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Imaging of Cerebrospinal Fluid Space and Movement in Mice using Near Infrared Fluorescence

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ABSTRACT

We developed an optical method for imaging the cerebrospinal fluid (CSF) space and the movement of CSF in mice using a near infrared fluorescence imaging methodology. Indocyanine green bound to high-density lipoprotein (ICG-HDL) was injected into the lumbar subarachnoid space of nude mice. The time course of CSF movement was followed over 48 hours. The imaging system was configured for epi-fluorescence measurements at near infrared wavelengths using the illumination light and narrow band excitation filtration with central wavelength of 755 nm. Emission light was filtered with a longpass filter with a cutoff at 798 nm. After the injection of ICG-HDL, a strong fluorescence signal clearly delineated the cisterna magna, bilateral supracerebellar/cerebello-pontine angle cistern, and interhemispheric cistern. Much of the fluorescent tracer was washed out within 24 hours after the injection. This study has therefore demonstrated that an optical method, employing near infrared fluorescence imaging, can determine the CSF space and the movement of CSF in nude mice.

Key words: Cerebrospinal fluid, Fluorescence, Imaging, Mice, Near infrared

INTRODUCTION

Fluorescence has long been employed as an investigational and diagnostic tool in medical and biology. The *in vitro* diagnostic techniques of fluorescence microscopy, fluorescence-activated cell sorting (FACS) and fluorescence in situ hybridization (FISH) are well established. *In vivo* applications of fluorescence are also employed although they mainly involve local administration or detection of the fluorophore. Fluorescence fundus angiography (FAG) in ophthalmology and the intraoperative discrimination of glioma tissue during neurosurgical operation using fluorescence imaging (Haglund et al., 1996; Kuroiwa et al., 1998; Stummer et al., 2000) are two such applications. Systemic *in vivo* imaging of small animals using fluorescent tracers is a technique that has recently emerged (Sweeney et al., 1999; Ntziachristos et al., 2003).

Clinically, dynamic cerebrospinal fluid (CSF) imaging is performed using radioisotope (RI) or computed tomography (CT) cisternography or cine-mode magnetic resonance imaging (cine MRI). RI cisternography has low spatial resolution and involves the complicated handling of radioactive materials. CT cisternography has high spatial resolution with axial tomographic images, however sagittal images are not directly accessible. There is also a possibility of adverse reactions induced by intrathecally-injected metrizamide (Drayer and Rosenbaum, 1978). Both RI and CT cisternography have the disadvantage of exposure to ionizing radiation. Cine MRI is non-invasive and has high resolution, however the entire flow dynamics, including wash out from the CSF space, are not evaluated.

To overcome these limitations, we have developed an optical method for imaging CSF space using a novel near infrared (NIR) fluorescence imaging methodology. The objective of this study was to establish the technique and employ it to image the CSF space and evaluate physiological CSF movement in mice. We also compared fluorescent images to anatomical images and identified the anatomical structures responsible for the most prominent fluorescent signals.

MATERIALS AND METHODS

Compounds, animals and procedures

The Institutional Animal Care and Use Committee of Harvard Medical School approved all procedures in accordance with international guidelines. All efforts were made to minimize the number of animals used and their suffering.

Indocyanine green (ICG) is a tricyanocyanine dye, which has a major absorption peak at approximately 780 nm and fluorescent emission peak at approximately 820 nm. Following the methodology of Sakatani et al. (Sakatani et al., 1997), ICG (Sigma-Aldrich, St. Louis, MO) was prepared as a 6.45mM stock solution in 0.8% NaCl. Human high-density lipoprotein (HDL) (Sigma-Aldrich, St. Louis, MO) was prepared as 10 mg/ml solution in phosphate buffered saline

(PBS, Sigma-Aldrich, St. Louis, MO). The final compound was prepared by mixing 5 μ l of the 6.45mM ICG stock solution with 0.5 ml of prepared 10 mg/ml human HDL solution. The rationale for the ICG-HDL complex was based on the fact that most ICG in circulating blood is bound to lipoprotein (Baker, 1966).

We examined the stability over time of two ampules of ICG-HDL solution, one maintained at room temperature and the other maintained at -80°C and only thawed a few minutes prior to each measurement. Samples from these ICG-HDL stocks were diluted 20:1 in methanol and the fluorescence intensity of diluted solution measured by the imaging apparatus. The fluorescence intensity of ICG-HDL kept frozen was relatively constant, only 10% decline over 3 months while the sample maintained at room temperature showed continuous decline, about 70% decline over 3 months (data not shown).

CD-1 nude mice, 7 weeks old (NU/NU, Charles River Laboratories, Wilmington, MA) about 25g, were anesthetized by intraperitoneal injection of a mixture of Ketamine (100mg/kg) and Xylazine (10mg/kg). A total of 10 mice were used for the imaging study. Under a stereoscopic operating microscope, the 5th lumbar lamina was removed and the spinal cord covered with dura matter was exposed. Then, 5 μ l of ICG-HDL solution thawed from frozen stock was injected into the subarachnoid space using a Hamilton microsyringe (Hamilton, Reno, Nevada). All animal procedures were done by one of the authors (Y.S.).

Imaging hardware

Anesthetized mice were positioned in the optical imaging system developed in our laboratory (Figure 1). The system was configured for epi-fluorescence measurements at NIR wavelengths using the illumination light from a 450 W Xe arc lamp (Oriel Instruments, Stratford, CT) and narrow band excitation filtration obtained by an interference filter (Omega Optical, Andover, MA, custom order) with a bandwidth of 33 nm and central wavelength of 755 nm. Emitted light was filtered with a longpass optical filter (Omega Optical) with a cutoff at 798 nm.

Whole-body images were digitized by a high resolution, cooled, CCD digital camera (ORCA-ER, Hamamatsu, Hamamatsu City, Japan) attached to a Pentium III-based personal computer running LabVIEW 6 system integration and automation software (National Instruments, Austin, TX).

Image acquisition and processing

The progress of ICG tracer was monitored by the imaging system as evidenced by the intensity of the NIR fluorescence signal emitted. Dynamic measurements of CSF flow were obtained by repeating the imaging procedure at 0.5, 1, 2, 4, 6, 24 hours post injection. In addition, 2 mice were imaged at 30 and 48 hours following injection of the tracer. An effort was made to position the head and feet at the same points on the stage prior to each image acquisition. Quantitative assessment of CSF movement was obtained by placing regions of interest (ROIs) on emission images and plotting the time-intensity profiles. ROIs were placed on the cisterna magna and bilateral cerebellopontine angle.

All images were acquired with the lens aperture set to F/1.6 and in the full resolution (1280 X 1024 pixels), non-binning, frame-blanking acquisition mode of the Hamamatsu camera. Exposure time for all images was 1.2 sec.

Each image was acquired using LabVIEW 6 software in PNG format. These images were processed and converted to TIFF format using custom software running on MATLAB release 12.1. (The Mathworks Inc., Natick MA). Further image manipulation, compression to 8-bit scale and annotations were performed with Photoshop 6.0 software (Adobe systems Inc., San Jose, CA).

Physiological CSF movement was investigated in normal adult mice. A total of 8 nude mice were used for serial imaging. Flow patterns were established and time-intensity curves were analyzed to establish baseline measurements. Anatomical correlation with near infrared imaging was addressed by sacrificing 2 mice at 2 times post injection and comparing macroscopic findings with near infrared imaging. In these cases the near infrared image of the

brain was taken after the skin and skull were removed. Furthermore after the brain was removed, the near infrared image of the upper and lower brain surfaces were taken and later compared with macroscopic findings. In order not to alter fluorescence distribution, we carefully removed convexity and skull base piece-by-piece using microsurgical techniques, so that minimal pressure was applied to the subarachnoid space.

All mice were sacrificed at the end of each experiment. The whole brain was then removed and fixed in 10% (v/v) formaldehyde solution. Axial sections of the fixed brain were stained using hematoxylin and eosin and evaluated histologically under an optical microscope.

RESULTS

Comparison of near infrared imaging and macroscopic findings

In order to identify anatomical structures that contribute a strong fluorescence signal to the images, we compared NIR imaging results with macroscopic findings. Figure 2 shows serial near infrared images taken after the injection of ICG-HDL into the lumbar spinal subarachnoid space. In these images we observed 1) dye accumulation in the occipital wide area, 2) bilateral strong signals in front of this wide dye accumulation, and 3) a midline longitudinal linear signal in the frontal part of the head. Figure 3 shows the white-light image and near infrared image of the mouse sacrificed 4 hours after ICG-HDL injection and these images were acquired after the upper skull was removed. A strong signal from the cisterna magna and supracerebellar cistern becomes more evident. Figure 4 shows the white-light image and near infrared image of the lower surface of the brain of the same mouse. We observed a very strong signal at the bilateral cerebello-pontine (C-P) angle, prepontine cistern and basal cistern. From these findings we conclude that 1) the dye accumulation in the occipital wide area corresponds to the cisterna magna, 2) the bilateral strong signal in front of this wide dye accumulation corresponds to the supracerebellar/C-P angle cisterns, and 3) the midline longitudinal linear signal in the frontal part of the head corresponds to the basal cistern.

Serial near infrared imaging

Following lumbar laminectomy, a nude mouse was injected with 5 μ l of ICG-HDL into the subarachnoid space. Figure 2 shows serial images of a typical mouse. Image quality was sufficient and image acquisition was rapid and repeatable. The fluorescent signal was strong enough and the noise was sufficiently suppressed. As early as 120 minutes post injection the fluorescence emission signal was evident in the posterior part of the head, emanating from the cisterna magna. The signal was strongest at 240 or 360 minutes post injection. In these images, the signal from the bilateral C-P angles was clearly observed. After this time, all signals gradually decreased. As illustrated in Figure 2, in some mice, the signal was still detectable above the background 48 hours post injection.

Three regions of interest were placed over the cisterna magna and bilateral supracerebellar/C-P angles (Fig. 2). The contrast was calculated as the difference in signal intensity relative to the signal in the control image acquired at time 0. Figure 5 shows the temporal sequence of the mean ROI contrast from 6 normal mice. The contrast peaked at approximately 4 hours post injection and then the tracer gradually washed out. The contrast at the bilateral supracerebellar/C-P angles was stronger than at the cisterna magna. ROI data were fit to a biexponential expressions of the form

$$S_{\text{ROI}}(t) = \frac{k}{(\alpha_2 - \alpha_1)} \left(e^{-\alpha_1(t-T_0)} - e^{-\alpha_2(t-T_0)} \right)$$

involving a scale factor, k , time shift, T_0 , and two exponential decay constants, α_1 and α_2 .

Smooth curves constructed from the least-squares solution are shown in the graph of Figure 5.

Histological study

The brain tissue of each mouse was examined following imaging and showed no histological abnormality under optical microscopy with hematoxylin and eosin staining.

DISCUSSION

Optical method for CSF imaging

Our results showed consistent findings of normal CSF movement in mice. This method is useful in determining the rate of CSF turnover, i.e. production and drainage. The cisterna magna and the supracerebellar/C-P angles are two of the largest non-ventricular CSF spaces. In this study of mice with expected normal CSF flow, ventricular CSF spaces were not externally detectable. This is expected because under normal conditions CSF reflux into ventricles does not occur. CSF is absorbed in the sagittal sinus. In our study however, CSF movement into the sagittal sinus was not demonstrated. This is likely due to the dilution of the ICG-HDL. Precise quantification of CSF flow rate using our data is not easy, because signal intensity was affected by multiple factors. A method to compensate for other factors influencing signal intensity is under development.

Other methods for CSF imaging

RI and CT cisternography as well as our NIR fluorescence imaging method all require intrathecal injections of contrast material. The advantages of cine MRI are that no invasive procedure is employed and no contrast material injected.

In RI and CT cisternography, relative quantification of CSF flow is possible, however absolute measurement of CSF flow rate or velocity is impossible. In cine MRI, absolute measurement of CSF flow velocity is possible for a limited region of interest, however there are many artifacts that influence this measurement. Kim measured CSF flow velocity in normal subjects and in patients with hydrocephalus using MRI with a two-dimensional cine phase contrast sequence (Kim et al., 1999). The results showed substantial variation of amplitude parameters in both normal subjects and patients and was therefore thought to be unreliable. In order to measure absolute CSF flow rate or velocity in NIR fluorescence imaging, further

investigation is needed. Correlation between NIR fluorescence imaging and cine MRI may be helpful for that purpose.

In the full assessment of CSF dynamics and the detection of abnormal convexity flow, RI cisternography is superior to CT cisternography (Piepgras et al., 1978; Tamaki et al., 1978). Cine MRI cannot show full CSF dynamics in one image. At present, only RI cisternography can demonstrate the entire CSF dynamics in a single image. Our methodology, which uses NIR fluorescence, could become an alternative to RI cisternography. RI cisternography is less susceptible to suboptimal images due to motion, a problem with patients suffering from dementia (Enzmann et al., 1979). This lower susceptibility to motion artifact also applies to our CSF flow measurement technique using NIR fluorescence.

Technical limitations of the method

Because the spinal CSF space in mice is very narrow, it is impossible to inject the dye directly. We injected the dye into the spinal cord and the back flow of the dye is sufficient to show intracranial CSF flow. Because of this complicated method of injection, the intracranial signal intensities were somewhat variable between mice, although we injected the same quantity of dye in each experiment.

The signal intensity and noise are very sensitive to the position and the angle of the mouse head. Although an effort was made to reposition mice accurately prior to each acquisition, invariably errors due to misregistration will show up as intensity variations. To completely eliminate these errors the head would have to be repositioned exactly in three dimensions prior to each image acquisition. It might be feasible to secure the head for the full duration of the study if continuous inhalation anesthesia were employed. In this study we used nude mice, because hair scatters the light and degrades the images. Application to other mice would require mechanical or chemical hair removal.

Usefulness and limitation of fluorescent imaging

The advantages of optical imaging are noninvasiveness, except for the injection of the fluorescent probe, rapid and repeatable image acquisition, no need for complex or expensive equipment and the possibility for future advanced applications involving multiple fluorescent probes emitting at varying wavelengths. The disadvantages are a high degree of scattered photons contributing to the degradation of image resolution and the limited ability for deep tissue penetration. Our study clarified the usefulness and limitations of this technique for CSF flow imaging in mice. This method will be useful to investigate CSF flow in small animal. Because human skin and skull are much thicker than that of the mouse and because this methodology doesn't scale dimensionally, the adaptation of this method to CSF flow imaging in humans is not straightforward. However the equipment needed to perform measurements is compact and so it provides the possibility of a new diagnostic tool for CSF flow measurement that might be applied at the bedside. Local cerebral blood flow in humans has indeed been measured using NIR at the bedside (Edwards et al., 1988; Kusaka et al., 2001; Villringer et al., 1993).

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Figure legends

Fig. 1: Optical imaging system block diagram

Fig. 2: Temporal sequence of near infrared images from the same mouse following the injection of ICG-HDL into the lumbar spinal subarachnoid space. The top right image shows ROI placement on the 6 hour image.

Fig. 3: White-light image (left) and near infrared image (right) acquired 4 hours after the injection of ICG-HDL into the lumbar spinal subarachnoid space and with upper skull removed. Note the strong signals from the cisterna magna and supracerebellar cistern.

Fig. 4: White-light image (left) and near infrared image (right) of the lower surface of the brain of the same mice. Note the very strong signals at the bilateral cerebello-pontine angle, prepontine cistern and basal cistern.

Fig. 5: Signal intensity (mean \pm SEM) for three regions of interest as marked together with the smooth curves obtained by least-squares fitting to a biexponential model.