INVESTIGATING THE EFFECTS OF SHEAR STRESS ON THE PROTEIN EXPRESSION OF LYMPHATIC ENDOTHELIAL CELLS

An Undergraduate Research Scholars Thesis

by

TAWFIK MOHAMMED HUSSEIN

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ABSTRACT

Investigating the Effects of Shear Stress on the Protein Expression of the Lymphatic Endothelial Cells

Tawfik Mohammed Hussein
Department of Biomedical Engineering
Texas A&M University

Research Advisor: Dr. Michael R. Moreno
Department of Mechanical Engineering
Texas A&M University

The lymphatic system plays three main important roles: Its cells are primarily responsible for the immune response of the human body, it represents a separate circulatory system, and it is involved in the transport of select nutrients from the digestive system to the circulatory system. All of these functions rely on the generation and regulation of the lymph flow along the lymphatic network. Any malfunction in the flow within the lymphatic network could potentially lead to an anomaly in the body as whole. Moreover, any imbalance within the fluid reabsorption of the interstitial fluid could lead to edema, which is a common problem worldwide. The lymphatic vasculature acts also as a conduit for cancer metastasis. My research will investigate the effects of shear forces on gene expression within lymphatic endothelial cells. Identification of factors that trigger gene expression in LECs also has implications for cancer metastasis as well as the pathophysiology of lymphatic edema. The expected outcomes for this research project is that it may identify changes in shear forces that may result in altered gene expression in lymphatic endothelial cells that may have a role in lymphatic edema.

DEDICATION

I dedicate this work to all those who suffer from lymphatic disease, and to future scholars who will pursue this work that will carry on the benefit to all mankind.

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I would like to thank my research advisor, Dr. Michael Moreno, for his continuous support and giving me the opportunity to be a part of this amazing field. I also would like to thank my graduate mentor, Mr. Caleb Davis, for teaching me everything I know about research and for his dedication to my success.

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NOMENCLATURE

BEC	Blood Endothelial Cells
BSA	Bovine Serum Albumin
CD-31	Cluster of Differentiation 31
DI	Deionized
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
FBS	Fetal Bovine Serum
LEC	Lymphatic Endothelial Cells
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
PROX-1	Prospero Homeobox Protein 1
VE	Vascular Endothelial

CHAPTER I

INTRODUCTION

The lymphatic system plays three main important roles: it is important in immune response, it returns fluids to the circulatory system, and it is involved in the transport of select nutrients from the digestive system to the circulatory system. All of these functions rely on the generation and regulation of the lymph flow along the lymphatic network. Any malfunction in the flow within the lymphatic network could lead to an anomaly in the body as whole. For example, imbalance of reabsorption of the interstitial fluid could lead to edema, which is a common problem worldwide. The lymphatic vasculature also acts as a conduit for cancer metastasis. Research currently done in the field includes the study of the alteration of the mechanical forces by edemagenic conditions, evaluation of the lymph endothelial cell sensitivity to changes in shear stress, and the measurement of stretch and flow in vivo.

It is increasingly recognized that the lymphatic vessels exist in a unique and highly variable mechanical environment (Dixon et al., 2006). As in the blood circulation, alteration of the forces experienced in the lymphatic vessel (e.g. shear stress or vessel stretch) produce measurable responses in the lymphatic endothelial cells (LEC) which may regulate lymphatic flow (Jafarnejad et al., 2015). Unlike in the blood vessels, lymphatic vessels actively contract to propel lymph, and this contraction also involves flow- and pressure-dependent responses (Gashev et al., 2004).

Despite the extensive study of the effects of shear and stretch on blood endothelial cells (BEC) (Davis et al., 2015), very little study of the effects of the same forces on LEC has been published. So far, in vitro studies of LEC have not considered physiological shear stress

waveforms or effects of combining shear and stretch. Our approach is to conduct shear-stress studies with a more realistic approach. I will, furthermore, study the effect of shear stress on LEC in vitro and observe its impact on gene expression in LEC to better understand its impact on the lymphatic network. In addition, cell alignment will be measured as an indication of the LEC response to shear stress. I expect the results from these studies of LEC response to physiologically-relevant mechanical forces to inform understanding of the mechanoregulatory mechanisms of lymphatic contraction and flow, potentially identifying targets for therapeutic intervention in cases of lymphatic dysfunction.

CHAPTER II

METHODS

PDMS Microchannels

Manufacturing the Polydimethylsiloxane (PDMS) Microchannels

In order to mimic the lymphatic vessels, PDMS microchannels were made. The role of the PDMS microchannels is to facilitate the shear stress that is made by the lymph flow on the lymphatic endothelial cells (LEC). Furthermore, this microchannel will be used to harbor cultured LEC and will be connected to a pump for conducting shear traction experiments. To make the microchannels, a solution of a siloxane base along with a curing agent was prepared. Furthermore, the ratio between the base and the curing agent was 8:1 such that the curing agent will allow the dimethyl siloxane to crosslink in order to form PDMS.

Upon mixing the base with the curing agent, it is then put under a pressure pump to degas it completely from air bubbles, which usually takes from 30 to 60 minutes. Then, the degassed mixture is poured over the microchannel scaffold (until the scaffold is completely covered by the solution), and the solution is then put in the oven under 60^0C to solidify for 24 hours.

Once the microchannels have solidified, they are then cut so that each microchannel is separate from the other. To clean them, tape can be used to detach dust particles from the surface of the microchannel in addition to rinsing them with distilled water. In addition, using a hole puncher, an inlet and an outlet for the microchannels can be punctured on each end of the microchannel. Furthermore, to be able to connect the microchannels with a pump system and to be able to inject cell media into the channel, pipette tips can be attached to both the inlet and the

outlet and to prevent the air from entering the channel as well as secure the tips in their respective inlet or outlet, a sealant is applied around the tips.

Plasma Processing the PDMS Microchannels and the Glass Slides

To close the channel from underneath, a glass slide must be attached securely to the bottom of the microchannel. This is done via plasma processing. Furthermore, both surfaces (the microchannel and the glass slide) must be free of dust particles. The glass slides are put in first into the plasma cleaner and it should be allowed to completely degas for ten minutes. Once that is accomplished, oxygen is pumped into the plasma cleaner and until a "glow" is observed, which indicates the plasma state. Upon observing the glow, the glass slides should remain in the plasma cleaner for 5 minutes and the PDMS microchannels should remain inside the plasma cleaner for 40 seconds. Once the bonding process is complete, DI water should be injected into the microchannels followed by immersing them in DI water. For sterilization, the bound microchannels are autoclaved in a cycle of at least 120^{o}C.

Cell Culture

The cells used for the shear-stress experiments are lymphatic endothelial cells (LEC), harnessed from rat mesentery, which were obtained by another lab that does primary harvests from the rat lymphatic vessels. Furthermore, they are cultured and adhered to the PDMS microchannels so that so that they can undergo wall shear stress. To culture the cells, the microchannels must first be coated with sterile 4% porcine gelatin solution in DI water to ensure cell adhesion. Moreover, two pipettes were used: a 10-microliter pipette and a 500-micron pipette to create a positive pressure for the inlet and a negative pressure for the outlet respectively. Via the 10-microliter pipette, gelatin is injected into the inlet until the inlet is full. Following that, by inserting the 500-microliter pipette into the outlet, the gelatin must be sucked

out slowly to prevent the formation of air bubbles within the microchannel, which can be detrimental to the cells. Once, the gelatin is spread, the gelatinized microchannels should be placed into the incubator for 30 minutes to ensure that the microchannel is fully coated with the gelatin. Afterwards, to rinse the gelatin that did not adhere to the surface of the microchannel, the microchannel should be rinsed with DPBS by injecting it into the inlet and pulling it from the outlet.

To infuse the gelatin-coated microchannel with LEC, two pipettes are used: a 10-microliter pipette and a 1000-microliter pipette. The 10-microliter pipette is used for injecting the cells into the inlet, and the 1000-microliter pipette is used to create a negative pressure in the outlet (with a slow rate of suction) to allow the cells to stream through the microchannel and not allow any air bubbles behind. The cell solution is prepared from a T75 flask of LEC of at least 80% confluence. Cells are removed from the flask via enzymatic digestion with Trypsin-EDTA, then centrifuged and reconstituted in cell media. The media used is DMEM with 20% fetal bovine serum (FBS) and a 1x antibiotic/antimycotic solution. Cell solution is approximately 300,000 cells/mL when seeding microchannels.

Shear Stress

Shear stress for a fluid is defined as the traction applied by the flowing fluid on the plane with respect to which the fluid is deforming. Since the flow of interest is lymphatic flow, and the plane of interest is the surrounding lymphatic endothelium, the shear stress experiments will aim to mimic lymphatic flow. Furthermore, a fluid will be pumped through the microchannel to produce shear stress upon the underlying LEC. Both the inlet and the outlet of the microchannel of interest will be connected via tubes to a flow pump that receives its source of fluid from a flask that will in turn be pump the fluid through the tubes into and out of the microchannel of

interest. The pump used is a precision-calibrated peristaltic pump that produces the required flow rate. The experiments will be run on four groups, one control and three experimental. The control group serves for the flow to be studied at very low values (< 0.1 dyne/cm^2), which is just enough to ensure perfusion. The other three experimental groups will serve for steady flow to be studied at shear stress 1, 3, and 10 dynes/cm^2, which correspond to low, average, and high levels of shear flows respectively within the physiological range (Jafarnejad et al., 2015). For each group, three sets of experiments were conducted. To further quantify the amount of shear flow that corresponds to each value of wall shear stress, Equation 1 was used (Humphrey and Delange, 2004).

$$\tau_w = \frac{6\mu Q}{\pi w h^2} \quad (1)$$

Where τ_w is wall shear stress, μ is the viscosity of the fluid, Q is the magnitude shear flow, and w and h are the width and height of the microchannel respectively. Given that the height of the microchannel is 120 μm and its width is 800 μm, and the medium used (MCDB-131 medium) has a viscosity of 0.7 cP at 37ºC, the respective shear flows were found to be 0.0862 ml/min for a shear stress of 1 dyne/cm^2, 0.259 ml/min for a shear stress of 3 dynes/cm^2, and 0.862 ml/min for a shear stress of 10 dynes/cm^2. Therefore, the obtained values of shear flow will be used to input the value of shear flow for each group into the pump, and the pump will push the fluid to flow with the given shear rate.

Microscopy

Phase Contrast Microscopy

Phase Contrast Microscopy was used to observe cellular adhesion on the PDMS microchannels. Furthermore, the microscope used is an inverted phase contrast microscope with both 10x and 20x objectives. It has a camera adapter for ease of recording images.

Immunofluorescence

Immunofluorescence was used to observe and compare expression of the following proteins: LYVE-1 (an LEC marker), VE-Cadherin (tests for cell-cell adhesion), Prox1 (which tests for lymphangiogenesis), and CD31. The procedure for immunofluorescence starts by making the primary antibody dilution buffer with the following concentrations (1X PBS, 1% BSA, 0.3% Triton X-100). To achieve that, Triton X-100 is added first then stirred with BSA. To make the blocking buffer (1X PBS, 10% serum, 0.3% Triton X-100), normal serum from the same species as the secondary antibody (e.g. goat) is used. In addition, 0.3% Triton X-100 (30 µL for 10mL solution) is stirred after adding the serum. The solution of 16% paraformaldehyde is diluted to 4% in PBS. For formaldehyde to be stable, the solution is made fresh and is stored in the dark at 4^{o}C. The cell culture surface is then washed three times with PBS for approximately 5 minutes. Following that, 4% paraformaldehyde solution is added and placed in the refrigerator at a temperature of approximately 4^{o}C for 10 minutes. The cell culture surface was washed three times with PBS for approximately 5 minutes. Furthermore, the blocking buffer was added to the cell culture surface and is kept at room temperature was added to the cell culture surface and is kept at room temperature for 1 hour. The primary antibody is diluted at a ratio of 1:200 in the primary antibody dilution buffer. Afterwards, the blocking buffer was removed and the primary antibody was added to the cell culture surface and placed in the refrigerator for approximately

4^{o}C overnight. The cell culture surface is then washed three times with PBS for approximately 5 minutes. The second antibody is diluted at a ratio of 1:200 in PBS and is added to the cell culture surface and kept at room temperature for 1 hour. Moreover, the cell culture surface is washed three times with PBS for approximately 5 minutes. Phalloidin should be diluted from stock to a ratio of 1:40 and added to the cell culture surface and should be kept at room temperature for 20 minutes. To view the sample, the sample was mounted onto a microscope slide with Prolong Gold and the coverslip edges is sealed with nail polish.

CHAPTER III

RESULTS

Polydimethylsiloxane (PDMS) Microchannels

Approximately 45 microchannels were manufactured with a width of 800 µm and a height of 120 µm. The microchannels were closed from the beneath using thin glass slides that would have the least diffractive effects on the light rays used in microscopy via the process of plasma treatment. Pipette tips were used as inlets and outlets for the cell culture media and gelatin as seen in Figure 1.

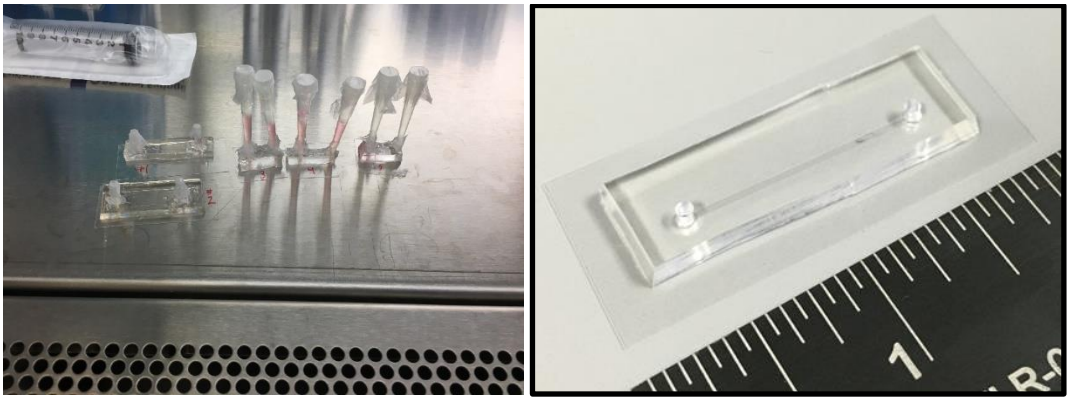

Figure 1. Microchannels

Cell Culture and Microscopy

Phase Contrast Microscopy

The following images were obtained via phase contrast and immunofluorescence for cells cultured on a petri dish. The triangular shape of the cells seen in Figure 2 indicates that the cells are fully adhered to the surface and adhered to their neighboring cells. This is achieved by having sufficient amounts of gelatin that allows for cellular adhesion. Furthermore, the cells exhibit a proliferative profile which is achieved by having cell media. On the other hand, the

circular bright spots are indicative of cells that haven't adhered to the surface of the plate. This could pertain to that the cell media was not sufficient to sustain the viability of the detached cells.

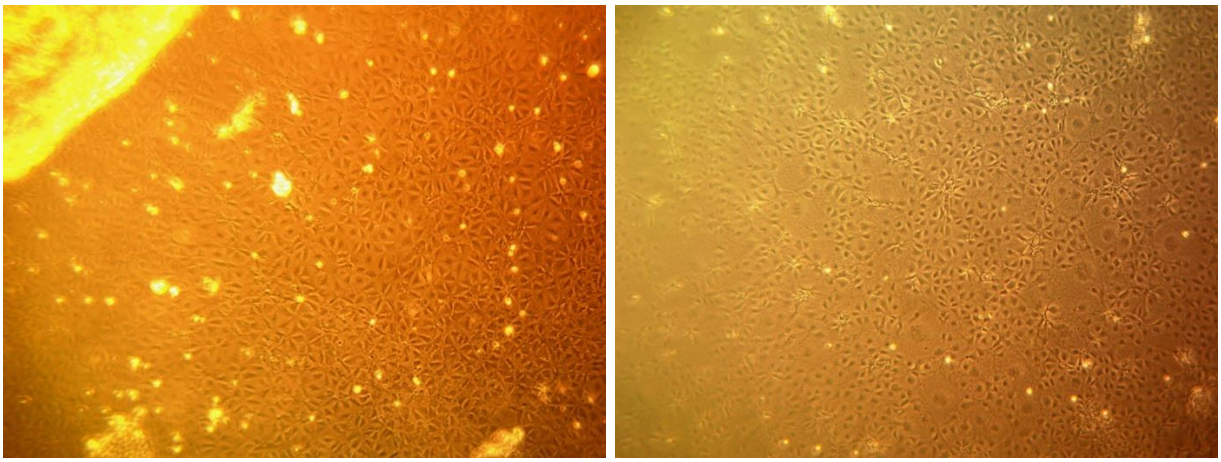

Figure 2. Phase Contrast Microscopy

Immunofluorescence Microscopy

The following images were obtained via immunofluorescence to test for the expression of the following proteins: LYVE-1 (Figure 3), CD31 (Figure 4), PROX-1 (Figure 5), and VE-Cadherin (Figure 6).

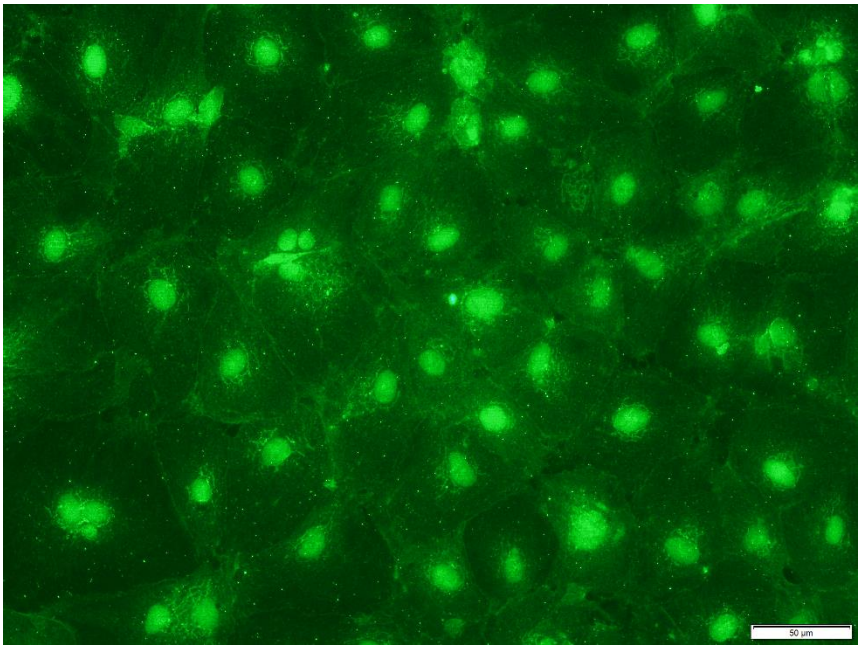

Figure 3. LYVE-1 Expression for LEC

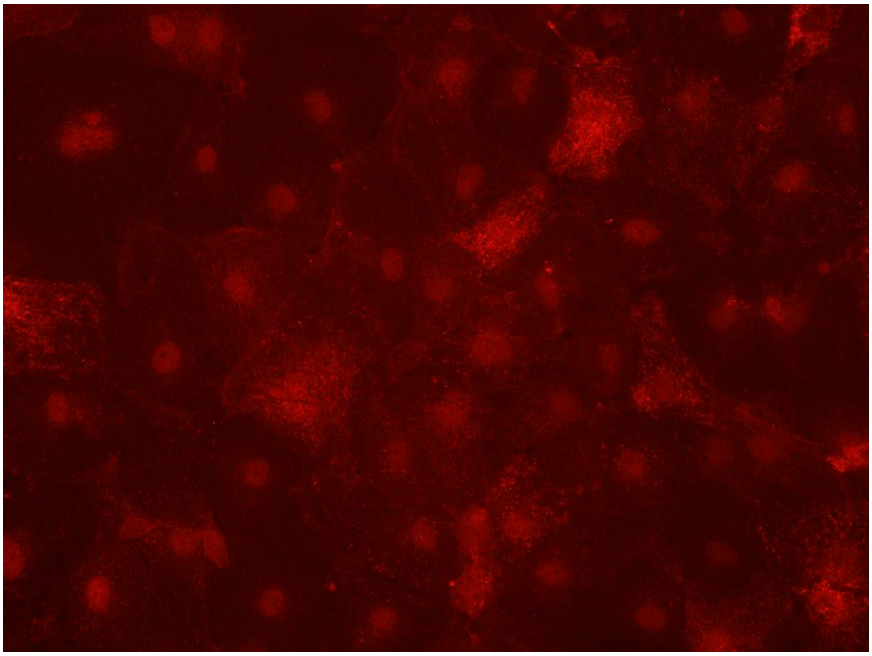

Figure 4. CD31 Expression for LEC

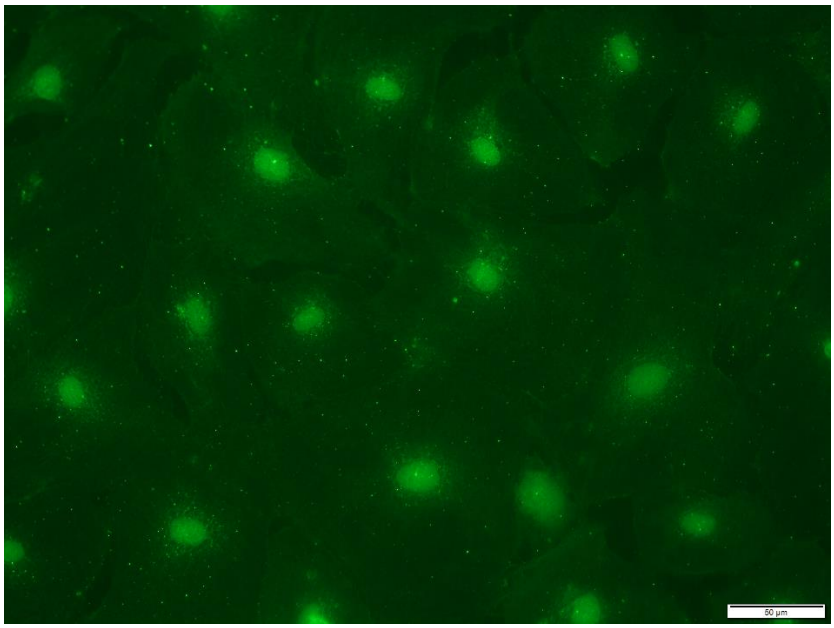

Figure 5. PROX-1 Expression for LEC

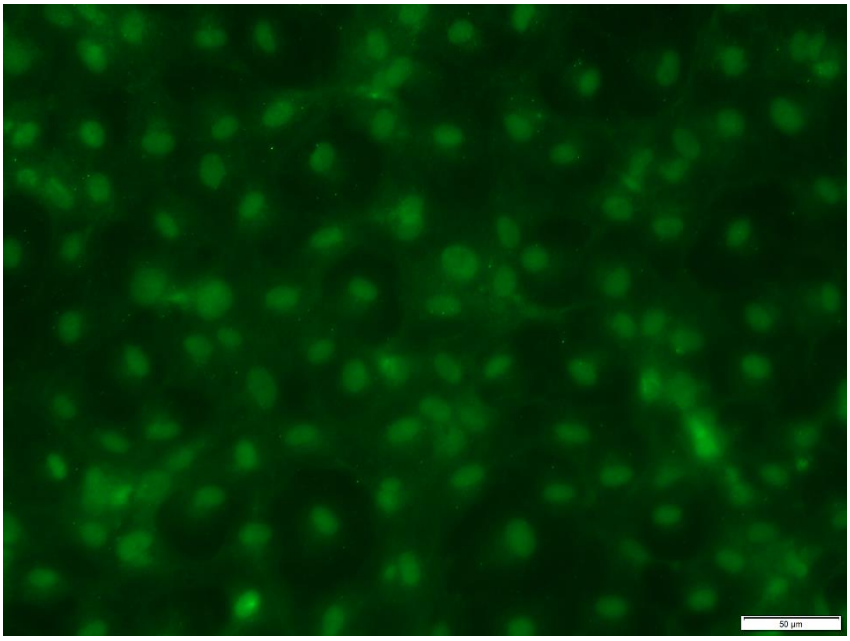

Figure 6. VE-Cadherin Expression for LEC

CHAPTER IV

DISCUSSION

The goal of this research was to study the effects of shear stress on the lymphatic endothelial cells. Upon manufacturing the PDMS microchannels, it was found to be more effective to degas both before and after pouring the silicone mixture upon the PDMS mold to completely devoid the microchannels of gas bubbles. Furthermore, to ensure an equal thickness throughout the microchannel, the silicone mixture should be poured and spread put equally on all parts of the mold.

Upon plasma treating the adhesion surface of both the glass slide and the microchannel and injecting the media within the microchannel, it was found that the media evaporated from within the microchannel after some time. Furthermore, further investigation revealed that the evaporation is likely due to the hydrophobicity of the wall of the microchannel. To solve this, after plasma treatment, D.I. water is injected into the microchannel and it is then stored in water until it is used for experimentations. This keeps it in a hydrophilic state, minimizing the evaporation of the cell media from within.

In addition, the gelatin traditionally used for cell adhesion is 2% gelatin solution. However, it was found that 4% gelatin was more effective for cell adhesion for LEC, so it was used later on for cell culture instead.

Shear stress experiments were not conducted due to the unforeseen problems in seeding the cells in the microchannels and maintaining their viability. However, given that the aforementioned issue was resolved and owing to the preliminary success with setting up the flow loop with the pump, manufacturing microchannels, cell seeding, and immunofluorescence, shear

stress experiments can be conducted to study the gene expression of LEC and the impact of shear stress on LEC deformation.

Rather than doing each part (cell seeding, shear stress, and microscopy) separately, future research can allow all the parts to be done within the same experiment. Furthermore, future research could be done to investigate the effects of strain (stretch) on LEC, given that it is also a mechanical event that occurs in vivo. Once that study has been done, the effects of both shear stress and stretch can both be done simultaneously as a more accurate study of what occurs in vivo. Therefore, to mediate such experiments, a flow bioreactor can be built to allow for such experimentations.

CHAPTER V

CONCLUSION

This research focused on studying the effects of shear stress on LEC. It can be achieved by synthesizing PDMS microchannel to harbor the cells. Afterwards, cells can be cultured on the LEC and once it is shown that the cells are proliferating normally, it can be connected to a pump to run the shear experiments using various magnitudes of flow. With phase contrast and immunofluorescence, the cells can be studied to observe the changes in their shape and biochemical response.

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