves in an orthograde direction (arrow). (C) Plot of fluorescent intensity profiles for four ROIs showing different patterns of propagating contraction waves in a collecting lymphatic vessel of a nude mouse. Asterisk denotes ILN and broken arrow ALN.

Figure 2C also shows the rapid dynamic decrease of fluorescent intensities due to the lymphatic contraction and the subsequent slow increase in the intensities due to the lymphatic relaxation and lymph “filling” within ROI 30. In addition, the passage of a packet of ICG-laden lymph through ROI 66 to the ALN is represented by intensity peaks as shown in Fig. 2C, reflecting the contractile assembly upstream emptying (contraction) and the subsequent downstream filling (relaxation). We observed the propagation velocity of $8.13 \pm 2.96$ mm/s between ROIs 30 and 66.

Intravital fluorescence imaging was also conducted after a midline incision of the abdominal skin from the inguinal to the axillary regions to confirm that ICG was picked up by the initial lymphatics and drained to LNs through the contractile, collecting lymphatic vessels.

Fig. 3. Overlay of white light and fluorescent images acquired by in vivo (A) and intravital (B) NIR fluorescent imaging after injection of ICG and a color image (C) after injection of EBD to a nude mouse. Asterisk denotes ILN. Arrows in A-C denote collecting lymphatic vessels. Scale: 1 mm.
Figure 3A shows an overlay of white light and fluorescent images that depict lymphatic trafficking of ICG along the branched internodal collecting lymphatic vessels. Figure 3B shows magnified images in the circle shown in Fig. 3A. As shown in Fig. 3B, the collecting lymphatic vessels run parallel to the blood vessels. After intravital fluorescence imaging, 10 μL of EBD was intradermally injected at the base of the tail, but several millimeters away from the previous ICG injection site for direct visualization of the lymphatics. Similar to ICG, EBD also binds to tissue macromolecules and is selectively taken up by the initial lymphatic vessels [21]. Figure 3C shows the lymphatic drainage of EBD along the lymphatic vessels, which is the same lymphatic flow pathway depicted by ICG fluorescence in Fig. 3B. As demonstrated in Fig. 3, non-invasive whole-body fluorescence imaging data correlated with intravital fluorescence and EBD images, demonstrating that both ICG and EBD were taken up by the initial lymphatics and drained from the ILN, through the contractile collecting lymphatic vessels, to the ALN.

More importantly, propulsive flow acquired from in vivo fluorescence imaging occurs along the lymphatic vessels. Figure 4 is an example from a C57BL/6 mouse, the background common to most transgenic mouse models. Figure 4 shows similar lymphatic architecture and function as described in nude mice. We observed the propagation velocity of 8.06 ± 0.74 mm/s in the inguinal efferent collecting lymphatic vessel. Propulsive lymph velocities and contraction frequencies ranged from 2.33 ± 0.51 to 8.06 ± 0.74 mm/s and 1.66 to 4.34/min, respectively, in 6 C57BL/6 mice.

We also imaged PROX1+/− mice using dynamic NIR fluorescent imaging following i.d. injection of ICG. It has been observed that most PROX1+/− adult mice were obese compared...
Fig. 6. Overlay (A) of white light and fluorescent images (B, Media 2) and quantification of abnormal lymphatic contractile function of Prox1+/− mice. Double arrows denote a tortuous collecting lymphatic vessel. (C) A 3-D plot of the fluorescent intensity as a function of time and distance. Arrow in C denotes orthograde propagating contraction wave, whereas broken arrow denotes retrograde propagating contraction wave. (D) Plot of fluorescent intensity profiles for four ROIs from which the distance between the ROIs and the transit time (Δt1 and Δt2) are obtained.

with their wild-type counterparts [3]. Figure 5 shows abnormal lymphatic drainage patterns from the injection site on the base of the tail. Diffused dye patterns as well as tortuous and ruptured lymphatic vessels were visualized as shown in Fig. 5. This phenomenon was also reported using an invasive method with injection of EBD [3]. However, lymphatic contractile function in Prox1+/− mice has not been investigated.

From our dynamic fluorescence imaging, we observed abnormal lymphatic contractile function as shown in Media 2. Figure 6A shows another example of overlay of white light and fluorescent images (Fig. 6B) from Media 2 depicting a tortuous fluorescent collecting lymphatic vessel indicated by a double arrow. A 3-D spatio-temporal map of fluorescent intensities in an inguinal efferent collecting lymphatic vessel indicates orthograde (arrow) and retrograde (broken arrow) propagation of contraction waves as shown in Fig. 6C. To quantify propagation velocity, four ROIs were selected. As shown in Fig. 6D, if we define Δt1 and Δt2 as the time delays between the fluorescence maxima or the onset of contraction of upstream and downstream regions of interest (i.e. Δt1 = t_{peak}(ROI 30) - t_{peak}(ROI 1) and Δt2 = t_{peak}(ROI 91) - t_{peak}(ROI 40)), we find negative values of Δt1 over the entire period of imaging, indicating that the contractions in ROI 30 preceded those in upstream ROI 1. The averaged Δt1 is −1.74 ± 0.16 s and the propagation velocity in the retrograde direction was found to be 6.56 ± 0.6 mm/s. We also observed positive values of Δt2, indicating propagation of contraction waves in the orthograde direction.

The averaged Δt2 is 2.81 ± 0.09 s and the propagation velocity in the orthograde direction was found to be 5.82 ± 0.2 mm/s. Persistent retrograde lymph flow, taken together with
tortuous and mispatterned lymphatic vessels, may contribute to adult-onset obesity caused by Prox1 haploinsufficiency. Using isolated and cannulated lymphatics, investigators have demonstrated contractile propagation in the orthograde and retrograde directions [22–24]. Dixon et al. [25] showed from intravital microscopic imaging that retrograde flow occurred in rat mesenteric lymphatics ranging from 2 to 18% of the time during imaging procedures. We observed the asymmetric contractility at junctions of branched lymphatic vessels and retrograde propagation of contractions in normal mice using ICG fluorescence (not shown) similar to that reported using invasive methods by others [23,24,26,27].

4. Conclusion

The physiological and pathophysiological importance of the lymphatic system could be characterized with non-invasive dynamic NIR fluorescence imaging. Indeed, we successfully demonstrated our ability to non-invasively and quantitatively image lymphatic architecture and contractile function in immunocompetent mice and in an animal model of lymphatic disease. While PROX1 mutations have not been identified in human disease, the use of non-invasive imaging to assess lymphatic function in mouse models of disease and to compare with human NIR fluorescence imaging could provide a clinically relevant diagnostic of lymphatic function, similar to how CT and MR angiography is employed in order to assess contribution of the blood vasculature in a variety of human diseases. Indeed, our group is currently conducting whole exome sequencing in humans to identify mutations that could be causative for specific lymphatic phenotypes. The use of transgenic animal models to match the imaging phenotypes found in humans with similar genetic defects could facilitate identification of disease causing mutations. In addition, if these genetic mutations point to specific pathways that could be pharmacologically mediated, then new therapeutics could be discovered for lymphedema. In addition, the ability to use the technique to conduct longitudinal imaging may enable assessment of the response to pharmacological agents as well as provide new insights into lymphatic biology.

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