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Endothelial Cell Culture Model of Carotid Artery Atherosclerosis

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Abstract—**Atherosclerotic lesions form non-randomly at locations in bends and bifurcations where the local flow can be classified as 'disturbed flow' and is associated with low shear stress oscillatory or reciprocating flow. Endothelial cells *in vivo* are constantly exposed to mechanical stimulation due to hemodynamic loading in the form of pulsatile pressure, cyclic stretch and shear stress to maintain phenotype and control function. In conditions like atherosclerosis, the pressure and strain loading remains the same whereas the local fluid flow behavior and shear stress are altered. Common *in vitro* models of atherosclerosis focus primarily on shear stress without accounting for pressure and strain loading. To overcome this limitation, we used our microfluidic Endothelial Cell Culture Model (ECCM) to achieve accurate replication of pressure, strain and shear stress waveforms associated with both normal flow seen in straight sections of arteries and disturbed flow seen atherosclerosis lesion susceptible regions. We specifically recreated mechanical stresses associated with the proximal internal carotid which is a major risk factor for stroke. Cells cultured using both conditions show distinct differences in alignment and cytoskeletal organization. In summary we recreated pressure, stretch and shear stress loading seen in straight sections and in the proximal internal carotid in a cell culture compatible platform.**

I. Introduction

Atherosclerosis or arteriosclerotic vascular disease is a pathological condition that results in increasing arterial wall thickness and stiffness due to buildup of monocyte derived macrophages, modified lipoproteins and lipids like cholesterol. In recent years, atherosclerosis has been recognized as a chronic inflammatory disease as opposed to a simple phenomenon of lipid deposition. This inflammatory process involves a complex interplay between lipoproteins, lipids, activated blood leukocytes and inflamed endothelium (endothelial cells (ECs) and smooth muscle cells (SMCs)) which dictates initiation and progression of wall thickening. In ECs, proinflammatory stimuli like hypercholesterolemia, hyperglycemia, hypertension, smoking and elevated levels of serum C-reactive protein trigger the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), cell surface integrins and selectins, causing circulating monocytes and lymphocytes to attach to ECs and transmigrate into endothelium within the arterial intima, monocytes differentiate into macrophages, internalize low density lipoprotein via scavenger receptor mediated endocytosis and amplify local inflammation via secretion of cytokines and growth factors. Growth factors induce SMC migration into lesions and deposition of extracellular matrix (ECM) proteins causing lesions to expand, form necrotic cores with cholesterol crystals, calcify causing arteries to narrow, decreasing blood flow and resulting in pain or limited function, as in conditions like angina, congestive heart failure and peripheral vascular disease. Plaques can also suddenly rupture, leading to thrombus formation and vessel occlusion increasing the risk of myocardial infarction and stroke, which together are responsible for ~ 50% of mortalities in developed nations.

Atherosclerotic lesions preferentially occur at vascular niches proximal to vessel branches and bends including several locations in the aortic arch, ascending and descending aorta and coronary and carotid arteries. At these locations, the local flow behavior is characterized as 'disturbed' and is associated with low shear stress recirculation, oscillation or lateral flow (average: < 4 dynes/cm^2). In comparison straight regions of arteries with 'normal' high shear stress laminar pulsatile flow (average: > 10 dynes/cm^2, maximum: < 100 dynes/cm^2). Several *in vitro* and *in vivo* studies[1-2] evaluating the effect on shear stress associated with disturbed flow have shown generation of ECs with polygonal morphology, random alignment of actin filaments, high EC turnover, slow EC migration, increased permeability, increased gene expression and expression of pro-inflammatory markers like platelet derived growth factor (PDGF), monocyte chemotactic protein-1 (MCP-1), VCAM-1, ICAM-1.

In addition to shear stress, ECs *in vivo* are also exposed to pulsatile pressure (normal: 120/80 mm Hg) and cyclic stretch (normal: ~ 6-11%) at around 80 bpm. Several recent studies have evaluated the response of ECs to both physiological and pathological levels of cyclic stretch[3-5]. The dynamics of EC response to cyclic stretch depends greatly on the magnitude, duration and frequency of application. Evaluation of cyclic stretch on ECs accomplished using flexible substrates reveals alignment of ECs perpendicular to the direction of stretch[6] (in the direction of flow in blood vessels) and stimulation of stretch-activated ion channels for Ca^{2+} ion transport.[7] *In vitro* studies demonstrate that short term stretch results in modulation of vessel tone through synthesis of superoxide[8] known to play a role in vasoconstriction whereas prolonged

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exposure to stretch resulted in increased generation of vasoactive mediators NO and ET-1[9], expression of angiotensin receptor II (ANG II-R)[10] and pro-inflammatory markers such as IL-8,[3] MCP-1,[3] ICAM-1[11] and VCAM-1.[11] Physiological levels of cyclic stretch have also been shown to significantly improve endothelial barrier function whereas increased levels of stretch seen in conditions like hypertension decreases barrier function.[12] Cyclic stretch also induces expression of matrix metalloproteinase (MMPs) 14 and 2 and increased production of tumor necrosis factor-α (TNF-α)[13]. Relatively fewer *in vitro* studies have focused on the direct effects of pressure on EC structure and function.[14] This can be attributed to the assumption that pulsatile pressure from blood flow causes the blood vessel to stretch and therefore the overall effect of pressure manifests itself primarily in the form of stretch. However, evaluation of pressure on ECs *in vitro* shows that pressure alone in the absence of stretch results in increased EC proliferation, cytoskeletal reorganization and synthesis of ECM proteins.[15-17] Further, hydrostatic pressure indirectly affects cultured EC monolayer permeability via NO[18] and Ca^{2+} signaling.[19] Elevated hydrostatic pressure mediates an increase in Ca^{2+} transport into cultured ECs, which in turn reduces the permeability of the cultured EC monolayer.[19] Cyclic stretch, particularly in environments with low shear stress may play an important role in atherogenesis.

Therefore it is important to accurately replicate mechanical stresses associated with both physiological and pathophysiological conditions to fully recreate the *in vivo* environment. To accomplish this we have developed an Endothelial Cell Culture Model (ECCM) that can be used to culture cells under normal and disturbed flow conditions seen in atherosclerosis while maintaining pressure and stretch at normal levels. Using this model we demonstrate culture of human aortic endothelial cells (HAECs) under fluid flow and mechanical loading conditions associated with carotid artery atherosclerosis which is a significant risk factor in stroke.

II. Materials and Methods

Endothelial Cell Culture Model

The ECCM **(Fig 1.)** is composed of a peristaltic pump to induce and control flow, a cell culture chamber with a compliant thin membrane that mimics a vessel wall, a pneumatically driven pulsatile chamber, a one-way valve, 3 tunable flow resistance elements to adjust preload and afterload, and 3 tunable compliance elements that represent arterial and venous compliance. The peristaltic pump flow rate determines the average levels of shear stress within the chamber. The cell culture chamber is a rectangular channel 0.75 mm x 5 mm x 70 mm made of PDMS and consists of rigid walls on three sides and 500 μm thick flexible membrane at the bottom on which cells are cultured. This thin (500 μm) membrane allows generation of physiological levels of stretch (5-25% constant strain and 5-10% cyclic strain) to the cells in response to applied pressure within the chamber. The main component that introduces pulsatility or contractile function within the ECCM is a pneumatically actuated collapsible chamber. The applied pressure, percentage systolic/diastolic fraction and frequency (bpm) can be manipulated to alter frequency and amplitude of pressure and flow waveforms. In addition to this chamber, tunable compliance and flow resistance elements upstream of the inlet (pulmonary) and downstream of the outlet of the cell culture channel (aortic/systemic) allow modulation of flow resistance and modulation of shape and amplitude of attained pressure and flow profiles. A one-way valve placed between the pulsatile and cell culture chambers ensures prevention of retrograde flows.

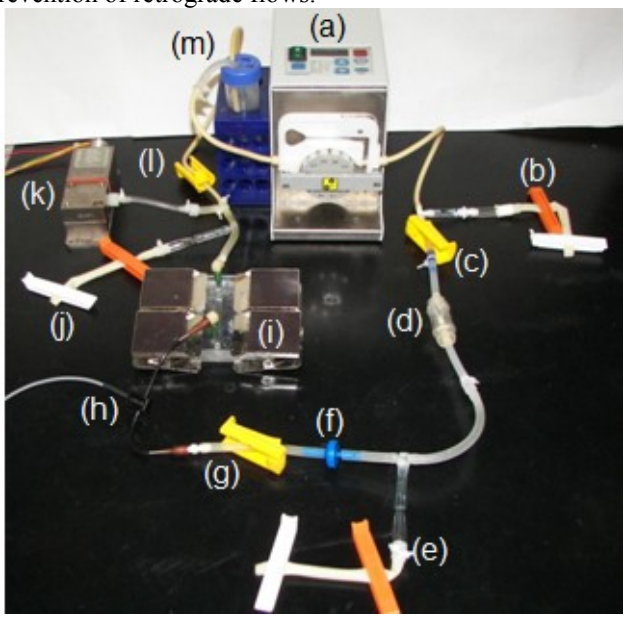

Fig. 1: Picture of the actual ECCM flow loop setup: **(a)** peristaltic pump, **(b)** pulmonary compliance 1, **(c)** pulmonary resistance 1, **(d)** collapsible chamber, **(e)** pulmonary compliance 2 **(f)** one-way valve (removed to generate disturbed flow), **(g)** pulmonary resistance 2 **(h)** inline flow sensor, **(i)** cell culture chamber, **(j)** aortic/systemic compliance, **(k)** inline pressure sensor, **(l)** aortic/systemic resistance and **(m)** medium reservoir.

Generation of Normal and Disturbed Flow

The setup mentioned above was used to generate both normal and disturbed flow patterns typically seen in normal (straight arteries) and in atherosclerosis susceptible like the proximal internal carotid. To recreate the disturbed flow, the one-way valve located prior to the cell culture chamber was removed. In addition, the flow rate of the pump was decreased to reduce flow velocity and wall shear stress.

Pressure, Stretch and Shear Stress Measurement

Pressure and flow measurements were accomplished using inline pressure and flow sensors (Validine, San Fransisco, CA and Transonics System, Ithaca, NY. respectively) as previously reported[20]. The strain values were measured using laser induced fluorescence to determine the membrane deflection under different applied pressure with the use of an imaging setup whereas shear stress was estimated based on channel dimensions, rate of pulsatility and flow rate similar to described previously[20].

Culture of HAECs within the ECCM

HAECs (Invitrogen, Carlsbad, CA) were initially cultured in standard tissue culture flasks with Medium 200 (Invitrogen, Carlsbad, CA) supplemented with Low Serum Growth Supplement (LSGS; Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin. Prior to seeding within the cell culture chamber in the ECCM, the surface of the cell culture chamber was treated with oxygen plasma for 30 second, then with 50 mg/ml of fibronectin for 12 hrs at 4^0C and for 30 min at 37^0C to promote cell adhesion. Then, fibronectin was removed by washing the device with cell culture medium, HAECs cells were seeded at a density of 0.5 million cells/ml. After, 4h, once the cells attach and spread, the medium was replaced with fresh medium and maintained in culture until they reached confluence (~24h). Then, the devices with the cells were assembled with the flow loop and gradually the fluid flow rate and pressures were increased until the values for the desired conditions were obtained. Further, the compliances, resistances and pulsatility were tuned to modulate the shape of the flow waveforms to recreate normal or disturbed flow conditions. Cells were cultured under normal and disturbed flow conditions for 24 hrs prior to analysis by confocal microscopy.

Evaluation of Shape and Alignment of HAECs under Normal and Disturbed Flow

Cell alignment and shape was determined following visual examination of phase contrast microscopy images. To determine F-actin microfilament arrangement, HAECs were fixed with 4% paraformaldehyde in 1x PBS for 20 min, washed two times with wash buffer (1x PBS containing 0.05% Tween-20 (Fisher Scientific, Fair Lawn, NJ)) and permeabilized with 0.5% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) for 2 min at room temperature. Then, cells were washed two times with wash buffer, and incubated at room temperature for 1h with TRITC-conjugated phalloidin (1:200; Millipore, Billerica, MA). Light Diagnostics mounting fluid (Millipore, Billerica, MA) was added to the cells and cells were examined using a Nikon Eclipse A1 Confocal Microscopy System (Nikon Instruments, Melville, NY).

III. RESULTS

Generation of Normal and Disturbed Flow within the ECCM

A setup similar to previously described was used to generate normal flow conditions whereas the setup was slightly modified by removal of the one-way valve and reduction of flow rate to generate reciprocating disturbed flow conditions. For normal flow, the ECCM was used to generate pressure, flow, strain and shear stress waveforms associated with normal (pressure 118/83 mm Hg systolic/diastolic, 13% constant strain, 6% cyclic strain, average flow of 28 ml/min, and average shear stress of 11 dynes/cm^2). As mentioned above, to obtain retrograde flow, the one-way valve located prior to the cell culture chamber was removed. The flow rate of the pump was also decreased to reduce flow velocity and wall shear stress. For these experiments, the pressure and strain within the system remained similar to the normal condition 120/80 mm Hg (systolic/diastolic) and 6-11% respectively. Shear stress however was significantly different with the disturbed flow condition (Mean ~ -0.5 dynes/cm^2, Max ~ 4 dynes/cm^2, Min ~ - 7 dynes/cm^2) in comparison to the normal condition (Mean ~11 dynes/cm2, Max ~ 57 dynes/cm^2, Min ~ - 7 dynes/cm^2). Transient negative values for shear stress in the normal condition does not represent significant retrograde flow; rather it represents the closure of the one-way valve, which is very similar to closure of the aortic valve *in vivo*. Unlike the disturbed flow condition, the negative values are not sustained as can be seen from the flow and shear stress plots **(Fig. 2)**.

Evaluation of HAECs within the ECCM

To evaluate the ability of the ECCM to recreate disturbed flow conditions and confirm ECs cultured under these conditions exhibit morphological changes that indicates the presence of disturbed flow, HAECs were cultured within the ECCM under normal and disturbed flow conditions as defined previously. HAECs were maintained under these conditions to allow fluid flow induced structural remodeling. After 24h, the cells were fixed, permeablized and evaluated by phase contrast microscopy and confocal microscopy. Cell size, shape, orientation along with staining for F-actin clearly indicate that cells cultured under disturbed flow exhibit a randomly oriented polygonal phenotype with expression of low levels of F-actin, characteristic of proatherogenic phenotype **(Fig. 3B and D)**. On the other hand, cells cultured under normal condition attained an ellipsoidal shape and show good alignment of F-actin in the direction of flow **(Fig. 3A and C)**.

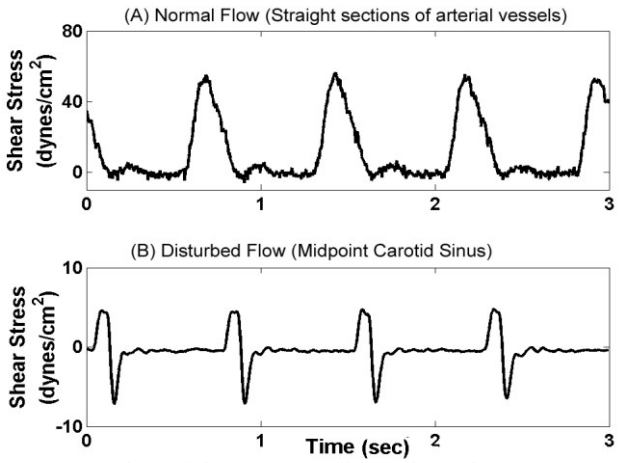

Fig. 2: Comparison of shear stress profiles used to culture HAECs under **(A)** Normal and **(B)** Disturbed flow conditions. The shear stress waveform closely mimics the shear stress waveform seen in the proximal internal carotid.

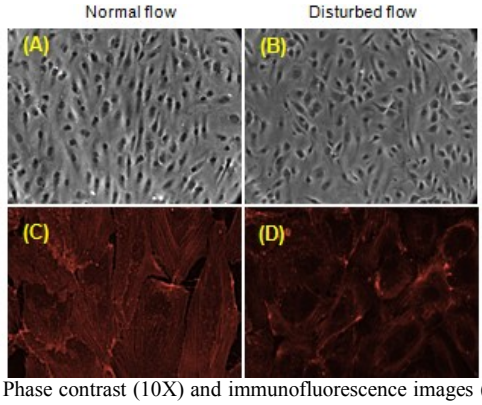

Fig. 3: Phase contrast (10X) and immunofluorescence images (40X) of HAECs cultured within the ECCM for 24 h. Phase contrast images of cells cultured under conditions of **(A)** normal and **(B)** disturbed flow cultured. Immunofluorescence microscopy images of HAECs to visualize actin microfilaments under **(A)** normal and **(B)** disturbed flow conditions. It can be clearly seen from the images that HAECs cultured under normal flow exhibit thick, aligned and evenly distributed F-actin in comparison to HAECs cultured under disturbed flow conditions that show short and randomly oriented F-actin localized closer to the cell periphery.

IV. Discussion

The coronary artery, carotid artery and various locations in the aorta and the aortic arch have high incidences of atherosclerotic lesions. This study focused on carotid artery atherosclerosis which is associated with the high risk for stroke. The flow waveforms close to the midpoint carotid sinus are unique with both positive and retrograde flow. To achieve this waveform, we removed the one way-valve and modulated the resistances and compliances. The shape and magnitude of shear stress values attained with the ECCM matches i*n vivo* observed values to within ± 5%. Also, the pressure and stretch values remained at normal levels for both the normal and disturbed flow conditions. Our preliminary proof of concept studies demonstrates the ability of the ECCM to accomplish HAEC culture under flow, pressure and stretch conditions seen in carotid artery atherosclerosis seen near the midpoint carotid sinus. We also clearly demonstrate visual differences between HAECs cultured under normal and disturbed flow conditions. Cells cultured under disturbed flow conditions are randomly oriented with randomly oriented actin cytoskeletal filaments.

In summary, we developed and validated the ECCM platform as a suitable model system for culture of endothelial cells under normal and disturbed flow conditions seen in atherosclerosis regions. By mimicking mechanical stresses seen in atherosclerosis susceptible regions in addition to replicating the fluid flow behavior, we demonstrate the ability to accurately mimic the mechanical loading environment *in vivo*. This system can be used to culture endothelial cells and recreate mechanical loads associated with various pathological conditions.

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