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Oswald Physical and Engineering Sciences First Place: An Improved in vitro Model for the Study of Endothelial Cells Using Micropatterned Surfaces

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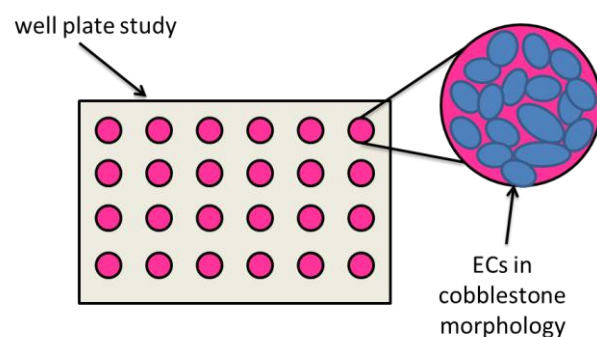
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Abstract

Sickle cell anemia, malaria, and cancer are a few of the deadly diseases that utilize blood vessels as a means of migration throughout the body. Adhesion of harmful cells to the endothelial lining of the circulatory system is an integral step in the metastasis of blood borne diseases. As a result of shear stress produced by blood flow through veins and arteries, the endothelium undergoes a distinct morphological change resulting in a more elongated and unidirectional morphology. It has recently been suggested that such changes in cell morphology can affect surface expression profiles, which in turn affects cell-cell binding and interaction to the endothelial wall. Currently, most researchers are using *in vitro* flow models or static well-plates to culture endothelial cells. However, traditional *in vitro* flow systems take approximately 24 hours to obtain a valid morphology, and static well plate studies result in cobblestone morphology more random in orientation than *in vivo* endothelial cells. In this study, we are investigating the use of micropatterned glass surfaces to statically culture human vein endothelial cells (HUVECs) in the desired elongated and unidirectional morphology and this system's effects on surface chemistry of the HUVECs. Microscopy and flow cytometry were used to compare the morphology and surface expression of HUVECs grown on control blank slides, on micropatterned grooves, and under flow conditions. HUVECs cultured on micropatterned grooves demonstrated the desired elongated and unidirectional morphology. Morphology analysis showed that HUVECs cultured on micropatterned grooves were statistically more elongated and unidirectional than HUVECs cultured on control blank slides.

Introduction

Sickle cell anemia, malaria, and cancer are a few of the deadly diseases that utilize the blood vessels as a means of migration throughout the body.[4] The circulatory system is lined with a thin layer of endothelial cells (ECs). Adhesion of cells to the endothelium is an important step in the migration of harmful diseases throughout the body. Within the vessel, the endothelium is exposed to fluid shear stress produced by blood flow (Figure 1). As a result of this shear stress, the endothelium undergoes a distinct morphological change resulting in a more elongated and unidirectional morphology. Most researchers to date are using simple *in vitro* flow models such as a parallel plate flow chamber or simplified models of *in vitro* static well-plate studies.[9,12,13] To attain this morphology in a flow system, researchers must expose a monolayer of cultured ECs to approximately 24 hours of flow.[12,13] In a static well plate study, this morphology cannot be obtained, and consequently, ECs will culture in a cobblestone morphology (Figure 2).
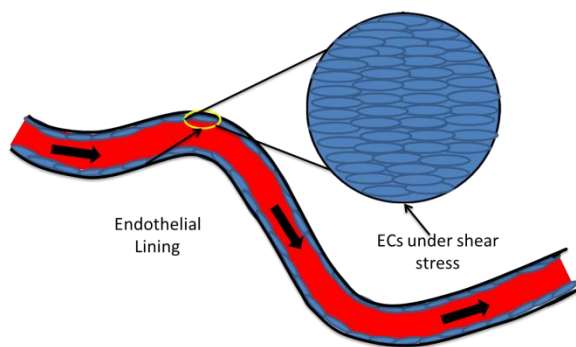
Figure 1: Endothelial Cells (ECs) *in vivo*   Figure 2: Endothelial Cells (ECs) in cobblestone morphology

Aside from morphology, the endothelium exhibits unique surface adhesion molecules: ICAM, VCAM, PECAM, E-selectin, and P-selectin.[4,9] It has been suggested that a correlation exits between ECs morphology and the adhesion molecules on the surface.[8,9] In addition to increases in adhesion molecule expression, it has also been suggested that under fluid flow conditions, the deforming of cells into an elongated state increases the number the number of

contact areas tumor cells have with the endothelium during the process of metastasis.[3] Although little study has been done on the effect of flow on surface expression, much has been done to show a correlation between gene expression and fluid flow.[17,18,21] Thus, a comparison of EC surface expression for different methods of EC culture, as well as *in vitro* model system that more accurately mimics endothelial cells *in vivo* without the arduous task of flow adapting for extended period of time would represent a significant contribution for the *in vitro* study of vascular cell migration and adhesion.

This study presents the use of a static, artificially induced flow adaptation method. In recent years, significant advancements have been made on the integration of microfabication and cell culture.[5,6,7] Recent studies have shown that the use of microfabricated microscale grooves promote the elongation and unidirectional grow of ECs seen *in* vivo.[19,20,21] In this study, microscale grooves were created using a process involving SU-8 2000.5, an epoxy-based negative photoresist.[1,2,11,14] It is hypothesized that using these microscale grooves will force ECs to grow in the desired unidirectional morphology statically, and hence, ECs cultured on this micropatterned substrate will generate a more similar surface expression profile to ECs grown *in vivo*. Thus, the main objective of this work is to develop a microfabricated system of culturing ECs that better mimics the morphology and chemistry of ECs grown *in vivo*. More specifically, surface expression profiles will prove a correlation between the elongated, unidirectional morphology and surface expression of adhesion molecules present on and EC monolayer. This system will then reduce the need for researchers to partake in arduous and lengthy methods of flow adaption system and allow more time to be dedicated to adhesion trials to inhibit the migration of deadly blood-borne diseases.
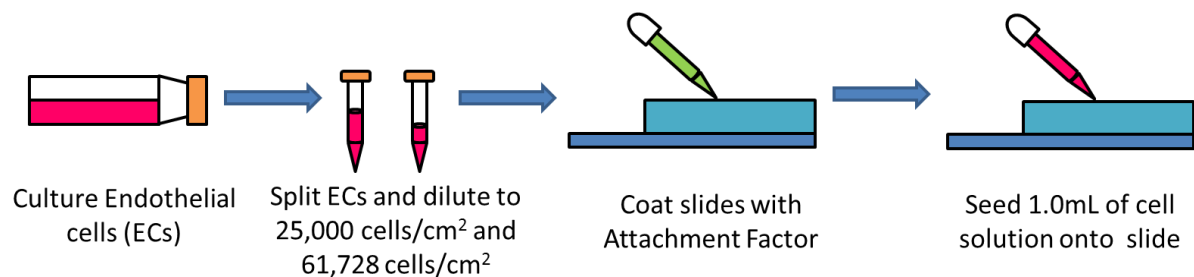
Materials and Methods

Cell lines

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Lonza (Basel, Switzerland) at passage 1 and utilized in adhesion studies from passage 4 up to passage 8. The HUVECs were grown in 75 cm^2 Costar tissue culture flasks and sustained with EGM-2 media (Lonza) supplemented with fungisome and penicillin streptomycin.

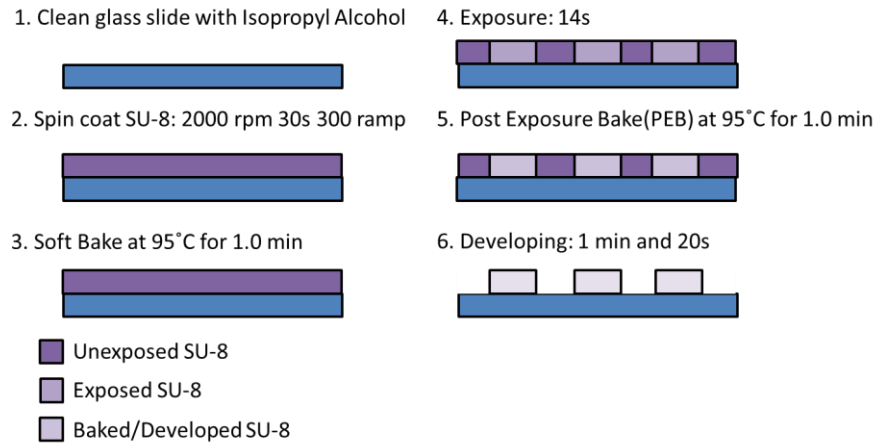*Preparation of the Control Endothelial Cell Monolayers*

The HUVECs were seeded onto patterned and blank glass slides (Fisher) with oxygen plasma cleaned polydimethylsiloxane (PDMS) chambers.[14] Attachment factor (Sigma) was applied prior to seeding. Low and high cell densities of 25,000 cells/cm^2 and 61,728 cells/cm^2 were seeded to reach a confluent monolayer. The endothelial cell monolayers were sustained in their respective culture media and maintained under the same conditions described in the previous section. The endothelial cell monolayers on the static control culture and the micropatterned culture were allowed to reach confluency on the slides in 24 hours. Figure 3 demonstrates the seeding protocol for both the control and micropatterned substrates.

Culture Endothelial cells (ECs) → Split ECs and dilute to 25,000 cells/cm^2 and 61,728 cells/cm^2 → Coat slides with Attachment Factor → Seed 1.0mL of cell solution onto slide

Figure 3: Seeding Protocol for HUVECs on micropatterned grooves and control blank slides

Microfabrication: Patterning of SU-8 2000.5

Patterns were created using SU-8 2000.5, a widely used negative epoxy-based photoresist. [1,2,11,14] Glass slides were first cleaned with Isopropyl alcohol (IPA) and placed on a 95°C hot plate until completely dry. After cooled, glass slides were placed in a spin coater and spun at 2000 rpm for 30 seconds with an acceleration of 300 rpm/s. [1] Following the spin coating, slides were placed on a hot plate at 95°C for one minute. After allowing the slide to cool completely to room temperature, slides were exposed to 14 seconds of UV light with an intensity of 10 mJ/cm in order to harden exposed portions. After exposure, slides underwent a post exposure bake (PEB) at 95°C for one minute. Again after cooling, slides were developed in fresh SU-8 developer

1. Clean glass slide with Isopropyl Alcohol

2. Spin coat SU-8: 2000 rpm 30s 300 ramp

3. Soft Bake at 95˚C for 1.0 min

4. Exposure: 14s

5. Post Exposure Bake(PEB) at 95˚C for 1.0 min

6. Developing: 1 min and 20s

Unexposed SU-8
Exposed SU-8
Baked/Developed SU-8

Figure 4: SU-8 2000.5 Protocol

followed by an IPA wash. Slides were then sprayed with IPA and blown dry. Figure 4 illustrates the entire process. Atomic Force Microscopy or AFM was used to produce a known groove height and to obtain accurate images of the grooves.

Four patterns on a chrome mask were used for exposure. Pattern A was designed to have alternating groove widths of approximately 1 micron and .5 microns. Pattern B was designed to have alternating groove widths of approximately 1.5 microns and .5 microns. Pattern C was

measured to have the same widths as Pattern A but reversed peaks and trenches. Similarly Pattern B has the same dimensions as Pattern D but with reversed peaks and trenches.

Preparation of PDMS Chambers for Cell Culture

Approximately 20.0 grams of Sylgard 184 silicone elastomer base and 2.0 grams of Sylgard 184 silicone elastomer curing agent were mixed together to form polydimethylsiloxane (PDMS). After mixing, PDMS was pouring into a rectangular mold and placed in an oven at 65°C for 70 minutes. Once cooled, rectangular cutouts of an approximate 2.5 centimeter length and 1.0 centimeter width were removed to create a chamber to cover the grooved pattern. Chambers were then bonded to glass with oxygen plasma treatment to ensure a watertight seal. Figure 5 shows the completed chambers.

1. Cast PDMS   2. Cut out chamber   3. O₂ plasma to grooved surface
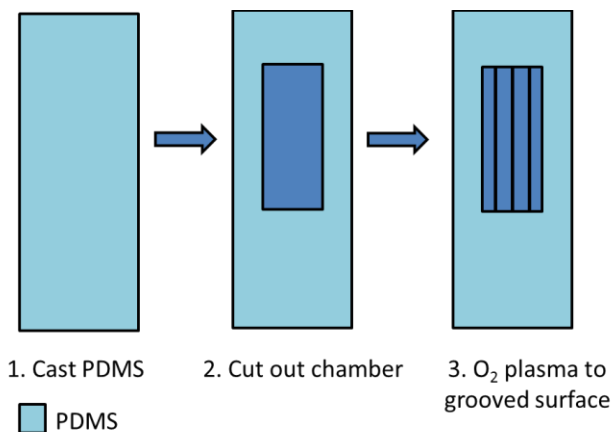
PDMS

Figure 5: PDMS chamber protocol

Evaluation of Morphology

After reaching confluency, cells were imaged on a Nikon Eclipse LV100 microscope. Once an image was obtained NIS elements software was used to characterize aspect ratios of cells on the control and micropatterned substrates. Using length measurement software, lengths and widths were determined. Lengths and widths were then used to determine aspect ratios, which were

used for comparison.   Orientation angles were measured by the cell's deviation from the horizontal. Histograms of orientation angles were made and analyzed in Minitab.

Texas red-X phalloidin a high affinity F-actin probe (Invitrogen) was used to stain the stress fibers of HUVEC cells. Cells were first fixed with a 3.7% paraformaldehyde fixing solution. After washing with phosphate buffered saline (PBS), cells were then soaked with acetone at -20˚C. After another PBS wash cells were stained with the F-actin stain diluted in PBS for 20 minutes. Cells were again washed with PBS and then imaged on a Nikon Eclipse LV100 microscope.

*Surface Expression Comparison Assay*

Immunohistochemical staining was used to tag specific adhesion molecules present in the metastatic cascade, ICAM, VCAM, PECAM, and E-selectin. Initial studies used VCAM-1 to analyze surface expression of HUVECs grown on the control blank slide and HUVECs grown on the micropatterned grooves.[23,24] Cells were first fixed with 4% paraformaldehyde fixing solution and blocked with 5% Bovine Serum Albumin (BSA).   They then were incubated with approximately 50 microliters of anti-VCAM-1, clone P3C4 primary antibody (Millipore) for one hour at room temperature.   Cells were then washed with phosphate buffered saline (PBS). Cells were then incubated with approximately 300 microliters of goat anti-mouse IgG (Fc), fluorescein conjugated secondary antibody (Fisher) for one hour at room temperature.[25]   After another PBS wash, cells were imaged on a Nikon Eclipse LV100 microscope.

Results and Discussion:

AFM Characterization of Micropatterned Grooves

Atomic force microscopy (AFM) was used to characterize heights and widths of the SU-8 2000.5 microscale grooves. The desired height based on spin curves was a half micron.[1] Hill to valley measurements from generated AFM traces indicated an average height of 500 nm or .5 microns. Two dimensional (Figure 6) AFM images allowed for width ratio consistency to be established in each pattern. AFM data and Gwyddian software generated three dimensional images to demonstrate topographical qualities to obtain data (Figure 6).
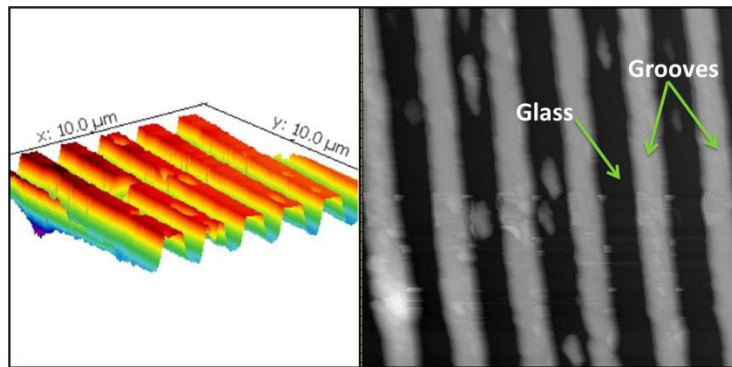

Figure 6: AFM images. **A:** 3D **B:** 2D

Evaluation of Morphology of HUVECs Cultured on Micropatterned Grooves

Image Comparison of HUVECs on Micropatterned Grooves and HUVECs on Control Slides

Seeding trials were conducted at low and high densities of 25,000 cells/cm^2 and 61,728 cells/cm^2. Low and high densities were hypothesized to have varying effects on elongation and orientation; however, the main difference observed throughout the morphology analysis was seen in confluency. After one day of culture high density cultures of both control and micropatterned HUVECs reached a more confluent monolayer than the lower density. Monolayers for both cultures reached confluency 24 hours after seeding; all of the following data was obtained from day one cultures. Bright field imaging of live monolayers provided a distinct morphological

difference between the HUVECs grown on control blank slide and the HUVECs grown on micropatterned grooves (Figure 7). F-actin staining was then used on a fixed monolayer to fluoresce stress fibers of the cells to better determine aspect ratios and orientation angles (Figure 7).
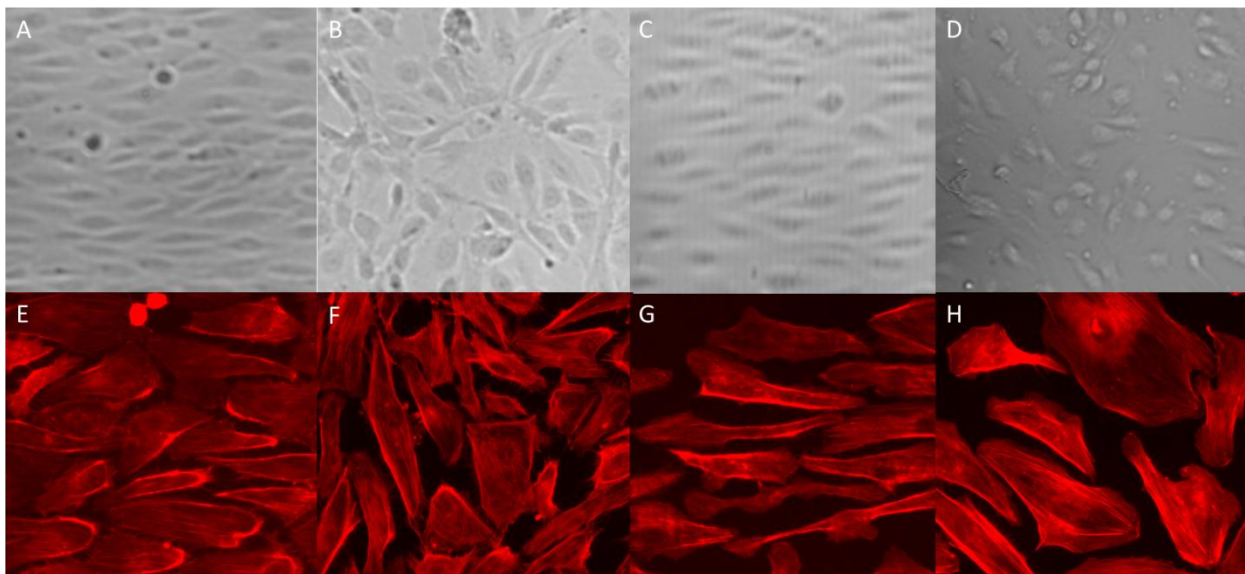
Figure 7: Bright-field images (**A-D**) and F-actin images (**E-H**). **A,E:** 61,728 cells/cm^2 cultured on micropatterned grooves. **B,F:** 61,728 cells/cm^2 cultured on control blank slide. **C,G:** 25,000 cells/cm^2 cultured on micropatterned grooves. **D,H:** 25,000 cells/cm^2 cultured on control blank slide.

*Aspect Ratio Comparison of HUVECs on Micropatterned Grooves and on Control Slides*

Lengths and widths of cells on micropatterned grooves and cells on control slides were taken to determine aspect ratios. A comparison of average aspect ratios of both micropatterned and control cultures at high and low is illustrated in Figure 8, seen below. High and low densities of cells grown on micropatterned grooves yielded mean aspect ratios of 4.184 and 4.000, whereas controls of the same density resulted in mean aspect ratios of 2.461 and 2.232. From aspect ratio comparison in Figure 8, it is evident that HUVECs cultured on micropatterned grooves were more elongated in nature that the HUVECs cultured on a control blank slide.
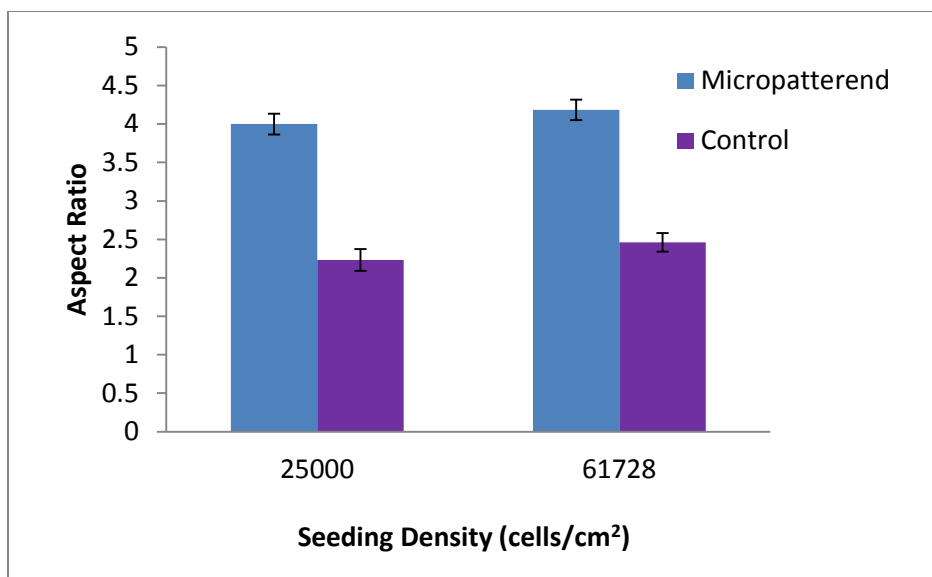
Figure 8: Elongation of HUVECs on micropatterned grooves versus on control. Controls: n=60. Micropatterned: n=138, n=114. Error bars represent SE.

Orientation Angle Histograms of HUVECs on Micropatterned Grooves and on Control Slides

Orientation angles were measured as a deviation from the horizontal, where a positive angle indicated an upward orientation and a negative angle indicated a downward orientation. A horizontal deviation of 0 degrees was the desired orientation due to its parallel orientation indicating unidirectional growth. Once angle deviations were measured and recorded, histograms (Figure 9) were constructed to analyze the distribution orientation angles. Cells grown on micropatterned grooves had a small distribution between ±40 degrees with a high frequency of cells between 0 and 20 degrees. The distribution was consistent between the low and high concentrations. Cells grown on the control blank slide had a large distribution between ±100 degrees with no high distributions in a specific bin. This data indicates that the control culture has cobblestone morphology with cells growing in varying directions, whereas the micropatterned culture has a more unidirectional morphology with small deviations from the horizontal.
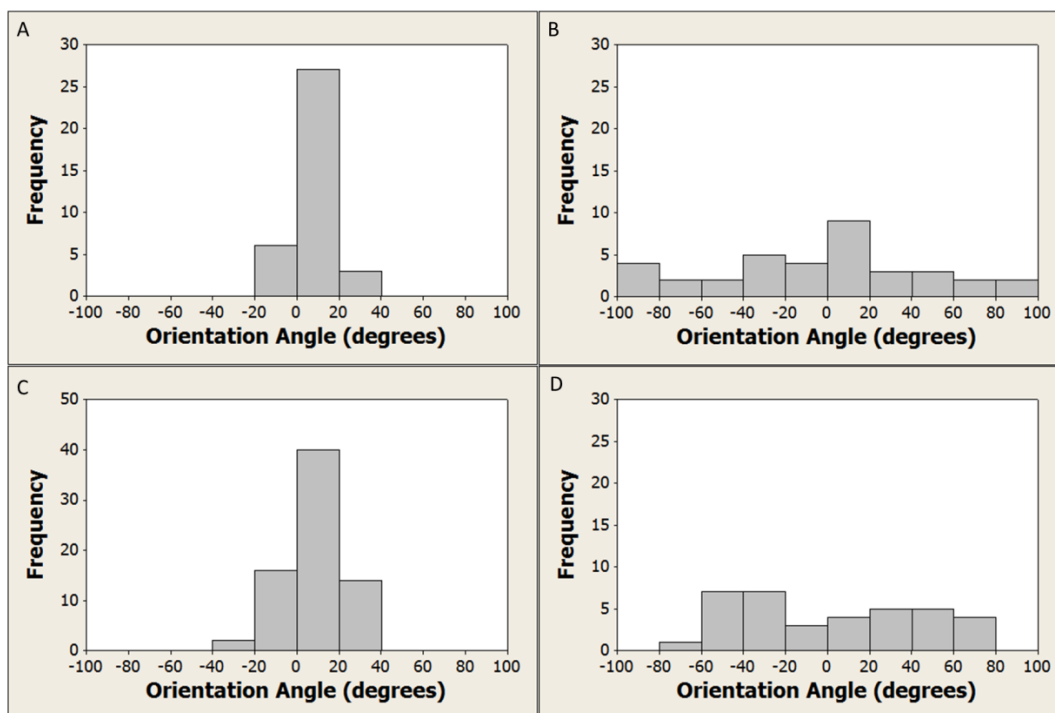
Figure 9: Histograms of orientation angles. **A:** Micropatterned 61,728 cells/cm^2 **B:** Control 61,728 cells/cm^2 **C:** Micropatterned 25,000 cells/cm^2 **D:** Control 25,000 cells/cm^2 Results are significantly different compared with controls with a t-t test (P<0.05)

Surface Chemistry Analysis
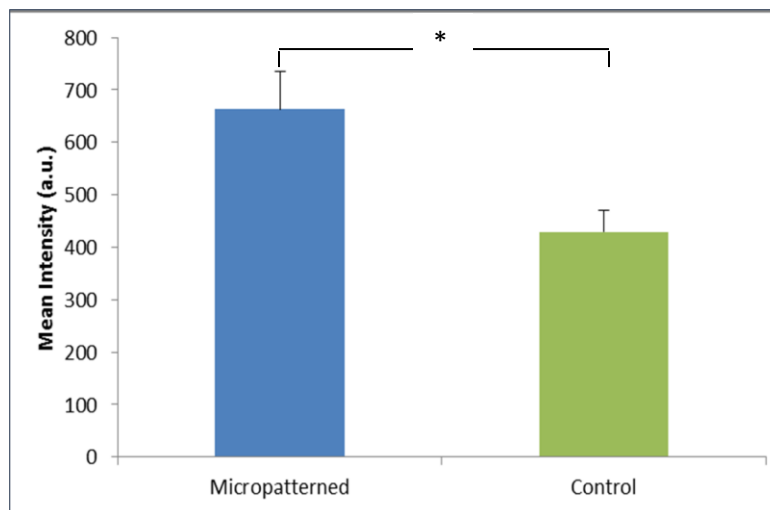
VCAM-1 Expression of HUVECs on Micropatterned Grooves and on Control Slides

VCAM-1 (vascular cell adhesion molecule) was chosen to analyze similarities and differences in surface chemistry between HUVECs grown on micropatterned grooves and HUVECs grown on the control blank slide. Preliminary images of fluorescent intensity data is presented below in Figure 10.
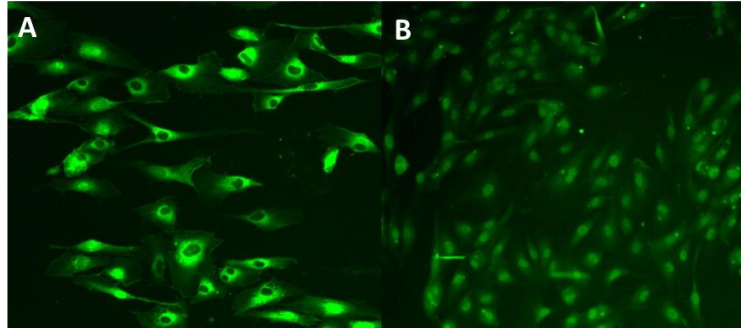
Figure 10: VCAM-1 Fluorescent staining. **A:** Micropatterned
62,000 cells/cm^2 **B:** Control 62,000 cells/cm^2

Cells were first stained with primary mouse IgG anti-VCAM-1 antibodies and then stained with secondary anti-mouse goat IgG antibodies. The primary antibody has a specific affinity for VCAM-1 adhesion molecule present on the surface of the endothelial monolayer; the secondary antibody is a nonspecific fluorescent conjugated antibody which binds to the primary antibody to produce a measurable intensity that is correlated to the amount of VCAM-1 present on the surface of each monolayer. An initial trial was run using the high density of cells (Figure 10). Measurements of mean fluorescent intensity were taken using Nikon LX100 imaging software. The mean fluorescence of images were taken and compared; collected data is below in Figure 11.

Figure 11: Surface expression data. Error bars represent SE,
n=7. *p<0.05

Results indicated a statistical difference in the mean intensities of the HUVECs cultured on micropatterned grooves and the HUVECs cultured the unpatternd glass slides. As seen the in Figure 11, HUVECs cultured on micropatterned grooves had a statistically larger mean intensity than HUVECs cultured on unpatterned glass slides. This indicates an up-regulation of VCAM-1 on the more elongated and unidirectional morphology that better mimics the morphology of endothelial cells *in vivo*.

Conclusion

Endothelial cells cultured on microfabricated microscale grooves are more elongated and more unidirectional in orientation than endothelial cells cultured on unpatterned glass substrates. This morphological distinction has led to an inquiry as to morphologies effect on the presence of adhesion molecules on the surface endothelial monolayers. Preliminary surface expression data indicates an up-regulation of VCAM-1 surface adhesion molecule on the surface of HUVECs cultured on micropatterned grooves. Though initial surface expression studies showed promising fluorescent images, future studies using flow cytometry in conjunction for image analysis will need to be used to produce more accurate and reliable results. However, this preliminary data does indicate the need for multiple assays to test other adhesion molecules pertinent in metastatic cascades of cancer and other blood borne diseases. If in fact this morphology influences an up-regulation of multiple adhesion molecules a device of allowing for culture on micropatterned grooves will promote more accurate results of interactions of harmful cells with the endothelial lining. Also, future studies will also be needed to conclude whether culture on micropatterned grooves displays similarities in morphology and surface expression to endothelial cells grown under flow. Overall studies to date have concluded that culturing on microfabricated grooves allows for a simpler and more accurate method of modeling the morphology and surface

chemistry of the endothelium *in vitro*, and that a device using these grooves for quick and easy

culture would result in faster and more accurate data for the study of deadly diseases like cancer.

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