Engineering the Endothelial Microenvironment

by

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ABSTRACT

Changes to the microenvironment of the endothelium can produce significant changes in the response of endothelial cells to stimuli. Human Aortic Endothelial Cells (HAECs) are tested in vitro for their fluid shear stress response when their substrates, and the solute concentrations of the fluids to which they are exposed, are modulated, and for their nitric oxide expression when they are exposed to hyperglycemic conditions. ImageJ is used to quantify either the degree of cellular alignment and elongation with the direction of flow, or the relative NO expression using the fluorochrome DAF-2. First, the results of Brower, et.al. are replicated: HAECs under normal glucose (4mM) conditions align and elongate with flow ($p<<0.05$), while high glucose (30.5mM) conditions negate this effect ($p<<0.05$) and is likely the result of Advanced Glycation End-products (AGEs). Then, in this study it is found that substitution of fibronectin for gelatin substrates does not impair flow ($p<<0.05$), indicating that fibronectin likely does not participate in the initiation of vascular lesions. High palmitic acid also does not prevent HAEC shear response ($p<<0.05$), which is consistent with Brower's predictions that AGEs are responsible for impaired elongation and alignment. NO production is significantly increased ($p<<0.025$) in HAECs cultured 24 hours under high glucose (30.5mM) conditions compared with normal glucose (4mM) conditions, indicating the presence of inducible nitric oxide as part of an inflammatory response. Aminoguanidine (5mM) added to high glucose concentrations reduces, but does not eliminate NO production ($p<<0.05$), likely due to insufficient concentration. Modulation of the endothelial microenvironment leads to pronounced changes in HAEC behavior with regards to NO production under hyperglycemic conditions. Diabetic model rat aortas are

explanted and imaged for the purpose of detecting aortic endothelial cell alignment and elongation; improvements in this method are discussed. A microvessel chamber used with explanted human tissue is re-fit to reduce required volumes of solutions and allow more effective experimentation.

DEDICATION

This work is dedicated in memory of my grandmother, Luella Eldridge, a loving woman, and a type I diabetic.

This work is also dedicated to the family and friends of the anonymous organ donors whose names and lives I will never know, but whose cells made this study, and countless other medical research, possible.

ACKNOWLEDGMENTS

I would like to thank Dr. Michael Caplan for his mentorship, advice, and guidance in the preparation of this thesis, and in my academic formation. You go above and beyond for your students, and I have learned much from both your words, and your example.

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I would like to thank Dr. Richard Herman for providing the diabetic rat model aorta, and Dr. Karen Swazea for explanting it. I would like to thank Dr. Christine Pauken for sharing her wealth of practical laboratory knowledge, and for teaching me how to spot a "happy" cell.

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CHAPTER 1

INTRODUCTION

Why Study the Endothelial Microenvironment?

The vascular endothelium is a thin layer of cells that lines the interior of blood vessels and arteries. Its essential functions include forming a selective blood barrier, and maintaining vascular homeostasis through its participation in blood clotting, angiogenesis, vasoconstriction, vasodilation, and inflammation (Behrendt, 2002). However, these functions may be disrupted, or disease states may occur when the microenvironment surrounding the endothelium is fundamentally changed, through modulations of the basement membrane, or variations in the concentration of biomolecular compounds in the blood. Endothelial microenvironmental changes, such as hyperglycemia, are often associated with diseases, such as diabetes, whose physiologic impact may be partially explained at the cellular level. For this reason, a better understanding of how endothelial cells respond to microenvironmental changes may help explain the characteristics of certain diseases, and aid in the design of better treatments.

An *in vitro* model of the vascular endothelium is an appropriate means to study the function of the endothelial layer because the endothelium exists in tissue in a largely two-dimensional environment (Behrendt, 2002). Few cell types found in tissue exist in two-dimensional environments, but endothelial cells do: the culture of endothelial cells, on protein-coated glass, that are exposed to physiologic shear stress resulting from flow, provides an analog to endothelial cells that grow a single layer thick on the luminal surface of a vessel carrying blood. This analog is offered in contrast to the culture of most cell types, which takes cells that exist in three-dimensional tissues and causes them

to proliferate in two-dimensional spaces, potentially altering any number of their behaviors, responses, or phenotypic expressions.

Why Study the Explanted Endothelial Microenvironment?

The cost of developing new drug treatments is now estimated at well over $1 billion USD per successful drug brought to market (Adams, 2010). The tremendous cost and time investment in developing new drugs is a result, at least in part, of the vast screening and elimination process of new targets, beginning at the cellular level and progressing through many stages prior to human clinical trials (Adams, 2010). Once a molecular compound has shown promise at the cellular level, it may then be tested in animal models such as mice and rats; then tested in a larger animal analog such as a dog or pig, and finally in three stages of human clinical trials. Thousands of drugs may be identified as targets at the cellular level, but fewer than ten of those targets will show safety and efficacy in the animal models and through the process of clinical trials (Adams, 2010). It has been speculated that one reason for this sharp narrowing of potential candidates is that drug screening at the cellular level does not represent a picture of the overall biological system. Moreover, at the animal level, candidate drug interactions with animal systems may or may not accurately represent the interactions that drug would have with a human system.

Recent studies using explanted tissue samples may provide a partial remedy for the cost of drug development by allowing researchers to explore drug targets at the tissue level, potentially without expensive animal trials (Cahill, 2012). Specifically, our collaborators at the Phoenix Veteran's Affairs hospital have developed a method for

obtaining microvessel samples from fat biopsies of enrolled patients. The ability to study drug targets using human tissue, without requiring a human clinical trial that may expose human subjects to additional risk, could substantially reduce the barriers of time and cost in identifying candidates for new treatments.

CHAPTER 2

ENGINEERING THE IN VITRO MICROENVIRONMENT

Introduction

The Endothelial Microenvironment and High Glucose Concentrations. Previous studies in the Caplan laboratory group have shown that disease states such as hyperglycemia can modulate the *in vitro* response of endothelial cells that are exposed to physiologic shear stress (Brower, 2009 and Kelso, 2011). Brower, et. al. showed that endothelial cells cultured under high glucose (30.5mM) conditions for 24 hours and then subjected to physiologic shear stress (8 dynes / cm^2) through fluid flow lose their characteristic ability to align and elongate with the direction of flow (2009). The loss of this ability may be related to the buildup up of atherosclerotic plaques seen in diabetes; briefly, plaques typically accumulate in regions of low shear stress within the vasculature, but in diabetics these plaques become disseminated. Lack of tight cell junctions, which can develop in flow-aligned endothelial layers, may allow for easier penetration of LDL cholesterol (Brower, 2009). That is, physiologic shear stress is atheroprotective, but low shear stress (like that seen in the junctions of arteries) is atherogenic, but the atheroprotection offered by physiologic shear stress is apparently diminished in diabetics.

Brower also showed that aminoguanidine, an inhibitor of advanced glycation end products, restored the ability of endothelial cells to align and elongate with flow, even after culture in high glucose conditions (2009). Kelso, et. al. later showed that pyridoxine, which shares key functional groups with aminoguanidine, also restored this cellular ability; pyridoxine is metabolized into vitamin B6 in the body (2010). It should be noted that Brower and Kelso saw the most pronounced effect on endothelial cellular elongation

and alignment when the concentrations of glucose were at the upper end of what is seen clinically (30.5 mM is approximately 549 mg / dl).

To validate the flow approach used in this study, a first aim is to replicate the results found by Brower and Kelso with regards to endothelial response to shear in high and normal glucose conditions. It is then reasonable to explore other blood solutes that may affect endothelial cell response. One of these is palmitic acid, a saturated free fatty acid that may be used to simulate the high level of fatty acids associated with vascular disease and diabetes; this study will explore the effects of palmitic acid on endothelial shear response as another of its aims.

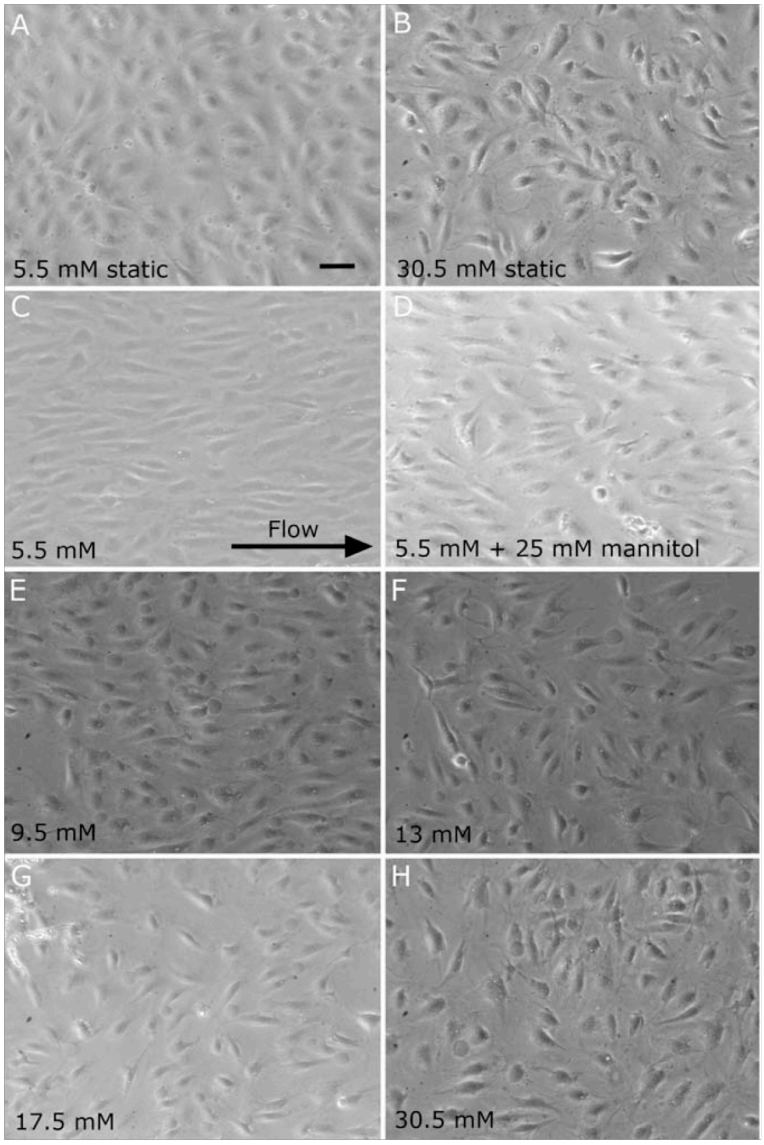


Figure 2.1: High Extracellular Glucose Concentrations Impair HAEC Alignment in Response to 8 dynes / cm^2 Fluid Shear Stress (Brower, 2009). Brower, et. al. show that high concentrations of glucose impair the alignment response of Human Aortic Endothelial Cells (HAECs) at concentrations greater than 17.5 mM, though the most pronounced impairment is seen at 30.5mM. Static cultures show no substantial alignment; normal glucose cultures at 5.5 mM placed under shear show a substantial degree of elongation (2009).

Figure 1 is from the thesis dissertation of Dr Jeremy Benjamin Brower.

The Endothelial Microenvironment and Changes in the Basement Membrane. Endothelial cells rest on a layer of extracellular matrix proteins, known as the basement membrane, that consists primarily of laminin and collagen IV (Hahn, 2009). However, in the lesions of atherosclerotic plaques, the basement membrane has been observed to include deposits of fibronectin (Orr, 2005). A recent study has shown that flow-exposed cellular signaling of JNK, a kinase involved in the inflammatory response, is upregulated in areas of fibronectin deposits and in atheroprone regions, indicating that fibronectin may play an important role in the endothelial cell response to flow-induced shear stress (Hahn, 2005). Given that fibronectin (Fn) may be associated with atherosclerotic lesions, it is hypothesized that cells cultured on fibronectin substrates will show similar impairment of endothelial cell shear stress response. One aim of this study is to explore this hypothesis.

Self-Modulating Environment: The Endothelium and Nitric Oxide Expression. Endothelial cells play an important role in maintaining vascular homeostasis through their secretion of nitric oxide (NO), an important signaling molecule that inhibits the proliferation of smooth muscle cells and causes vasodilation (Davignon, 2004). Specifically, NO production leads to the synthesis of cyclic GMP (cGMP), which leads to muscle relaxation. NO is secreted from endothelial cells when endothelial nitric oxide synthetase (eNOS) catalyzes the breakdown of L-arginine to L-citrulline and NO (Davignon, 2004). The nitric oxide synthetase family includes at least three members. eNOS and its complementary nNOS (which acts on neurons in signal transduction) are

both calcium-dependent synthetases that produce comparatively low amounts of NO. iNOS, or inducible nitric oxide synthetase, however, produces high levels of NO that may exceed 1000-fold those produced by eNOS or nNOS (Feng, 2001 and Beck, 1999). iNOS is thought to be produced primarily by macrophages (though other cell types may have the ability) as part of a cytotoxic response to kill bacteria, cancer cells, or parasites, but high NO concentrations also increase cell apoptosis and have been shown to increase mortality in mice (Feng, 2001). NO expression may thus be an important metric of cellular behavior under microenvironmental changes, and indeed forms a part of the response itself.

Detection of nitric oxide expression has been developed using the fluorochrome 4,5-diaminofluorescein diacetate (DAF-2), which reacts with NO to produce green-fluorescent triazolofluorescein. DAF-2 is membrane-permeable and can detect NO with a sensitivity of up to 5nM; traizolofluorescein is non-permeable and thus remains within the cell (Kojima, 1998). Using the NO-detection capabilities of DAF-2, an objective of this study is to determine what NO production differences, if any, are found between cells cultured under normal glucose, high glucose, and high glucose + aminoguanidine conditions. It is hypothesized that high glucose will result in increased NO production, and that aminoguanidine, a known eNOS inhibitor, will cancel this effect (Corbett, 1992).

Methods

Cell Culture. Human Aortic Endothelial Cells (HAECs) are cultured at 37°C and 5% CO_2 in Gibco® Medium 200 (Life Technologies M-200-500) with Low-Serum Growth Supplement (Life Technologies S-003-10), and with 1% L-glutamine and 1% penicillin/streptomycin. Media is changed every 48 hours. Baseline media glucose concentration is 4mM.

For flow experiments, cells are cultured on glass chamber slides (LabTex® II Chamber Slides #177372) that have been treated with gelatin or fibronectin at a density of 6.3 mg / cm^2 (Brower, 2009). For high glucose cultures, a 1M solution of D-glucose (Sigma #D8375) is added to change the culture concentration to 30.5mM. For high palmitic acid cultures, bovine serum albumin is added at a concentration of 4mM to increase affinity for the palmitic acid and allow endothelial cells to interact with the fatty acid. Palmitic acid is added immediately after the bovine serum albumin at a concentration of 200μM. All flow cultures are allowed 24 hours of incubation at experimental conditions prior to exposure to flow at 80-100% confluence.

For NO expression experiments, cells are cultured in a 12-well flat-bottom plate (BD Falcon #353043) for 24-hours at experimental conditions prior to running the assay at 70% confluence. High glucose cultures are at 30.5mM as described above; high glucose + aminoguanidine cultures included a 5mM solution of aminoguanidine bicarbonate (Sigma #A7259) in addition to the high glucose.

Flow Experiments. Cells were subjected to 8.0 dynes / cm^2 of shear stress for 12-hour periods using culture media treated at experimental concentrations, with the addition of HEPES Buffer Solution (Life Technologies #15630-106) to 10mM to maintain pH

balance. Shear is calculated as $\tau = 6\mu Q/h^2 b$, where τ is the shear stress at the chamber slide, μ is the fluid viscosity (8.50×10^{-4} Pa s), Q is the volumetric flow rate (5.833×10^{-8} m^3 s^{-1}), h is the flow chamber height (250×10^{-6} m), and b is the flow chamber width (6.0×10^{-3} m). A peristaltic pump is used in conjunction with an upstream and downstream reservoir that serve as flow dampeners that permit laminar flow. The chamber slide is mounted to an Immunetics® flow chamber, held in place by vacuum. Pump, reservoirs, flow chamber, and connecting tubing are kept in a 37°C incubator for the duration of the experiment.

Cells are imaged pre-and post-experiment at 10x magnification on a phase contrast light microscope. Each cell is analyzed as a separate data point using ImageJ to trace cell boundaries and calculate major and minor axes and angle relative to the horizontal, from 0 - 180°. For angles greater than 90°, the formula *new* = 180 – *old*, where "new" is a normalized angle of alignment and "old" is the original measurement was applied; this gives all values under 90° relative to flow and allows for comparison to a projected random average of 45°. Statistical significance is set at alpha = 0.05 and unpaired t-tests (one-tailed) are used to check for cell aspect ratio and cell angle relative to flow. A Bonferroni correction is used for increasing replicates. Briefly, a test for significance with significance at 0.05 predicts one of twenty replicates will be erroneously found to be insignificant. Thus, the p-value should be halved as a Bonferroni correction for each additional replicate, to minimize the possibility of erroneous tests for significance. When two replicates are conducted in this study, significance is reduced from alpha = 0.05 to alpha = 0.025. Note that the number of replicates is independent of sample size.

NO Expression Assays. After the 24-hour incubation period in experimental conditions, cells are imaged at 10x magnification under phase contrast and FITC fluorescence to establish baseline auto-fluorescence. DAF-2 is then added at a concentration of 5μM and cells are imaged in both phase contrast and FITC fluorescence. Because baseline NO expression is close to the noise floor of the assay, a stimulant is added to magnify differences between NO expression in test conditions. This stimulant is calcium ionophore A23187 (Sigma #21045 FLUKA). After 10min and 30 min of incubation with A23187, cells are imaged in both phase contrast and fluorescence. Using the phase contrast images to identify individual cells, the total integrated brightness of fluorescence of each cell is measured using ImageJ, and background auto-fluoresence is subtracted. A 2-tailed unpaired t-test is used to determine significance of the mean cell fluorescence at alpha = 0.05, with a Bonferroni correction employed as described above for increasing number of replicates.

Results

Endothelial Cells Under Physiologic Shear Stress. As previously found by Brower, et. al., HAECs cultured on gelatin under normal glucose (4mM) conditions responded to physiologic shear stress through cellular alignment and elongation parallel to the direction of flow ($p<<0.05$ for both alignment and elongation). Similarly, HAECs cultured under high glucose conditions (30.5mM) failed to align ($p=0.826$ for a test of average angle of orientation is not 45°) and showed reduced elongation in comparison with the normal glucose culture ($p<<0.05$).

HAECs cultured on gelatin under high concentrations of palmitic acid (200μM) did not show a response that was statistically different from that of the normal glucose culture; that is, these cells both aligned and elongated in the presence of shear ($p<<0.05$ for both alignment and elongation greater than that of the high glucose culture).

When the culture substrate was changed from gelatin on glass to fibronectin on glass, the elongation response of the HAECs was unchanged ($p<<0.05$), but cellular alignment with the direction of flow was reduced relative to the normal glucose on gelatin culture ($p<<0.05$), though alignment and elongation was improved relative to that of high glucose on gelatin conditions ($p<<0.05$).

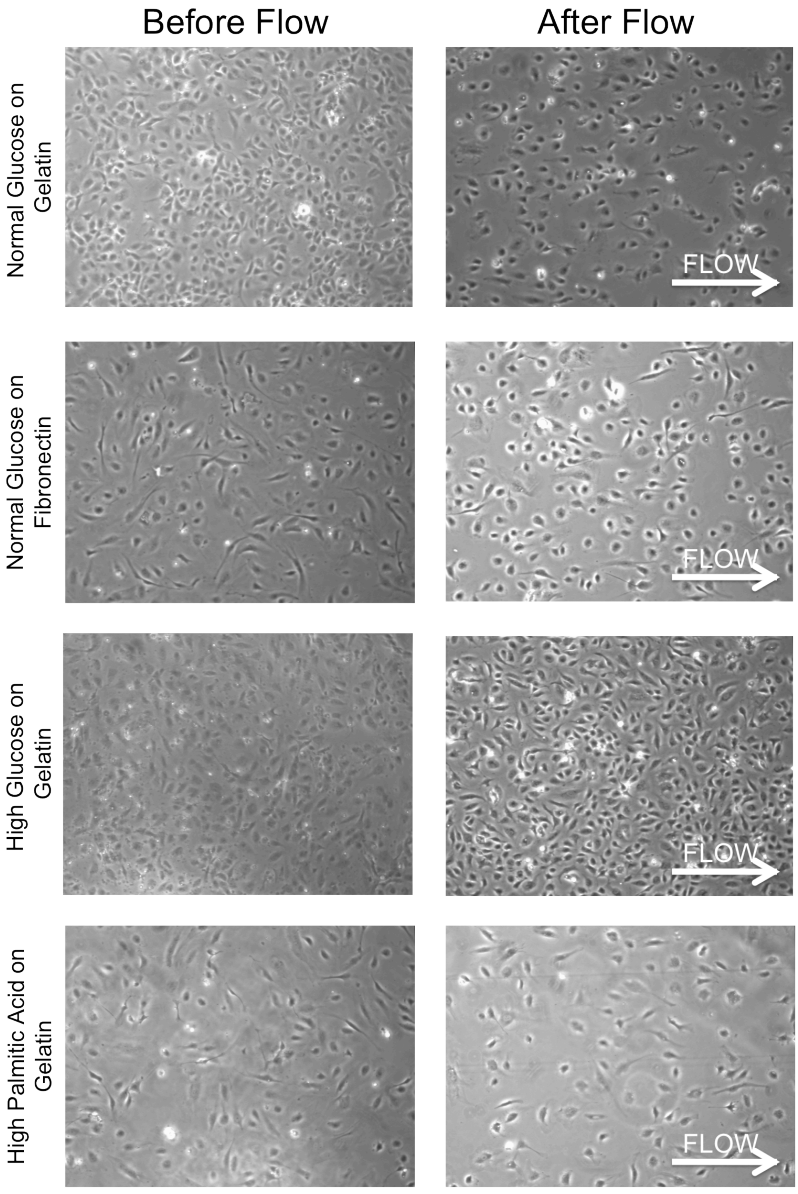
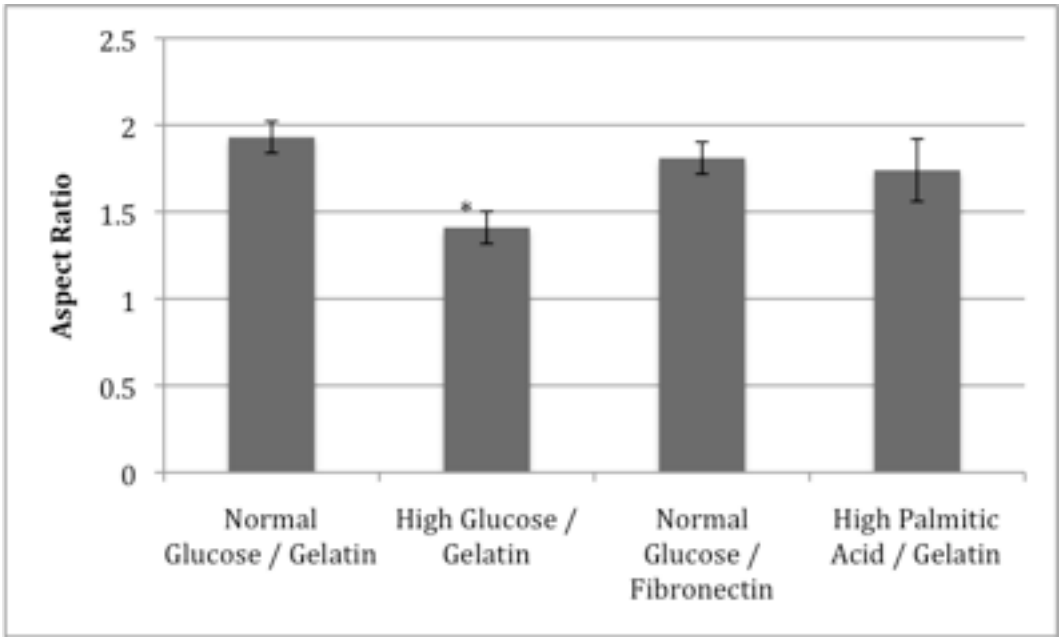


Figure 2.2: Human Aortic Endothelial Cell Alignment and Elongation under Physiologic Shear Stress from Flow. HAECs are cultured on gelatin or fibronectin-treated glass chamber slides in normal glucose (4mM), high glucose (30.5mM) or high palmitic acid (200μM) conditions. Cells are subject 8.0 dynes/cm^2 of shear stress from flow using media containing normal glucose, high glucose, or high palmitic acid concentrations. When cultured under normal glucose conditions, HAECs align and

elongate with the direction of flow ($p<<0.05$). High glucose concentrations destroy this effect ($p=0.826$). Changing the substrate of culture from gelatin to fibronectin preserves alignment and elongation ($p<<0.05$). Cells cultured under high palmitic acid conditions retain their ability to align and elongate with flow ($p<<0.05$). Direction of flow is indicated. Images are phase contrast at 10x magnification.



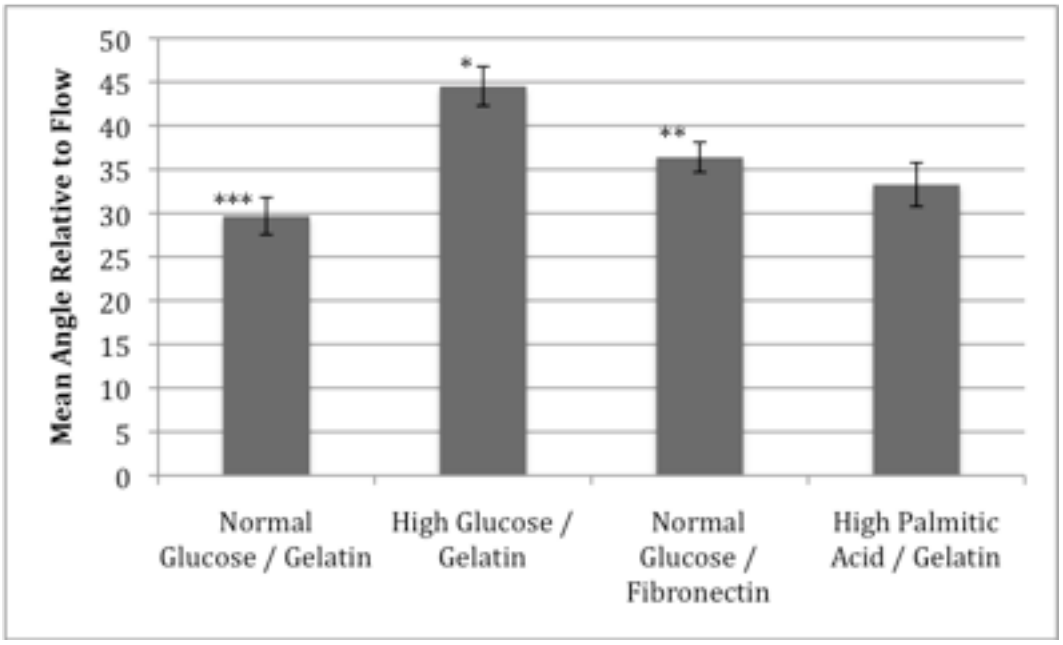


Figure 2.3: Human Aortic Endothelial Cell Aspect Ratio and Angle of Alignment under Physiologic Shear Stress from Flow. In **Figure 2.3A** (top), the aspect ratio of cells grown under a combination of glucose or palmitic acid concentration and substrate are shown: normal glucose / gelatin (n=165), high glucose / gelatin (n=138), normal glucose / fibronectin (n=134), and high palmitic acid / gelatin (n=98). Bars show mean aspect ratio, plus or minus the standard error of the mean. High glucose / gelatin treatment of HAECs shows reduced aspect ratio compared with the other three treatments. Alignment, but not elongation, is reduced when HAECs are cultured on fibronectin and exposed to flow. In **Figure 2.3B** (bottom), the mean angle of orientation of the HAECs, relative to flow, is shown, plus or minus the standard error of the mean for normal glucose / gelatin (n=165), high glucose / gelatin (n=138), normal glucose / fibronectin (n=134), and high palmitic acid / gelatin (n=98) conditions. Randomly-oriented cells are assumed to have an angle of 45° relative to the horizontal (the direction of flow); the high glucose / gelatin combination does not vary significantly from this random orientation. All other treatments have angles that are significantly different from 45°, indicating mean cellular alignment with flow.

Nitric Oxide Expression. Nitric oxide (NO) expression was measured for cells grown under normal glucose (4mM), high glucose (30.5 mM) and high glucose + aminoguanidine (30.5mM and 5.0mM, respectively) conditions for a 24-hour period, using the fluorochrome DAF-2 (4,5-diaminofluorescein diacetate) to selectively bind NO. Mean fluorescence per cell was tested for each treatment, with the normal and high glucose treatments repeated. High glucose culture conditions resulted in the highest level of NO expression by HAECs ($p<<0.025$), with high glucose + aminoguanidine as the

second-highest, statistically different level ($p<<0.05$), and finally normal glucose conditions leading to the least amount of NO expression ($p<<0.025$).

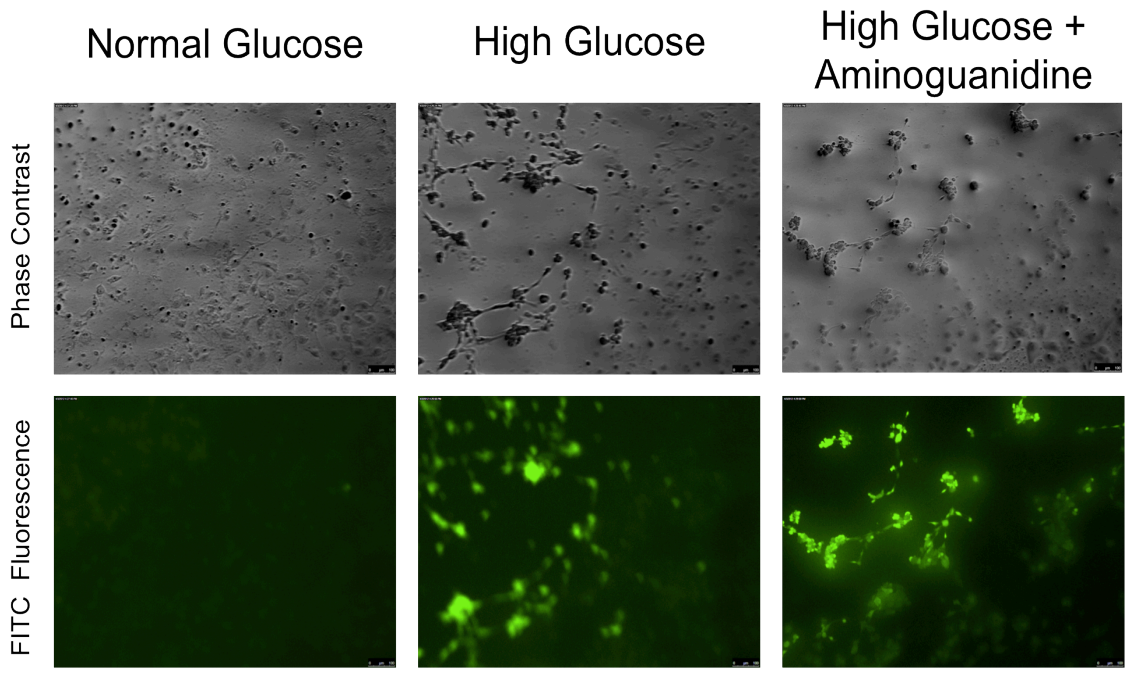


Figure 2.4: Human Aortic Endothelial Cell Nitric Oxide (NO) Expression using DAF-2 Fluorescence. HAECs are cultured under normal glucose (4mM), high glucose (30.5mM), or high glucose + aminoguanidine (30.5mM and 5mM, respectively) conditions for a 24-hour period. NO expression is measured through the fluorescence of DAF-2 (4,5-diaminofluorescein diacetate) which fluoresces selectively for NO. HAECs grown under normal glucose conditions show the lowest NO expression ($p<<0.025$). High glucose conditions led to the highest level of NO expression by HAECs ($p<<0.025$). High glucose + aminoguanidine conditions produced a moderate level of NO expression by HAECs, greater than that of the normal glucose conditions but less than that of the high glucose conditions alone ($p<<0.05$). Top images are captured at 10x magnification

using phase contrast microscopy; bottom images are using FITC settings at 10x magnification. Contrast of the images in this figure has been enhanced post-analysis.

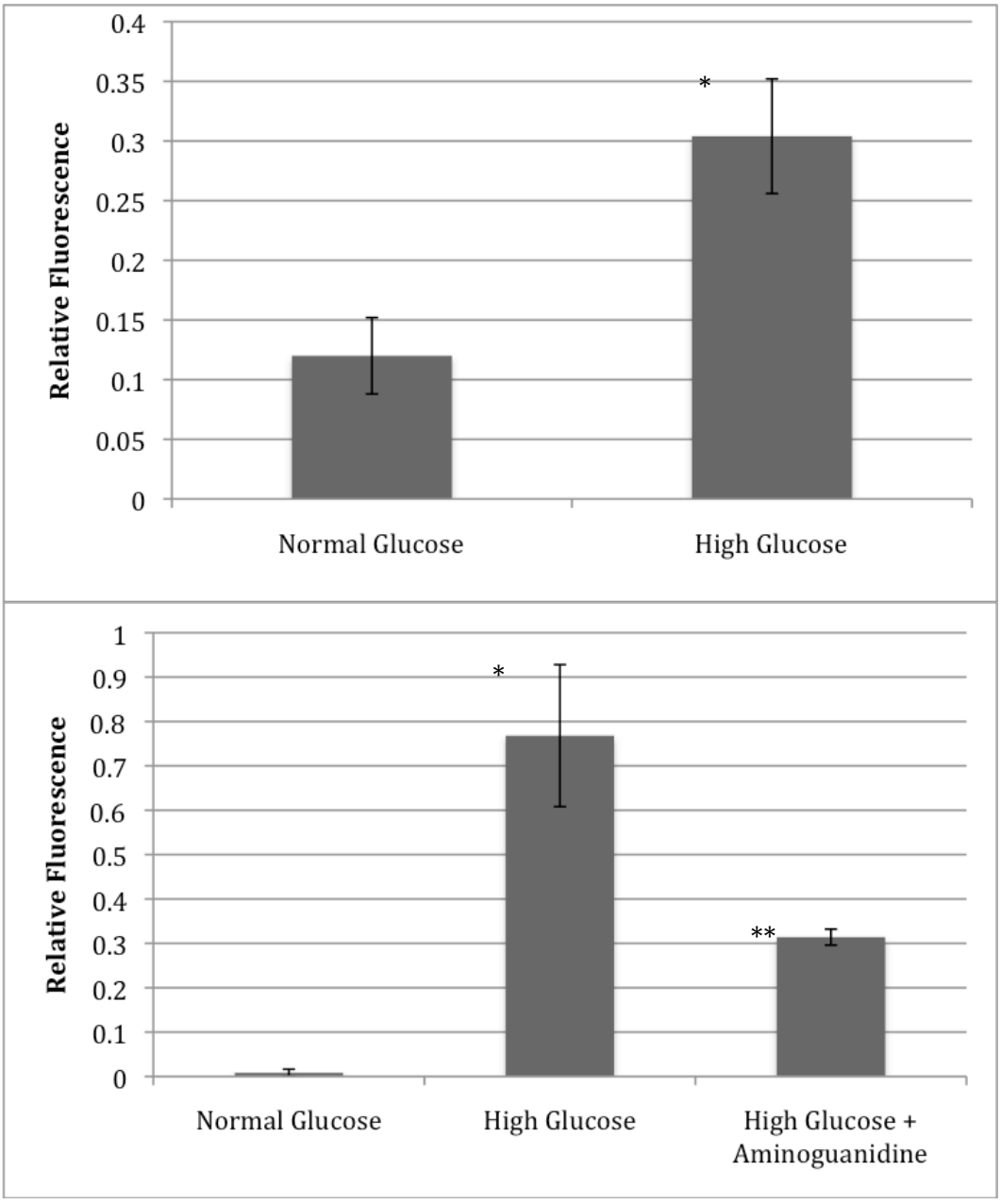


Figure 2.5: Human Aortic Endothelial Cell Relative NO Expression under High Glucose, Normal Glucose, and High Glucose + Aminoguanidine Conditions. In

Figure 2.5A (top), the relative fluorescence of HAECs cultured under normal glucose (4mM, n=22) and high glucose (30.5mM, n=86) conditions is shown as a mean value per cell, with the error bars showing the standard error of the mean. Fluorescence shown is relative to the background level of dish auto-fluorescence, and should not be considered a meaningful quantification, other than the relative significance of greater NO expression of HAECs under high glucose ($p<<0.025$). In **Figure 2.5B** (bottom), the relative mean fluorescence per cell, plus or minus the standard error of the mean, is shown for a second replicate of normal glucose (4mM, n=22) and high glucose (30.5mM, n=35), and a single replicate of high glucose + aminoguanidine (30.5 mM and 5mM, respectively, n=140) culture conditions. Normal glucose conditions show essentially zero relative fluorescence, indicating zero relative NO expression ($p<<0.025$). High glucose conditions led to the greatest relative expression of NO ($p<<0.025$), while aminoguanidine with high glucose reduced the comparative NO expression ($p<<0.05$) relative to the high glucose results, though it was still significantly greater than that of the normal glucose conditions alone ($p<<0.05$).

Discussion

The experimental approach for the flow system was validated in the replication of the results of Brower, et. al. from 2009, with normal glucose concentrations leading to HAEC alignment and elongation with flow, and high glucose concentrations destroying that effect. After multiple failed attempts at duplication of the flow setup created by Brower and Kelso, this was an important step forward in the study.

The use of fibronectin as the culture substrate for cells exposed to flow did not produce a significantly different effect on elongation, relative to cells cultured at normal glucose conditions on a gelatin substrate, but it did reduce the degree of cellular alignment with the direction of flow. Brower found that cells cultured on fibronectin show increased integrin expression, leading to tighter cellular binding to the substrate (Brower, 2009). This increased binding may explain the comparatively lower degree of cellular alignment in response to flow.

While the deposits of fibronectin in areas with vascular lesions or atherosclerotic plaques may be related to disease states, it does not appear that the fibronectin itself is responsible (or can be responsible) for the failure to align and elongate that may be associated with the propagation of these lesions. Brower explored the possibility that the modulation of cell shear response was due to the presence of advanced glycation end products (AGEs), which impaired the function of heparin sulfate proteoglycan, a transmembrane protein that may have been involved in the mechanotransduction of fluid shear stress (Brower, 2010). It would appear that fibronectin alone does not have this same effect on heparin sulfate proteoglycan, or any other transmembrane protein, cell surface receptor, or other signaling pathway. This is consistent with the proliferative behavior of cells bound to fibronectin under various circumstances (Schwarts, 2001). Fibronectin deposition may not be part of the problem, as it were, but part of the attempt at a solution by encouraging the growth of new endothelial cells to cover the lesion. This finding should be duplicated if possible, and cell shear response to high glucose while cultured on fibronectin may also be tested to determine if fibronectin has any benefits to cells already damaged by AGEs.

That the palmitic acid would also fail to modulate the HAEC shear response is logical in context of the Brower AGE finding. While high concentrations of saturated free fatty acids may lead to a variety of health concerns, they would not necessarily be able to act along the same pathways as glucose, in terms of the ability to glycosylate membrane-bound or membrane-associated proteins and produce the AGEs that appear to impair the shear response. Whether the palmitic acid may have a synergistic effect with glucose concentrations is unclear. Future studies should examine the possibility that the presence of palmitic acid may otherwise cause endothelial cells to be more susceptible to the effects of AGEs, such that a lower concentration of glucose may impair the shear response if palmitic acid is also present. This is especially relevant given the high incidence of diabetics with high triglycerides (Triglycerides, 2011).

NO production in endothelial cells cultured under high glucose is significantly increased relative to production under normal glucose conditions; production under high glucose + aminoguanidine is significantly greater than that of just normal glucose, but less than that of high glucose alone. The disparity in expression, especially between normal and high glucose conditions, is more indicative of the quantities of NO produced by eNOS and iNOS , respectively, since iNOS is known to produce 1000-fold more than eNOS[9]. The literature suggests that eNOS is the primary NO synthetase of endothelial cells, and that iNOS is primarily found with macrophages; however, several studies indicate that endothelial cells also produce inducible NO (Fukumura, 2006 and Jens Kroll, 1998). It would then appear that the normal glucose conditions led the HAECs to produce only what is typical of eNOS, while the high glucose conditions resulted in the activation of iNOS and the production of significantly greater amounts of nitric oxide.

Some possible explanations for this phenomenon include increased vascular stiffness with high glucose, resulting in increased NO production to relax smooth muscle tissue, or the more probable NO production as part of an inflammatory response.

The reduction of cellular NO production in the presence of aminoguanidine, but lack of restoration to the levels seen in normal glucose controls, is unexpected, and the cause of this phenomenon is unclear. One possible explanation is that there may have been insufficient aminoguanidine concentrations to completely reduce NO expression back to the levels seen in normal glucose cultures. Aminoguanidine was added at the same concentration used to rescue the effects of advanced glycation end products, but these may not necessarily be the cause of the induced NO production. It is known, however, that aminoguanidine is an inhibitor of both eNOS and iNOS (Fukumura, 2006 and Jens Kroll, 1998). With sufficiently high concentrations, it is possible that aminoguanidine would shut down all NO production. These tests should be reproduced with increased concentrations of aminoguanidine to see if this is the case. Additionally, the question of whether it is the AGEs, or another effect of the high glucose concentrations causing the increased NO production, may be addressed by culturing cells with glycosylated bovine serum albumin, a known AGE, and then testing NO production.

CHAPTER 3

ENGINEERING THE EX VIVO MICROENVIRONMENT

Introduction

In the previous chapter, the behavior of endothelial cells in the *in vitro* environment was explored, but the value of studying endothelial cell behavior in an *ex vivo* environment is clear when the costs –both time and money—of bringing treatments to market is considered. Studying cells in tissue may reveal interactions and behaviors that are not elicited in the *in vitro* environment.

The Ex Vivo *Endothelial Microenvironment*. Endothelial cells in tissue exhibit adverse responses when subjected to diabetic conditions: Previous studies have shown substantial changes in the morphology of vascular endothelial cells in diabetic models of both rabbits and mice (Haddock, 1991 and Yanga, 2001). These changes include hyperplasia and cell blebbing, along with a general thickening of the tunica intima (Haddock, 1991 and Yanga, 2001). Based on the endothelial alignment and elongation behaviors found by Brower (2009), it is hypothesized in this study that the endothelial cells of a diabetic model rat will show a failure to respond to *in vivo* shear stress. A subsequent aim of this thesis is then to explore *in vivo* endothelial alignment and elongation for diabetic model rats, through the use of *ex vivo* imaging of vascular endothelial tissue from diabetic models.

Experimental Constraints in the use of Human Tissue to Study Endothelial Cell Behavior. Physicians and researchers at the Phoenix Veteran's Affairs hospital have developed techniques to recover human microvessels from fat biopsies, and to mount and pressurize these vessels for use in testing whole-vessel response to stimuli such as nitric

oxide. The experimental apparatus used to maintain these vessels during the course of hours-long experiments requires extensive volumes, relative to the size of the microvessels themselves: 100 mL each of perfusate and superfusate are required for vessels that are typically a centimeter or less in length, and no more than 150 microns in diameter. Reagents used in these experiments are frequently individually costly, and are often intended for use with volumes that are smaller by an order of magnitude or more. To facilitate more cost-effective research, one aim of this thesis is to engineer an appropriate re-fit of the microvessel apparatus to accommodate a reduction of volume while maintaining or improving performance.

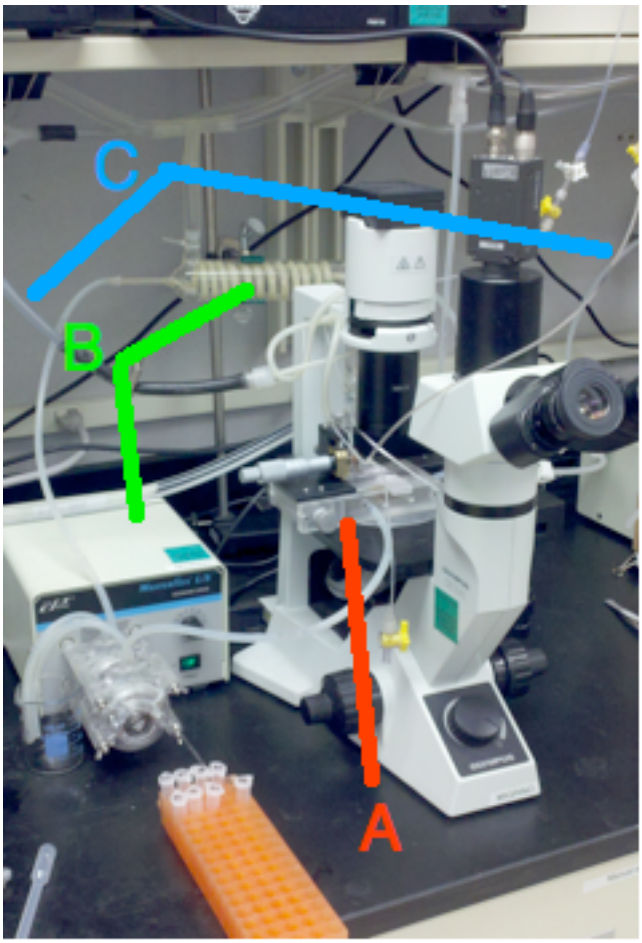


Figure 3.1: Microvessel Imaging System at the Phoenix Veteran's Affairs Hospital. The chamber marked at **A** contains the glass cannulae mounts for the microvessel , as well as a chamber in which the microvessel sits, and tubing connections to either end of the cannulae (where the perfusate enters), and to the chamber (where the superfusate enters). In **B**, the pump and heat exchange apparatus that supply warmth to the superfusate require 100mL of volume in the tubing and pump reservoirs. The tubing marked at **C** contains the perfusate, and is connected on either end to 100-mL syringes (not shown), partially filled and held at an appropriate height to maintain a 60 mmHg pressure within the microvessel.

Methods

Vessel Explant and Imaging. High-fat diet (HFD) model diabetic rat aortas are explanted post-mortem and fixed with 2% para-formaldehyde in phosphate-buffered saline, then permeabilized with fixing solution plus 1% Triton-X100 detergent. ZO-1 polyclonal antibody (Invitrogen #617300, rabbit) is used to detect the outer membrane of individual cells via the ZO-1 antigen. Aortas are also stained with ToPro3 for DNA detection. Images are acquired using FITC confocal microscopy.

Microvessel Chamber Engineering Re-Fit. Following discussions with the researchers at the Phoenix VA Hospital, regarding the requirements for their experiments, and the ideal function of an improved microvessel chamber, design specifications were established. Idea generation and selection then occurred, with special effort paid to incurring minimal cost while maintaining or improving functionality. Following the review and selection of a final design, components were acquired and the re-fit

assembled, followed with delivery to the Phoenix VA researchers for their validation and use.

Results

Rat Model Aortic Endothelial Cell Characterization. Following incubation with the ZO-1 polyclonal antibody and ToPro3, images were acquired of the inner luminal space of the rat aorta, including the endothelial lining of the vessel. Analysis of cell alignment and elongation was not conducted as the bounds of individual cells are not clearly defined.

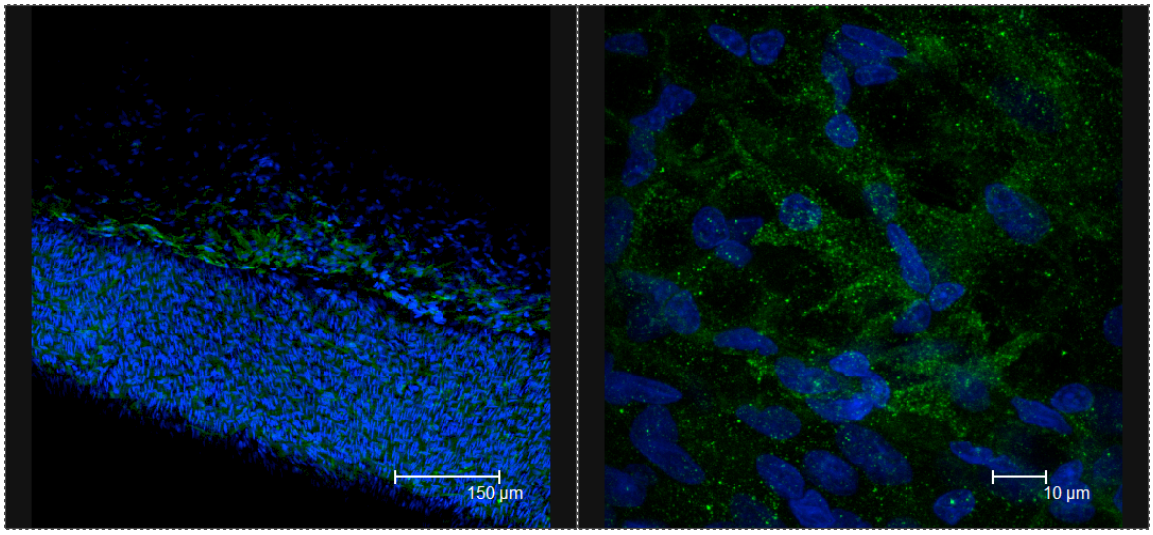


Figure 3.2: Characterization of Rat Aortic Endothelial Cells. Green 488-nm excitation shows the ZO-1 antibody binding sites indicating the boundaries of cells. Blue ToPro3 excitation identifies the nuclei of individual cells. In **3.2A** (left), the 10x image reveals thousands of cells arranged in the endothelial layer. In **3.2B** (right), approximately two dozen endothelial cells are visualized at the vessel luminal surface.

Microvessel Chamber Re-Fit. The volume of the superfusate system is reduced to 3 mL, and the volume of the perfusate system is reduced to 500uL. The two 100-ml syringes previously required to maintain 60 mmHg pressure are replaced with a 1-mL syringe on the leading end of the vessel tubing, and a 5 psi (272 mmHg) pressure gauge. Depressing the syringe manually or with a syringe pump can achieve the desired 60 mmHg (1.1psi) pressure with as few as 500uL in the syringe. The microvessel chamber for superfusate is sealed off, and the heat exchanger for the superfusate is re-purposed to heat the chamber itself, taking advantage of internal channels that were previously unused. Adequate superfusate aeration is ensured through air bubbling via a 20-gauge needle connected to an in-house air tank; hemoglobin may be added as necessary to increase oxygen tension.

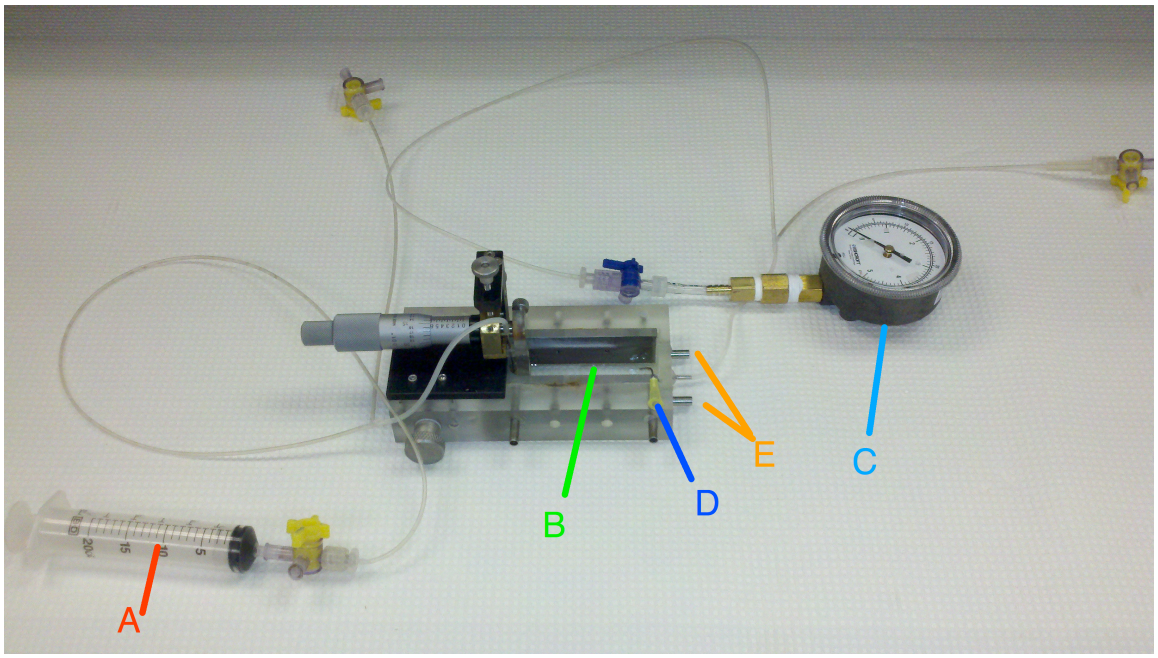


Figure 3.3: Microvessel Chamber Re-Fit for Phoenix VA Hospital. A single syringe **(A)** replaces one of two 100-ml reservoirs to provide pressure to the interior of the

microvessel. The chamber where the microvessel is mounted, and the superfusate surrounds the vessel, is shown in **B**. In **C,** a fluid pressure gauge replaces the other 100-mL reservoir. The gauge is rated to 5 psi, or ~272 mmHg. Brass fittings provide compatibility with the small tubing diameter of perfusate system. A needle hookup at **D** provides aeration of the superfusate, and the internal channels indicated at **E** are connected to the previous heat exchange system, warming the superfusate without passing the superfusate itself through the 100 mL of tubing.

Discussion

Rat Aorta Endothelial Cell Characterization. The explanted aortas from the diabetic rat model provide an opportunity to begin to characterize endothelial cell behavior at a broader level, in the context of the surrounding cell types and tissues. The extraction from a recently deceased animal allows close study of physiologic phenomena, and may permit future studies to explore the cellular-level findings discussed in the previous chapter of this thesis.

The results of the imaging of the aorta thus far provide a framework for the development of techniques to successfully image the interior of the aorta. Phalloidin, which will detect actin within a cell, may be able to provide a clearer representation of the boundaries of cells. This in turn would allow the same kinds of alignment and elongation analyses that are performed *in vitro* in this thesis, to also be performed on the *ex vivo* aortas.

The images acquired to date do not permit this analysis because individual cell boundaries are not clearly defined, though the nuclei of individual cells are. To the

author's best knowledge, no one in the literature has applied the ZO-1 antibody to a vessel, rather than cells, and this technique may require additional time to permeabilize and incubate. An alternative to antibody incubation would be the use of transgenic mice with an endothelium that expresses, as an example, green fluorescent protein (GFP).

Microvessel Chamber Re-Fit. The newly refitted microvessel chamber meets the design specifications for the VA researchers and permits substantially reduced quantities of reagents to be used to conduct human tissue explant experiments. Total perfusate volume is reduced from 100mL to 500uL (a factor of 200) and total superfusate volume is reduced from 100 ml to 3mL (a factor of 33). Aeration and heat are kept constant. The use of a syringe in combination with a syringe pump opens up the possibility of conducting flow experiments, by using a valve at the pressure gauge to allow fluid to drip out of the system. Commercially-available microvessel chambers with similar specifications cost upwards of $1200; total cost to re-fit this chamber was $87.

CHAPTER 4

CONCLUSIONS

Modulations of the endothelial microenvironment can result in affected cellular response to stimuli. This study replicates the findings of Brower, et. al. showing that hyperglycemia impairs the endothelial cell shear response. Change of culture substrate from gelatin to fibronectin does not affect HAEC elongation response, though it does reduce alignment relative to culture under normal glucose on gelatin. Culture with high concentrations of palmitic acid, a saturated free fatty acid, does not affect the shear stress response. Nitric oxide production is increased in HAECs cultured under high glucose conditions, which may indicate the presence of inducible nitric oxide as part of an inflammatory response. NO production is reduced, but not restored to levels found in normal glucose controls, when HAECs are cultured under high glucose with aminoguanidine. Thus, the modulations in microenvironment did induce differences in cellular response, especially with respect to hyperglycemia and cellular NO production.

Explants of vascular tissue, both animal and human, provide the opportunity to study cellular behavior at the tissue level. A microvessel chamber is successfully re-engineered to meet the requirements of reduced volumes while maintaining an equal level of functionality and a low cost. An imaging framework for rat aortas is established that leads to the ability to test *ex vivo* endothelial response to diabetic and high fatty acid conditions.

CHAPTER 5

FUTURE WORK

Palmitic Acid, High Glucose, and the Buildup of Atherosclerotic Plaques. An important long-term aim of this study is to explore the potential link between endothelial cell response and the build-up of atherosclerotic plaques. It is hypothesized that the failure to align and elongate with the direction of flow leads to the buildup of plaques by providing more cross-sectional surface area for the adsorption of LDL cholesterol, and by preventing the formation of tight junctions that might otherwise prevent LDL cholesterol from reaching the basal side of the endothelium.

In the studies conducted by Brower and Kelso, the impairment of the alignment and elongation response of *in vitro* HAECs was most pronounced at levels of high glucose concentrations (30.5mM). These concentrations are generally higher than the day-to-day glucose levels of a majority of diabetics, including those who are being treated. Yet, atherosclerotic buildups are still seen. One hypothesis to further explore is that free fatty acids may have a "synergistic" effect with high glucose concentrations in impairing the alignment and elongation response. Free fatty acids may absorb into the lipid membranes of endothelial cells, providing points of inflexibility and overall contributing to increased cellular stiffness. Cells, once stiffened, may then be more susceptible to a failure to align and elongate once exposed to levels of glucose that are heightened relative to normal, but are not at such an extreme as 30.5 mM glucose.

Aortic Endothelial Alignment and Elongation Ex Vivo. Both the rat aortic diabetic model and the human microvessel models provide an unique opportunity to determine if the *in vitro* alignment and elongation behaviors are also true in animals and people who

are diabetic, or who eat high-fat diets, or both. An improved method for imaging individual cells – perhaps with phalloidin-based detection of actin – is needed, but the acquisition and subsequent imaging of tissue from diabetic rats and humans could support or disprove the roles of endothelial cell alignment and elongation in the buildup of plaques.

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APPENDIX A

PROTOCOL FOR PREPARING FLOW CHAMBERSLIDES

Materials & Equipment

- Gelatin stock (7.94 μg/μL). Bovine.
- Sodium carbonate/ sodium bicarbonate / sodium azide buffering solution. Prepare in stock solutions of 50-100mL.
    - 0.35M $NaHCO_3$
    - 0.15M Na_2CO_3
    - 0.03M NaN_3
- Glass chamber slides (LabTex® II Chamber Slides #177372)
- 37°C bath
- Biosafety cabinet

Gelatin-Coating the Chamber Slides

1. Warm gelatin aliquot in 37°C bath until homogenous and transparent. Vortex as needed.
2. Warm sodium carbonate/bicarbonate/azide buffering solution to 37°C.
3. In a sterilized biosafety cabinet, pipette gelatin and sodium carbonate/bicarbonate/azide solution into 15-mL tubes according to the following proportions:

Number of Chamber Slides to Treat	Quantity of Gelatin (**μL**)	Quantity of buffering solution (**mL**)	Total Volume (**mL**)
1	7.141	2	2.007141
2	14.282	4	4.014282
3	21.423	6	6.021423

4	28.564	8	8.028564
5	35.705	10	10.035705
6	42.846	12	12.042846
7	49.987	14	14.049987
8	57.128	16	16.057128
9	64.269	18	18.064269
10	71.41	20	20.07141

*These proportions result in a surface gelatin concentration of each chamber slide of 6 µg per square centimeter.

4. Working quickly, vortex or invert capped centrifuge tube to homogenously mix contents.
5. Pipette 2mL of combined and mixed solution into each chamber slide.
6. Seal chamber slides with Parrafin film.
7. Rock 4 hours to overnight.
8. If not to be used within 24 hours, refrigerate at 4°C. Do not keep for more than 7 days, even if refrigerated.

Plating Cells onto Chamber Slides

1. When you are ready to plate cells directly into the chamber slides (e.g. you have already counted and re-suspended your cells), remove any Parrafin film and open sterilized chamber slides in a sterilized biosafety cabinet.
2. Using suction (such as a Pasteur pipette with vacuum), remove the buffering solution from the chamberslide via the side wall of the

chamber slide. Do not directly suction the bottom of the chamber slide, as this will remove the gelatin coating.

3. Rinse the chamber slide once with pre-warmed, 37°C, sterile phosphate-buffered saline (PBS).
4. Plate cells at a concentration of 150,000 – 200,000 cells **per chamber slide**. Cells plated at this density will typically reach confluence within three to four days. If possible, add cells drop-wise and evenly across the surface of the chamber slide.
5. Gently rock the chamber slide back-and-forth and side-to-side to aid even cell spacing.
6. Replace chamber slide cover. Place chamber slide in one-half of a Petri dish if desired for ease of transport. Incubate under desired conditions.

APPENDIX B

PROTOCOL FOR FLOW STUDIES

Materials & Equipment

- 25mL of HAEC flow media
    - Normal HAEC media with 10mM HEPES Buffer
- 1M stock of HEPES Buffer
- 50mL of 1M Phosphate-buffered saline (PBS)
- Flow system
    - Peristaltic pump
    - Flow cell capable of fitting a standard microscope slide
    - Flow dampener
    - Vacuum (house vacuum acceptable)
    - Tubing, 1/16" inner diameter. Silastic™ acceptable.
    - Incubator or 37°C room
- Modifying reagents, as desired (e.g. glucose, palmitate, etc.)
- Paper towels; 10% bleach.

Preparing the Flow System

1. Pre-warm the flow system, PBS, and HAEC flow media to 37°C.
2. Connect flow system with blank microscope slide, begin flow, and flush system with 25 mL of PBS. Flow may be adjusted for rapid exchange of PBS. Ensure that the flow system does not run dry by continuously adding PBS at the input point.
3. Exchange flow media for PBS. Drip PBS into waste container; add media in segments of 3-5 mL. Once drip waste becomes the color of media, flush additional 3-5mL out of the system.

4. Check the flow system for the presence of bubbles. Gently tap them along the direction of flow to the outlet point.
5. Cover inlet with aluminum foil as necessary.

Preparing the Cells for Flow

1. Ensure that cells have reached minimum 80% confluence before staring a flow experiment. Desired modifying reagents (glucose, etc.) should be added a sufficient incubation period in advance of conducting a flow experiment.
2. Collect images of the cells on the slide at 10x magnification, ensuring that the major axis of the slide is parallel to the bottom horizontal of the image.
3. Carefully remove the chamber from the chamber slide, using the push-tong device provided by LabTek (see Appendix A). Do not allow slide to become dry; add pre-warmed media dropwise as necessary.

Starting the Flow Experiment

1. Stop flow.
2. Raise flow cell to a level slightly below the inlet.
3. Break the vacuum.
4. Allow media from the inlet reservoir to flood into the space on top of the flow cell. Ensure that this area is completely wet.
5. Invert microscope slide such that the cell surface faces the media pool on top of the flow cell.

6. Firmly press the microscope slide against the wet surface of the flow cell, taking care to not introduce any air bubbles to the system.
7. Connect the vacuum. Ensure a clean seal.
8. Begin flow at the appropriate volumetric flow rate (e.g. 3.5 ml/min).
9. Allow flow experiment to run min. 12 hours, max. 16 hours.

Ending Flow Experiment

1. Have a petri dish with pre-warmed media available.
2. Stop flow (record the time).
3. Break the vacuum.
4. Quickly remove the microscope slide and place in the petri dish with pre-warmed media.
5. Quickly ensure that media from the flow system is directed towards the waste container as it drains.
6. Quickly begin collecting images of the microscope slide. Make note of the direction of flow, and keep the major axis of the slide parallel with the bottom of the image.
7. After images have been collected, dispose of the microscope slide in appropriate waste container.
8. Flush flow system with PBS; store in PBS.
9. Sterilize surfaces and properly dispose of any cell-contacted media.

APPENDIX C

PROTOCOL FOR IMAGING EXPLANTED RAT AORTAS

Materials and Equipment

- Fixation Media
    - 2% para-formaldehyde in PBS
- Permeabilization Media
    - 2% para-formaldehyde in PBS
    - 1% Tween or Triton-X
- Antibody Wash
    - 45 mL DI/purified water
    - 4.5 mL 10x PBS
    - 0.45g Bovine Serum Albumin (BSA)
- Phosphate-buffered saline
- Forceps / tweezers
- 6-well plates; centrifuge tubes

Procedure

1. Immediately rinse explanted vessel in ice-cold PBS. 2-3 washes, or until PBS is clear post-wash.
2. Fix explanted vessel in fixation media for 30 min.
3. Transfer explanted vessel to permeabilization media for 30 min.
4. Wash 3x in antibody wash; 6-well plate or centrifuge tubes may be used.
5. Dilute primary antibody in antibody wash (typically 1:250, but follow manufacturer's specifications).

6. Incubate explanted vessel in primary antibody / antibody wash 8 hours to overnight at 4°C.
7. Wash 3x with antibody wash.
8. Dilute secondary antibody in antibody wash (typically 1:500). Protect from light.
9. Incubate vessel in secondary antibody/antibody wash minimum 1 hour at room temperature, to overnight at 4°C. Protect from light
10. Wash 3x PBS.
11. Add DAPI or ToPro3 as desired for DNA labeling; incubate 15 min at room temperature.
12. Wash 1x PBS.
13. Mount vessel on microscope slide with vectashield.
14. Image using appropriate laser excitation.

BIOGRAPHICAL SKETCH

Eric is a young engineer and entrepreneur with a passion for good ideas that can make a difference in our communities' biggest and oldest problems. A dual bachelor's and master's student at Arizona State University, Eric studies biomedical engineering and the interactions of endothelial cells with their microenvironments. He has collaborated with the Phoenix VA, and conducted research at MIT in the summer of 2011. Outside the lab, he leads the FlashFood team as they innovate to reduce food waste and hunger in new ways in their local Phoenix, Arizona community. His team has won the U.S. Microsoft Imagine Cup and been accepted to the ASU-Edson Entrepreneurship Accelerator. Eric hails from Tucson, Arizona, where he grew up loving hiking, camping, and Mexican food. An avid traveler, he has, in the past year, been to Prague, NYC, Sydney (Australia), Ljulbljana (Slovenia), Seattle, and Anchorage. He studied in Granada, Spain and speaks fluent Spanish. Eric enjoys playing guitar (he is self-taught), strategy boardgames, and his wife's cooking. He is a microbrew (beer) aficionado, and looks forward to what the future holds for him.