THE DEVELOPMENT OF 3-D, *IN VITRO*, ENDOTHELIAL CULTURE MODELS FOR THE STUDY OF CORONARY ARTERY DISEASE

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Abstract: The sensitivity of the vascular endothelium to wall shear stress plays a central role in the development and progression of atherosclerosis. While current studies investigate endothelial response using idealized *in vitro* flow chambers, such cell culture models are unable to accurately replicate the complex *in vivo* wall shear stress patterns arising from anatomical geometries. In this study we have created both a simplified/tubular and an anatomically realistic model of the human right coronary artery. Following endothelial cell culture, and the application of steady flow, cell morphology was analyzed. In both models a progressive elongation and alignment of the endothelium in the flow direction was observed following 8, 12, and 24 hours. This change, however, was significantly less pronounced in the anatomical model (as observed from morphological variations indicative of localized flow features). Differences were also observed between the inner and outer walls at the disease-prone inlet region. Since morphological adaptation is a visual indication of endothelial shear stress activation, the use of anatomical models in endothelial genetic and biochemical studies may offer better insight into the disease process.

Introduction

Cardiovascular disease is a leading cause of mortality and hospitalization in North America [1]. Atherosclerosis, a condition of arterial wall thickening, has been shown to occur at focal locations throughout the human vasculature, such as regions of curvature and bifurcation [2]. This predilection has not been explained by lifestyle or genetic risk factors and has been linked to the response of endothelial cells (ECs) lining the luminal surface of blood vessels, to complex hemodynamic forces, such as wall shear stress and pressure gradients [3]. Due to the difficulty of studying the endothelium *in vivo*, a number of *in vitro* systems have been developed with the aim of replicating the *in vivo* hemodynamic environment over a cultured monolayer of ECs in a controlled environment, including: cone and plate viscometer systems, parallel plate chambers, and 3-D straight tubular flow models. The sensitivity of ECs to wall shear stress has been well documented in such idealized models. These devices expose ECs to simple, uniform, 1-D flow fields. However, blood flow characteristics are defined principally by the tortuous, 3-dimensional geometry of the vessel, and, therefore, i*n vivo* wall shear stress patterns are poorly replicated by these simplified cell culture models. To date no model has accurately replicated the geometry of an artery for the creation of an anatomically accurate cell culture model. This model would better replicate the *in vivo* flow characteristics and shear stresses. The goal of our study was to create such a model of the human right coronary artery (RCA), and to culture ECs within it. An idelaized straight tubular model was also developed, in parallel, for comparison. Anatomical models, such as the one presented in this study, will play an essential part of further studies into the activation of endothelial cell biochemical pathways leading to atherosclerosis, and intravascular device testing.

Materials and Methods

For the preparation of geometrically accurate models the entire left ventricular outflow tract (including the ascending aorta, the aortic root, and the coronary ostia; as well as the inlet, proximal and acute marginal regions of the coronary arteries) of mildly diseased and healthy post-mortem human hearts were cast at physiologic pressure using Batson's No. 17 anatomical casting (Polysciences Inc.). The most successful cast was then used to create Sylgard™ 184 silicone elastomer models for cell culture, using a low melting point alloy as an intermediate negative mold. Sylgard™ straight tubular models, 3.2 mm in diameter, were created, using a custom made mold (Figure 1).

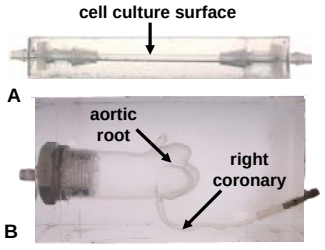

Figure 1: (A) simplified straight tubular model (B) anatomically accurate model

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Models were then prepared for cell culture by hydrophilization in 70% sulphuric acid, followed by sterilization, and coating with fibronectin (40 µg/ml) (Sigma-Aldrich). Human abdominal aortic endothelial cells (HAAEs) (ATCC), pass five, were cultured within the models in a cell culture incubator under rotation at a density of $4.48\pm0.8\text{x}10^4$ cells/cm^2 for 4 hours. For flow experiments, the models were connected into a perfusion loop composed of a media reservoir and a low-pulsatility peristaltic pump (Ismatec). The reservoir contained EC culture media (PromoCell) supplemented with 10% dextran (Sigma-Aldrich), to increase its viscosity to 3.5 cP. The entire apparatus was placed in a cell culture incubator, and cells were subjected to steady flow (119.5 ml/min, Re=233) for varying time periods: 8, 12, and 24 hours. At the end of each experiment cells were fixed in 4% buffered formalin, and stained with crystal violet for visualization under a light microscope.

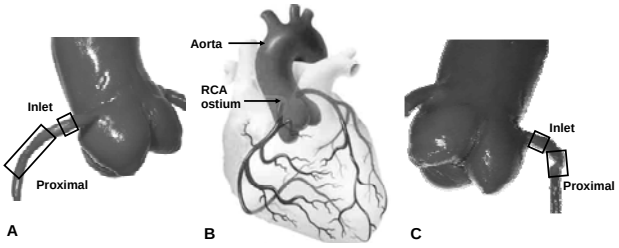

Figure 2: The approximate location of the inlet and proximal RCA regions indicated on anatomical cast; (A) ventral view (C) dorsal view

High resolution images captured at 100X magnification were acquired using a DC camera (Leica) and processed for image analysis. Approximately 280-790 cells per experiment were analyzed. For the anatomical model these photographs were taken at the inlet and proximal RCA on both the inner (myocardial) and outer (pericardial) walls (Figure 2). For the particular model used in this study, the inlet was defined as the nearly straight region from the ostium to the beginning of the first curvature (~ 7 mm). The proximal RCA was defined as the region extending from the end of the inlet and incorporating the first curvature (~18 mm).

To select cells within each image an interactive Matlab 7.0 (Mathworks, CA) software was created. This software displays a filtered image and allows the user to select individual cells. A second algorithm was designed to processes the resulting cell information using Matlab image processing routines to compute the morphological parameters of each cell. These included the perimeter and area (used to calculate the cell shape index, SI) and the angle of orientation (θ). The SI value is defined as:

$$\text{SI} = \frac{4\pi A}{P^2} \qquad (1)$$

where A = cell area and P = cell perimeter

The SI characterizes the degree of cell elongation and is equal to 1 for a circle and 0 for a straight line. The angle of orientation is the angle between the cell major axis and the longitudinal axis of the model.

Shape index data was averaged and reported as means ± standard deviations. Statistical analysis was performed by parametric tests. Mean values were compared using a one-way analysis of variance. If a significant difference was found among the means, multiple comparisons were performed using a Bonferroni post-processing test with a 95% confidence interval. A "p" value less than 0.05 was considered statistically significant. To compare two means a two tailed parametric t-test was used. For the angle of orientation, distributions variances were tested for statistical significance using an F ratio test.

Results

HAAECs were successfully cultured in both models. Under static (no flow) conditions, the structure of each cell was observed to be similar in the models to that seen in cell culture flasks. For flow experiments, progressive evidence of cell alignment in the axial flow direction was seen starting at 8 hours. This was followed by further cell elongation and alignment at 12 and 24 hours (Figures 3 and 6).

Within the simplified model a significant difference in the cell mean shape index, a measure of cell elongation, was found between the static, 8, 12, and 24 hour experiments ($p<0.001$ for all pair-wise comparisons, 1-way ANOVA, Bonferroni post-test) (Figure 4).

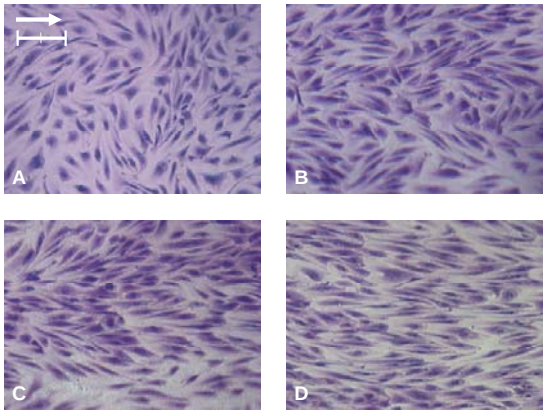

Figure 3: Light microscope images of ECmorphological changes in the simplified Sylgard^{TM} model. HAAECs were subjected to a steady laminar shear stress of magnitude 22 dynes/cm^2 for 8, 12, and 24 hours, (B-D) respectively. (A) represents the no flow control. (Bar = 100 µm, Magnification = 100X) The arrow points in the direction of net flow.

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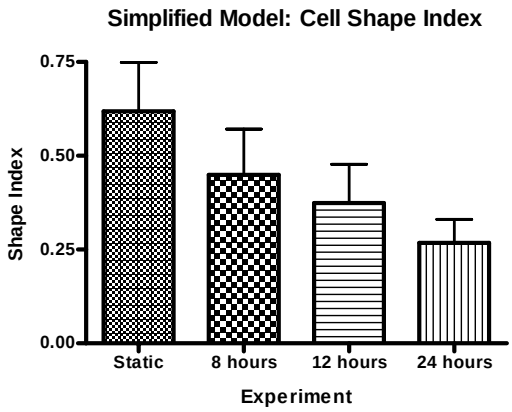
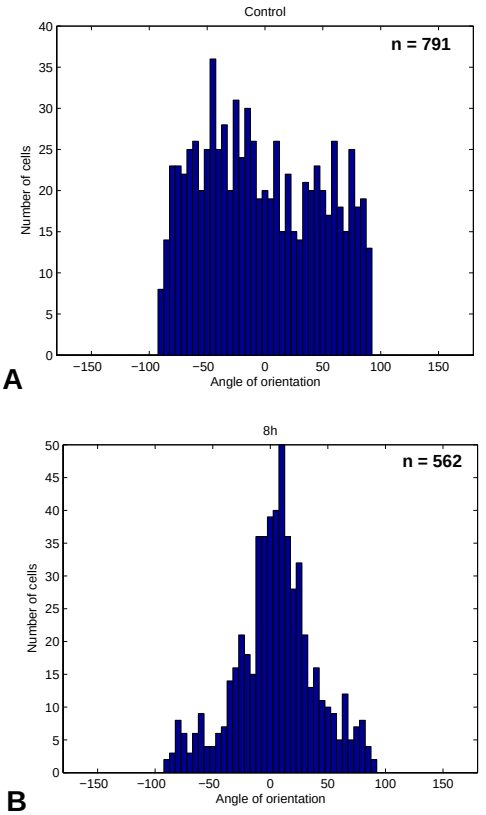


Figure 4: Cell shape index time history for simplified model experiments; SI = 1 corresponds to a perfect circle, while SI = 0 corresponds to a line; all values are expressed as mean ± standard deviation

A significant decrease in the variability of the cell orientation angle was also observed ($p<0.05$ for all pairwise compariosns, F-ratio test). Alignment angle distributions exhibited a bell-shaped curve around the 0º horizontal flow direction (except for the control where a large dispersion of values was observed, as would be expected for randomly oriented cells). A significant narrowing of the distributions around 0º occurred as experimental flow time increased (Figure 5).



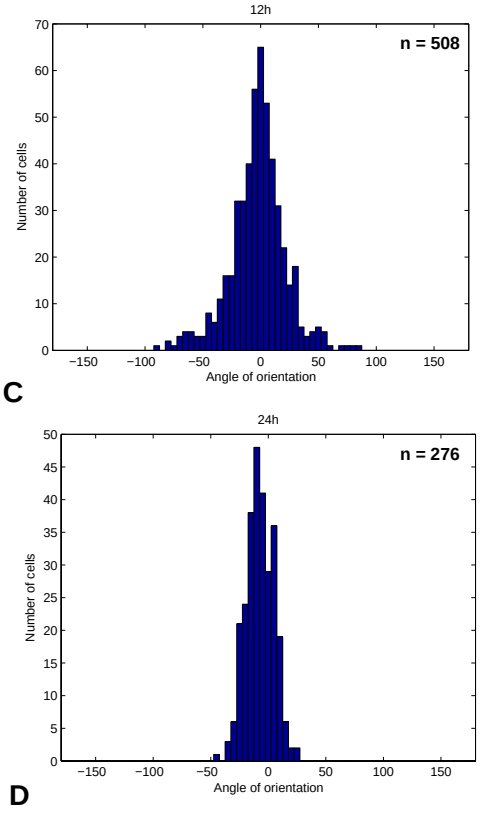


Figure 5: Histograms illustrating the distribution of cell angle of orientation for the control (A), 8 hour (B), 12 hour (C), and 24 hour (D) simplified model flow experiments [n = sample size]

For anatomical model experiments the pattern of HAAEC elongation and alignment in the direction of flow at the relatively straight inlet region was less pronounced (Figure 6 and Table 1).

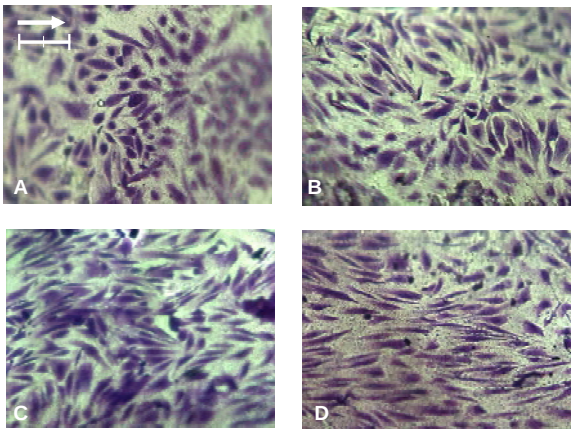


Figure 6: Light microscope images of ECs in anatomical Sylgard™ models following 8, 12, and 24 hours, (B-D) respectively. (A) represents the control. (Bar = 100 µm, Magnification = 100X) The arrow points in the direction of net flow. Certain images locations are blurred due to the local curvature.

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Shape index results showed that the mean cell shape at the inlet of the anatomical model following 24 hours of flow was not statistically different from the 12 hour simplified model experiment ($p>0.05$). The anatomical 12 hour experiment was also not different from the 8 hour simplified model experiment ($p>0.05$). As expected, the controls of the two models are also not statistically different ($p>0.05$) (Figure 7 and Table 1).

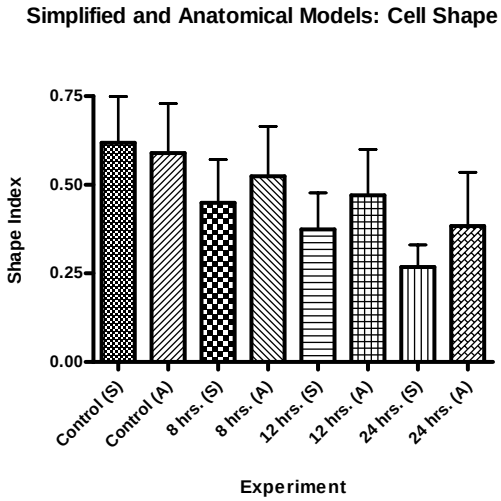

Figure 7: Bar graph illustrating SI time history for both the simplified (S) and anatomical (A) models

Inlet region alignment angle histograms for anatomical model experiments were observed to be more widely distributed around 0°. In general, there was a significant decrease in the angle variability with flow; however, for the anatomical model this was true only following 8 hours. When comparing the two types of models all timed experiments showed significantly different results ($p<0.05$), indicating that in the simplified model global cell alignment occurred faster (Table 1).

Table 1: Significance test results for comparisons between the anatomical and simplified models

Experiment	*SI: Bonferroni test*	*Angle: F-ratio test*
Control (S) vs. Control (A)	P > 0.05	P > 0.05
Control (S) vs. 8 hrs. (A)	P < 0.001	P<0.05
Control (S) vs. 12 hrs. (A)	P < 0.001	P<0.05
Control (S) vs. 24 hrs. (A)	P < 0.001	P<0.05
8 hrs. (S) vs. Control (A)	P < 0.001	P<0.05
8 hrs. (S) vs. 8 hrs. (A)	P < 0.001	P<0.05
8 hrs. (S) vs. 12 hrs. (A)	P > 0.05	P<0.05
8 hrs. (S) vs. 24 hrs. (A)	P < 0.001	P<0.05
12 hrs. (S) vs. Control (A)	P < 0.001	P<0.05
12 hrs. (S) vs. 8 hrs. (A)	P < 0.001	P<0.05
12 hrs. (S) vs. 12 hrs. (A)	P < 0.001	P<0.05
12 hrs. (S) vs. 24 hrs. (A)	P > 0.05	P<0.05
24 hrs. (S) vs. Control (A)	P < 0.001	P<0.05
24 hrs. (S) vs. 8 hrs. (A)	P < 0.001	P<0.05
24 hrs. (S) vs. 12 hrs. (A)	P < 0.001	P<0.05
24 hrs. (S) vs. 24 hrs. (A)	P < 0.001	P<0.05

The inlet and proximal regions of the anatomical model were further analysed to investigate possible differences in cell morphology between the inner (myocardial) wall and the outer (pericardial) wall. A significant difference in shape index was found at the inlet region, with cells on the inner wall displaying a more elongated morphology ($p<0.0001$, unpaired t-test) (Figure 8). A similar trend was observed with the angle of orientation (not presented). In contrast, no such difference was observed for cells located at the proximal region ($p>0.05$, unpaired t-test).

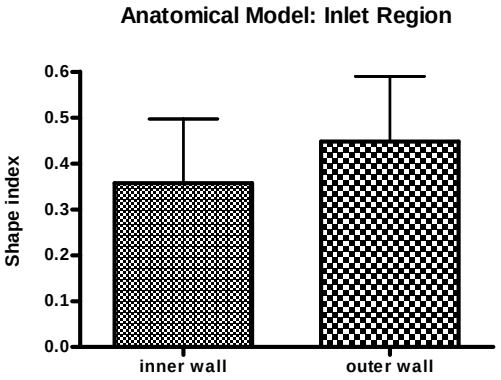

Figure 8: Bar graph illustrating cell shape index variation between the inner and outer walls of the anatomical model inlet

Discussion

In this study, we have shown that both simplified and anatomical models of very small diameter are able to support the culture of human abdominal aortic endothelial cells under static (no flow) conditions. In addition we proved that these cells remain adherent under a physiologic magnitude of wall shear stress during steady flow. To our knowledge, the anatomical cell culture model developed in this study is the first physiological cell culture model with arterial geometry. It is also the first model to replicate the coronary ostium and the inlet region.

As a control, a straight tubular model was created with the same inner diameter. Such a model was designed to expose cells to a uniform level of wall shear stress, while the anatomical model was designed to recreate the complex *in vivo* shear stress patterns seen in the human RCA.

In both models we observed a progressive elongation of cell shape, as well as a progressive orientation of the cells, in the axial direction following 8, 12, and 24 hours of flow. However, we observed significantly less cell elongation and cell alignment in the flow direction in the anatomical models than predicted by the corresponding identical experiment in the simplified model. These results suggest that such dramatic endothelial elongation as seen in simplified models may not be representative of the true EC behaviour.

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The comparison of the simplified and anatomical model at the inlet region serves to emphasize the importance of arterial geometry on the nature of flow and hence endothelial morphology. Even when comparing regions of the anatomical model that are relatively straight and have similar diameters, significant differences exist due to the dependence of local flow characteristics on upstream geometries. Therefore, in constructing an RCA model it is essential to try to preserve the true *in vivo* anatomy. This geometry has implications in cell-cell signalling, upstream release of cytokines and blood component/EC interaction.

Several present *in vitro* systems are limited to investigating the effect of one particular level of uniform shear stress on the endothelium. Using such models, EC remodelling and the regulation of gene expression by uniform laminar shear stress has been well documented [4]. It is well know, however, that wall shear stress patterns in arteries vary dramatically. Our results show that this causes endothelial cell structural variability. Furthermore, genomic analysis has confirmed the differential expression of endothelial genes under non-uniform shear stress. Simplified models fail to mimic the complex *in vivo* arterial wall shear stress patterns and the resulting biomechanical environment. By exposing all cells to the same level of shear, these studies mask local cell-cell signalling interactions present *in vivo* [5]. Our model provides an excellent opportunity to better test these interactions.

Our study is not the first to subject ECs to non-uniform wall shear stress; however, it is the first to use an anatomical geometry to accurately reproduce *in vivo* conditions. Dai *et al.* [6] simulated temporal gradients in shear stress by using time varying shear stress waveforms obtained computationally at two anatomical locations of the human carotid bifurcation. Although simplified, such studies are an improvement toward endothelial modeling incorporating variations in shear stress.

Idealized spatial gradients in wall shear stress have also been investigated. Particularly, modified parallel plate chambers have been used to generate shear gradients present in flow separation and reattachment. In these studies a rectangular barrier is placed perpendicular to the flow direction to try to create three defined areas of disturbed flow (reversal, reattachment, and recovery). It is difficult, however, to compare the cell morphology created by this relatively non-physiologic model with our results, as no flow separation or recirculation is likely to occur in the RCA [7].

At the inlet region of our anatomical model, an area prone to disease, we observed a difference in overall cell alignment between the inner (myocardial) wall and the outer (pericardial) wall, with cells on the inner wall showing a more elongated morphology. As opposed to the inlet region, at the mid-proximal curvature we observed no difference in cell morphology between the inner and outer wall.

Ojha *et al.* [8] found eccentric thickening, a precursor to atherosclerotic focal lesions, in the proximal region of the RCA along the inner wall. The inner wall was cited by other researchers to be the most prone region for the development of atherosclerosis due to its perceived lower wall shear stress [5]. Kirpalani *et al.* [9] reported spatial asymmetry in wall shear stress in the proximal region, with axial shear stress at the inner wall being much lower than that at the outer wall. Joshi *et al.* [7], however, observed no correlation between intimal thickening and wall shear stress patterns after performing computational flow studies on four geometrically different human RCAs. Contrarily, our results suggest that at the model inlet the inner wall, which showed greater cell alignment, is exposed to more uniform flow than the outer wall. Furthermore, we observed no difference in cell morphology on the inner and outer proximal walls. Such a difference would be expected from low/high wall shear stress patterns assumed to be present in these locations. In the aforementioned studies, however, an anatomical cast with sightly different geometry was used. The cast inlet was not captured but rather replaced with a straight section incapable of replicating the true *in vivo* upstream fluid dynamics. The inlet and proximal vessel dimensions were also different.

Understanding how the endothelium cell morphology is affected by local flow patterns is the first step in defining its response to flow. The findings of our study emphasize that a meaningful investigation of the vascular endothelium has to take into consideration the complexity of the flow environment to which these cells are exposed *in vivo.* This flow environment is dominated by the patient-specific arterial geometry. Therefore, *in vitro* systems designed to accurately model this geometry will help to more clearly define the functionality of the vascular endothelium under both physiological and patho-physiological conditions.

However, in the creation of such a model, a number of assumptions were made. These assumptions constitute limitations to the present study. We have used a monolayer of ECs to approximate the arterial wall, ignoring interactions with other vascular cells. We have also used cell culture media as a blood substitute, and have not considered the effects of blood components.

Large variations exist in the anatomical structure of human coronary arteries [7]. Therefore, it is difficult to generalize our patient-specific findings to all the cases that may be encountered in the human coronary arterial tree. To be able to draw such conclusions the current study should be repeated with several other casts.

We have assumed steady flows, and have not considered the time varying characteristics of blood flow, geometrical changes due to the motion of an artery during the cardiac cycle, or the effect of branches. Branching patterns, particularly in the RCA, can vary significantly between individuals. However, compared to the left coronary artery, branches in RCA tend to be small relative to main trunk of the artery.

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Lastly, by the application of steady flow we have chosen to investigate the effect of shear stress on EC morphology. Although, the model we have created is fabricated from a material with distensible properties, we have not attempted to quantify or replicate transmural pressure forces such as cyclic strain.

Conclusions

Endothelial cells play an important role in the development and progression of atherosclerosis. The morphology of these cells is known to correlate with the local hemodynamic environment in arteries *in vivo*. The remodeling of the vascular endothelium in response to flow is a highly dynamic process that is dependent on the flow environment. *In vivo*, this environment is dominated by arterial geometry and plays an essential role in the activation of mechanosensitive signaling pathways linked to atherosclerosis. In order to study this role it is necessary to develop realistic and dynamic EC culture models. Although present simplified tubular models have evolved to more accurately replicate the *in vivo* hemodynamic environment, geometrical considerations are still largely ignored. In this study we have presented the groundwork for a new anatomically realistic *in vitro* cell culture model which can be used to better simulate the complex *in vivo* wall shear stress patterns present in the human RCA. This model showed significant differences in EC morphology even when compared to the most advanced idealized flow systems. Since structure and function are intimately linked, it is concluded that realistic wall shear stress patterns created by anatomic geometries are vital to the study of shear induced atherosclerosis. Lastly, our model will be beneficial not only in further elucidating the role of ECs in atherosclerosis, but also in the design and testing of vascular constructs.

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