Quantification of cerebrospinal fluid transport across the cribriform plate into lymphatics in rats

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Nagra, Gurjit, Lena Koh, Andrei Zakharov, Dianna Armstrong, and Miles Johnston. Quantification of cerebrospinal fluid transport across the cribriform plate into lymphatics in rats. Am J Physiol Regul Integr Comp Physiol 291: R1383–R1389, 2006. First published June 22, 2006; doi:10.1152/ajpregu.00235.2006.—A major pathway by which cerebrospinal fluid (CSF) is removed from the cranium is transport through the cribriform plate in association with the olfactory nerves. CSF is then absorbed into lymphatics located in the submucosa of the olfactory epithelium (olfactory turbinates). In an attempt to provide a quantitative measure of this transport, 125I-human serum albumin (HSA) was injected into the lateral ventricles of adult Fisher 344 rats. The animals were killed at 10, 20, 30, 40, and 60 min after injection, and tissue samples, including blood (from heart puncture), skeletal muscle, spleen, liver, kidney, and tail were excised for radioactivity assessment. The remains were frozen. To sample the olfactory turbinates, angled coronal tissue sections anterior to the cribriform plate were prepared from the frozen heads. The average concentration of 125I-HSA was higher in the middle olfactory turbinates than in any other tissue with peak concentrations achieved 30 min after injection. At this point, the recoveries of injected tracer (percent injected dose/g tissue) were 9.4% middle turbinates, 1.6% blood, 0.04% skeletal muscle, 0.2% spleen, 0.3% liver, 0.3% kidney, and 0.09% tail. The current belief that arachnoid projections are responsible for CSF drainage fails to explain some important issues related to the pathogenesis of CSF disorders. The rapid movement of the CSF tracer into the olfactory turbinates further supports a role for lymphatics in CSF absorption and provides the basis of a method to investigate the novel concept that diseases associated with the CSF system may involve impaired lymphatic CSF transport.

olfactory nerves; arachnoid granulations and villi; hydrocephalus; pseudotumor cerebri

THE DRAINAGE OF CEREBROSPINAL fluid (CSF) from the brain plays an important role in maintaining homeostasis within the cranium. In the classical concept, CSF is absorbed from the subarachnoid compartment by arachnoid villi and granulations. However, considerable evidence exists to support the notion that lymphatic vessels external to the cranium play an important role in this process (reviewed in Ref. 25). Although there are no lymphatic vessels within the parenchyma of the brain, CSF moves through the cribriform plate foramina in association with the olfactory nerves and is taken up by an extensive network of lymphatic vessels located within the olfactory submucosa. Once CSF has entered the absorbing lymphatics, it is conveyed in progressively larger ducts through various lymph nodes and is deposited ultimately in the venous system at the base of the neck (44). This concept is supported by studies in many mammalian species (25), including humans (8, 19, 28, 42) and nonhuman primates (6, 18, 30, 43).

Quantitative support for a lymphatic role in CSF transport has come mainly from studies in sheep (2, 3, 32, 35). These studies used radioactive protein tracers, infusion protocols, and surgical techniques to block flow through the cribriform plate. Overall, these experiments demonstrated that lymphatics have the major function in removing CSF from the cranium.

With this concept gaining momentum in the literature, it is now worth considering whether an impediment to CSF uptake by lymphatics contributes to the pathogenesis associated with disorders of the CSF system. In this regard, experimental approaches in sheep will likely continue to provide important information on CSF parameters. However, studies in rodents have several advantages. Apart from cost considerations, CSF dynamics in rats have been studied extensively (9, 12, 15, 22, 29), and a strong connection between CSF and extracranial lymph has already been made in this species (4, 19, 23, 26, 40). Additionally, the rat has proven to be an important species for the study of CSF disorders such as hydrocephalus (1, 10, 20, 21, 24, 27, 31, 34, 39). Nonetheless, many of the experimental protocols designed for use in sheep are difficult to apply to rats because of the relatively small size of the latter.

With these factors in mind, our objective in this study was to develop a relatively simple quantitative approach to assess lymphatic CSF absorption in rats. Once CSF is taken up by lymphatics adjacent to the cribriform plate, it enters a network of vessels in the olfactory turbinates, and consequently, it seems reasonable to expect that the turbinate tissues would have high concentrations of a CSF tracer. This assumption was supported by the studies presented in this report, and we conclude that the turbinate protocol will provide a routine measure of lymphatic CSF absorption that can be applied to studies of the pathophysiological implications of lymphatic CSF transport.

MATERIALS AND METHODS

A total of 60 adult Fisher 344 rats (148–248 g) and two 9-day-old pups were used for this investigation (purchased from Charles River, Saint-Constant, QC, Canada). The adult animals were fed lab rat chow (LabDiet 5001) until death. All experiments were approved by the ethics committee at the Sunnybrook Health Science Centre and conformed to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

Rats were anesthetized initially by placement in a custom-built rodent anesthesia chamber using halothane (4–5%) in oxygen. For the

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Experimental procedure, they were maintained with 2–2.5% halothane in oxygen delivered by a nose cone (rat anesthesia mask, Kopf, Model 906, Tujunga, CA). The animals were placed on a heating pad (Fine Science Tools, Vancouver, BC) and fixed in position in a small animal stereotoxic device (Kopf, Model 900). The skin over the cranium was removed and the junction of the sagittal and coronal sutures was identified. A small high-speed microdrill with a rounded tip (Fine Science Tools) was used to grind away the bone to expose the dural membrane.

A 50-μl Hamilton syringe (Fisher Scientific, Toronto, Ontario) with a 30-gauge needle was used at the location of 0.92 mm posterior and 1.4 mm lateral to the bregma, and 3.3 mm deep to the dural membrane at the left or right hemisphere in a stereotoxic apparatus. The needle was loaded with 125I-human serum albumin (HSA), and the tip was lowered into one of the lateral ventricles (the right and left ventricles were used randomly in this study). The coordinates were noted from the stereotoxic instrument and adjusted according to the reference values from a rat brain atlas (37). Fifty microliters containing 500 μg of 125I-HSA (0.93 MBq/ml, 10 mg/ml, Drax Image, Quebec) was injected into one of the lateral ventricles. The needle was removed after 1–2 min, and the needle path sealed with bone wax. After 10, 20, 30, 40, or 60 min, the animals were killed by injection of 1.0 ml euthanyl intraperitoneally. Immediately after death, a blood sample was taken from the heart. A kidney and the spleen were removed, and samples from liver, skeletal muscle, and tail were collected. In five animals in the 30-min group, the lymph nodes in the neck region, including the cervical nodes (6–8), as well as the popliteal (2) and mesenteric nodes (2–3), were excised. All tissue samples were placed into preweighed glass test tubes for weight determination using a Mettler BB2400 balance. The carcasses were then frozen for at least 24 h in a freezer.

To facilitate the assessment of radioactivity in the olfactory submucosa and to prevent potential post mortem tracer contamination from the CSF compartment, a portion of the turbinates were cut from frozen tissue (illustrated schematically in Fig. 1E). A diagonal line was marked on each animal at a 45° angle relative to the orbitomeatus line rostral to the orbit. A second line was drawn 1.0 cm anterior and parallel to the first line, and the selected coronal tissue block was excised with a fine-tooth saw. The frozen tissue section was placed flat under a dissecting microscope (Stereomaster, Cole-Parmer, Anjou, Quebec) and measured top to bottom. This number was divided by 4 and the top one-fourth, bottom one-fourth, and middle half were assessed separately for radioactivity. The upper quarter represented partial superior cells of the ethmoid turbinates, along with cartilage and soft tissues of the nasal wall. The middle portion contained the main portion of the turbinates (henceforth termed middle turbinates). The bottom section consisted of a fraction of the posterior cells of the turbinates and part of the hard palate. The ethmoid turbinate samples were weighed as described above. All tissue samples were monitored for radioactivity in a multichannel gamma spectrometer (Compugamma, LKB Wallac, Turku, Finland).

Visualization of transport across the cribriform plate. We used two methods to visualize the movement of CSF across the cribriform plate. In five adult rats, 100 μl Evans blue dye (Fisher Scientific, Toronto, Canada; 2% in sheep plasma) was injected into the lateral ventricle using methods similar to those used for the radioactive tracer. After 20 min, the rats were killed and frozen. After decapsulation, the heads were sectioned in either a sagittal, coronal, or axial plane. The tissues were examined under a dissecting microscope (Meiji EMZ-TR, Cole Palmer), and images were captured on a Nikon digital camera (Coolpix 995).

In a second group of five adult and two 9-day-old pups, yellow Microfil (MV-122, Flow Tech, Carver, MA) was injected into the cisterna magna post mortem. The best results were obtained using a preparation that was more dilute than that recommended in the product literature. In adults, 3 ml of diluent was used for every 1 ml of yellow Microfil, and the material catalyzed with 10% (of total volume) of MV Curing Agent. In 9-day-old rat pups, 2.5 ml diluent was used with 2.5 ml Microfil. Immediately after death in adults, a laminectomy was performed at the C7 cervical-thoracic level of the vertebral column and a 26-gauge angiocatheter inserted in the cisterna magna. Microfil (2.5–3 ml) was infused into the cranial subarachnoid space. In pups, 0.2 ml was injected suboccipitally. The Microfil was allowed to set for 20 min. The animals were frozen overnight, and sagittal, coronal, or axial slices were made of the head in preparation for photographic and histological studies. Cranial slices for all studies were performed to reveal the olfactory bulb and olfactory turbinates using a planar cooled with dry ice clamped to a bench.

For histology, the tissues were harvested from 9-day-old pups and fixed in 10% formalin. Good sections of the cribriform plate area were difficult to achieve in adult animals because of the proximity of bone and soft tissues that caused disruption of the area of interest. Consequently, for histology, we used younger animals. The soft cartilaginous cribriform plate in these specimens proved much easier to section. The tissues were paraffin-embedded and cut into 4-μm sections and stained with hematoxylin and/or eosin. Histological assessments were performed using a Motic Digital Microscope (DMBS), and images were acquired using Motic Images Advanced 3.0 software (GENEQ Montreal, Canada).

Analysis of data. Tracer recoveries in all tissues were expressed as percent injected dose/g tissue. All data were expressed as the means ± SD. The results were analyzed with a two-factor repeated-measures ANOVA (Group×Tissue) followed by the post hoc Tukey Studentized Range Test. Additionally, to compare the differences between the tissues at each time point, post hoc single degree-of-freedom contrasts were performed. We interpreted P < 0.05 as significant.

RESULTS

Visualization of CSF transport across the cribriform plate. Twenty minutes after the injection of Evans blue dye into the CSF compartment, the dye was observed to distribute throughout the CSF compartment and was found in the subarachnoid space around the olfactory bulbs and adjacent to the cribriform plate (Fig. 1, A and B). The cribriform plate lies at the base of the brain and supports the olfactory bulbs. Fig. 1F illustrates the cribriform plate in an adult rat after removal of the brain and olfactory bulbs. In addition, Evans blue dye was observed external to the cranial within the olfactory turbinates (Fig. 1, A and B). This indicated that material injected into the CSF space was transported rapidly through the cribriform plate and formed the basis of the quantitative studies that were to follow. We did not observe any obvious dye at other locations, although it was likely that some would be present within the venous system, as was suggested by the vascular entry of the 125I-HSA (see below).

The dye could not be observed within individual lymphatic vessels macroscopically, presumably because of the dissociation of the dye-protein complex. In addition, the tissues with Evans blue were not amenable to microscopic analysis, as the dye was lost during preparation for histology. However, Microfil administered into the CSF space could be found in the olfactory submucosa in an extensive lymphatic network (Fig. 1C). Individual lymphatic vessels containing this contrast agent were clearly visible. On histological examination in young rats (see MATERIALS and METHODS), Microfil appeared in lymphatic vessels throughout the submucosa (example in Fig. 1D).
Comparison of tracer concentrations in various tissues over time. We observed considerably higher tracer concentrations in the olfactory turbinates compared with those measured in blood (Fig. 2). The highest concentrations of $^{125}$I-HSA were found in what we have termed the middle turbinate area, which represented the bulk of the olfactory turbinates. The lower turbinates (which include some of the posterior cells of the turbinates and part of the hard palate) also contained high concentrations of the tracer. The radioactivity in the upper turbinates (representing partial superior cells of the ethmoid turbinates along with cartilage and soft tissues of the nasal wall) was similar to that observed in blood. The recoveries of CSF tracer in all olfactory turbinates peaked 30 min after injection and at this time, the average radioactive recoveries in the middle (9.4%) and lower turbinates (5.9%) were 5.9 and 3.7 times higher than the blood average (1.6%), respectively.

In five of the animals in the 30-min group, various lymph nodes were excised and assessed for radioactivity. Averaged recoveries in the pooled neck lymph nodes were high, being in the same range or even greater than those in the middle turbinates (inset in Fig. 2). These nodes are known to be interspersed along lymphatic CSF drainage pathways. In contrast, there is no evidence to suggest that the popliteal and mesenteric lymph nodes collect lymph/CSF from the subarachnoid compartment, and tracer recoveries in these nodes were very low.

CSF ultimately drains into the blood through lymphatics and possibly to some extent through arachnoid projections. In this regard, blood levels of the radioactive protein peaked 40 min after tracer installation but also showed an initial concentration spike at 10 min (Fig. 3; the blood recoveries from Fig. 2 have been plotted here with adjustment of the y-axis scale). Tracer recoveries in skeletal muscle, spleen, kidney, liver, and tail were much lower but seemed to mirror those in blood.

**DISCUSSION**

By sampling a tissue through which lymphatic vessels are transporting CSF, we hoped to capture a time when the radioactive “signal” was sufficiently high to warrant the development of a routine method to assess CSF movement across the cribriform plate. Focusing our study on the extracranial olfactory tissues seems to have achieved this goal as the concentration of the CSF tracer was much higher in the turbinates than in the other tissues monitored.
MATERIALS AND METHODS), we found it difficult to assess whether this concept. For technical reasons (as described), in this report, we included images from 9-day-old postnatal pups (Fig. 1D). However, in nonprocessed tissues (Fig. 1C) Microfil particles were noted within discrete lymphatic vessels but were not commonly observed in the surrounding tissues.

A second limitation of using CSF tracer concentrations in the olfactory turbinates to reflect lymphatic uptake relates to the nature of the measurements themselves. Ideally, we would like to quantify the total mass of CSF tracer that had entered the olfactory submucosa over time. For example, we could have estimated the mass of tracer in each tissue by multiplying the cpm/g by tissue weight. However, this approach would have been misleading. Over time, CSF transports from the subarachnoid compartment into the turbinate tissues and is conveyed ultimately to the venous system. At any given time, the amount of tracer in the turbinates would simply represent the tracer that was caught in transit through this tissue and would in no way reflect the total mass of tracer that had transited from the CSF space through this intermediate compartment on its passage to venous blood.

The quantitative significance of lymphatics in CSF transport has been established by previous studies from our group using different experimental approaches. In rats, we compared the mass transport of radioactive HSA to plasma before and after obstruction of the downstream cervical lymphatic vessels (4). Plasma recoveries were reduced by ~50% after the interruption of cervical lymph transport. Similar results were observed in sheep (2, 3). Additional support for cribriform-lymphatic CSF absorption was obtained by combining infusion approaches with a method to seal the cribriform plate extracranially (32, 35). Combined, these experiments demonstrated a significant role for lymphatic vessels in cranial CSF transport. Indeed, at the theoretical opening pressure at which CSF absorption would be initiated, the data suggested that more than 80% of cranial drainage occurred through the cribriform plate (32, 35). We also assessed the direct entry of a protein
tracer into the cranial venous system in sheep and observed that tracer entry into the superior sagittal sinus only occurred at high intracranial pressures (36, 46). One possibility is that the arachnoid projections divert CSF from the cranium when intracranial pressure is transiently or chronically elevated.

To summarize, the technique used in this report provides a time-dependent snapshot that reflects a small portion of the physiological transport of CSF that had occurred through the olfactory turbinates. However, as discussed later, this approach may be helpful in assessing the possibility that absorption of CSF by lymphatics may be compromised in certain CSF disorders.

Physiological characteristics of tracer movement across the cribriform plate. On the basis of the available information, the most important connections between the subarachnoid compartment and extracranial lymphatics appear to exist at the level of the cribriform plate. Lymphatic vessels within the olfactory submucosa form a distinctive association with the olfactory nerves, and this anatomical relationship serves to facilitate CSF removal from the subarachnoid space (19, 23).

Once instilled in ventricular CSF, the radioactive albumin entered the olfactory turbinates rapidly with peak concentrations being achieved 30 min after injection. At this time, radioactivity in the middle turbinates was nearly 6 times that of blood. At 40 min, turbinate tracer concentrations declined but levels of $^{125}$I-HSA were always greater than those monitored in blood and other tissues. Presumably, the relatively small radioactive signal in the nasal muscle, spleen, liver, kidney, and tail simply represents the presence of the tracer within the blood of these tissues.

The rapid appearance of albumin in the turbinates is consistent with relatively quick CSF disappearance rates from the brain measured in other studies. For instance, the half-lives in CSF of soluble amyloid β peptide, sucrose, or PEG4000 were ~8 min, ~60 min, and ~60 min, respectively (12, 13). About 80% of IGF-1 was cleared from the brain 30 min after injection into the ventricles (33). It is of interest to note that the rapid removal of IGF-1 was not temporally related to its appearance in blood. The authors speculated that the delay in plasma appearance might have been due to the entry of IGF-1 into the lymphatics with some time required for transport to blood through the cervical lymphatic vessels. Additionally, experiments in rats using X-ray microscopy have indicated that the X-ray contrast medium reached the cribriform plate about 7 min after installation in the cisterna magna followed by the appearance of the agent in the nasal cavities (7). In this same study, the authors noted that Indian ink similarly administered, reached the cervical lymph nodes 20 min after injection.

The appearance of the tracer in blood is of course expected since all CSF removed from the cranium is ultimately transported to the bloodstream either through a lymphatic/cribriform plate route, drainage through the perineural spaces of other cranial/spinal nerves and/or clearance across the arachnoid villi and granulations into the cranial veins. The initial spike in blood tracer concentrations may have been due to direct transport of HSA into the cranial veins, as the intracranial pressure would be expected to increase temporarily due to the tracer injection into the ventricle. In past studies, we observed some transient direct CSF-cranial venous transport when intracranial pressure was elevated abruptly to high levels (36, 46).

However, the 10-min delay in the peak concentration of tracer in blood (40 min) compared with the turbinates (30 min) seems consistent with the view that a significant amount of the tracer made its way into blood after first traversing the lymphatic network in the olfactory turbinates, moving through the cervical lymphatics, and finally transporting into the venous system at the base of the neck. This is supported by the data that indicates high levels of CSF tracer in the lymph nodes in the neck region (Fig. 2). Presumably, the leveling off of blood tracer concentrations reflects the balance between the declining tracer entry from lymphatics (and possibly other sources) and tracer removal from blood as albumin disappears continuously from the vasculature by filtering into the myriad noncentral nervous system’s interstitial compartments throughout the body.

Advantages of a routine method to assess CSF transport across the cribriform plate. Because increases in CSF outflow resistance have been measured in many of the common CSF disorders and in some of the animal models used to simulate these conditions, it seems reasonable to ask whether the impediment to CSF absorption occurs at the level of the cribriform plate. There is already some reason to believe that this is the case. In mice, the introduction of transforming growth factor-β1 into the ventricular system produces hydrocephalus (41). Of relevance to lymphatics is the observation that the time taken for intracisternally administered Indian ink to stain cervical lymph nodes in hydrocephalic animals was enhanced significantly compared with controls, indicating that CSF transport across the cribriform plate was likely impaired. However, the full impact of an impediment to CSF transport across the cribriform plate is unknown at this time. There are other anatomical locations at which subarachnoid CSF and lymph may come in contact. An example of this would be the lymphatic absorption of spinal CSF (5). Additionally, although it is easy to conceptualize that an increase in CSF pressure would occur when CSF uptake into lymphatics is compromised, it is not clear how this would contribute to the expansion of the ventricles in hydrocephalus.

Some answers to these questions may come from studies in rodents. A considerable amount of information on CSF formation, distribution and absorption is already available in rats. For example, the movement of CSF from the ventricular system to various locations in the brain has been mapped with autoradiographic methods (12). CSF pressure and outflow resistance have been measured in rats at various ages from fetal animals to adults (22). Additionally, developmental studies have revealed that CSF-lymphatic associations become functional around the time that significant volumes of CSF are being produced by the choroid plexus (26).

In addition, there are numerous rat models of CSF dysfunction. The kaolin-induced hydrocephalus model in rats has proven valuable in understanding the disease and its relevance to brain extracellular fluid (39), vascular parameters (24), axonal damage (10), gene expression (1), and CSF dynamics in both the acute and chronic phases of the disease (27). Several genetic models of hydrocephalus have also provided interesting insight on the disease. In this regard, several genes have been postulated to contribute to the development of ventriculomegaly in the H-Tx rat, which exhibits the noncommunicating form of hydrocephalus (21, 31). Obstruction to CSF flow/absorption in this model is associated with abnormal develop-
ment of the cerebral cortex (34). The LEW/Jms hydrocephalus rat model has also been characterized, and the pathological changes may involve more than one gene (20). Finally, several models of subarachnoid hemorrhage have been developed for use in this species (38).

The ability to acquire a quantitative measure of CSF drainage at a discrete absorption site (the cribriform plate) has the potential to add a new dimension to the investigation of the causes of CSF system disruption. Any reduction in the movement of tracer from the CSF space to the ethmoidal system could signal some problem in lymphatic CSF absorption. Recognition of the lymphatics as an important component in CSF absorption may provide the foundation on which to develop new concepts related to the pathogenesis of hydrocephalus. Rapid distribution of intraventricularly administered sucrose into cerebrospinal fluid cisterns via subarachnoid fluid cisterns via subarachnoid velae in rat. Neurosci 75: 1271–1288, 1996.

Perspective on CSF absorption. It is generally assumed that CSF absorption occurs through the microscopic arachnoid villi and macroscopic granulations, which represent extensions of the arachnoid membrane into the lumen of cranial venous sinuses. However, there is relatively little convincing in vivo evidence to support this contention, and, consequently, this textbook view is increasingly being challenged (11, 14, 16, 17, 18, 19). There is abundant evidence to suggest that the lymphatic circulation plays an important role in volumetric CSF absorption. A CSF absorption deficit is believed to contribute to disorders of the CSF system, such as pseudotumor cerebri and hydrocephalus. Recognition of the lymphatics as an important component in CSF absorption may provide the foundation on which to develop new concepts related to the pathogenesis of congenital or acquired CSF anomalies.

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