Stimulation of Angiogenesis through Collagen Gel by Applying Shear Stress and Interstitial Flow

By

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Abstract

The induction of angiogenesis has been documented in many diseases such as cancer, arthritis, and diabetes. Angiogenesis can be stimulated by a variety of signals including a mechanical stress applied to the apical side of endothelial cells. To get a better understanding of this mechanical stimulus, a parallel plate flow chamber was designed and tested to elicit a constant laminar flow onto a monolayer of endothelial cells. The goal of this research was to develop a more physiologically similar *in vitro* system to study the effects of shear stress on endothelial cells. Unlike prior flow chamber apparatus, this chamber allows the cells to be seeded on collagen gel rather than a rigid substrate to more closely mimic *in vivo* environment. A shear stress of 1 Pa was applied to the endothelial cells for a duration of 36 hours. Results show that after flow was initiated for this duration, a change in shape of the endothelial cells can be seen when compared to the static condition. Elongation and alignment of cells plated on collagen gel can be seen in the direction of flow, though not at pronounced as the elongation typically seen from cells plated on rigid substrates. Nuclear and F-actin staining also revealed similar results. The nuclear staining revealed a confluent monolayer formation occurred prior to flow and was maintained throughout the experiment. A change in organization of the F-actin fibers, from radially protruding out from the nucleus during static condition to a more ordered arrangement after the flow was implemented could also be seen. The changes in cell appearance illustrates that the mechanical stimulus of a shear stress has an effect on endothelial cell arrangement and suggests that this effect depends to some degree on the cell adhesion substrate stiffness. Furthermore, new research in this area can look at both the signaling that leads to these morphological changes as well as the factors that control angiogenesis.

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Chapter 1: Introduction

Angiogenesis and the role of Endothelial Cells

Angiogenesis, the growth and formation of new blood vessels, has been researched in connection to many diseases such as cancer, arthritis, and psoriasis (6). However, angiogenesis is also an essential function for development, maintaining adequate tissue oxygenation, and wound repair. Studying the biological factors, both mechanical and chemical, that influence angiogenesis will thus provide a better understanding of these important physiological and pathophysiological processes.

Blood vessels are tube-like structures that transport nutrients and oxygen to tissue throughout the body. These vessels are lined internally by a monolayer of endothelial cells. During new blood vessel formation, endothelial cells divide and migrate in a direction dictated by biochemical and mechanical cues in order to form protruding "branch-like" structures from the original vessel. Surrounding the blood vessels is the extracellular matrix composed mainly of collagen, proteoglycans, and fibronectin. Cells migrate to spaces in the matrix where they divide and move to form new vessels and networks of branching vessels, using the extracellular matrix for structural support (figure 1).

With emerging biological engineering technologies, a better understanding of angiogenesis is possible. *In vitro* experiments where a layer of endothelial cells is plated on hydro gels, such as collagen, poly-lactic acid (PLA), and poly-glycolic (PGA), provide a more physiological environment compared to prior experiments where the endothelial cells are plated on rigid substrates. Collagen is widely used because it is a natural macromolecule and is a major part of the extracellular matrix (22). Similar to *in vivo*

conditions, confluent cells are able to invade the gel and form tube-like structures (30). The gel acts as the extracellular matrix providing structure and support during migration and proliferation in that direction. (31).

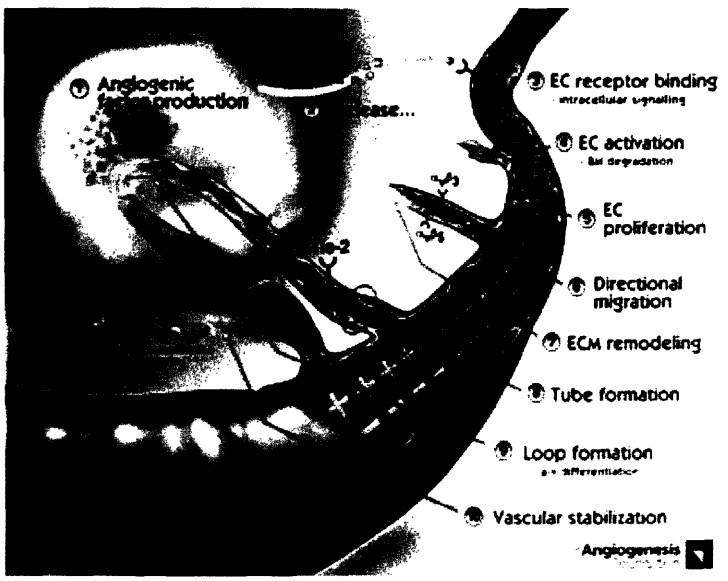


Figure 1: Endothelial cells undergo angiogenesis to produce new networks of blood vessels. The steps of angiogenesis are (1) stimulation of endothelial cells by angiogenic factors; (2) Degradation of the capillary basal lamina by activated endothelial cells; (3) Capillary sprout formation and migration of endothelial cells; (4) New vessel maturation.

Mechanical Stimuli

As mentioned earlier, angiogenesis is initiated in a variety of different diseases such as cancer and during the progression of arthritis. As a result of this link between blood vessel formation and disease progression, research has been done in determining the biological basis of angiogenesis. Details concerning the steps in which the blood vessels form have been confirmed by many different studies (21). The induction of vessel formation by chemical stimuli has been thoroughly studied. It has been shown that adding different cell growth factors, such as vascular endothelial growth factor (VEGF)

and platelet derived growth factor (PDGF), while a cell is immersed in a three-dimensional extracellular matrix-like environment causes capillary formation in all directions (26).

One area of fundamental and extensive research is mechanotransduction; the biochemical response of cells to external mechanical stresses and strains. Using mechanical knowledge of material properties and physics, researchers are inducing forces and strains, and generating flow past cells to investigate a variety of physiological processes. One such process that is being researched is the connection between mechanical forces and angiogenesis. Angiogenesis has been proven to be stimulated when a shear stress is applied to a layer of endothelial cells and also when flow is induced normal to (through) an endothelial monolayer. Vascular sprouting is enhanced by fluid shear stress (18). The stress applied on a specific face of the cell is presumably transduced into biochemical signals that allow more control over the direction in which cells migrate and thus the direction in which capillaries are formed.

Hemodynamic Effects

Endothelial cells *in vivo* are constantly exposed to flow along their apical surface, the magnitude and time-variance depending on the location within the vasculature. In vascular studies, it has been shown that endothelial cell function is dependant on the shear stress that arises as a result of frictional forces opposing the flow (9). From basic mechanics, shear stress is the tangential force divided by the surface area. For vascular systems, shear stress is determined as the product of the velocity gradient normal to the surface of the endothelial monolayer where the fluid is acting and the fluid viscosity,

assuming a Newtonian fluid. A gradient of fluid velocity forms because at the wall fluids do not slip, making the velocity zero, while flow along the center of the chamber is moving with the maximum velocity in the vessel. Determination of the velocity profile is done by using the conservation of momentum equations (see fluid dynamics section). Shear stress can then be calculated, by evaluating the gradient.

Much research has demonstrated that blood vessels in the human body change their phenotype in response to different levels and time-varying patterns of shear stress applied to them from the flow of blood in the circulatory system. Depending on the rate of flow, blood vessels either constrict or dilate, which is caused by a signal from the endothelium sent to the vascular smooth muscle to contract (19). In addition to *in vivo* studies, many *in vitro* studies have also shown effects of flow on endothelial cells (1,13). Through these studies, it is now clear that mechanotransduction has a tremendous effect on tissue morphology and function, including angiogenesis.

Overview of thesis

The motivation behind this thesis is to better understand the effects of shear stress on angiogenesis when applied to a monolayer of endothelial cells, focusing on the mechanical cues that initiate cell migration and tube formation. In order to investigate initial cellular changes such as movement and elongation, we developed a parallel plate flow chamber as our *in vitro system,* to apply a shear stress of physiologically equivalent magnitude to a monolayer of endothelial cells grown on a collagen gel and monitored their alignment. Previous studies have shown that endothelial cells subjected to shear stress initially experience changes in shape (elongation) and alignment in the direction of

flow whereas cells that are not subjected to shear stress are polygonal with no identifiable alignment (10). This is an example of endothelial cell mechanotransduction, where F-actin filaments and cell morphology reproducibly aligns with the direction of flow under physiologic arterial shear stress (4).

A distinguishing factor of this research compared to prior flow chamber studies is that the cells are plated on a collagen gel rather than a rigid substrate, such as glass, in order to mimic physiologic extracellular matrix stiffness and allow for a three dimensional (3-D) response rather than a 2-D response. The change in environment may alter how the cells respond to external force, possibly resulting in differences in elongation and alignment of cells compared to rigid substrates. Accordingly, cells were imaged at different time-points to investigate the progression of endothelial alignment and elongation under shear stress, as monitored with F-actin fluorescent staining techniques.

CHAPTER 2: Parallel Plate Flow Apparatus

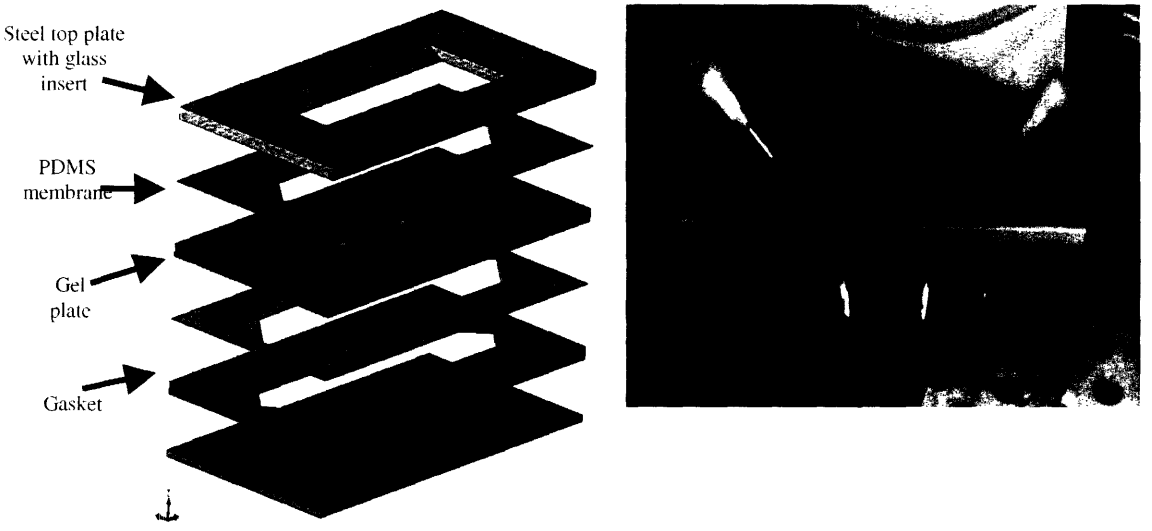
When trying to mimic *in vivo* environments with *in vitro* experiments, one tries to diminish the amount of variables and differences between the two. In studies dealing with the vascular system and the effects of blood flow on endothelial cells, it is quite often too difficult to use *in vivo* experiments, as a result much research is being conducted to produce *in vitro* similar environment. In order to create a sustained laminar flow, most *in vitro* systems use either a parallel plate flow chamber or a cone and plate designs (5). Both systems have their advantages along with disadvantages. In the parallel flow chamber, cells can be imaged in real time and the shear stress can be easily calculated compared to the plate and cone design where at high velocities abnormal flow patterns are generated (5, 29). Because of this, we will be developing and implementing a parallel plate flow chamber in our experiments. The flow chamber will allow us to study the effect of shear stress on the monolayer itself. Furthermore, we will be able to examine the influence the adhesion substrate has on the cells' response to shear stress.

Flow Chamber Assembly

The prime motivating factor in designing this flow chamber is that the cells are grown on collagen instead of a rigid substrate like prior flow chamber designs. As mentioned earlier, this allows a 3-D effect to take place because the cells could now move through the collagen. In addition, collagen more closely mimics the extracellular matrix compared to rigid substrates such as glass. In order to achieve this, a multi-layered parallel plate flow chamber was designed (see figure 2).

The chamber in which fluid is flowing has a surface area of 12.5 cm^2 (5 cm x 2.5

cm). The collagen plate comprises the bottom of the chamber and is the only region we are interested in seeing the effects of shear stress. The walls of the chamber were first made out of a thin silicone sheet with a thickness of 100 microns. This thin film was placed on the top of the gelling plate and then was sandwiched with the top plate creating the entire chamber. The top surface of the chamber is constructed out of steel, but has a glass insert directly above the collagen. This allows for visualization and imaging of the cells and collagen. Imaging also played a role in determining the thickness of the chamber. Because there is maximum distance at which a microscope can image, the height of the chamber was strictly regulated (figure 2).



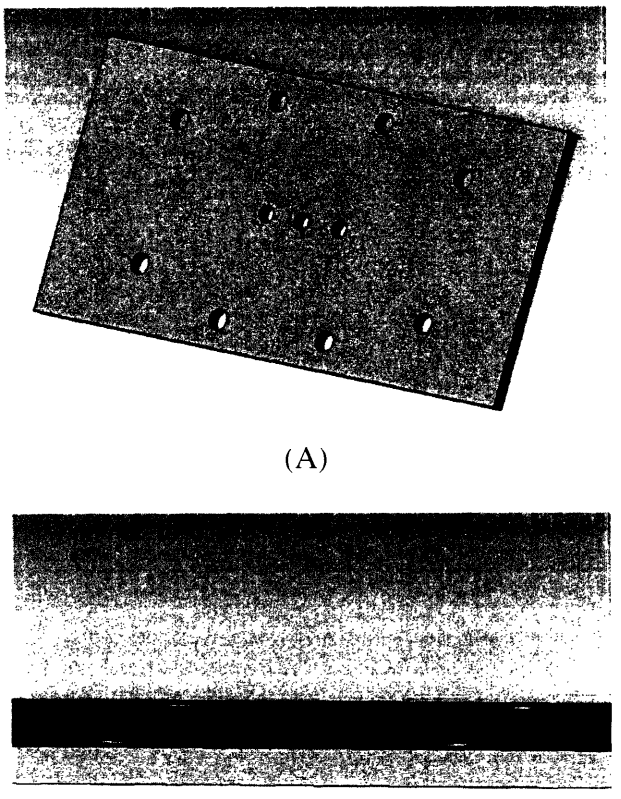
(A) (B)

Figure 2: (A) Exploded view shows the composition of the flow chamber. The top plate contains a glass insert for microscopy. A 200 micron layer of PDMS constitutes the walls of the flow chamber. A polycarbonate piece containing a rectangular cut out for is the apparatus used for collagen gelling and cell seeding. A rubber gasket is used at the bottom in order to get a constant force to seal the chamber. (B) Assembled flow chamber with inlet and outlet tubes.

However a modification that had to be made after testing was that an increase in height of the chamber was needed. One reason for this change was for handling and sterile purposes. The thin silicone sheet collected dust particles and would cling to materials due to static charges. And so a new material, polydimethylsiloxane (PDMS), was used. A thin layer of this material, roughly 200 microns, was spun onto polycarbonate plates and cut into specific gasket flow channel dimensions. Another more important reason the height had to be increased dealt with air bubbles. Air bubbles sporadically appear in the system due to a variety of ways: the pump, loosened tubing, or valves being opened and closed. With the height of 100 microns, these bubbles would get trapped in the chamber on top of the collagen, having detrimental affects on the collagen substrate. Increasing the height of the chamber allowed the bubbles to flow through the chamber and eventually exit the system. Because the increase in height was only 100 microns, imaging was not hindered except at high magnifications.

Design of Collagen and Cell Seeding Plate

The gelling plate was initially designed to be made out of polycarbonate and have three small cylindrical holes of diameter 3mm in the center (figure 3). The reasoning behind using small cylindrical holes placed in the center of the plate was to neglect complex flow near the chamber side walls. Because the holes' diameters were substantially small compared to the length and width of the chamber, a constant laminar flow existed over the entire surface of the collagen.



(A)

(B)

Figure 3: (A) Polycarbonate gelling plate with three holes of diameter 3 mm for gelling. (B) View of meniscus forming over the three holes as a result of surface tension in the collagen. This led to the design of a new gelling piece.

However after initial testing of the flow chamber, it became evident that changes to this plate must be made. The most urgent concern that had to be addressed was the ability to form a flat monolayer where the flow interacted. The small diameter of these three holes caused a meniscus to be formed on the upper edge of the plate (figure 2b). As a result the collagen eventually would erode due to the non-uniform channel height causing local regions of high shear stress.

Instead of having three separate holes to gel, the new design contained a rectangular cut out of the polycarbonate piece that contained a shoulder for better collagen adherence. The cut out had a length of 2.0 cm and a width of 1.0 cm. A small hole with diameter 4mm was placed in the center of the cut out to allow for interstitial flow (figure 4). The size of the well is far larger than the prior design. The ability to gel more collagen into the well, due to the increase in depth, length, and width, allows for better stability. The increase in length and width of the well made a substantial contribution to eliminating the formation of the meniscus by allowing the gel to become flat before coming in contact with the opposite side of the well.

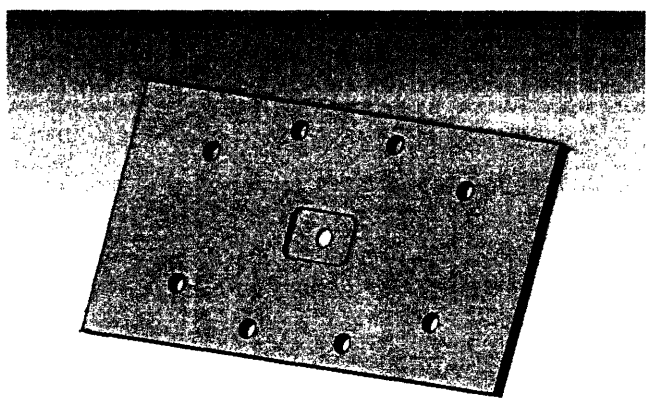


Figure 4: The gelling compartment was cut out of polycarbonate. It contains a rectangular cut out with dimensions 10mm by 5 mm by 4 mm. In the center of the piece, there is a cylindrical hole with diameter 3 mm.

Fluid Dynamic Control

In order to obtain the desired physiological shear stress, we must derive a relationship between shear stress and flow. The amount of shear stress is proportional to

the amount of flow, the height of the chamber, as well as the viscosity of the fluid. Using a simplified version of the Navier – Stokes equation we obtain an initial equation relating viscosity of the liquid (DMEM media):

$$\mu \cdot \frac{d^2}{dy^2} V_x(y) = \frac{dP(x)}{dx} \tag{1}$$

we can solve for the flow as well as the wall shear stress for a parallel plate flow chamber. Integrating this equation twice and using the boundary conditions

1. No-slip condition: Vx = 0 at y = h/2
2. Shear rate zero at axis of symmetry: dVx/dy = 0 at y=0

We get an equation for the velocity profile in the y direction

$$V_x(y) = \frac{1}{2 \cdot \mu} \cdot \left(\frac{-dP}{dx} \right) \cdot \left[\left(\frac{d}{2} \right)^2 - y^2 \right] \tag{2}$$

With this equation we can solve for the flow rate of a parallel plate chamber, whose height is h and viscosity, μ,

$$Q = \frac{h^3 \cdot w}{12 \cdot \mu} \cdot \left(\frac{-dP}{dx} \right) \tag{3}$$

Flow can then be related to the wall shear stress of a parallel plate flow chamber by the equation

$$\tau(y) = \frac{6 \cdot \mu \cdot Q}{w \cdot h^2} \tag{4}$$

As mentioned earlier, the physiological shear rate is 1 Pa, so in order to obtain that shear with a viscosity of .001 Pa*s, width of 15 mm and a height of 200 microns, a flow of 62.5

ml/hr is needed (12). The flow ran for a total of 36 hours. At different time points, the cells were imaged and analyzed.

CHAPTER 3: Applying Shear Stress to Endothelial Monolayers

Materials and Methods

Collagen gelling

To mimic the extracellular matrix, the cells were seeded on top of a collagen gel. The collagen gel was made with Type I Rat-tail Collagen (BD Biosciences, Bedford, MA, 35-4236). Phosphate-buffered saline (PBS) was added to get a concentration of 10%. An initial amount of NaOH was used that was proportional to the volume of collagen added (volume of collagen multiplied by 0.023). Distilled water was then added to obtain the desired volume. The mixture was then titrated using NaOH in order to achieve a pH of 7.4. Different concentrations of collagen were tried to find the optimal environment for the cells to adhere and grow. The final concentration of collagen used was 0.4%. After the mixture was made, the liquid was pipetted into the well and incubated for 40 minutes at 37 degrees Celsius. After the collagen formed a gel, 2 ug/ml fibronectin (Invitrogen, Carlsbad, CA, 33016-023) in PBS was placed over the entire chamber surface (including collagen and polycarbonate) for four hours and incubated at 37 degrees Celsius in order to provided a binding surface for the cells on the polycarbonate surrounding the collagen gel.

Endothelial cell culture

Bovine aortic endothelial cells (BAEC), passages 9-12, were seeded onto the collagen gel and cultured in Dulbecco's modified Eagle's medium (DMEM, Cambrex, East Rutherford, NJ) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

Cells were seeded with DMEM at a density of approximately 72,000 cells/cm^2 on both the collagen and polycarbonate surfaces. The cells were grown overnight in order to form a confluent monolayer and also to allow for attachment to the collagen and fibronectin.

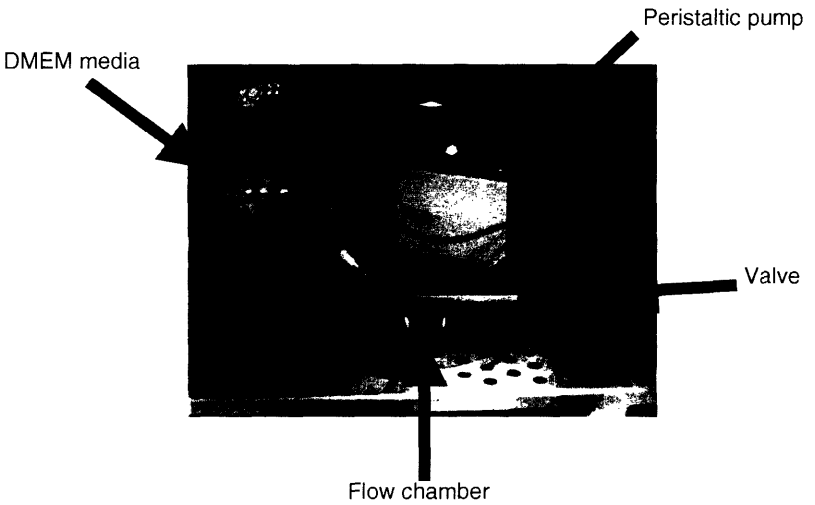
Flow System

After the flow chamber was assembled, wingnuts are used to tighten and seal the chamber to a rubber gasket. The entire device is placed in the incubator in order to maintain constant temperature and carbon dioxide condition required for cell survival. A peristaltic pump (Pharmacia Fine Chemicals P-3) was placed within the incubator, and generated the constant flow of DMEM with 10% FCS and 1% P/S through the flow chamber (see figure 5 for complete flow loop).

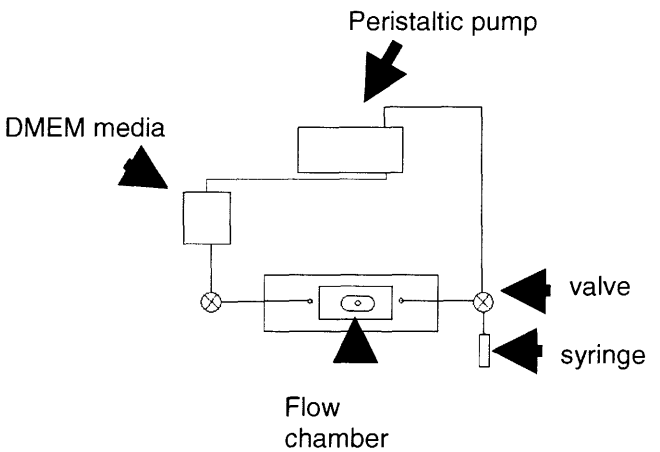
F-actin and nuclear staining

Phalloidin was used to stain all F-actin fibers. Phalloidin works by binding to actin at the junction of monomers of G-actin (20). To stain the nucleus, the fluorescent reagent DAPI (4'-6-Diamidino-2-phenylindole) was used. DAPI works by forming complexes with natural double stranded DNA, making it very concentrated in just the nucleus. In order to stain both F-actin and the nucleus, the cells had to be first washed with PBS twice. They were then fixed using 4% paraformaldehyde at room temperature for 30 minutes and then washed again with PBS. In order for the phalloidin and DAPI to enter the cell, the cell membrane had to be permeablized using 0.1% Triton-X solution in PBS for 15 minutes. After a thorough wash with PBS, the cells were then incubated at

room temperature with phalloidin at a concentration of 12.5 ug/ml and DAPI at a concentration of 0.5 ug/ml for 30 minutes.



(A)



(B)

Figure 5: (A) The set up of the flow chamber is a simple loop. The flow source is a peristaltic pump which flows media through the flow chamber. Two valves are located on either side of the chamber with a syringe attached to the inflow valve. These valves are used to eliminate bubbles that form as well to stop the flow in order to image the cells. (B) A schematic view of the flow loop.

Microscopy

Cells were imaged using both fluorescence and non-fluorescence techniques with an inverted light microscope (Nikon Eclipse TE-300). Images were obtained with objectives ranging from 4x to 20x in magnitude. A CCD camera (Hamamatsu Orca C4742-95-ER) was attached to the microscope which was linked to an Apple computer using OpenView software. Images were taken first without fluorescence at 4x to notice physical attributes of the monolayer. A comparison was made between the monolayer that was on collagen and the monolayer that was on the polycarbonate piece. At 10x and 20x, images of small groups of cells or individual cells were taken to notice any alignment or elongation that had occurred. Images were then taken at 10x and 20x using fluorescence. The images were used to qualitatively capture the amount and direction of F-actin formation in cells that were exposed to a shear and with cells that were not. Nuclear staining was done in order to distinguish individual cells.

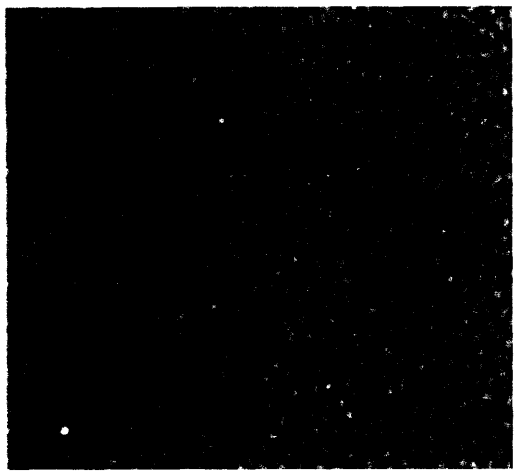
RESULTS

Concentration of Collagen

The concentration of collagen played a large role in both cellular and polycarbonate attachment. A range of collagen from 0.2% and 0.4% was tested under static and shear environments to determine the optimal amount. It was found that the best volume percentage to use was 0.4%. At low percentages (0.1-0.3%), the collagen would not adhere well to the polycarbonate. When fibronectin (in PBS) was added, the collagen would sometimes lift up and completely detach from the polycarbonate. When this concentration of collagen was exposed to a flow, the collagen had a tendency to slightly tear at the edges of the collagen and polycarbonate leading to discontinuities between the two surfaces. This causes the collagen to rip from the well as time passes.

Cells under static conditions

To asses the effect of shear stress has on endothelial cell migration and shape, we used the control of a static situation on both collagen and polycarbonate. After 36 hours of incubation at 37°C, the cells formed a confluent monolayer over both surfaces. The cells were imaged at different magnifications to assess monolayer formation and general shape of cells. Using 4x magnification, it could be seen that a confluent monolayer had been formed with little discrepancies between the two surfaces (figure 6a and 7a). The sections towards the edge of the polycarbonate did not have confluent monolayers. But because they were located towards the edge of the chamber, the flow was not affected.



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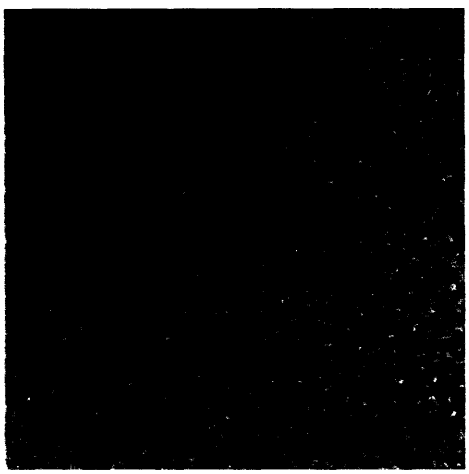

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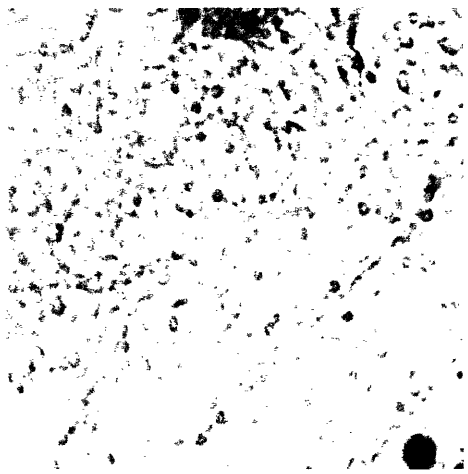
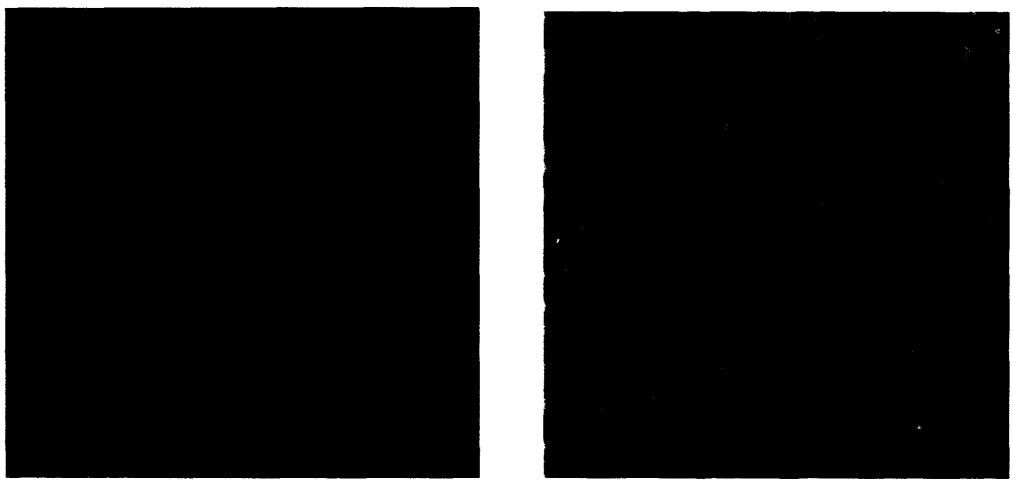


(C)

Figure 6: Cells were grown on the polycarbonate that was coded with 2% fibronectin under different conditions. (A) ECs were grown under static conditions for 36 hours in order to have a control to compare experimental results. (B) The ECs after 24 hours of shear stress of 1 Pa. Cells are not aligned nor show signs of elongation. (C) After 36 hours of flow, the endothelial cells still do not show signs of elongation or alignment on the polycarbonate.

Under 10x magnification, small groups of cells were imaged to get a better view of cellular shape (figure 7b). Cells on both the polycarbonate and collagen looked very similar. The cells had no alignment and were round in shape. The cells were then fixed and stained with DAPI and phalloidin. Images were taken at 20x. By the amount of

nucleus staining, it could be seen that a monolayer had formed consistently over the collagen (figure 9a). Actin staining was evident in these images. The F-actin looked uniform throughout the cell and formed radially out from the nucleus (figure 9b).



(A) (B)

Figure 7: In addition to the polycarbonate, the cells were also grown on a collagen gel for 36 hours. (A) Under 4x magnification, these cells, under static conditions, show a well confluent monolayer. (B) Individual cells are not aligned or elongated under these circumstances.

Cells under constant shear stress

To examine the effect of shear stress on endothelial cells, a constant flow was applied to the cells for durations of 18, 24, and 36 hours. After 18 hours of flow, more elongation could be seen over the spectrum of the gel. Alignment was still not prevalent, but sections of cells showed some alignment along with elongation (figure 8b). Furthermore, areas of the collagen had been slightly disrupted and depressed, while others remained flush with the polycarbonate piece (figure 8a).

Images were also obtained at 24 hours. At 4x magnification, it can be seen that the confluent monolayer is still present on both the polycarbonate piece as well as the collagen gel section. A comparison between the two areas shows that the cells are denser

on the collagen than the polycarbonate piece. Modifications in size and shape can be seen for endothelial cells on the collagen. Elongation in the flow direction seems to be evident in different areas of the collagen, yet still not complete over all the cells. Alignment can be seen more so than at 18 hours, but still in select sections of the collagen compared to the entire area (figure 8a and 8b). Cells seeded on the polycarbonate, however, were not fully aligned or elongated at this point either. Cells were still mainly round in shape and stretched in all directions (figure 6b).

The final time point that was taken was at 36 hours of shear stress exposure. Images were taken at different magnitudes in order to get the full spectra of the effects of flow on the endothelial cells. Looking at 4x magnification, one can see differences in the shape of the cells on polycarbonate (figure 6c) compared to collagen. The cells on the polycarbonate do not seem aligned and are less dense than those on the collagen (figure 8e). Most ECs on the collagen are not aligned, yet some areas are aligned with the direction of the flow. At 10x magnification, cells on the collagen can be seen to show more elongation in the flow direction compared to cells on the polycarbonate (figure 8f).

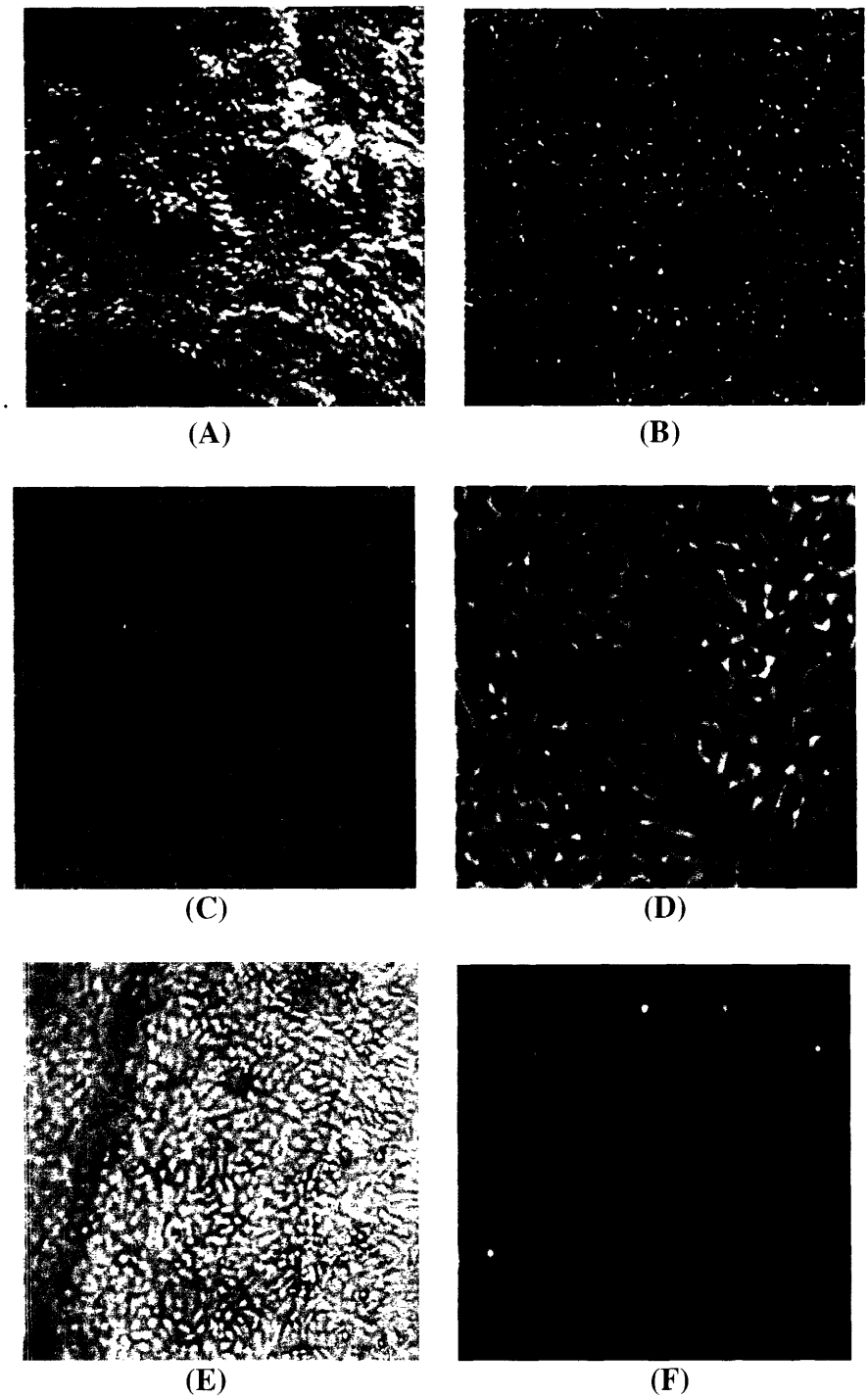


Figure 8: Under a shear stress of 1 Pascal, endothelial cells seeded on collagen gel alter in shape as a function of time. (A) After 18 hours of flow, the collagen and cells are intact despite non-uniformities in height. (B) At 18 hours, a confluent monolayer that shows small groups of elongated cells. (C,D) Cells are beginning to align and still have a confluent monolayer at 24 hours of flow. (E) 36 hours flow reveals patches of cells that are both aligned and elongated. (F) At 10x magnification after 36 hours of flow, elongation can be seen quite easily.

The cells were then fixed and stained with DAPI and phalloidin to exhibit changes in monolayer and F-actin stress fiber formation, respectively. Images of just DAPI staining show that cells still cover the surface of the collagen gel. However, the monolayer has been disrupted. Cells seem to have migrated partially through the gel, shown by cells being on top of one another (figure 9c). Compared to static conditions, more F-actin is present at this time point. F-actin formation is more prevalent in the direction of flow compared to the perpendicular direction (figure 9d).

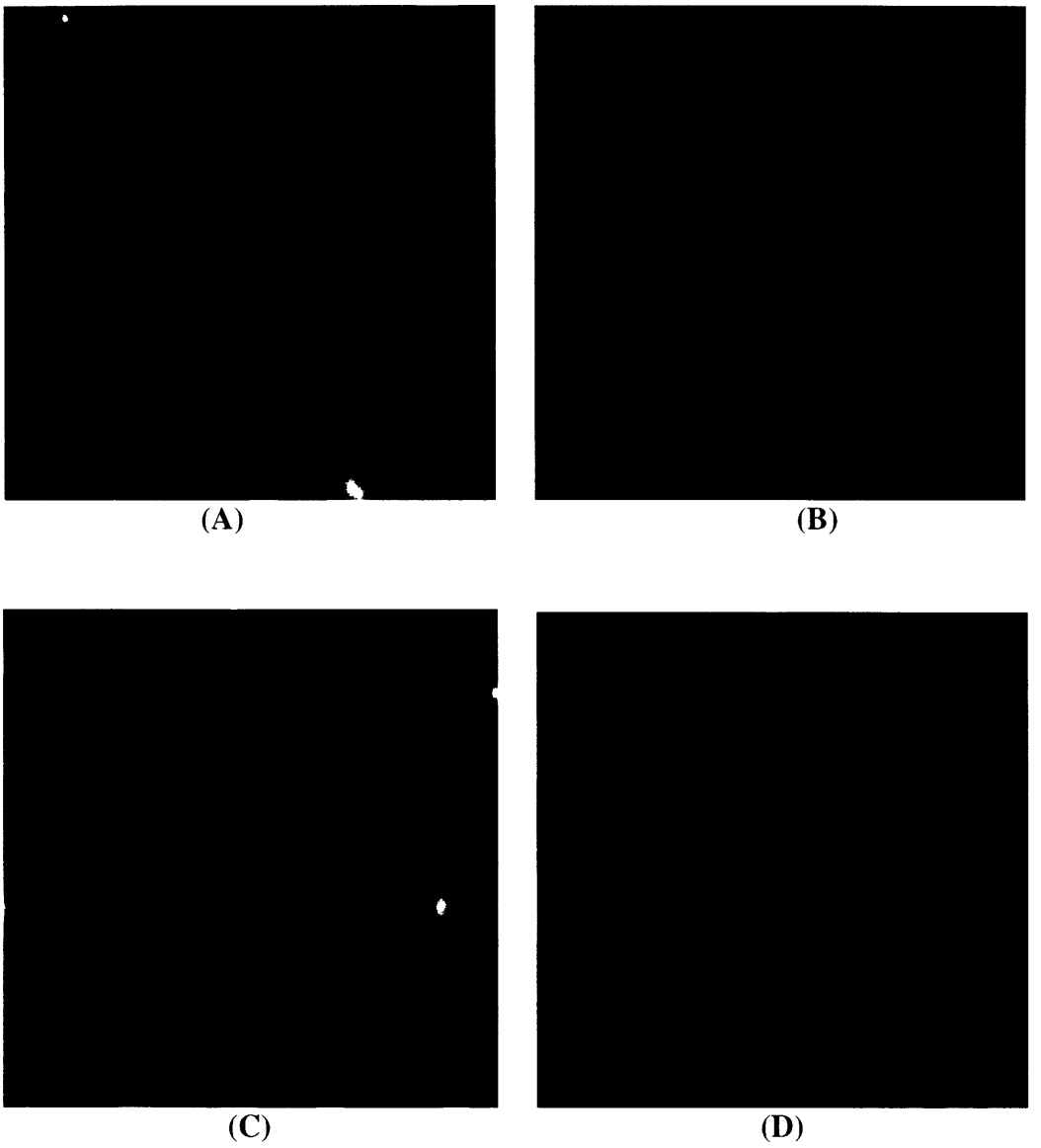


Figure 9: (A) Nucleus staining under static conditions (B) Nucleus and F-actin staining under static conditions (C) Nucleus staining of nucleus after 36 hours (D) Actin and nucleus stating after 36 hours

Discussion

With the new modifications of the flow chamber done here, we were able to more accurately study the effects of shear stress on endothelial cells. The development of the flow chamber allowed us to apply a constant, laminar flow over the cells for multiple hours in order to elicit a morphological response. Preliminary results possibly show a difference in cellular response when cells are plated on collagen gel compared to rigid substrates.

Effects of shear stress vs. static conditions

After applying a shear stress for 36 hours on a monolayer of endothelial cells, we recognized substantial alterations to the cells during that time frame. It was shown that cells first became elongated and then began to align in the direction of flow. Cells is the vascular system are the most prone to effects of shear stress because they are constantly acted upon by blood flow. A key attribute of endothelial cells that form capillaries is their ability to migrate and divide into the extracellular matrix making more capillaries for the blood to flow. This process is called angiogenesis (21). In order for cells to migrate through the matrix, cells must first become elongated and then align in the direction of flow. Furthermore, others have shown this endothelial reshaping has to alter gene expression profile, including the location of growth factors in the cell (16) and adhesion molecules (24).

It was shown in this experiment that endothelial cells undergo morphological changes when stimulated with a shear force, even when plated on a substrate with physiologic stiffness. Under static conditions, the cells appear round and displayed a

cobble-stone pattern characteristic of un-stressed endothelium. However, a change in shape occurred when a shear stress was applied: the cells started to show directionality (in the direction of flow) as well as some alignment in the same direction. This is very similar to the effects seen *in vivo* (14, 25). Migrating to the direction of flow allows for proteins and growth factors to be localized in specific areas of the cell as well as reducing the amount of shear gradient along the shear surface (2).

This study also examined the change in F-actin organization as a result of shear stress. Prior to shear, the F-actin filaments were sparse and in a radial direction outward. This suggests that cells are producing a tensile force to preserve shape and compression (23). After the shear, the F-actin looked more aligned in the direction of the flow. Malek and Izumo (23) showed that F-actin realignment due to shear is initiated by an increase in intracellular calcium. This was done by using quin2-AM which chelates [Ca2+]. The result revealed that actin stress fibers did not align in the direction of flow. Shear stress induces an increase in intracellular calcium in two-fold. Initially, calcium is released from compartments within the cell, and then later stretch induced ion channels located on the cell membrane are opened allowing extracellular calcium to enter (11). Consequently, signaling pathways related to calcium may have a role in the actin organization changes seen here.

Shear effects on collagen vs. polycarbonate

The flow chamber setup allowed for a comparison between the cellular effects on collagen vs. polycarbonate. The media enters the chamber where cells are plated on fibronectin coated polycarbonate for 20 mm before reaching the collagen region. It then

interacts with the collagen gel, which may also have some fibronectin bound to its surface, for 15 mm before transitioning back to the fibronectin-coated polycarbonate before leaving the chamber. This design allows study of both the collagen and polycarbonate seeded cells in response to the same flow condition. Examination of the results presented here shows that under static conditions, both materials have a confluent monolayer. Yet when a shear is induced on that monolayer, different results occur. After 36 hours of flow, cells were too disrupted to draw any conclusive comparison, most likely due to an inability of adhesion protein adsorption onto the polycarbonate surface, while those grown on collagen showed some alignment and elongation. If the adhesion proteins did not absorb onto the polycarbonate surface, this would cause a problem for cells to attach. When cells are first seeded on collagen gel, extracellular matrix proteins interact with cell receptors initiating a signaling pathway. This pathway stimulates the transport of more extracellular matrix receptors to the cell surface and thus increasing the binding to the collagen (7). This cycle does not occur on the polycarbonate because of the minimal amount of extracellular matrix proteins. If a valid comparison was possible, it would have been expected that cells grown on the polycarbonate substrate would have shown more alignment and stress fiber formation compared to those grown on the collagen gel.

Collagen also has the benefit of allowing movement in three different directions compared to 2-D movement on polycarbonate. This was seen in the image of DAPI staining after 36 hours of flow. In the center of the image you can see, nucleus staining of different magnitudes overlapping one another. This is evident that two cells are

located at different heights in the same place. On polycarbonate, this is not possible because it is impermeable.

Future Studies

As mentioned earlier, the apical side of the cell has a blood flow across it while the basal side is attached to this extracellular matrix. This lack of uniformity polarizes the cells. By controlling biochemical and mechanical variables on both sides of the polarized endothelium, our eventual goal is to better understand how these cells function *in vivo* and apply this knowledge to the pathophysiology of disease progression, as well as angiogenesis. In particular, a part of initial efforts of working towards that goal, we explored how mechanical stimulation affected the morphology of the endothelium grown on a collagen substrate.

The research conducted here lays a solid foundation for further research in the effects of shear stress on endothelial cells. In the immediate future, modifications to the flow chamber such as increasing the amount of shear in order to see formation and possibly alignment of the F-actin stress fibers. Another modification that has large physiological implications is the ability to induce an interstitial flow. Blood only flows on one side of the cells; as a result a pressure gradient is formed through the cell layer. This variable is mainly neglected in most flow experiments because of lack of ability to induce an interstitial pressure; the device that we currently use has this ability, but requires modification of having an area of the collagen unsupported by the polycarbonate (over a hole). This would allow fluid and pressure to flow through the collagen. This adjustment will allow us to simulate *in vivo* conditions more precisely. Another alteration that can be made to mimic physiological characteristics is to generate a physiologic flow rate wave form. In the vascular system, blood is not pumped in a constant uniform way; it is instead pumped in a pulsating manner. Programming a

peristaltic pump to stimulate arterial and venous wave forms; we could analyze the effects of pulsatile flow on cell growth and migration over compliant substrates.

As mentioned earlier, mechanical stimuli causes morphological effects in endothelial cells. These effects initiate signals from receptors in the cells in order to elicit a response. A more in depth understanding of propagation of signals, mainly a force on the cell membrane, is an avenue that is currently being addressed. One of the signaling areas being researched is MAP kinase signaling. Early results from studies have shown that some connection does exist, but much is still unknown. MAP kinase pathways along with G protein signaling has numerous effects in the cell and so examining a possible link between the two are of much interest (9). As with this example and countless other genes (PDGF-B, VCAM-1) that seemed to be affected by mechanical stimuli, determining the pathway in which the genes are regulated is important. Do the mechanical stimuli follow the same pathway as chemical signals or are there additional signaling pathways, such as through the cytoskeleton, that have the ability to warrant a response (27)?

Much research is also being done in this area in a clinical aspect because of the correlation between shear stress on endothelial cells, disease progression, and angiogenesis. Angiogenesis has been linked to many diseases such as cancer, diabetes, and arthritis. Studying the mechanisms that initiate angiogenesis could then be used to develop cures for the above listed diseases. An obvious clinical use of this research would be the development of engineered blood vessels to be used as substitutes for by-pass surgery. Another important section of research that this could possibly stem or even add to is quantification of vessels migration due to the blood flow. Visualizing cellular

movement either through the collagen or even in the direction of flow could explain many details about cellular characteristics. The ability to construct capillaries *in vitro* will also provide a platform for studying vascular diseases, without the problem of having to attain live specimens. Thus, developing an environment that mimics *in vivo* conditions to study biological processes as well as disease is beneficial to both basic scientific research and clinical research.

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