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Research paper

Fluorescence imaging of lymphatic outflow of cerebrospinal fluid in mice

Sunkuk Kwon [a,*], Christopher F. Janssen [b], Fred Christian Velasquez [a], Eva M. Sevick-Muraca [a]

[a] Center for Molecular Imaging, Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center, Houston, TX 77030, United States
[b] Center for Laboratory Animal Medicine and Care, University of Texas Health Science Center at Houston, Houston, TX 77030, United States

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ABSTRACT

Cerebrospinal fluid (CSF) is known to be reabsorbed by the lymphatic vessels and drain into the lymph nodes (LNs) through peripheral lymphatic vessels. In the peripheral lymphatics, the contractile pumping action of lymphangions mediates lymph drainage; yet it is unknown whether lymphatic vessels draining cranial and spinal CSF show similar function. Herein, we used non-invasive near-infrared fluorescence imaging (NIRFI) to image (i) indocyanine green (ICG) distribution along the neuraxis and (ii) routes of ICG-laden CSF outflow into the lymphatics following intrathecal lumbar administration. We demonstrate lymphatic contractile function in peripheral lymphatics draining from the nasal lymphatics to the mandibular LNs. In addition, we observed afferent sciatic lymphatic vessels, which also show contractile activity and transport spinal CSF into the sciatic LNs. This drainage pattern was also visualized by NIRFI following intrathecal thoracic injection. *In situ* intravital imaging following intrathecal lumbar injection of blue dye shows similar distributions to that seen *in vivo* with ICG. NIRFI could be used as a tool to probe CSF pathology including neurological disorders by imaging CSF outflow dynamics to lymphatics.

1. Introduction

Cerebrospinal fluid (CSF) surrounds the central nervous system (CNS) and plays an important role in mechanical and immunological protection of the CNS (Ransohoff and Engelhardt, 2012). CSF is mainly produced by the choroid plexuses of the lateral, and the third and fourth ventricles (Sakka et al., 2011), from where it normally flows into the subarachnoid space and circulates to the cerebral hemispheres in rostral direction and to the spinal subarachnoid space in the caudal direction. In humans, nearly 0.5 l of CSF are produced each day and its efflux from the CNS is known to be from (i) absorption through subarachnoid and spinal arachnoid villi for return to the venous circulation and (ii) drainage through the cribriform plate and nasal mucosal lymphatics to the lymph nodes (LN) in animals and humans (Johnston et al., 2004; Veening and Barendregt, 2010; Hladky and Barrand, 2014).

Recent studies in animals have shown that CSF is transported from the subarachnoid space into the brain parenchyma along perivascular arterial spaces (PAS, or Virchow-Robin spaces) (Iliff et al., 2012; Louveau et al., 2015). It has been hypothesized that the water channel protein, Aquaporin-4 (AQP4), located on the foot processes of astrocytes that line the PAS, facilitates CSF transport into the brain parenchyma where it exchanges macromolecules with interstitial fluid (ISF) while moving across the brain parenchyma before emptying into the perivenous spaces and finally into the lymphatics (Iliff et al., 2012; Louveau et al., 2015). Disrupted glymphatics has recently been demonstrated in an animal model of Alzheimer's disease (AD) (Tarasoff-Conway et al., 2015; Peng et al., 2016). CFS efflux has also been demonstrated from lymphatic vessels found in the dura, which drain CSF into deep cervical LNs (DCLNs) *via* foramina at the base of the skull (Louveau et al., 2015; Aspelund et al., 2015). Therefore, CSF outflow from the brain is directed to the LNs draining the head and neck region, which constitute a major part of the peripheral lymphatic system. It remains to be determined Whether or not disrupted CSF outflow into the lymphatics has implication for human diseases that involve neuroinflammation.

Previously, our group has developed methods to non-invasively image the anatomy of peripheral lymphatic vasculatures in humans and animal models of human disease, as well as the active lymphangion pumping function that is responsible for peripheral uptake and lymphatic delivery to the venous system using near-infrared fluorescence imaging (NIRFI) (Sevick-Muraca et al., 2014). While the dynamics of CSF flow have typically been measured with invasive, surgical studies with tracer injection into the cisterna magna and parenchyma, herein we explore the *in vivo* dynamics and routes of CSF outflow into the peripheral lymphatics following intrathecal administration of ICG using

Abbreviations: AD, Alzheimer's disease; CNS, central nervous system; CSF, cerebrospinal fluid; DCLN, deep cervical lymph node; EBD, evans blue dye; ICG, indocyanine green; ISF, interstitial fluid; LN, lymph node; MLN, mandibular lymph node; MeILN, medial iliac lymph node; NIRFI, near-infrared fluorescence imaging; PAS, perivascular arterial spaces; SLN, sciatic lymph node.

* Corresponding author at: Center for Molecular Imaging, Brown Foundation of Molecular Medicine, University of Texas Health Science Center, 1825 Pressler Street, SRB 330F, Houston, TX 77030, United States.
E-mail address: sunkuk.kwon@uth.tmc.edu (S. Kwon).

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NIRFI. Our results show that in addition to CSF drainage into the mandibular lymphatics, there is transport of CSF from the spinal subarachnoid space directly into the truncal lymphatics, which is confirmed by *in situ* intravital imaging. These results may suggest new strategies to impact CSF outflow and neuroinflammation through the manipulation of lymphatic function.

2. Materials and methods

2.1. Animals

Animals were maintained in a pathogen-free mouse facility accredited by The Association for Assessment and Accreditation of Laboratory Animal Care International. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center-Houston. All experiments were performed in accordance with institutional guidelines. Five to seven month old female and male CD-1 mice (33–50 g; Charles River, Wilmington, MA) were housed and fed irradiated pelleted food and purified, acidified water.

2.2. Tracer administration

Animals were clipped and residual hair removed using depilatory cream 24 h before imaging. Mice were anesthetized with isoflurane and maintained at 37 °C on a warming pad. ICG (0.25 mg; Akorn, Inc., Buffalo Grove, IL) was dissolved in 50 μl of distilled water and diluted in 0.45 ml of artificial CSF. For intrathecal injection, the fifth-sixth lumbar vertebrae (L5–L6) area was disinfected using betadine and a small incision (~1 cm) made between the L5 and L6 vertebrae for better visualization of surface landmarks (such as spinous process) and thus accurate intrathecal injection. Since the last rib cannot be easily identified without surgical exposure, spinal segmental levels were identified by palpation of surface landmarks on the dorsum in reference to the iliac crest. Thoracic vertebrae were also identified by palpation, since T10 is transitional vertebra (Juan and Ruddle, 2003). At the last imaging, computed tomography (CT) and *ex vivo* imaging as described below was performed to confirm our landmarks. A 31-gauge needle (BD UltraFine™ II Short Needle or Hamilton syringe) was inserted between L5 and L6 vertebrae and a tail flick response was referenced as indication of correct position of the needle into the intradural space (Njoo et al., 2014; Stokes et al., 2011). Intrathecal injection of imaging agents was performed immediately after a tail flick. A volume of 10 to 30 μl (0.33 to 0.6 μl/g body weight) of 645 μM of indocyanine green (ICG; Akorn, Inc.), or a mixture of 10–30 μl (0.33 to 0.6 μl/g body weight) of ICG (15 μl) and evans blue dye (EBD; 15 μl) was injected and the incision closed with surgical glue (3M Vetbond, 3M Animal care products). The injection site was covered with the black electrical tape to prevent oversaturation of the camera. In addition, a different group of mice received intrathecal thoracic injection between the 10th and 11th thoracic vertebrae (T10–T11) as described above. In order to demonstrate that fluorescent signals in peripheral lymph nodes arose from CSF into the lymphatics rather than hemovascular delivery through the high endothelial venules in the lymph nodes, another group of mice ($n = 2$) intravenously received 100 μl of 645 μM of ICG.

2.3. Fluorescence imaging and computed tomography

Dynamic whole-body NIRFI was performed before, immediately, and for up to 20 min after injection of the tracers using a custom-built fluorescence imaging system (Kwon and Sevick-Muraca, 2007, 2011) using excitation and emission wavelengths of 785/830 nm for ICG, respectively. In addition, dynamic NIRFI imaging of afferent cervical and spinal lymphatic vessel function was conducted for 10 min at 30 min post injection. After NIRFI, intravital color and EBD fluorescence imaging using fluorescence stereomicroscope (Leica M205 FA) was performed. LNs were collected and imaged. The nomenclature used in Van den Broeck et al. was used to identify LNs (Van den Broeck et al., 2006).

Computational tomography (CT) imaging was also performed using an INVEON multimodality CT (Siemens Medical Solutions, Knoxville, TN, USA). The CT imaging parameters were X-ray voltage of 80 kV with an anode current of 500 μA, and an exposure time of 260 ms of each of the 120 rotation steps over the total rotation of 220° at medium system magnification. CT images were reconstructed using a Feldkamp cone-beam algorithm with a ramp filter cut off at the Nyquist frequency. CT images were analyzed using INVEON Research Workplace (Siemens Preclinical Solutions, Knoxville, TN).

2.4. Analysis of lymphatic vessel function and statistics

ImageJ (National Institutes of Health, Washington, DC) was used to analyze the fluorescence imaging data (Kwon and Sevick-Muraca, 2011). To reveal lymphatic contractility, fixed regions of interest (ROIs) in fluorescent lymph channels were defined on fluorescence images. The mean of the fluorescence intensity within each ROI in each fluorescence image was then calculated and plotted as a function of imaging time to demonstrate contractile lymphangion activity.

Data was presented as average values ± standard error (SE). Statistical analysis was performed with Prism 5 (Graphpad Software, Inc.). The average fluorescent intensity data from individual time points were analyzed by unpaired 2-tailed Student's *t*-tests. The significance level is set as $p < 0.05$.

3. Results

Fig. 1 shows representative white light, NIRF, and merged images of a mouse at 30 min post injection of 0.33 μl/g (10 μl in 30 g of a mouse) of ICG between L5 and L6 vertebrae. Due to the drainage of ICG-laden CSF from the cribriform plate region into nasal lymphatics, we observed strong NIR fluorescent signals in the endoturbinates (open arrow), which makes eyes and upper hard palate in the mouth appear to be fluorescent (Fig. 1). ICG fluorescence was also detected in cisterna magna and supracerebellar cistern (red circle in Fig. 1). The liver was fluorescent due to ICG clearance (arrowhead in Fig. 1). In addition, two fluorescent lymphatic vessels parallel the facial vein draining to mandibular lymph nodes (MLNs) were visualized. Our dynamic NIRFI demonstrated propulsive lymphatic function in the two peripheral lymphatic vessels draining to the MLNs (Fig. 2 and Video 1), as evidenced by dynamic changes of fluorescent intensities in two ROIs selected in the peripheral vessels as shown in Fig. 2B. Our quantification data showed that the average contraction frequency of the vessels draining to the MLNs 30 min after intrathecal lumbar injection was 5.2 ± 0.5/min ($n = 5$).

We imaged mice longitudinally in order to examine ICG distribution along the neuraxis over time. Fig. 3 shows representative fluorescent images of a mouse at 0.5, 1, 3, 5, 7, 24, 48, and 72 h post injection. ICG fluorescent signal diffused in both rostral and caudal directions after injection. As shown in Fig. 3A, ICG moved rostrally towards the brain as evidenced by fluorescent signal in the cisterna magna at 30 min after injection. ICG fluorescence was found in the MLNs and between the fourth sacral and first coccygeal vertebrae (S4 and C1) 30 min post-injection. NIRFI data showed an increase in the fluorescent intensity in the MLNs and cisterna magna for up to 3 h post injection and then a slow decrease over 72 h post injection. (Fig. 3B). We observed that fluorescent intensities in the cistern magna were significantly higher than those in the MLNs at 1, 3, 5, 7, 24, and 48 h post injection (Fig. 3B).

We also sought to investigate how injection volume affects CSF drainage. When a 0.6 μl/g bolus of ICG was injected, we observed lymphatic drainage to the sciatic LN (SLNs) from the vertebral segment (Fig. 4). NIRFI demonstrated rapid caudal spread of ICG all the way down to the first coccygeal vertebra immediately after intrathecal injection and then into the SLN *via* lymphatic vessels (Fig. 4). Dynamic