Ex Vivo Model of Cerebrospinal Fluid Outflow across Human Arachnoid Granulations

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PURPOSE. The brain’s arachnoid membrane with granulations is an important biological barrier whose responsibilities include the transmission of cerebrospinal fluid (CSF) and the regulation of pressure. Membrane disturbance may cause changes that are difficult to replicate with animal models, suggesting the need for a model using human arachnoid membrane with granulations for the study of conditions such as Alzheimer disease, hydrocephalus, and pseudotumor cerebri. The authors detail the development and validation of an ex vivo model of CSF outflow across human arachnoid granulations (AGs) as an approximation of in vivo conditions.

METHODS. Human AGs were perfused at normal physiological pressure in physiological and nonphysiological directions for permeability data. Fluorescent particle perfusion with electron microscopy identified outflow pathways through the AGs.

RESULTS. This human ex vivo model demonstrated in vivo properties of unidirectionality, particle transport, and ultrastructure, similar to our 2005 in vitro model. The average baseline hydraulic conductivity in the physiological direction \((n = 20)\) was 1.05 ± 0.15 \(\mu\)L/\(\text{min/mm Hg/cm}^2\) compared with 0.11 ± 0.03 \(\mu\)L/\(\text{min/mm Hg/cm}^2\) in the nonphysiological direction \((n = 3)\) under statistically equivalent \((P = 0.46)\) average normal physiological pressures \((5.88 ± 0.22 \text{ mm Hg and 6.14 ± 0.23 mm Hg, respectively})\).

CONCLUSIONS. The ex vivo model is feasible and herein demonstrated. These findings agree with in vivo CSF outflow. This model increases understanding of the clearance not only of CSF but also of metabolites through the arachnoid membrane. Additional evidence suggests, but does not yet prove, that CSF outflow may occur in a similar manner in the arachnoid membrane adjacent to the granulations, in addition to the flow through the AGs. This is a topic for further investigation.


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ARTICLES

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addition, we established an imaging technique to quantify visible AGs morphologically that is applicable to future research.

**METHODS**

**Tissue Procurement**

Experiments were conducted in accordance with guidelines and regulations set forth by the Office of Responsible Research Practices Institutional Review Board for human subjects at The Ohio State University (IRB Protocol 2002H0318) and The Declaration of Helsinki. Human tissue samples (n = 28; subject age range, 23–91) were collected within 24 hours of death. Samples were collected only from subjects free of infectious disease, advanced neurologic or hydrodynamic disorders, subarachnoid hemorrhage, and brain tumors. Arachnoid membrane and underlying cortex were dissected from the areas adjacent to the longitudinal fissure. Samples were stored in sterile Dulbecco modified Eagle medium/Ham F-12 nutrient medium (50:50 vol/vol) (Cellgro Mediatech, Herndon, VA) supplemented with L-glutamine (Cellgro Mediatech), 10% newborn calf serum (Invitrogen Gibco, Carlsbad, CA), and penicillin, streptomycin, and amphotericin B (all Cellgro Mediatech) and were stored at 20°C until final dissection and perfusion. All samples were perfused within 24 hours of death.

**Perfusion Experiments**

AGs and adjacent membrane were removed from cortical tissue, trimmed to 5- to 6-mm diameter, and secured in a modified Ussing chamber (model CHM4; World Precision Intruments, Inc., Sarasota, FL) with a 4-mm O-ring. A controlled pressure gradient was maintained across the arachnoid membrane in the physiological basal-to-apical (B→A) or apical-to-basal (A→B) direction using the same media and was maintained in the selected perfusion direction. The apparatus was incubated and thermally equilibrated. An in-line pressure transducer (Transpac IV; Abbott Critical Care Systems, North Chicago, IL) continuously monitored the pressure, as described elsewhere. LabView (version 6.1; National Instruments, Austin, TX) was used for transducer data collection. Pressure gradients (5 mm Hg) were maintained by adjusting the fluid reservoir height relative to the membrane in the chamber, simulating normal in vivo pressures. Ambient pressure was monitored to correct for atmospheric pressure changes throughout all experiments. Perfusate was collected and measured at the conclusion and was used to calculate an evaporation correction factor, as previously described.

**Microparticles**

FITC-conjugated polystyrene microparticles suspended in PBS (0.002–0.0002% vol/vol; particle size 0.1, 0.2, 0.5, 1.0, and 2.0 μm; Molecular Probes, Eugene, OR) were introduced to the media upstream of the AGs. The microparticle solution was allowed to perfuse across the AGs for 40 to 45 minutes before fixation. Perfusate containing microparticles was sampled and qualitatively examined using an inverted microscope (Axioim; Carl Zeiss Meditec, Inc., Dublin, CA) equipped with DAPI, FITC, and Cy3 filter sets or was quantitatively analyzed (Zetasizer Nanoseries ZS; Malvern Instruments, Worcestershire, UK) by a particle counter with commercial software (Novell; Dispersion Technology; Waltham, MA).

**Membrane Viability Protocol**

Sample viability was assessed by staining membrane from the same donor with a viability/cytotoxicity kit (Live/Dead; Molecular Probes). Whole pieces of AG and membrane were submerged in the viability/cytotoxicity solution (Live/Dead; Molecular Probes) for 45 minutes at 37°C. AG samples were mounted on slides and viewed under an inverted microscope equipped with DAPI, FITC, and Cy3 filter sets.

**Postperfusion Morphologic Imaging**

En face images of perfused, fixed membrane were taken for surface area quantification of visible AGs using a 35-mm camera (FM2; Nikon) with a 55-mm, 1:2.8 lens (MicroNIKKOR; Nikon) stabilized and focused at a fixed distance. Two images were taken of the membrane in the chamber. The film (35-mm 100 ASA Elite Chrome; Kodak, Rochester, NY) was processed and scanned on a 35-mm slide scanner (Sprintscan 4000; Polaroid). Pixel resolution was approximately 60 μm, suggesting an individual AG would be approximately 5 pixels in diameter. Two investigators independently used Adobe Photoshop to manually identify the visible AGs (Fig. 1). The AG surface area was calculated as a percentage of total area available for outflow by counting the number of positive pixels in the image compared with the total perfused area. Linear regression was used to assess reproducibility between investigators.

**Postperfusion Arachnoid Membrane Analysis**

Membrane was fixed under pressure with 10% neutral-buffered formalin (Richard Allen Scientific, Kalamazoo, MI), staged with ethanol and xylene (Fisher Scientific, Pittsburgh, PA) dehydration, and embedded.
in paraffin. Paraffin blocks were sectioned (6–10 μm) and stained with hematoxylin and cosin (Sigma, St. Louis, MO).

For frozen sections, membrane was fixed with 4% paraformaldehyde and infiltrated with 20% sucrose (Sigma) solution overnight, then embedded in OCT compound (Miles Inc., Elkhart, IN) and snap frozen in liquid nitrogen. Frozen sample blocks were stored at −80°C until sectioning with a cryostat (7–10 μm; Bright Instrument Company Ltd., Huntingdon, UK) for immunohistochemical staining.

Slides were dried for 50 minutes, fixed for 5 minutes with iced acetone, dried for 30 minutes, rinsed with PBS, and blocked with 10% newborn calf serum in PBS for 30 minutes. Sections were incubated with FITC-conjugated mouse anti–ZO-1 antibody (1:100; Zymed, South San Francisco, CA) or Alexa Fluor 555 phalloidin (1:400; Molecular Probes) in PBS for 1 hour, rinsed with PBS, and dried completely. Sections were counterstained with 4',6'-diamino-2-phenylindole (DAPI), mounted with an antifade reagent (Prolong Gold with DAPI; Invitrogen), and visualized using an inverted microscope equipped with DAPI, FITC, and Cy3 filter sets. Negative controls were processed using the same protocol but with the primary antibody omitted.

Electron Microscopy

For transmission electron microscopy (TEM) processing, membrane was fixed with 3% glutaraldehyde (Sigma) and placed in 0.1 M phosphate buffer solution for 45 minutes in 5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer with 0.1% ruthenium red (SPI Supplies, West Chester, PA) for 2 hours, followed by three buffer (0.1 M phosphate with 0.1 M sucrose, pH 7.4) rinses for 5 minutes each. Secondary fixation was completed with 1% osmium tetroxide (Sigma) in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sucrose for 1 hour. The sample was then rinsed, stained (2% uranyl acetate for 1 hour), and dehydrated in a graded series of ethanol. Samples were incubated in propylene oxide for 15 minutes, infiltrated with Spurr resin and polymerized at 60°C overnight. Embedded membrane was sectioned with an ultramicrotome (EM UC6; Leica, Wetzlar, Germany) at 70 nm. Sections were placed on 100-mesh Formvar-coated grids stained with 2% uranyl acetate and Reynolds lead acetate and were examined under a transmission electron microscope (G2 Bio TWIN; FEI Tecnai, Hillsboro, OR) at 80 kV.

Hydraulic Conductivity Calculations

Hydraulic conductivity \( (L_p) \) is a measure of the amount of fluid that passes across the membrane; it depends on the surface area \( (A) \), volumetric flow rate \( (Q) \), and pressure gradient \( (\Delta P) \). The equation used to calculate \( L_p \) in these perfusion experiments was \( L_p = Q/(A\Delta P) \).

### Results

#### Feasibility Studies: Histology and Transmission Electron Microscopy

Hematoxylin and cosin (H&E)-stained paraffin-embedded membrane sections permitted examination of the membrane morphology. Images confirmed the removal of all cortical tissue, leaving only the arachnoid membrane with granulations. A typical AG is seen in Figure 2, with arachnoidal cells around the outer surface and more predominantly at the apex of the granulation. These cells were also found dispersed throughout the collagenous fibrous capsule, lining the apical side of the AG, and clustered at the cap cell area of the AG.

Preliminary viability/cytotoxicity (Live/Dead; Molecular Probes) stains of perfused AG samples confirmed viability of the membrane up to 24 after death. AGs stained immediately after collection showed viability, whereas postperfusion AGs exhibited no viability 24 hours after death.

Frozen sections of perfused AGs were immunohistochemically stained for ZO-1 and fluorescence microscopy. These sections stained positively for ZO-1 (Fig. 3, arrows) indicating tight junctions in the membrane after perfusion. The Figure 3 inset shows ZO-1 staining around a nucleus, indicating numerous tight junctions. A negative control for ZO-1 appropriately did not stain, confirming the specificity of the ZO-1 antibody (results not shown).

TEM images of physiologically oriented, perfused AGs showed features consistent with those described in earlier morphologic studies, including intracellular vacuoles, extracellular cisterns, desmosomal cell-to-cell junctions, and pinocytotic vesicles. Figure 4 shows extracellular cisterns ranging from 1 to 4 μm in diameter. Additionally, an extended extracellular cistern was seen at the apical edge of the perfused AG. Optically empty spaces not clearly enclosed by an intact cellular membrane were classified as cisterns; some had desmosomal junctions at either end. Figure 5 shows an intracellular...
lar vacuole in a position of exocytosis (arrow) from the apical side of the AG. In addition, this same image shows the presence of numerous intracellular vacuoles throughout the arachnoidal cells, consistent with pinocytotic activity. Figure 6 shows postperfusion AGs with numerous desmosomal cell-cell junctions characteristic of cells of arachnoidal origin.

**Microparticle Transport Results**

Preliminary physiological basal-to-apical (B→A) perfusion experiments showed the presence of 0.5-μm particles in the perfusate when examined by fluorescence microscopy (data not shown). This result was replicated with multisized microparticles (0.1–2.0 μm), and passage was confirmed but not quantified by visual comparison to the microparticle stock solution using fluorescence microscopy (data not shown). Light-scattering analysis of experimental perfusate examined the passage of 0.5- and 2.0-μm particles across the arachnoid membrane by light-intensity data. The first intensity peak in Figure 7 at 460 nm and the second peak at 2497 nm corresponded to 0.5- and 2.0-μm particles in the perfusate. The difference from the expected particle size and the obtained light-intensity peak might be attributed to the presence of a hydrated radius surrounding the suspended particles. Additionally, light-scattering analysis enabled particle counts, revealing a ratio of 99:1 of 0.5- to 2.0-μm particles in the perfusate solution. The ratio of 0.5- to 2.0-μm particles in the original suspension introduced to the system was 64:1.

In conjunction with histologic techniques, microparticle location helped to determine fluid outflow pathways. In physiologically perfused AGs, microparticles were located on the basal side of the AG, within the stem (arrow), core capsule (arrow), and cap cell regions (arrow) of AGs. Figure 8 shows an AG with microparticles in the central core and at the apical edge and cap cell cluster.

With nonphysiologically perfused (A→B) arachnoid membrane, the perfusate did not contain microparticles (data not shown). Figure 9 shows the location of particles in such AGs. Compared with physiologically perfused AGs, microparticles in AGs did not penetrate the AG. In addition, the AGs appeared crushed rather than distended, as in Figure 8, suggesting membrane damage.

**Fluid Directionality Perfusion Experiment Results**

Human AGs were perfused at normal (physiological) pressure with the AGs oriented physiologically (B→A) or nonphysiologically (A→B). A summary of the average pressure drop across the AGs, average volumetric flow rate, and average hydraulic conductivity is provided in Table 1. The average pressure drop across the AG samples was statistically equivalent regardless of AG orientation during the perfusion experiment. Nevertheless, the resultant flow rate and average hydraulic conductivity were significantly different.
Visible AG Surface Area

To show the applicability of the technique, physiologically perfused AG surface area \((n = 10)\) was quantified using a modification of the imaging technique previously described. Figure 1 shows an image of perfused membrane and an Adobe Photoshop rendering of the location of the visible AGs. The identification of AGs was completed by two independent researchers with an agreement of the location of AGs of \(r^2 = 0.82\), indicating reproducibility. The average area of perfused visible AGs calculated was 0.02082 cm\(^2\) (range, 0.0104–0.0358 cm\(^2\)), corresponding to an average total AG area available for outflow of 16.9%, assuming that fluid flow occurs only through the visible AGs as is classically reported. For paired experiments, adjacent AG sections with similar morphologic appearance were perfused.

**FIGURE 7.** Light-scattering analysis of perfusate after physiological perfusion of AGs. First light-intensity peak (larger) corresponds to 0.5-\(\mu\)m particles, whereas the second light-intensity peak corresponds to 2.0-\(\mu\)m particles.

**DISCUSSION**

A successful ex vivo model needs a viable, intact, isolated AG segment. This was confirmed by staining and viability tests for up to, but not beyond, 24 hours.

ZO proteins are peripheral membrane proteins associated with tight junctions in the blood-brain barrier, where they help anchor the actin cytoskeleton. Cultured AG cells have demonstrated positive immunohistological staining of ZO-1. Positive immunohistologic staining of tight junction components for ZO-1 (Fig. 3) in postperfusion ex vivo AGs showed maintenance of intrinsic properties essential for regulating fluid flow in vivo.

In arachnoid membrane, desmosomal cell-to-cell junctions serve as anchoring sites for the intermediate filament vimentin. Desmosomes work to hold adjacent cell membranes together, increasing the tensile strength of the cell layer.
through the intermediate filaments they anchor and, in turn, the strength of the membrane.\textsuperscript{57} As seen in Figure 6, desmosomes were identified based on their distinct morphology. The presence of desmosomes provides support for the maintenance of AG and arachnoid membrane integrity throughout the experiment. In combination with ZO-1 fluorescence staining, the presence of cell-cell junctions strongly suggests evidence for the preservation of AG and membrane integrity.

Previous studies have used TEM to examine arachnoid membrane and AG ultrastructure of animal and human tissue, leading to proposals of multiple fluid outlet mechanisms.\textsuperscript{18,25} TEM studies by Yamashima et al.\textsuperscript{25} of human arachnoid membrane showed the presence of large extracellular cisterns, microcisternocytotic vesicles, and intracellular vacuoles in the arachnoid cell layer suggesting dual mechanisms of CSF transport (transcellular and paracellular). TEM analysis of perfused human arachnoid membrane suggests the presence of ultrastructural features, in agreement with observations by Yamashima et al.\textsuperscript{25} in whole tissue and in cells cultured from human AG membrane.\textsuperscript{31} Large optically empty spaces in Figure 4 may correspond to extracellular cisterns associated with the paracellular transport pathway. A fused vacuole leaving the apical side of the arachnoid membrane and the numerous intracellular vacuoles (Fig. 5) illustrate evidence for a transcellular mechanism of CSF transport. Together these characteristics are consistent with previous ultrastructural studies proposing a dual transport mechanism for CSF outflow while also demonstrating that the perfused membrane maintains intrinsic fluid transport pathways.

A limiting factor for TEM with the ex vivo model is fresh tissue, requiring a maximum of 12 hours from death to TEM processing. Despite prompt processing, tissue necrosis can be identified in TEM images. The necrosis is mainly limited to cell organelles, and the extent is similar between different samples processed within the 12-hour time frame. However, the critical intact cell membrane remains, allowing for classification of cellular ultrastructural features using TEM.

The ex vivo model permits fixation of the AGs under a constant physiological pressure gradient. This is difficult to accomplish in situ. Previous studies demonstrated the need for a positive CSF pressure gradient for flow across secured AGs.\textsuperscript{39,38,59} Our results of AG viability, integrity, and ultrastructural evidence verify that an ex vivo model of CSF outflow across human AGs is feasible.

Animal studies have used microparticles as tracers of physiological fluid flow across the arachnoid membrane and granulations, as described earlier.\textsuperscript{17,20,22,23,40,41} In our study, the passage across the AGs of FITC-conjugated microparticles (0.1–2.0 μm) agrees with those previous results and indicates that AG transport mechanisms remained intact throughout the experiment. Additionally, perfusate of nonphysiologically perfused AGs did not contain microparticles, supporting the important unidirectional fluid flow requirement for an AG transport system that mimics in vivo functionality.

In our ex vivo model, quantitative light-scattering analysis of the perfusate supported particle passage and demonstrated a technique that may be used to further analyze particle distribution passed under variable conditions. These perfusate analyses will be useful in future studies. Previous work by Davson et al.\textsuperscript{54} showed that alterations in the colloidal osmotic pressure of CSF did not alter the rate of absorption at the arachnoid membrane so that the addition of microparticles to the perfusate solution should not alter intrinsic transport mechanisms across the membrane in the ex vivo model.

Intra-AG distribution and location of microparticles allowed for additional investigation of membrane morphology and fluid flow pathways through AGs. Figure 8 shows more particles at the arachnoid cap cell cluster at the apex rather than at other places in the AG, suggesting that this AG region is an important locus along the CSF outflow pathway. This is consistent with previous observations regarding fluid transport across the arachnoid membrane.

Particles were not distributed through nonphysiologically perfused AGs (Fig. 9). They appeared trapped on the nonphysiologically pressurized apical side of the AG. The AG was not distended but was compressed. These findings add support for unidirectional fluid transport through the ex vivo AG model. Microparticle experiments indicated the passage of multisized particles, in agreement with previous research, and offer evidence to confirm the unidirectional passage of fluid through AGs.

In healthy adult humans, the pressure drop of 3 to 5 mm Hg between the subarachnoid space and venous sinuses provides the driving force for CSF outflow across the arachnoid membrane to the venous drainage system in a basal-to-apical direction.\textsuperscript{43,44} In cases of increased venous pressure with blocked CSF outflow, blood did not flow back into the subarachnoid CSF. This finding supported the concept of an intrinsic unidirectional property of the membrane.

To examine unidirectional properties of the ex vivo model, the AGs were perfused in the physiological (B→A) and nonphysiological (A→B) directions. Analysis showed that for experiments performed at normal pressure, a significant difference in the calculated average hydraulic conductivity existed between samples perfused in the physiological and nonphysiological directions (P < 0.001) at statistically equivalent pressures (P = 0.46). In addition, the respective flow rates for A→B perfusions were essentially zero. These results indicate that the AGs used for the ex vivo model replicated the unidirectional flow of CSF in vivo at normal pressure. These results agree with our previous in vitro studies using cells cultured from human AGs,\textsuperscript{50} and they provide clear evidence in favor of the traditionally described CSF absorption route at physiological pressure.

An assumption made in the calculation of hydraulic conductivity was that the total perfused AG area was equal to the total surface area of the perfused tissue. This does not take into account the morphologic differences between AG samples. It is possible that AGs have different hydraulic conductivity values than arachnoid membrane containing microvilli. This surface area assumption led to the calculation of only the baseline, minimum value of the hydraulic conductivity through the AGs. An all-inclusive morphologic assessment of each membrane sample would be required for the calculation of hydraulic conductivity through AGs compared with arachnoid membrane without visible AGs. This assessment would aid in deter-

### Table 1. Comparison of Hydraulic Conductivity for AGs Perfused in Physiological (B→A) and Nonphysiological (A→B) Directions at Physiological Pressure

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<th>Pressure (mm Hg)</th>
<th>Flow Rate (μL/min)</th>
<th>Average L&lt;sub&gt;P&lt;/sub&gt; (μL/min per mm Hg/cm²)</th>
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<tbody>
<tr>
<td>Average A→B</td>
<td>3</td>
<td>6.14 ± 0.23</td>
<td>0.09 ± 0.04</td>
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<tr>
<td>Average B→A</td>
<td>20</td>
<td>5.88 ± 0.22</td>
<td>0.76 ± 0.11</td>
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<tr>
<td>P</td>
<td>—</td>
<td>0.46</td>
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mining the total functional transport units associated with fluid outflow across the human arachnoid membrane and would allow for comparisons of area available for outflow to smaller mammals. We have devised a variation of an imaging technique previously described by our group to quantitatively analyze the surface area of perfused AGs in arachnoid membrane.

Based on our viability assessment, the working time window to use AGs after death was narrow, which limited the number of samples that could be perfused from a single donor. Efforts were made to collect AG samples from regions corresponding to the lateral lacunae, shown to contain the largest concentration of AGs. Nevertheless, the morphology of every sample used for experimentation was inherently unique, with differences in the number of visible AGs and microscopic arachnoid villi. It has been shown that the AG distribution varies between donors and is dependent on demographics such as age, sex, and race. Previous ex vivo animal studies have studied arachnoid membrane general morphology, but techniques to incorporate an AG surface area analysis were not used, making this a unique addition to our ex vivo model.

We have successfully used an established imaging technique to identify total surface area of grossly visible AGs on the arachnoid membrane, with high reproducibility among independent researchers. Figure 1 shows a sample image used for analysis. This technique can directly compare morphologic differences between tissue donors and can determine the relationship between AG surface area and hydraulic conductivity.

Because of the clinical connection attributed to basal obstruction in congenital hydrocephalus, there is great interest in a new successful model developed in the adult rat. Extraventricular (communicating) obstruction of the basal cisterns in one group and obstruction of the superior parasagittal subarachnoid space (presumably the AGs) in another group was achieved using critically placed subarachnoid kaolin. In both groups it was shown that CSF obstruction was obtained and that communicating ventricular dilatation followed. Of interest was that basal cistern obstruction produced greater ventriculomegaly and that it did so more quickly than the obstruction produced by the localized superior suprasagittal kaolin injections, though they are not fully comparable. Nevertheless, it verifies that CSF outflow obstruction can be produced by either technique, confirming that both avenues are operable and significant in their adult rat model. This work must be seriously considered in the developmental and clinical aspects of hydrocephalus in infants.

In conclusion, we have shown and herein demonstrated that our ex vivo model is feasible. These findings are in agreement with those shown in our earlier in vitro model and with in vivo CSF outflow physiology. This model will increase our understanding not only of the clearance of CSF but also of metabolites through the arachnoid membrane.

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**References**


