A NOVEL TECHNIQUE FOR THE CHARACTERIZATION OF ENDOTHELIAL CELL MECHANICS

A Senior Honors Thesis

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ABSTRACT

A Novel Technique for the Characterization of Endothelial Cell Mechanics

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Circumferential distension of the arterial wall during the cardiac cycle regulates endothelial cell (EC) morphology and function. We have previously shown that cyclic stretching of ECs leads to activation of signaling events relevant to atherosclerosis, but that this signaling subsides when the cells align perpendicular to stretch. We hypothesized that the purpose of this cellular response is to maintain a homeostatic stress level within the cell. Stresses within the cell are both born and created by cytoskeletal components. Stress fibers create tension that is transmitted to substratum via integrin connections. To test if this alignment response affects the forces applied to the substratum by stretching the ECs, we are developing a system to quantify substrate

deformation caused by stretching ECs. The technique uses a poly(ethylene glycol) diacrylate hydrogel having an elastic modulus appropriate for traction microscopy and embedded with fluorescent beads. The traction microscopy system was comprised of computer controlled actuators and a clamping mechanism used to stretch the fluorescent bead embedded hydrogels under a confocal microscope objective. Image analysis via cross-correlation of bead displacements was used to generate the displacement field of the hydrogel immediately below and surrounding the cell. The preliminary results obtained indicate a successful proof of concept and show the method to be sound in principle. The system provides for the development of unique experimental conditions including the ability to perform uniaxial and biaxial stretching. In conclusion, the development of a novel method for the characterization of endothelial cell mechanics appears to be possible. However, the technique must be further developed and refined in order to increase efficacy and repeatability.

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I INTRODUCTION[1]

Endothelial cells (ECs), long thought to be mere barriers lining the lumen of blood vessels throughout the body to control the diffusion of nutrients, have now been shown to be a very dynamic system and a major participant in the formation of early stage atherosclerotic plaques. Atherosclerosis is a slow progressive disease characterized by the accumulation of lipids within the arterial wall and an uncontrolled inflammatory immune response. Atherosclerosis can lead to a variety of critical conditions, which constitute a large portion of the deaths in the United States, including angina, myocardial infarction, and stroke. It has been found that mechanical stimulation of ECs, via hemodynamic shear stress and cyclic stretching, may be a major contributor to the underlying cause of the topographical distribution of atherosclerosis in distinct regions within the body's arteries. A better fundamental understanding of the mechanical nature of these ECs is needed in order to gain a complete understanding of atherosclerotic plaque formation. The purpose of this study is to develop a method to quantify the traction forces applied by ECs to their substrate under a variety of loading conditions.

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[1] This thesis follows the style and format of *The Journal of Cell Biology.*

Atherosclerosis and Endothelial Cells

Cardiovascular disease accounts for the majority of the deaths in United States, Europe and parts of Asia (Braunwald, 1997). The long held paradigm of atherosclerosis disease formation has been that of a slow accumulation of lipids within the arterial wall leading to eventual blockage of the vessel lumen and the subsequent cardiovascular conditions including angina, myocardial infarct, and stroke (Ross, 1999). Since the early 1990s, however, the paradigm has shifted to that of a set of highly specific cellular and molecular responses which more closely resemble an autoimmune or inflammatory response gone awry (Ross, 1999, Libby, 2002, Schoenfeld, 2001).

It has become increasingly evident that injury to the vascular endothelium, a monolayer of cells lining the lumen of blood vessels, plays a critical role in the development of early stage atherosclerotic plaques. As shown in Figure 1, endothelial cells (ECs) are directly involved in the early stages of atherosclerosis through a variety of processes including increased endothelial permeability to low-density lipoprotein (LDL), and increased recruitment of monocytes (Ross, 1999). The localization of

atherosclerotic plaques near arterial branches has long been attributed to both the unique hemodynamic oscillatory shear stresses and the high-magnitude cyclic stretching which spatially correlate with areas having greater accumulation of LDL within the arterial intima (Kakis 2004, McMillan, 1985, Moore, 1994). As seen in Figure 2, the morphology of ECs observed at branched regions differs greatly from ECs found in long straight regions (Cornhill, 1974, Davies, 1995, Nerem, 1981, Silkworth, 1975). The difference in both the mechanical stretch and fluid shear stresses at arterial branches as compared to the straight regions affect many features of ECs, such that their morphology, growth, permeability, and gene expression, that may contribute to the preponderance of atherosclerotic lesion formation (Moore, 1994, Thubriker, 1995, Zhao 1995). However, the mechanisms by which the particular mechanical environment affects EC function remain to be determined.

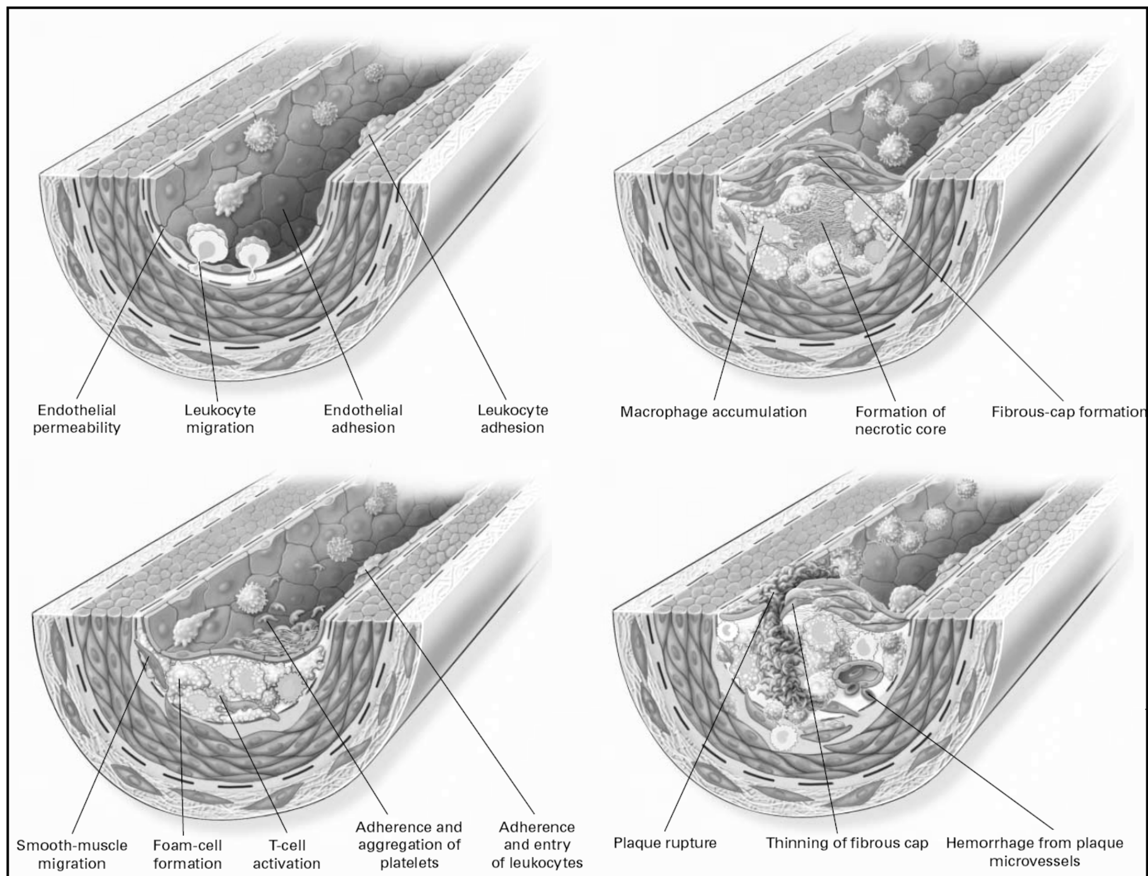

Figure 1: Stages of atherosclerotic plaque formation (Adapted from Ross, 1999. See Appendix A). Endothelial cells, being the only barrier between the blood and the underlying vessel wall, play a crucial role in early stage atherosclerotic plaque formation which includes LDL accumulation and monocyte adhesion and migration into the vessel wall.

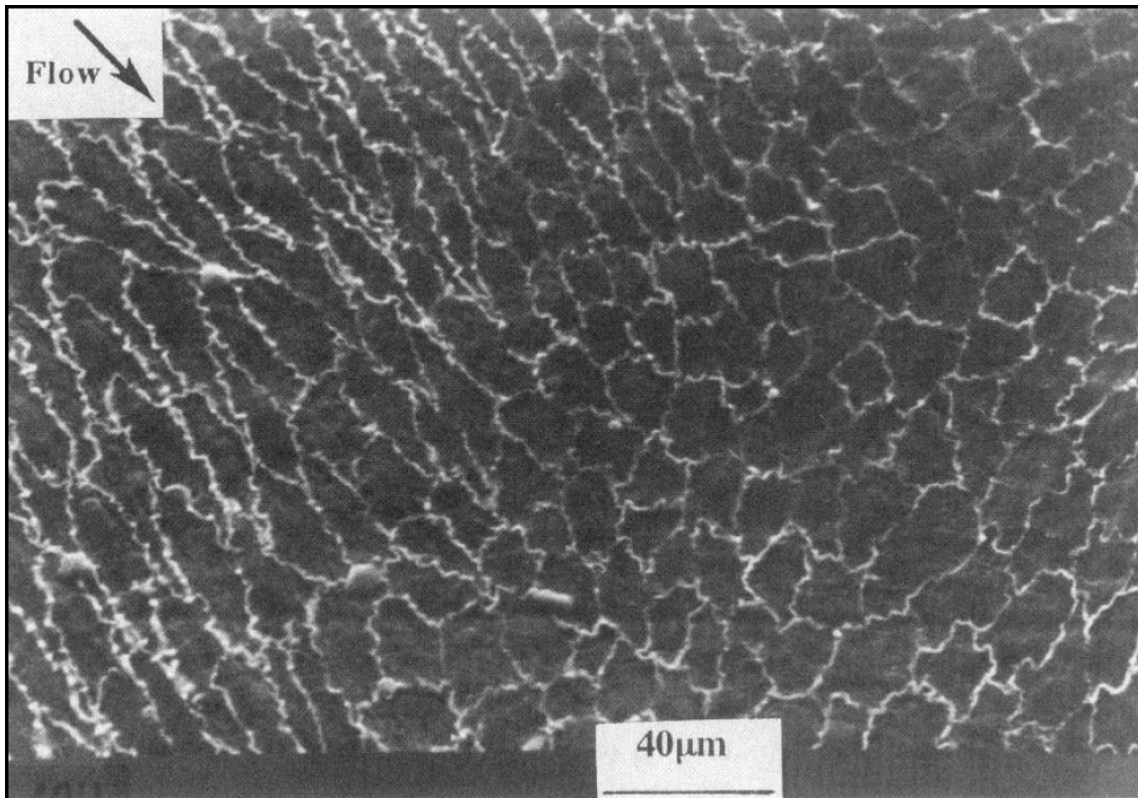

Figure 2: Endothelial cell morphology abruptly changes near arterial branches (Adapted from Cornhill et al., 1974. See Appendix B). ECs display a distinctly rounded morphology in branched regions as compared to a more polarized elongated morphology in straight regions.

Given the differences in the mechanical environment associated with straight versus branched regions of arteries, attempts have been made to determine the mechanism by which ECs adapt to these conditions. Studies have shown vascular ECs orient themselves and form stress fibers perpendicular to the direction of stretch (Kaunas, 2005). A recent *in vitro* study has shown that attached ECs respond to stress, induced by uniaxial substrate stretch, by aligning perpendicular to the direction of stretch

in a manner that appears to reduce the induced increase in intracellular stress. However, ECs exposed to biaxial stretch, an approximation of the mechanical environment at arterial branches, cannot align as the stress is non-directional and thus display a non-polarized morphology similar to static conditions as seen in Figure 3 (Kaunas, 2006). Elevated intracellular stress appears to activate c-jun N-terminal kinase (JNK), which regulates the AP-1 transcription factor, which in turn mediates several genes involved in the early stages of atherosclerosis including endothelin-1, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) (Cheng, 1996, Clinton, 1992, Davies, 1993, Lerman, 1993, Wang, 1993, Wang, 1995).

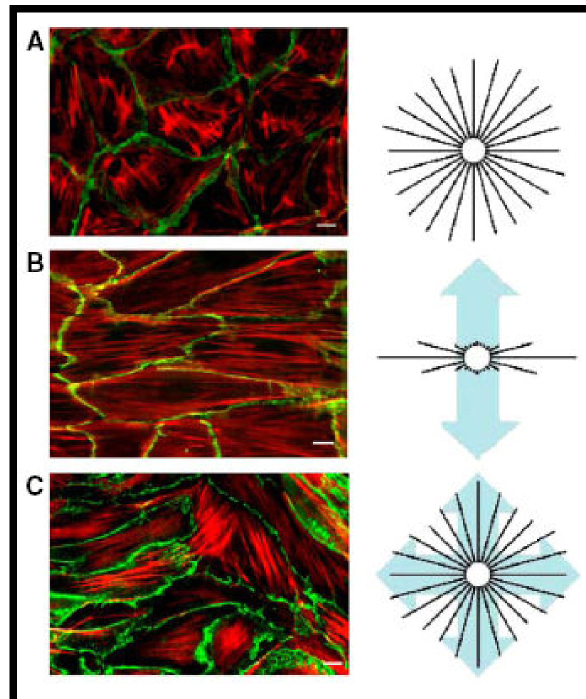

Figure 3: Endothelial cell morphology in response to static conditions (A), uniaxial stretch (B), and biaxial stretch (C) (Adapted from Kaunas et al., 2006). Straight arterial regions are modeled via uniaxial stretch while branched regions are modeled via biaxial stretch.

Cell Mechanics

Cell mechanics is a broad field in which the principles of physics and engineering are applied to study both the mechanical properties of cells and the effects of mechanical forces on a variety of cellular processes. Cellular mechanics is being recognized as equally important as cellular biochemistry in determining a cell's reaction

to stimuli and propensity to pathological development. For example, the notion that the endothelial cells lining blood vessels were just a passive anti-thrombogenic barrier was replaced with a mechanotransduction model. In this model, the endothelial cells respond to increased shear stress and decreased shear stress, by releasing nitric oxide (a vasodilator) or endothelin-1 (a vasoconstrictor), respectively, in order to normalize flow velocity, and hence stabilize shear stress on the arterial wall (Buga, 1991, Yoshizumi, 1989).

The complicated structure of the cell and its dynamic nature makes its mechanical properties difficult to elucidate and mechanical models difficult to conjecture and prove. Understanding the mechanical properties such as elasticity and viscoelasticity is crucial to developing constitutive equations to describe cellular deformations. Recent advances in bioimaging and force measurement tools with sufficient resolution for cellular study are helping advance the field. Methods like atomic force microscopy, magnetic twisting cytometry, optical trapping, micropipette aspiration, shear flow, and substrate stretching are allowing researchers to devise experiments that directly measure the cells response to applied stresses and strains (Bao, 2003, Mow, 1994). Quantitative measurements of these mechanical properties can then

be used to formulate mathematical models of cell mechanics. Basic continuum models assume the cell to be an homogeneous elastic solid or a viscous liquid encased in an elastic cortical shell (Hochmuth, 2000). A structure-based "tensegrity" model, developed by Donald E. Ingber, describes the cell in terms of load bearing struts (i.e. the cytoskeleton) counteracted by tension bearing cables (i.e. actomyosin filaments) which stabilize the cell; this model accounts for force transmission within the cell as well as mechanotransduction (Ingber, 1997). None of these models are capable of completely describing a cell's mechanical behavior, however. As new models are created and refined, researchers must resolve problems relating to anisotropy, heterogeneity, cellular remodeling, and cellular interactions with other cells and the extracellular environment.

Traction Force Microscopy

Traction microscopy, unlike methods such as cell aspiration and magnetic bead cytometry, is a techniques used to elucidate the mechanical properties of cells indirectly and without external interference with the cell. The goal of traction force microscopy is to provide a method by which the forces a cell exerts on the substratum to which it is

adhered can be indirectly measured and quantified. The first such method used an elastic substrate that would wrinkle under the traction forces of the adhered cells (Harris, 1980). These wrinkles could then be analyzed to qualitatively determine the forces being produced by the cells, however wrinkling provides a qualitative technique with poor spatial and temporal resolutions (Dembo, 1999). A method combining a deformable substrate with embedded fluorescent bead markers allowed for increased accuracy and true quantification (Lee, 1994). The fluorescent beads provide a point field that can be imaged before and after a cell is removed from the substrate. A comparison of these two images can then provide a displacement field which combined with the material properties of the particular substrate can be used to mathematically calculate the traction forces generated by the cell (Dembo, 1999).

Digital Particle Image Velocimetry

Digital particle image velocimetry (DPIV) was originally developed to measure fluid flow fields based on time-resolved displacements of tracer particles (Adrian, 2005). In traction microscopy, DPIV has been used to automatically calculate the displacements of the fluorescent beads embedded in a hydrogel substrate by a signal processing method

known as cross-correlation. The DPIV method divides an image into interrogation areas (IAs) that vary in size depending on the users needs. These IAs are cross-correlated by comparing individual IAs frame by frame to determine the similarity between subsequent images. IAs that share similar or identical groups of particles, as measured by the degree of cross-correlation, are matched and the displacement of the IA is calculated, as shown in Figure 4. The calculated displacement of IAs between images is used to calculate the displacement field.

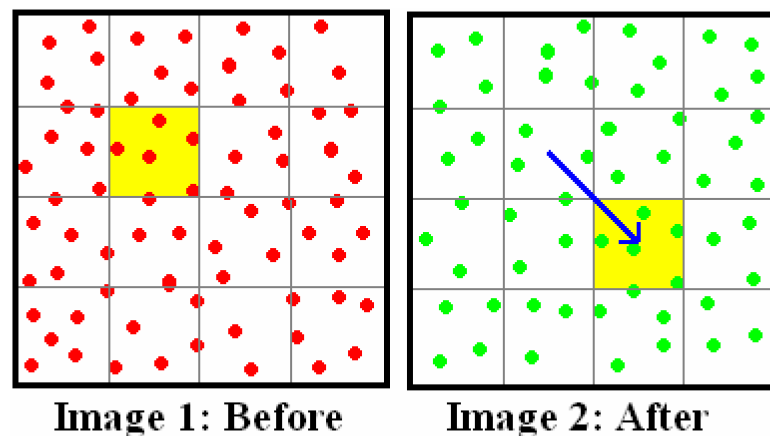

Figure 4: A simplified example of cross correlation and DPIV.

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III METHODS AND MATERIALS

Cell Culture

Bovine aortic endothelial cells (BAECs) and bovine aortic smooth muscle cells (BASMCs) were isolated from the aorta and cultured in DMEM (Gibco) containing 10% FBS, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin. Cells were cultured at 37°C in a humidified incubator containing 5% carbon dioxide.

Hydrogel Preparation

Poly(ethylene glycol) diacrylate (PEGDA) hydrogel was used because it has been found to support cell growth with proper surface treatment and is stretchable (Hahn, 2006). PEGDA of 10,000 molecular weight is synthesized from PEG powder (Fluka), acryloyl chloride (Lancaster 10363), and triethyl amine (Sigma) through a 3 day process (Delong, 2005). A mixture consisting of 0.1g PEGDA powder, 10µL acetophenone, and 1.5µL of 0.2µm red fluorescent spheres (FluoSpheres, Molecular Probes) per milliliter of deionized water is prepared and sterilized through a syringe driven 0.22µm filter (Millipore). Approximately 3mL of mixture is needed per hydrogel

being produced. A mold, consisting of two 75x50mm glass slides (Corning), two 10x50mm strips of 1/16″ porous hydrophilic polyethylene, and one 10x50mm strip of 1/16″ polycarbonate is assembled and held together by binder clips as seen in Figure 5. The entire assembly creates a 30x50x1.6mm vessel which is filled with the PEGDA mixture which also fills the porous polyethylene strips. The hydrogel is photocrosslinked by placing the entire assembly on an ultraviolet transilluminator for 2 minutes per side. As a result a hydrogel is created with attached polyethylene strips. These strips provide a secure grip for the clamping mechanism in order to effectively stretch and secure the hydrogel. Finished hydrogels surfaces are photocrosslinked with a 50μmol/mL solution of (ACRL)-PEG-peptide where the cell adhesive peptide is arginine–glycine–aspartic acid–serine (RGDS). This surface treatment assures proper cell adhesion to the surface of the hydrogel.

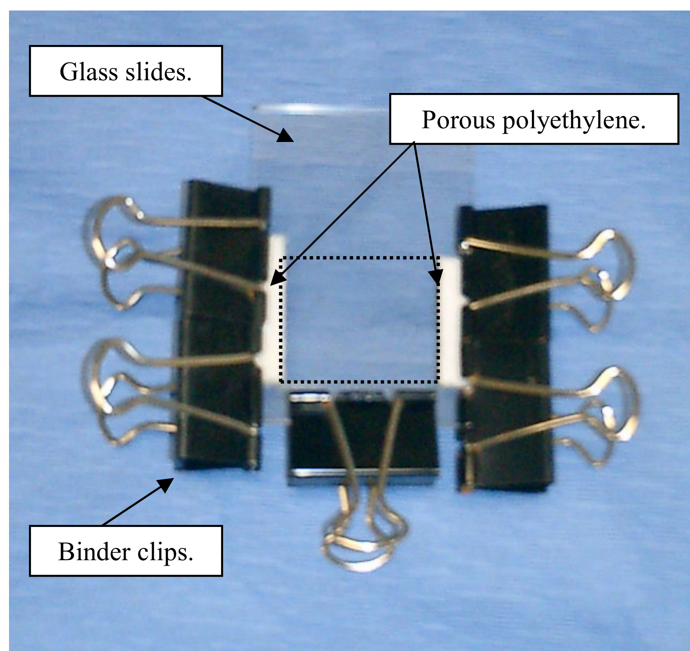

Figure 5: Hydrogel assembly mold consisting of binder clips, large glass slides, and porous polyethylene strips. The dashed square represents the area to be filled with PEGDA mixture and subsequently photocrosslinked to form the hydrogel.

Microscopy

A Nikon Eclipse FN1, fixed stage microscope, with a NIR APO 60x/1.0W DIC N2 objective combined with a Nikon D-Eclipse C1 confocal microscopy system were used to image both cell and bead positions. A confocal system was used in order to focus on the plane of beads just below the hydrogel surface. These beads will have the largest displacements and better represent the traction forces exerted by the cell.

Experimental Setup

As seen in Figure 6, the hydrogel with spread cells was submerged within media, the edges clamped either using 2 clamps for uniaxial stretching or 4 clamps for biaxial stretching. The clamping mechanisms are connected to computer controlled miniature positioners (Parker MX80S) which allow for the both accurate and precise stretching of the hydrogel substrate. Images are taken at each stage (a-d) of the experimental procedure described in Figure 7. An EC attaches to the hydrogel, produces traction forces and thus an initial hydrogel deformation and bead displacement (a). The gel is stretched and the EC responds with greater traction forces; a new bead displacement is produced (b). Trypsin (10x concentration) is used to remove the cell thus releasing all traction forces and images are taken in both stretched (c) and unstretched (d) positions. Experiments are conducted under both uniaxial and biaxial stretch conditions and under a variety of stretch lengths.

a)

b)

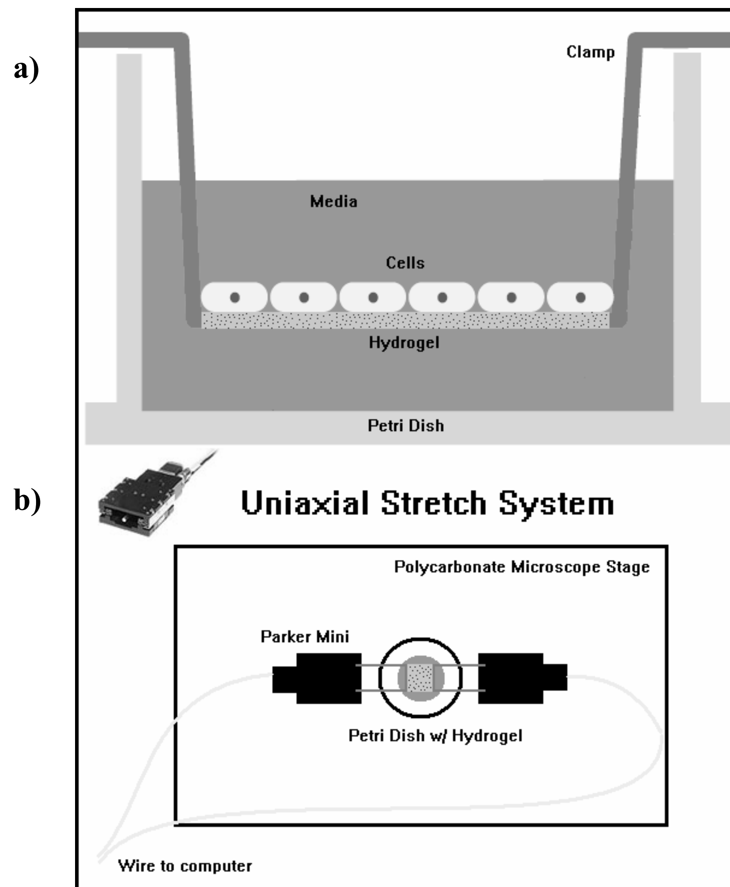

Figure 6: Experimental chamber with ECs, hydrogel, and clamping mechanism (a) nd a top view of the microscope stage for a uniaxial stretch experiment (b).

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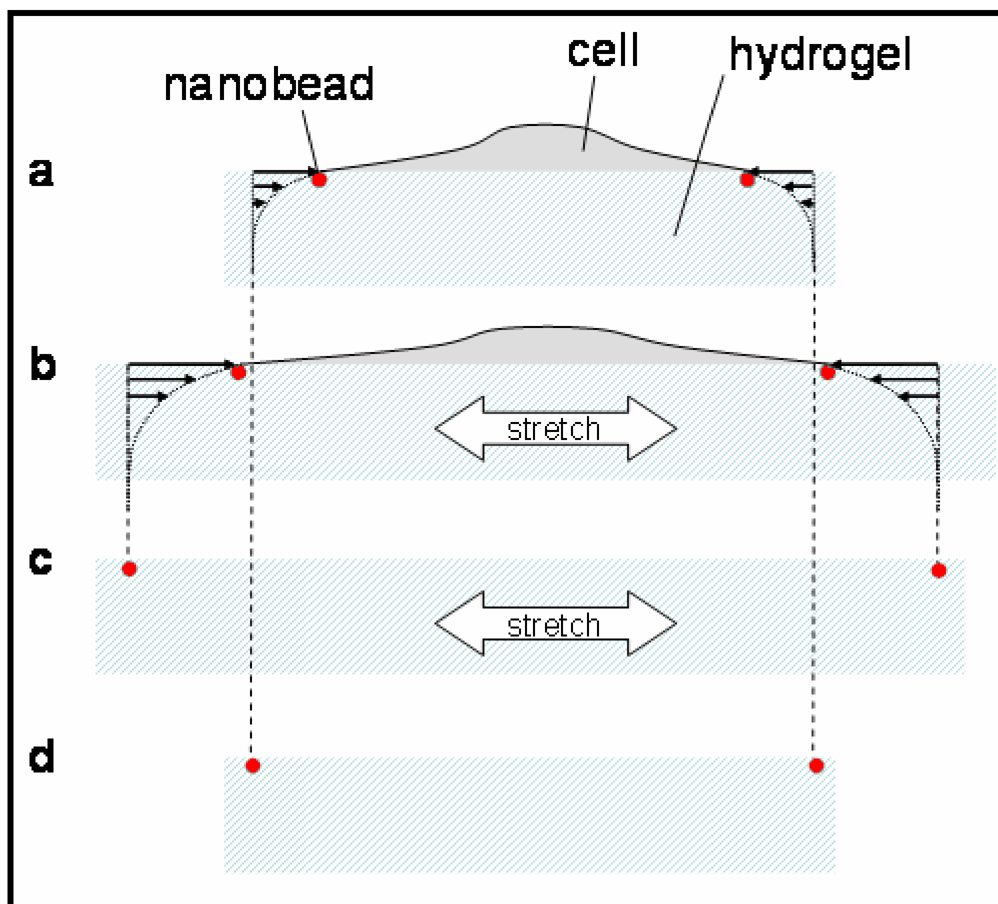

Figure 7: Traction forces induce hydrogel deformation throughout various experimental stages (a-d). Unlike typical traction microscopy where only two images are needed, in order to test stretched cells four images must be taken.

IV RESULTS

The majority of the work conducted thus far has focused on developing the experimental methods and building and testing the system. Therefore, only preliminary data has been gathered using the method described above and much work remains to be done in order to fully quantify the traction forces produced by ECs under loading conditions. Digital particle image velocimetry (DPIV) image analysis software (TSI Insight 6.0) was used to analyze the before and after images. An example of the preliminary data gathered can be seen in Figure 8. A merged fluorescent image illustrates the outline (white) of a cell extension, as well as beads before (red) and after (green) the cell was removed (Fig 8a). From this data, the displacement field was calculated using the DPIV software (Fig. 8b). This data demonstrates the efficacy of the experimental procedure and image analysis procedure. As can be seen, bead displacements are far greater near the cell pseudopodia indicating the cell is exerting a traction force on the underlying hydrogel. Beads farther from the cell pseudopodia appear to be yellow as a result of the red and green bead images coming together and forming the color yellow which indicates that these beads have not been displaced. The

displacement field is calibrated and displayed using a color gradient and exaggerated vectors to more clearly indicate magnitude.

Several difficulties were encountered throughout the experimental process. It was discovered that bead positions drifted over time which is likely due to the buoyant nature of the submerged hydrogel, as seen in Figure 9. Beads drifted at a rate of approximately 1μm per minute. This presents a problem since calculating accurate bead displacements is critical to determining the traction forces generated by the cell. This issue can be remedied relatively easily. The traction forces generated by the cell will cause the hydrogel to contract but these forces and the subsequent strains associated with them decay away from the cell, thus there is a point where these displacements are no longer relevant and the beads can be used as fixed markers to align the images before performing the DPIV analysis.

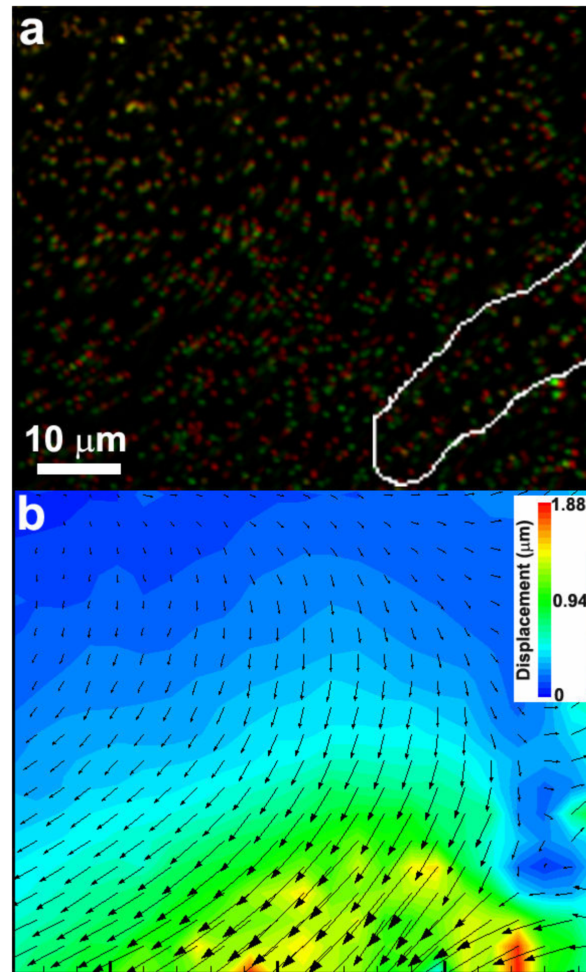

Figure 8: Example of preliminary data: bead displacement (a) and displacement field (b). Image (a) is generated by superimposing the before (red) and after (green) fluorescent bead images and an outline of the cell pseudopodia can be seen. Image (b) clearly indicates displacements near the cell to be greater and decay away from the cell.

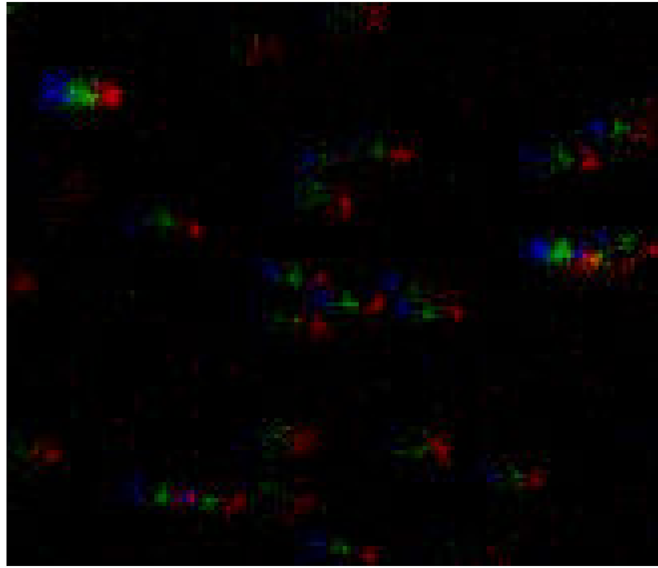

Figure 9: Bead displacements caused by fluctuations of the PEGDA hydrogel. Three images have been superimposed and false color has been applied. Beads moved consistently from left (red) to right (green then blue) throughout a period of 7.5 minutes. The rate of motion was calculated to be approximately 1μm per minute.

V DISCUSSION AND CONCLUSIONS

Discussion

A proof of concept for a novel technique for the characterization of endothelial cell mechanics has been demonstrated. The results indicate that traction microscopy utilizing a PEGDA hydrogel embedded with fluorescent beads does indeed work well and provides several unique benefits. PEGDA, when surface treated with a RGDS, provides an adequate surface for cell attachment. Unlike previous studies using traction microscopy, which have used a polyacrylamide gel attached to a glass slide, the PEGDA hydrogel can be stretched thus allowing cells to be subjected to both step and cyclic stretch. This advantage allows us to mimic the mechanical environment in the straight and branched regions of arteries using uniaxial or biaxial stretching respectively. Using the computer controlled actuators to control the rate and amplitude of stretch combined with the ability to stretch uniaxially or biaxially allows for a wide range of experimental conditions to be tested. The adaptation of ECs to these conditions as well as the traction force the ECs generate can then be studied and may further elucidate fundamental mechanical characteristics of ECs.

The technique presented although conceptually simple to understand, essentially consisting of imaging fluorescent beads and determining their displacement, is technically challenging. Several components must work seamlessly in order for the system to function properly. For example, the PEGDA hydrogel must interface with the computer controlled actuators through an effective clamping mechanism, and confocal microscopy requires the hydrogel plane being imaged to remain stable throughout the experimental process. The issue of hydrogel fluctuations between consecutive images was addressed by using beads ~50μm away from the cell as stationary reference points in order to align the images.

The continuation of this research will seek to further quantify the displacement fields produced by the ECs on their hydrogel substrate. In order for this to be accomplished several tasks must be completed. Firstly, the mechanical properties of the PEGDA hydrogel must be properly determined. The most important property is the elastic modulus (Young's modulus) or stiffness. Knowing the elastic modulus, the traction forces exerted by the ECs under a variety of loading conditions can be calculated and a set of physiologically relevant data can be produced.

Conclusion

A proof of concept for the novel technique for the characterization of endothelial cell mechanics which has been described above has been presented. This technique can now serve as a platform technology from which a variety of experimental conditions including uniaxial and biaxial stretching, both cyclic and step, at various rates and amplitudes. These conditions would be used to effectively mimic the mechanical environment in the straight and branched regions of arteries. The data gathered will provide the basis for a fundamental understanding of the effects of stretch and EC adaptation on the traction forces ECs exert. Further work is still required to improve the efficacy and repeatability of the technique as well as gather sufficient data to make informed conclusions on the characterization of EC mechanics.

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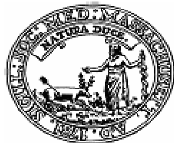

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April 27, 2007

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Texas A&M College of Engineering: Biomedical Engineering Department
(Fall 2005 to Present)

- Using digital particle image velocimetry (DPIV) to analyze deformations in cells caused by magnetic twisting cytometry in order to better understand the wave-like transmission of force through cells.
- Applying DPIV techniques to determine the homeostatic stress level within endothelial cells cultured on fluorescent bead imbedded polymer hydrogels. This research is being conducted as part of the Undergraduate Research Fellows Program.

MIT Summer Research Program: Harvard-MIT Division of Health Science and Technology (Summer 2006)

- Cell biocompatibility of parylene-C thin membranes for use in cell patterning, dynamic co-cultures, and high throughput applications.

Texas A&M College of Medicine: Medical Physiology
(Fall 2004 to Spring 2005)

- Develop software to track real-time micro-vessel diameter changes using a video feed and image processing techniques.