Modeling Endothelial Barrier Properties of Diseased Cerebral Vasculature

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Modeling Endothelial Barrier Properties of Diseased Cerebral Vasculature

Abstract

Cerebral cavernous malformations (CCM) is a genetic vascular disease that results in leaky, malformed blood vessels (lesions) in the brain. The identified problem with this disease is that there are currently no effective treatment or prevention methods due to a lack of information known about the disease mechanism. KRIT-1 (or CCM-1) is one of three genes responsible for the disease. However, while KRIT-1 has been confidently linked to CCM, its role in the cell signaling pathways that regulate endothelial behavior and morphology is not well understood. In this paper we utilized two main methods to inform our goal of developing a hydrogel model. First, we simulated the conditions of CCM lesion formation with a parallel-plate flow chamber, using wildtype cells and recently cells where KRIT-1 expression has been knocked down. Secondly, we used silencing RNA (siRNA) to knock down KRIT-1 protein expression for use in both flow chamber experiments. Our aim was to create a flow system that included a hydrogel model of a channel, in which we could implant endothelial cells. Our work identified morphological differences between wildtype and KRIT-1 knockdown cells, differences in endothelial cell behavior with and without flow, and created the first iteration of a hydrogel flow system. This flow system is the first step towards creating an in vitro model that will one day allow for this disease to be studied.

Keywords: cerebral cavernous malformations, KRIT-1, hydrogel, parallel-plate flow system

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Introduction

Cerebral cavernous malformations (CCM) is a genetic neurovascular disease characterized by the improper development of small blood vessels in the brain, resulting in leaky, pocket-shaped lesions[1]. These lesions are characterized by impaired blood-brain barrier function, which can lead to major neurological problems and cerebral hemorrhage[1]. The lesions that form are fragile and can bleed, leading to strokes, headaches and other neurological symptoms[2]. About 0.5-1% of people develop CCM with the lesion forming shortly after birth[3]. However, 25% of people will not develop symptoms. Without the development of symptoms, people with CCM will live their lives unaware that they have this disease[3]. Due to this, there is currently no effective prevention or treatment therapy available for CCM. Current treatment methods include prescribing medication to treat symptoms, MRI monitoring of lesion development, and an invasive surgery to remove the malformed blood vessels if the situation becomes dire[3]. The lack of effective treatment methods is due to there being a lack of information known about the disease mechanism of CCM.

KRIT-1 (or CCM-1) is one of three genes responsible for the disease, with the phenotype usually caused by a "two-hit" mechanism: a congenital mutation of one allele and a spontaneous mutation of the second to produce a homozygous loss-of-function mutation[4]. While KRIT-1 has been confidently linked to CCM, its role in the cell signaling pathways that regulate endothelial behavior and morphology is not well understood. While the genetic cause and the subsequent clinical presentation of CCM have been characterized, the mechanism that connects the two remains unknown, limiting potential for development of therapeutic treatments. The established involvement of KRIT-1 in shear stress regulated signaling pathways and the localization of CCM lesions to mostly low shear stress (usually venous) blood vessels suggests the hypothesis that lesion formation may be a result of abnormal shear stress response of the endothelial layer[5]. We utilized three aims to further investigate this hypothesis: silencing KRIT-1,

identifying differences in morphology in cells under flow, and developing an in vitro hydrogel model.

The first aim was to transfect bovine aortic endothelial cells (BAECs) using silencing RNA (siRNA) to knock-down KRIT-1 protein expression. BAECs were chosen for initial experimentation due to their durability and because their behavior under flow is well-characterized in previous literature, making them a good choice for a "proof of concept" model and for confirming the functionality of new protocols[6,7]. Transfecting BAECs allows for the replication of the loss-of-function mutation in KRIT-1 that occurs with CCM. These "disease state" cells can be exposed to flow and allow for the establishment of the possible role of KRIT-1 in endothelial shear stress adaptation.

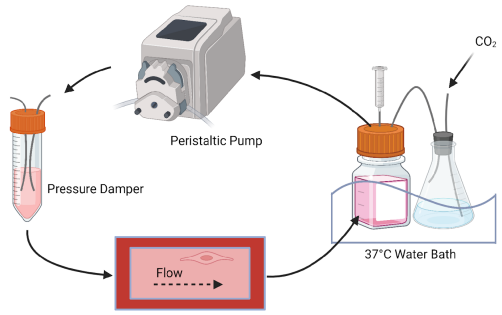

Fig. 1. A diagram of the parallel-plate flow chamber setup. This system allows for shear stress to be applied to cells to study the change in morphology and phenotype expression

The second aim was to use a parallel-plate flow chamber to identify differences in morphology in endothelial cells with and without flow. The flow experiment allows for BAECs to be exposed to a set shear stress while also regulating the temperature and pH of the system, both of which are essential for cell survival. The use of the parallel-plate flow system (Figure 1), allows for the confirmation that healthy wildtype endothelial cells behave as expected as seen in previous literature, and allows for the observation of behavior of cells with KRIT-1 knocked-down.

Results from the previous two aims informed the third aim of developing an in vitro model to study the disease mechanism of CCM. The development of a perfusable norHA hydrogel model of the affected vasculature will allow for the studying of 3D morphology of CCM lesions as they occur in blood vessels. This paper describes an initial iteration of this model. Once created, this will help identify how the dimensional aspects of cerebral blood vessels play into lesion formation and characteristic leakiness.

Development of this in vitro model will promote further understanding of the abnormalities in endothelial barrier properties and shear stress adaptation as a result of KRIT-1 mutations. Further, this model will provide insight into the signaling pathways involved in CCM lesion formation. Overall this hydrogel model is the first step towards being able to study this disease in a novel way, therefore opening the door to the future development of therapeutic treatment methods.

Results

SiRNA Transfection

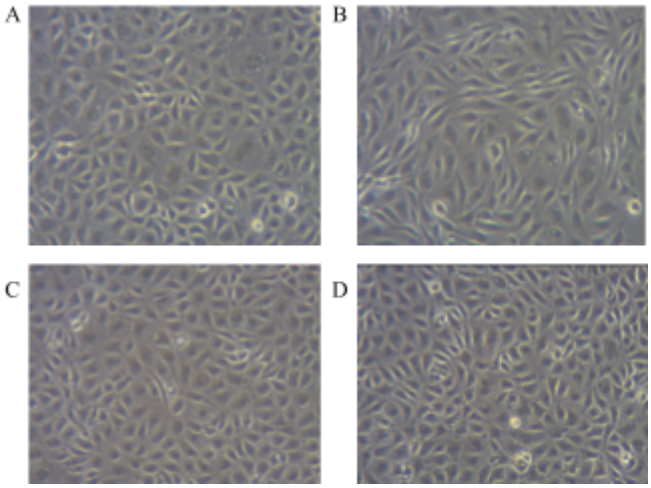

Fig. 2. A comparison of wild-type (A) and transfected (B) cells, along with two controls, transfection with a nonsense (non-targeted) siRNA sequence (C) and mock transfection using all transfection reagents but no siRNA (D). The wild-type cells and the controls appear cobblestone, while the transfected cells are more elongated and less densely packed, representative of a lower cell density. The controls allow for the confirmation that the change in phenotype is due to KRIT-1 being knocked-down and not another element of the transfection process.

As previously stated, the purpose of transfecting the BAECs with siRNA was to knock down KRIT-1 protein expression and mimic the CCM phenotype. Two days after transfection, transfected cells appeared more elongated and were less densely packed than wild type cells plated at the same density (Figure 2). In Figure 2, the difference in cell density can be interpreted as the

difference in the number of cells in the image frame. Figure 2A, the wild type cells, shows 197 cells, while Figure 2B shows only 143 cells. Both images are at 10X magnification. The transfection was done in conjunction with several controls, including a mock transfection using identical reagents but no siRNA, and transfection done using a non-targeted siRNA sequence. Only the cells transfected with KRIT-1-targeted siRNA showed this clear visual difference in phenotype. The control conditions were visually identical to the wild type cells.

KRIT-1 has been previously linked to signaling pathways involved in cytoskeletal function as well as cell-cell adhesion pathways involving Rap1[8,9,10]. It is hypothesized to be a Rap1 effector and a microtubule-associated protein, with downstream effects on cadherin-containing complexes, junctional protein expression, actin stabilization, and growth factors like VEGF. The phenotype exhibited by the transfected cells aligns with previous literature and with a hypothetical phenotype related to the disruption of these cytoskeletal and adhesion pathways.

Parallel-Plate Flow Chamber

The parallel-plate flow chamber allows for the adjustment of flow rate (and therefore shear stress) and cell-type, while also keeping the environment conditions constant in order to support the survival of the cells. Cells are plated on a glass slide and attached to a chamber with a gasket. The chamber is then connected to a container of cell medium, CO_2 for pH control, a peristaltic pump, and a pressure damper to protect the cells from any oscillating flow rate that could occur from the pump. Before using "disease state" cells with KRIT-1 knocked-down in the flow loop, wildtype BAECs were used as a control and proof of concept. Healthy endothelial cells are expected to align and elongate in the direction of flow and shear stress.

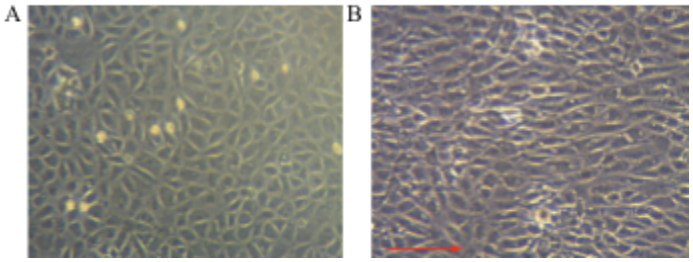

Fig. 3. A comparison of wild-type BAEC cells before being exposed to flow (A) and after being exposed to flow (B) in the parallel-plate flow system. The red arrow on the right indicates the direction of flow. The post flow cells are aligned and elongated in the direction of flow.

Multiple experiments were conducted to confirm this behavior, and the wildtype BAECs were imaged on a glass slide before and after being exposed to flow in the chamber. In their natural resting state without flow, the cells are round, fully confluent, and have a cobblestone appearance (Figure 3A). After the cells are exposed to shear stress, they elongate in the direction of flow, losing their round appearance, and orient themselves in that direction (Figure 3B).

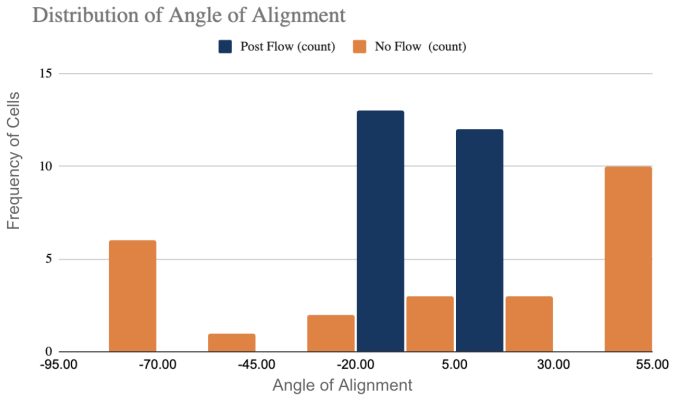

Fig. 4. Histogram displaying the distribution of angle alignment of the cells relative to the direction of flow. The x-axis is angles of alignment while the y-axis is the # of cells measured.

In order to quantify this alignment, ImageJ was used to calculate the angle of orientation of cells relative to the direction of flow, or the x-axis (Figure 4). The average angle of alignment for cells without flow was -6.01 degrees with a standard deviation of 49.53. The average angle of alignment for cells after being exposed to flow was 4.63 degrees with a standard deviation of 7.83. The standard deviation of cells without flow is almost 7 times greater than the standard deviation of the cells post flow, allowing for the conclusion that the cells have become more aligned after experiencing shear stress. The larger standard deviation corresponds to the random orientations of the no flow cells.

For the quantification of the elongation of cells, ImageJ was utilized to measure the major and minor axis lengths of the cells, and calculating the aspect ratios of the cells by dividing the major by the minor length (Figure 5). A rounder cell is expected to have an aspect ratio closer to 1 than that of a cell that has become elongated. The average major axis length of cells without flow was 78.1 with an average aspect ratio of 2.22. The average major

axis length of cells after being exposed to flow was 138.1 with an average aspect ratio of 3.61. As expected, these results indicate elongation in the cells that were exposed to shear stress.

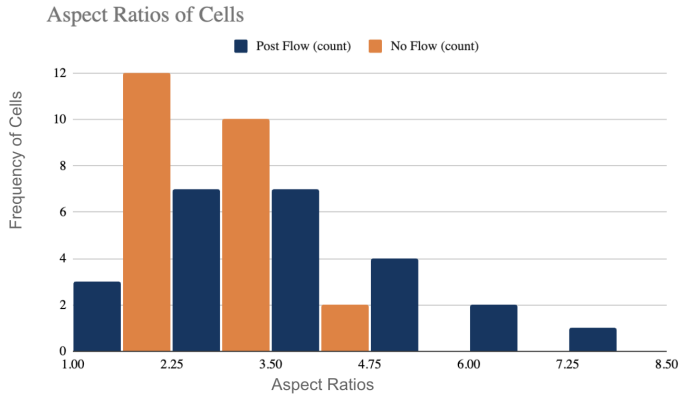

Fig. 5. Histogram displaying the distribution of the cells' aspect ratios. The x-axis is the aspect ratios while the y-axis is the # of cells measured.

After knocking-down KRIT-1 in BAECs, those cells were plated on a glass slide and exposed to shear stress in the parallel-plate flow chamber (Figure 6). After being exposed to flow there is no indication of alignment in these cells. The cells also have lost their healthy cobblestone appearance and instead have irregular shapes. Further flow experiments are required to quantify the behavior of these BAECs.

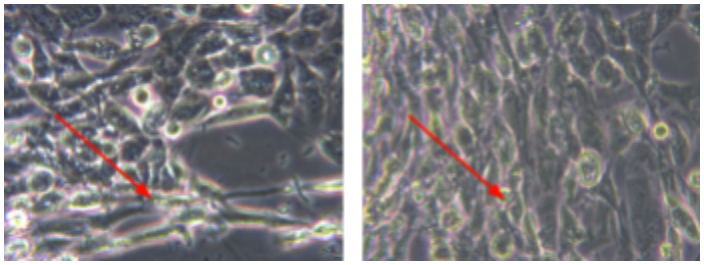

Fig. 6. Images from an initial trial of KRIT-1 knockdown cells on the parallel-plate flow chamber. The red arrows indicate the direction of flow. There is no visible alignment in the direction of flow.

Hydrogel Flow System

The hydrogel flow system required the cells to form an even monolayer around the channel and for flow to replicate shear stress in brain microvasculature. When first attempting to form the monolayer, the cells were not proliferating and thriving on the hydrogel. We attempted to plate the cells on a flat surface of hydrogel material and they still were not proliferating. We learned that the NorHA recipe that we had been using did not account for the thiolated RGD that was being added, so the NorHA was unable to bond to the RGD and there was no extracellular matrix protein in the hydrogel for the cells to adhere to. In the later versions of the hydrogel, the cells adhered to the inside of the channel (Figure 7C). Another change made in the final versions of the hydrogel system was that the cell density was increased to at least 50% confluence. We determined that the cell density needed to be high because more cells in the channel means they might have a better chance of adhering.

To be able to more evenly coat the channel with cells, we attempted to rotate the hydrogel in the hour after plating cells so they would adhere to all sides of the channel more uniformly rather than adhering to only one side due to gravity. Previous research used a device to rotate the hydrogel over a few hours after plating the cells[11]. In our attempt to manually rotate the hydrogel, some cell medium was lost after each rotation, and we were unsure if air would get into the channel because the medium was not deep enough to keep the hydrogel submerged the whole time. Even when rotating the hydrogel, the cells did not form a monolayer like we would have expected. For the next hydrogel flow systems we set up, we decided not to rotate the hydrogel, and instead to see if the cells would adhere and proliferate in the channel as cell medium flowed through.

Another change made in the final versions of the hydrogel system was that the cell density was increased to at least 50% confluence. We determined that the cell density needed to be high because more cells in the channel means they might have a better chance of adhering to the channel surface. If rotating the hydrogel, having a higher density of cells would give cells a better likelihood of attaching to surfaces all around the channel to help form a more even monolayer.

Once the cells were adhered to the hydrogel, we added flow to the channel so the cells would be able to start proliferating in an environment with flow, as they would in vivo. To do this, we estimated that the shear stress (τ) in cerebral capillaries was 10 dyn/cm^2. Dulbecco's Modified Eagle Medium (DMEM) was the primary ingredient in the cell culture medium, and the value we used for viscosity (μ) was 959.8 centiPoise (cP)[12], but this value does not accurately represent the viscosity of our medium so future hydrogel flow systems will assume the viscosity of the cell culture medium is closer to the viscosity of water. The outer radius (R) of the needle we used was 359 um. With these three values and Equation 1, we calculated the target flow rate (Q) of the medium through the hydrogel to be approximately 10.9 uL/hr (Equation 1)[13,14].

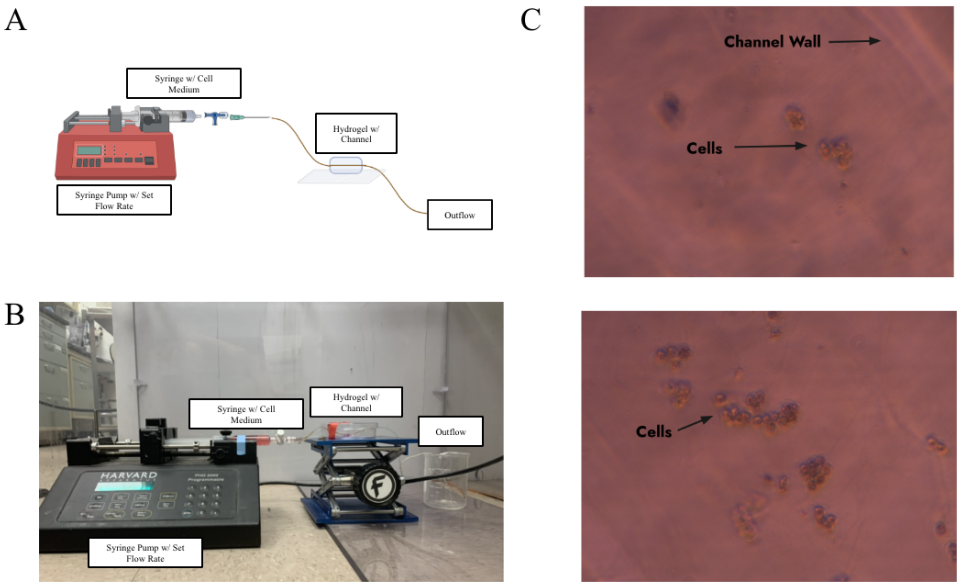

Fig. 7. A diagram (A) and image (B) of the final hydrogel system setup & images of the channel within the final hydrogel when cells have adhered (C). Initial prototypes consisted of a channel through the hydrogel with the goal of cell adhesion. Eventually flow was included at a rate of 10.9 uL/hr using a syringe pump. The cell wall indicates that the cells were plated inside of the channel.

Equation 1: $Q = (\tau * \pi * R^3)/(4 * \mu)$

Discussion

The transfected cells appeared to have a more elongated morphology, a lower cell density, and an overall different phenotype than the cubic cobblestone monolayer of the wild type cells under the same static conditions. Previous research has shown that KRIT-1 knockdown in vascular endothelial cells results in overexpression of vascular endothelial growth factor A (VEGF-A), which promotes endothelial proliferation and angiogenesis[8]. When allowed to grow past confluence for several more days, the transfected cells more quickly lost contact-inhibition and began growing overtop of each other, while the wild type cells and control cells remained a contact-inhibited monolayer. The loss of contact inhibition of the transfected cells allowed to grow past confluence may be explained by previous research showing that endothelial cells with impaired contact-inhibition are more responsive to VEGF-induced proliferative signaling[15]. The growth overtop of the monolayer may have been cells with poor cell-cell junctions responding to increased VEGF-A as a result of the KRIT-1 knockdown. β-catenin is a junctional protein that has been studied in connection to the impaired endothelial barrier function associated with CCM[16]. While KRIT-1 knockdown has not been previously shown to decrease overall expression of β-catenin, protein staining by Glading et al. showed that its localization to cell-cell junctions was disrupted in knockdown cells, indicating that KRIT-1 interaction may be important to the functional role of β-catenin in cell-cell junctions[10]. If KRIT-1 interaction with β-catenin is required for normal contact inhibition, this mechanism combined with VEGF overexpression may explain the behavior of the over-confluent knockdown cells, as well as the bright cell boundaries in the barely-confluent knockdown cells, which appear visually more distinct than cell boundaries between the wild type control cells (Figure 2). The apparent low cell density in the barely confluent knockdown cells may be a secondary effect of abnormal intracellular signaling resulting from impaired cell-cell junctions and cytoskeletal morphology.

The use of the parallel-plate flow experiments confirmed the change in morphology in endothelial cells once they are exposed to shear stress. The parallel-plate flow experiments also indicated a morphological difference between wild-type and KRIT-1-knockdown cells under flow. The elongated morphology of confluent knockdown cells also aligns with previous literature, including observations by Glading et al. that under static conditions siRNA-transfected KRIT-1 knockdown cells

exhibited more transverse actin stress fibers than control cells, in which actin was predominantly distributed around the circumference of each cell[10]. Interestingly, transverse actin stress fibers are observed in wild type endothelial cells exposed to fluid flow, which also adopt an elongated morphology (Figures 3 and 5). This relationship may be explained by findings of Jilkova et al. demonstrating that the CCM complex, including KRIT-1 and CCM-2, inhibits ICAP-1 activation of the mechanotransductive β-1 integrin, which can also be activated by shear stress[17]. In KRIT-1 knockdown cells, non-functionality of the CCM complex ICAP-1 inhibitor leads to β-1 integrin overactivation, resulting in a phenotype similar to shear stress-induced elongation. The experiments done by Jilkova et al. suggest that KRIT-1 and its associated complex are a crucial antagonist to β-1 integrin activation by shear stress. Especially in the lower shear stress capillaries typically affected by CCM lesions, where more inhibitory regulation may be required to maintain the appropriate degree of morphological change, the loss of the CCM complex may prevent appropriate endothelial adaptation to shear stress.

Discussion of the Hydrogel Flow System

The results of the hydrogel flow system indicated that cells should be plated at a high cell density to have a better chance of survival and proliferation and contain extracellular matrix protein (RGD). In the paper by Mannino et al., they infused cells in the hydrogel in a similar way, but their methods varied slightly[11]. They also used a hyaluronic acid based gel and a PDMS hydrogel. Their hydrogel itself contained B-cells, macrophages, and T-cells in an attempt to simulate a tumor. Unlike our system, this lab was able to form a confluent layer of cells within the channel[11]. Using the same type of hydrogel would not be helpful in our hydrogel flow system, but perhaps it would be worth comparing the elastic moduli or other properties of the hydrogel to understand why our cells were not proliferating as expected.

Previous models of hydrogels made of NorHA were successful in creating monolayers in the channels[18]. These hydrogels also had RGD added to the NorHA to improve the likelihood of cell adhesion just as we did for our hydrogel model. For the material itself, Song et al. explain that they modified the hyaluronic acid with adamantane and beta-cyclodextrin. Understanding more about the effects that these additions cause both directly and indirectly could help us understand why the cells were not proliferating on our hydrogel.

Broader Impacts

By developing an in vitro model that mimics the cells in brain microvasculature, CCM can be studied in a novel way. The hydrogel flow system gives researchers the ability to alter certain conditions of the model, such as flow rate and shear stress, channel diameter, and cell protein expression, and measure the resulting permeability of the blood brain barrier. The ability of this model to alter so many variables can help future researchers understand what physiological and hemodynamic properties are affecting the formation of CCM, if any. This model could even be altered to study other genetically linked diseases that affect vessel permeability both in the brain or in other vasculature.

The hydrogel flow system model could also be used to help develop more effective therapeutic treatments for CCM. Not only can the research done using the device inform the development of these therapeutics, but they can also be tested in the hydrogel system. This can be done by monitoring the difference in blood brain barrier permeability with fluorescence to see if permeability of transfected cells treated with the therapeutic drug is less than transfected cells without it. The cells treated with the drug can also be compared to wild-type cells to determine the effectiveness of the drug.

Limitations

The cells used so far in this project were BAECs, but the cells that are affected by this disease are human brain microvascular endothelial cells (BMECs). BAECs were used because so much is known about them through previous research and they are much easier to handle, but they come from cows rather than humans. Furthermore, BAECs are from aortas meaning they are accustomed to higher shear stresses, more variable flow, and different substrate properties than endothelial cells from brains. BMECs are the actual cell types that are affected by CCM,

so using these cells in the experiments would more accurately represent the disease.

Since the hydrogel's channel is created using a needle, the channel can only be straight or else there is the risk of shearing the hydrogel itself. For this reason, only linear flow can be modeled by this hydrogel flow system. The closest approximation we could do to studying how different shear stresses affect cells in the same capillary is varying the amount of shear stress on the cells in the channel by changing the flow rate of cell culture medium.

The smallest cerebral microvasculature can be as small as 5 micrometers[19]. Our model used a 22 gauge needle to create the channel, which has an outer diameter of 718 um. Using a needle to create the channel and inject the cells means we won't be able to make a channel smaller than the smaller needle we have access to. We also need to use caution when making the channel smaller because the already very low flow rate would reduce even more, which might not be possible on the syringe pump.

One hallmark of CCM is that new blood vessels are malformed as they develop. The way the current hydrogel model is designed, we can study the cell-cell junction connections and permeability of the monolayer, but we can't see microvasculature create new branches using cell signaling. A better model might allow the cells to form their own paths through the hydrogel so we could understand if there is a correlation between KRIT-1 knockdown, different levels of shear stress, and the typical CCM popcorn-like cluster forming.

Future Work

The next step to further this project would be to complete a series of western blots to confirm the changes we saw in the transfected cell's phenotype were actually due to a knockdown of KRIT-1. The four main conditions that should be run are wild-type cells with and without flow and transfected cells with and without flow. From the western blots we would use actin as a control and we would expect to see broad bands of the same thickness for all conditions. We would expect to see less KRIT-1 protein present in the transfected cells compared to the wild-type cells.

After confirming the transfection with the western blot, the transfected cells should be plated in the hydrogel flow system. This would allow these cells to be studied in a three dimensional environment under flow, more similar to how they would exist in human brains. Once a confluent monolayer of transfected cells can be formed, junction proteins can be stained to observe differences in distribution and concentration between the wild-type and transfected cells. Fluorescence staining can also be used in the cell medium so the amount of medium able to permeate the monolayers can be quantified. These tests can give insight into the extent to which knocking-down KRIT-1 in endothelial cells allows leakiness across the blood brain barrier.

The fluorescence experiments should also be done on BMEC cells, because this would provide the best representative model of CCM. The cells would first need to be transfected to knockdown KRIT-1 and then studied under flow in the parallel plate flow chamber. When plating the cells in the hydrogel channel, it is possible that adjustments to the properties of the channel will need to be made to accommodate the BMECs, which tend to be more sensitive than BAECs. For example, the RGD concentration can be varied, the elastic modulus of the channel can be adjusted, and RGD can be introduced into the channel itself by coating the needle that forms the channel in it.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) from the VEC-4 cell line were used in all experiments. Cells were cultured in BAEC Complete cell culture medium (Dulbecco's Modified Eagle Medium (Gibco, 4.5 g/L D-glucose, L-glutamine, without sodium pyruvate), 10 percent heat-inactivated calf serum, 2.92 mg/mL L-glutamine, and 50 ug/mL each penicillin-streptomycin). For passaging, cells were rinsed with Dulbecco's phosphate-buffered saline at pH 7.1 without calcium or magnesium, separated using trypsin-EDTA (Gibco) and centrifuged for 5 minutes at 250*g* before resuspension in BAEC Complete medium. BAEC Complete medium without penicillin-streptomycin was used to plate for siRNA transfection.

SiRNA Transfection

The transfection mixture was prepared by gently combining 30 pmol siRNA (ThermoFisher) diluted in 250 uL Opti-Mem (Gibco) with 5 uL RNAiMAX (ThermoFisher) also diluted in 250 uL Opti-MEM. The mixture of these solutions was incubated for 20 minutes prior to its use.

One day prior to transfection, cells were plated on 6-well plates (9.6 cm^2 per well) at 2.5 x 10^5 cells/well in 2.5 mL complete growth medium (DMEM, heat-inactivated calf-serum, and L-glutamine) without antibiotics.

For the transfection, 0.5 mL of the transfection mixture was added to each well, for a total of 3 mL volume per well and a final 10 nM siRNA concentration. Cells treated using a nonsense (non-targeted) siRNA sequence and cells treated with all reagents except siRNA were used as controls. After transfection the cells were incubated and allowed to grow for 24-48 hours prior to being collected as lysates or replated on a glass slide for use in the flow chamber.

Parallel-Plate Flow Chamber

To mimic a physiological shear stress environment, cells were exposed to flow using a parallel-plate flow chamber. The parallel-plate flow chamber was composed of a chamber piece containing cells, a peristaltic pump, a pressure damper to decrease flow pulsatility, and cell culture medium and carbon dioxide reservoirs heated to 37 degrees Celsius in a warm water bath (Figure 1). The culture medium was circulated using sterilized L/S 16 PharmaPure® tubing (Cole-Parmer). All tubing was backfilled to eliminate air bubbles from the system. The chamber piece was made of polycarbonate plastic, 32 by 85 mm, with T channels on each end, 56.3 mm apart. Cells were plated on a 38 by 75 mm glass slide with a rectangular silicone gasket, leaving approximately 15 cm^2 of surface area for cell growth. The cells were allowed to grow to confluence. In preparation for a flow experiment, the slide was placed facedown on the polycarbonate chamber and secured with binder clips[20].

A flow rate of 86.7 mL/min was calculated based on a target shear stress of 10 dynes/cm^2, chosen to represent the lower end of average arterial shear stress and the values used in prior literature for studies of endothelial alignment[21,22].

Hydrogel Flow System

The polydimethylsiloxane (PDMS) mold for the hydrogel was made using 90 percent by volume liquid PDMS and 10 percent Sylgard 184 Silicone Elastomer Curing Agent, with inner dimensions 3.5 by 9.5 mm and wall thickness of 2 mm. The mold was plasma-bonded to a glass slide.

A norbornene-hyaluronic acid (norHA)-based hydrogel (26 mg/mL norHA (Lifecore), 15 ug/mL LAP photoinitiator, 0.54 mg/mL DTT, 1.7 mg/mL RGD) was used as the matrix for the channel. The channel was created using a blunt-tipped, sterile 22 gauge needle inserted lengthwise through the hydrogel and PDMS walls of the mold. The needle was attached to a syringe containing a suspension of 2.5 x 10^6 cells/mL in BAEC Complete medium. The needle was drawn carefully backwards out of the hydrogel, leaving a channel into which the suspension was simultaneously injected. As the needle was drawn out of the PDMS walls of the mold, sterile microfluidics tubing (Trajan Scientific, 0.793 mm outer diameter and 0.508 mm inner diameter) was inserted to prevent the PDMS from closing on either side of the channel (Figure 6A and B). On the inflow side of the channel, the microfluidics tubing was inserted into stretchier Tygon® tubing (Cole-Parmer) with the same inner diameter. A 25 gauge needle was inserted into the the other end of the Tygon® tubing. This needle was connected to a 10 mL syringe filled with BAEC Complete medium. As with the parallel-plate flow chamber, all tubing was backfilled prior to being connected to prevent air bubbles from entering the channel. A syringe pump was used to push medium through the channel at a rate of 10.9 uL/hr. The entire system was encased in a plastic chamber heated to approximately 37 degrees Celsius with a heat gun.

End Matter

Author Contributions and Notes

M.C.J adapted the parallel-plate flow chamber protocol and the design of the polycarbonate chamber from R.E.M's dissertation[20]. M.C.J conducted many flow

experiments with the parallel-plate flow chamber, and M.C.J and M.G.M captured the cell images included in this paper related to the parallel-plate flow experiments and the siRNA transfection. M.G.M and E.R.C also ran parallel-plate flow chamber experiments for imaging. M.G.M performed image analysis on the parallel-plate and siRNA transfection images. E.R.C did the hydrogel synthesis, and kept track of the materials and protocols related to the hydrogel. E.R.C, M.C.J, and M.G.M designed and prototyped the hydrogel flow system. M.C.J wrote the Materials and Methods section of this manuscript and the siRNA transfection sections of both the results and discussion. M.G.M wrote the Abstract, Introduction, and parallel-plate flow chamber section of the results. E.R.C wrote the hydrogel flow system sections of the results and discussion, as well as the parallel-plate flow chamber and limitations sections of the discussion. G.H.F and A.G.H helped with cell culture and contributed valuable ideas to the hydrogel prototype. G.H.F also made the invaluable contribution of helping to take apart and clean the parallel-plate flow chamber after use.

The authors declare no conflict of interest.

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