ENDOTHELIAL CELL RESPONSE AND LEUKOCYTE ADHESION IN AN ASYMMETRIC STENOSIS MODEL: ROLE OF FLUID WALL SHEAR STRESS GRADIENTS

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‘Anyone who has never made a mistake has never tried anything new’

- Albert Einstein
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ABSTRACT

The focal nature of atherosclerosis has been associated with complex geometries. The response of endothelial cells to hemodynamics is believed to be linked to atherosclerotic plaque development, progression and rupture. Great advances have been made in our ability to study cellular responses to mechanical forces. Unfortunately, the inability to recreate in vitro the realistic in vivo mechanical stimuli may be obscuring our understanding. In order to determine the mechanistic link between hemodynamics and plaque stability, three dimensional in vitro cell culture models were designed and biomolecular techniques were adapted to analyze endothelial cell function. Dextran was used as a supplement to increase the growth medium viscosity and its effects were characterized. Straight/tubular in vitro models were used to study the acute and long term response of endothelial cells to wall shear stress (WSS) of different magnitude. Anatomically realistic and asymmetric stenosis models were created in order to examine the morphological response of endothelial cells to complex hemodynamic forces. Within the stenosis models, the regional adhesive properties of neutrophils were tested as well as the localized expression of inflammatory molecules. Results show that appropriate time matched dextran containing static controls are required as this additive modified inflammatory marker cell expression both under static and flow conditions in a concentration and time dependent manner. Endothelial cells exposed to wall shear stress altered their morphology depending on the magnitude and duration of exposure. Morphological adaptation was sensitive to the spatial wall shear stress gradients present in both the asymmetric stenosis and the anatomically realistic models. Neutrophil adhesion and inflammatory marker expression differed in the spatial WSS gradient regions of the asymmetric stenosis models. This study highlights the possible role for spatial wall shear stress gradients in the development and progression of atherosclerotic plaques, through the localized analysis of inflammatory markers and neutrophil adhesion.
L’athérosclérose est une maladie qui se développe localement où le flux sanguin est perturbé. Les cellules endothéliales sont soumises en permanence à des contraintes mécaniques et leur réponse a été reliée au développement et à la progression de l’artériosclérose. Différentes chambres d’écoulement ont été développées in vitro afin d’étudier la réponse des cellules endothéliales aux forces de cisaillement et de grandes avancées ont été faites afin de mieux comprendre les réponses cellulaires à l’écoulement sanguin. Ces modèles ne peuvent reproduire adéquatement la complexité des conditions présentes in vivo. Ainsi, afin de déterminer le lien entre les forces hémodynamiques et la stabilité des plaques artérioscléreuses, des modèles tridimensionnels ont été développés ainsi que les techniques biomoléculaires nécessaire afin d’étudier la fonction des cellules à l’intérieur de ceux-ci. Du dextran a été utilisé comme supplément afin d’augmenter la viscosité du média de culture et son effet sur les cellules a été caractérisé. Des modèles tubulaires ont été utilisés afin d’étudier la réponse à court et à long terme des cellules endothéliales à des forces de cisaillement de diverses magnitudes. La réponse morphologique des cellules endothéliales, suite à l’exposition d’un débit constant, a été étudiée dans des modèles idéalisés de sténoses asymétriques et réalistes anatomiquement. L’expression régionale des cellules endothéliales et l’adhésion locale de neutrophiles dans les modèles sténosés ont été quantifiées en fonction de la durée et de la magnitude des forces de cisaillement. Les résultats obtenus démontrent que le dextran peut affecter la réponse des cellules et ainsi les contrôles appropriés doivent être utilisé. La morphologie des cellules endothéliales varie avec la durée et la force du cisaillement ainsi que dans les régions où des gradients sont présents. L’adhésion et l’expression sont modifiées dans les régions où l’écoulement est perturbé. L’utilisation de modèles réalistes et tridimensionnels, présentant différentes forces de cisaillement, augmente la compréhension de l’influence des forces hémodynamiques sur la réponse des cellules endothéliales, et conséquemment, sur le développement et la progression des maladies cardiovasculaires.
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PREFACE

This thesis was completed in the chemical engineering department of McGill University from September 2006 to August 2009. The research was performed under the supervision of Dr. Richard L. Leask at McGill University and at the Montreal Heart Institute. Since May 2003, I have undertaken the project of developing novel \textit{in vitro} models to study endothelial cell response to geometrically driven hemodynamics as they are believed to play a role in the development and progression of cardiovascular diseases. I have developed techniques to reliably expose endothelial cells to complex realistic three dimensional flow fields. I have attended national and international conferences and was awarded the best vascular research at the Quebec Vascular Society (November 2005, Montréal, QC, Canada, 1000$) for my MEng thesis work, a first prize poster in fundamental research at the Montreal Heart Institute Day (June 2006, Montréal, QC, Canada, 1000$), the 3\textsuperscript{rd} poster prize (Biofluids and Imaging) at the Summer Bioengineering Conference (ASME, June 2007, Keystone, CO, USA, 100$) and an oral presentation award at the Canadian Biomaterials Society (May 2009, Québec, QC, Canada, 100$).

During my BEng and MEng, I have been involved in several projects other than my main research. I participated in the mechanical testing of different tissues. These include mechanically testing aortic and pericardial tissue in order to characterize the regional mechanical properties in an attempts to understand pathology development and progression \textsuperscript{1}. I was also closely involved in the testing of genetically modified mouse skin, to verify the implications of fibulin-5 related diseases \textsuperscript{2}. I performed a study on the conservation, after formalin fixation, of the localized mechanical properties of canine descending thoracic aortas. Most of my experience is related to physiologically replicating \textit{in vivo} situations and studying pathologies such as atherosclerosis. My solid engineering background has helped me to develop more accurate and realistic ways of studying biomechanics. Also, I have gained a realistic view of academic and
institutional research, acquired a great amount of experience and learned to work efficiently in a laboratory.

During an NSERC USRA project under the supervision of Dr. Richard L. Leask in 2003, I started the development of three dimensional \textit{in vitro} cell culture models. I worked with Kate Markey, an Australian student which developed the initial moulds used to build the models. I continued my project during the school year, with Lindsay Lessard on the establishment of cell lines, cell cryopreservation and culture on Sylgard\textsuperscript{TM} coated petri dishes. During another NSERC USRA in 2004, I designed acrylic moulds to cast the lumen of the models, using low melting alloy, both for straight/tubular and asymmetric stenosis geometries (6 mm diameter). At the Montreal Heart Institute, I tested porcine aortic valves and coronary arteries which I dissected, got grips made for pressure inflation tests and learned on plaque stability and imaging techniques. I performed uniaxial tensile testing for a study on canine and bovine descending thoracic aortas with Nusrat Choudhury (MEng).

I finished my BEng in December 2004 and started my MEng in January 2005. I collaborated with Dr. Mehri at the Montreal Heart Institute, developing the experimental set-up to perform platelet adhesion assays, a project which was continued by Jeff Shrum (MEng). During my MEng, I designed physiological size (3.175 mm diameter) human coronary artery asymmetric stenosis and straight/tubular models. During my PhD (2006-2009), I extended the work I had done during my MEng, in the development of realistic \textit{in vitro} models for studying cardiovascular diseases and endothelial cell pathobiology. This thesis is composed of 5 manuscripts prepared during my PhD degree related to the study of endothelial cell response to hemodynamic forces.

When using \textit{in vitro} models, it is often required to modify the growth media properties using dextran. In order to fully examine the effects of this additive on the behaviour of endothelial cells, a study was performed. I measured dextran solution viscosity and density in 2006. At this point, we were autoclaving dextran in water before adding it to the cell growth media, a procedure I improved and modified by mixing dextran directly in growth media and filtering the
resulting solution. The effects of dextran on cell proliferation and response were performed with Raffi Afeyan in 2006 and completed with Joanna Rossi in 2009 (Article 1, Submitted).

I was involved in the development anatomically realistic in vitro models from the casting of in vivo geometries from pathologic samples, which was a project initiated by Monica A. Farcas (MEng) and continued by Helen Lentzakis (MEng). I contributed to the experimental troubleshooting and was responsible for the resubmission process of this article (Article 2, Submitted).

It is believed that there is a causal link between mechanical forces and atherosclerosis development and coronary plaque stability. During my MEng thesis, I developed straight/tubular and idealized coronary artery stenosis models. I performed morphological studies, which I completed during my PhD (Article 3, Submitted) as the computational and photochromic flow visualization studies were completed.

After the poster competition of the Quebec Vascular Society, Ian B. Copland (AccelLab INC) contacted us and provided cells line to examine neutrophil adhesion in the asymmetric stenosis models (Article 4, Submitted). This work was completed in 2008 after insightful discussions with Morton H. Friedman at the BMES conference in Saint-Louis on the endothelial cell preshearing effects on the adhesion in the different regions of the models.

Immunofluorescence techniques were developed to image the cells in the complex three-dimensional models during my MEng thesis, allowing the localized analysis of protein expression of inflammatory markers and vasoactive molecules. Biomolecular techniques, such as Western blotting and real-time PCR were developed to quantify expression of proteins and mRNA. These were adjusted to be applicable to the in vitro models. The analysis of the endothelial cell expression in straight/tubular models was achieved during the last year of my PhD (Article 5, In progress). Additionally, regional analysis of the local fluorescence intensity is currently being performed in order to be added to the manuscript to be submitted.
CONTRIBUTION OF AUTHOR

The content of this thesis is adapted from manuscripts that were submitted or that are in preparation in which the author made a substantial contribution. Unless noted otherwise, the experiments described in this thesis are the sole work of the author. Dr. Richard L. Leask had significant input into the experimental design and the editing of the manuscripts. Additionally, Lisa Danielczak provided assistance in cell culture. Joanna Rossi developed the mRNA analysis procedure and contributed to the design of experiments. In all cases, Louis R. Villeneuve assisted with the acquisition of the confocal images and Monica A. Farcas helped in the initial troubleshooting.

The work included in this thesis was presented at several conferences and resulted in 20 conference proceedings, including 9 oral and 11 poster presentations, in 4 submitted publications (Physical Review E, BioMedical Engineering OnLine, American Journal of Physiology – Heart and Circulatory Physiology and Journal of Biomedical Engineering) and in 1 publication to be submitted. It was awarded 4 research presentation prizes; additionally, a report of invention (ROI # 10021, McGill July 2009) has been filed regarding the three dimensional in vitro cell culture technique developed.
Article 1. Concentration and Time Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression in Vitro

Leonie Rouleau, Joanna Rossi, Richard L. Leask

Contributions
L.R. had the general idea for the publication, designed and performed the research in collaboration with J.R. and Raffi Afeyan, collected the viscosity and density data, analyzed and interpreted the proliferation, attachment, adhesion, protein (confocal and western blotting) and some mRNA expression data, performed statistical analysis and wrote the manuscript.
J.R. performed part of the research in collaboration with L.R., collected, analyzed and interpreted mRNA data, contributed to the troubleshooting, helped in the experimental design and revised the manuscript.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

This article was adapted from the version submitted August 25th 2009 (BMDB-S-09-001214) to Biomechanics and Modeling in Mechanobiology. (Submitted)
Article 2. The Development of 3-D, In Vitro, Endothelial Culture Models for the Study of Coronary Artery Disease

Monica A. Farcas, Leonie Rouleau, Richard Fraser, Richard L. Leask

Contributions
M.A.F. designed and performed the research, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.
L.R. helped editing the manuscript, gathered part of the data, resubmitted the manuscript and completed the response to reviewers.
R.F. provided access to human tissue.
R.L.L. helped editing the manuscript and interpreting the data, funded the research and contributed to the troubleshooting and the experimental design.

This article was adapted from the version submitted June 30th 2009 (MS ID: 1471216454286138) to BioMedical Engineering OnLine. (Submitted)
Article 3. Endothelial Cell Morphologic Response to Asymmetric Stenosis Hemodynamics: Effects of Spatial Wall Shear Stress Gradients
Leonie Rouleau, Monica A. Farcas, Jean-Claude Tardif, Rosaire Mongrain, Richard L. Leask

Contributions
L.R. designed and performed the research, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.
M.A.F. contributed to the development of the procedure used to grow the cells in the models and the making of the moulds.
J.C.T. provided funding and contributed to the initial troubleshooting of the experimental set-up.
R.M. designed the stenosis model geometry and provided funding.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

This article was adapted from the version submitted July 8th 2009 (BIO-09-1207) to the Journal of Biomechanical Engineering. (Submitted)
Article 4. Neutrophil Adhesion on Endothelial Cells in a Novel Asymmetric Stenosis Model: Effect of Wall Shear Stress Gradients
Leonie Rouleau, Ian B. Copland, Jean-Claude Tardif, Rosaire Mongrain, Richard L. Leask

Contributions
L.R. designed and performed the research, collected, analyzed and interpreted the adhesion data, performed statistical analysis and wrote the manuscript.
I.B.C. provided the NB4 cells and the general idea for the publication.
J.C.T. provided resources at the Montreal Heart Institute and contributed to the initial troubleshooting of the experimental set-up.
R.M. designed the stenosis model geometry and provided funding.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

This article was adapted from the version submitted July 15th 2009 (#: H-00666-2009) to American Journal of Physiology – Heart and Circulatory Physiology. (Submitted)
Article 5. Inflammatory Response of Human Aortic Endothelial Cells in Stenotic Hemodynamics Environment

Leonie Rouleau, Joanna Rossi, Richard L. Leask

Contributions
L.R. designed and performed the experiments, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.
J.R. developed the mRNA analysis extraction and quantification procedure as well as the cDNA and quantitative real-time PCR methods.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

This article has yet to be submitted (In progress). A quantitative regional analysis is in progress in order to compare the expression within the different regions of the model.
PUBLICATIONS

Published in referred journal


Submitted in referred journal


Manuscript in preparation


CHAPTER 1: INTRODUCTION

Coronary artery disease is one of the leading causes of death and hospitalization in North America. Atherosclerotic plaques, which may lead to vessel stenosis, develop at focal regions where blood flow is disturbed, such as bifurcations and curvatures. It has been hypothesized that mechanical forces may account for the non-random distribution of atherosclerosis. Hemodynamic forces are known to influence endothelial cells. These cells are known to mediate the development of coronary artery disease as they regulate vascular tone, inflammation, thrombosis and vascular remodeling. Wall shear stress alone can alter endothelial cell phenotype to one that promotes atherosclerosis and potentially thrombus formation.

Much of our knowledge on endothelial cell response to wall shear stress has been obtained from simplified cone-and-plate and parallel-plate in vitro models. Blood components have similarly been shown to be activated, in simplified models, by shear stress, creating conditions promoting thrombus formation. However, relating these results to clinical manifestations has been limited by the simplistic and unrealistic hemodynamic environment created in these models. Few in vitro flow experiments have been performed in realistic three-dimensional compliant geometries or examined spatial wall shear stress gradients.

Once an obstruction has developed in a vessel, blood flow is further disturbed and hemodynamic forces continue to play an important role as the stenosis progresses. Blood flow in stenotic vessels creates complex hemodynamic patterns with both positive and negative spatial wall shear stress gradients, usually absent in conventional in vitro models. As geometry is fundamental to reproduce realistic wall shear stress, it is not surprising that we have been unable to elucidate the mechanisms of endothelial cell shear activation in disease progression and plaque stability.

Straight/tubular transparent compliant models as well as anatomically realistic coronary artery and idealized eccentric atherosclerotic plaques in vitro models were designed. Dextran was used as a supplement to increase the growth
medium viscosity and its effects were characterized. The *in vitro* cell culture models were used to characterize the response of endothelial cells to spatial wall shear stress gradients, in terms of morphology and function, as well as the adhesion of neutrophils in a stenotic geometry in order to determine the mechanistic link between hemodynamics and plaque stability.
CHAPTER 2: OBJECTIVES

The underlying hypothesis of this work is that the spatial wall shear stress gradients created by a complex three dimensional coronary geometry can cause endothelial dysfunction. The response is hypothesized to trigger endothelial cell inflammatory pathways causing localized blood component adhesion linked to plaque rupture. Specifically, this work provides information on the inflammation response of endothelial cell in a novel three dimensional in vitro asymmetric stenosis hemodynamic environment.

The aim of this work was to assess the localized time dependent effects of wall shear stress magnitude and spatial gradients on vascular cells in a stenotic environment.

The specific objectives included:

- designing straight/tubular and symmetric stenosis in vitro models
- developing reliable endothelial cell culture and perfusion procedures
- adapting biomolecular techniques to analyze the cells within the models
- verifying the effects of dextran on endothelial cell function
- quantifying regional cell morphology in the models
- analyzing protein and gene expression dependence on the wall shear stress magnitude and duration of exposure in straight/tubular in vitro models
- observing localized cell expression through confocal microscopy in the spatial wall shear stress gradients present in the stenosis model
- characterizing localized neutrophil adhesion in the stenosis model.
CHAPTER 3: LITERATURE REVIEW

3.1 Cardiovascular System

3.1.1 Physiological Functions

The cardiovascular system includes the heart and blood vessels. It supplies nutrients and oxygen to the tissues and removes carbon dioxide as well as the by-products of metabolism. It maintains homeostasis and is responsible for body temperature regulation and fluid maintenance. The coronary circulation supplies the heart and maintains cardiac function, whereas the cerebral circulation services the brain.

3.1.2 Cardiovascular Diseases

Cardiovascular diseases are an important cause of mortality and morbidity in the World. Atherosclerosis may result in the partial or complete occlusion of blood vessels and cause several collateral pathologies. Imbalance in oxygen supply and demand due to partial obstruction of the coronary arteries can produce angina or myocardial ischemia. Severe myocardial infarction can result in cardiac arrest. In the cerebrovascular system, arterial occlusion can cause transient ischemic attacks or stroke. In all cases, occlusion is usually due to thrombotic emboli shed from an eccentric atherosclerotic plaque.

3.2 Artery Physiology

3.2.1 Anatomy of the Vascular Wall

The vascular wall consists of three layers, the tunica intima, media and adventitia, which vary in prominence in elastic and muscular arteries, Figure 3-1. Blood flows through the lumen of the artery in contact with the tunica intima. This layer consists of a monolayer of endothelial cells (EC) and is supported by an internal elastic lamina. With atherosclerosis, this layer thickens with smooth muscle cells migration and proliferation as well as increasing fibrofatty deposition over time. The thickest layer, the tunica media, largely defines the structural properties of the tissue. In coronary arteries, this layer is composed mainly of
vascular smooth muscle cells (VSMC) and extracellular matrix proteins, primarily elastin and collagen. Smooth muscle cells contribute to the vasoconstriction and vasodilatation of the arteries $^{39}$ and take part in vessel remodeling. The *tunica adventitia* is the outermost layer and lies outside of the external elastic lamina. It consists of dense fibroelastic tissue, mainly collagen and fibroblasts as well as small vessels referred to as the *vasa vasorum*.

![Endothelium location and blood vessel wall structure](image)

**Figure 3-1**: Endothelium location and blood vessel wall structure: *tunica intima, media* and *adventitia*.

### 3.2.2 Blood Components

Blood is a suspension of several cell types suspended in plasma. It contains red blood cells (erythrocytes), platelets (thrombocytes) and white blood cells (leukocytes). Red blood cells contain hemoglobin and are responsible of distributing oxygen throughout the body. Platelets are cell fragments circulating in the blood and are involved in coagulation $^{40}$. Leukocytes form a large family of cells divided into polymorphonuclear cells (granulocytes) and agranulocytes. The former are divided into eosinophils, basophils and neutrophils and the latter contain lymphocytes and monocytes. Blood components are important players in cardiovascular diseases as they regulate hemostasis and participate in the inflammation cascade. Neutrophils are the first white blood cells that adhere to a dysfunctional endothelium and monocytes participate in the progression of atherosclerotic plaques.
3.2.3 **Endothelium**

Endothelial cells form a continuous lining in blood vessels. They are flat, with a centrally located oval nucleus, slightly raised compared to the rest of the cell and elongated in the direction of blood flow. Under physiological conditions, the endothelium acts as a selective barrier controlling permeability and transport. It metabolizes factors to be circulated in the blood or acting locally in order to control the proliferation of underlying vascular smooth muscle cells and to regulate the adhesion and extravasation of neutrophils, monocytes and lymphocytes. Hence, it regulates homeostasis and thrombosis, vasoconstriction and vasodilatation as well as immune and inflammatory responses. ECs react to physical stimuli, such as mechanical forces, and chemical stimuli, such as blood borne, autocrine and paracrine mediators from within the circulation or from the vessel wall.

3.3 **Atherosclerosis**

Atherosclerosis is a chronic and progressive disease characterized by the hardening and thickening of blood vessels due to plaque formation in the inner vessel wall layer. Various factors such as genetics, cholesterol levels, hypertension, obesity, diabetes, smoking, lifestyle, age and gender are known to promote atherosclerosis. Atherosclerosis tends to remain clinically quiescent until late in the development of the disease. Indeed, vascular remodeling initially preserves the artery shape; however, atherosclerotic plaques can narrow the arterial lumen, causing the formation of a stenosis and leading to a reduction in the supply of blood to the myocardium. Plaque rupture can result in thrombus formation and occlusion of arteries, hence responsible for clinical events such as myocardial infarction and stroke.

3.3.1 **Focal Nature of Atherosclerosis**

The focal nature of atherosclerotic lesions suggests that mechanical forces and more specifically fluid shear stresses may play a major role in its
development. This distribution cannot be explained by lifestyle or risks factors alone as they are common throughout the vasculature. As presented by DeBakey et al. as well as Asakura and Karino, atheroprone regions include areas where blood flow is disturbed. Preferential sites in the arterial tree are the coronary arteries, the major branches of the aortic arch, the visceral arterial branches of the abdominal aorta and the terminal abdominal aorta and its branches \(^4,5\). These regions combine low and high wall shear stress magnitudes with large temporal and spatial fluctuations \(^54\).

Since atherosclerotic plaques are repeatedly found in disturbed flow regions, Ross et al. hypothesized that an injury to the endothelium can cause atherosclerosis \(^55\). It is now understood that local blood flow disturbances can stimulate endothelial cells creating a pro-atherogenic cell phenotype \(^7,9,17,56\).

### 3.3.2 Plaque Vulnerability

Plaque vulnerability is highly debated. Atherosclerotic plaques are believed to be vulnerable when filled with a lipid core and covered by a thin fibrous cap \(^57,58\). As the lesion progresses, inflammatory mediators cause expression of factors which can promote thrombus formation and the production of matrix metalloproteinases (MMP) that can weaken the fibrous cap, hence creating a vulnerable plaque \(^8,59\).

Susceptibility to rupture, fracture and fissure by mechanical stresses resulting from sudden changes in pressure, flow field or pulse rate is likely mediated by the response of endothelial cells within the plaque. Indeed, rupture of thin-cap fibroatheromas is the most common cause of arterial thrombotic events. Post-mortem studies have revealed that plaque disruption usually occurs at the shoulders \(^60-63\), where the cap is often thinnest and most heavily infiltrated with inflammatory cells \(^64\). It is believed that local hemodynamic factors and mass transport dictate plaque stability and rupture location.
3.3.3 Atherothrombosis

Endothelial dysfunction is considered the earliest process in the transition from a normal to a pathological state, which may lead to the formation of vulnerable plaque \(^8\). Activation of the endothelium leads to a cascade of reactions, which include leukocyte activation, rolling/tethering, adhesion and transmigration into the vessel wall. Neutrophils are one of the first white blood cells to adhere to the dysfunctional endothelium. Monocytes are attracted to the inflammation site and transform into macrophages, which become lipid-laden foam cells by engulfing low-density lipoproteins. Inflammatory cytokines and growth factors can cause vascular smooth muscle cell migration and proliferation \(^{48}\).

![Diagram of atherothrombosis](image)

**Figure 3-2:** Activated endothelial cell expression increase leukocyte adhesion and transmigration through the vessel wall, followed by monocyte transmigration and transformation into foam cells, accentuating atherosclerosis and eventually leading to atherothrombosis (Figures were produced using Servier Medical Art).

Atherothrombosis, characterized by an atherosclerotic lesion with a superimposed thrombus formation, is the major cause of acute coronary syndromes and cardiovascular death \(^{63}\), Figure 3-2. Clinically, atherosclerosis manifests through luminal narrowing or vessel occlusion. Disruption of the fibrous cap can expose the underlying thrombogenic fibers and the components constituting the plaque \(^{36,37}\). These thrombotic changes result from the activation of the clotting cascade by tissue factor, resulting in platelet activation and thrombin formation, potentially causing sudden occlusion \(^{36}\).

3.4 Mechanical Stresses

Vascular cells are known to respond to cyclic strain and hydrostatic pressure \(^{7,65-67}\), however, shear forces appear to have a dominating effect.
Mechanical stresses in the vessel are influenced by different factors such as the rheological properties of blood, vessel geometry, pulsatility, tissue mechanical properties and hydrostatic pressure.

### 3.4.1 Fluid Dynamics

For an incompressible Newtonian fluid, wall shear stress (WSS) (τ) is defined as the tangential force per unit area experienced at the fluid-wall interface. It is proportional to the dynamic viscosity (µ) of the fluid and the shear rate (γ), hence τ = µγ. The wall shear rate can be estimated by the slope of the local velocity distribution very close to the wall γ = dv / dr in one dimensional flow. For laminar steady fully developed flow in a tubular vessel, the shear rate can be estimated as γ = 32Q / πD³ where Q is the volumetric flow rate and D is the diameter.

Fluid parameters can be non-dimensionalized in order to reproduce physiological conditions. This is often required to make in vitro analysis possible. For steady flow, the Reynolds number (Re), which represents the ratio of inertial over viscous forces Re = ρV D / µ (where ρ is the density and V is the velocity) is of primary importance.

### 3.4.2 Blood Rheology

Blood is a suspension and may behave as a non-Newtonian fluid. Its viscosity varies with plasma viscosity, red blood cell deformability, aggregation and hematocrit. Extensive work has been done to characterize blood viscosity and different mathematical models were developed, such as the Carreau, the power law, the Casson and the generalized power law models. Blood behaves as a Newtonian fluid at shear rates greater than 200 s⁻¹ and has a viscosity of approximately 3.5 cP (0.0035kg/(m·s)) at low wall shear rates, blood viscosity increases dramatically, an effect due to the cross-linking of red blood cells. This occurs specially in capillaries. Wall shear rates in the human vasculature range from 50-2000 s⁻¹ with corresponding wall shear stresses between 5 and 200
dynes/cm$^2$ and can be as high as 3000 dynes/cm$^2$ at branches, curvatures and in stenosed arteries $^{69,70}$. Computational studies performed in realistic human right coronary arteries have validated the Newtonian approximation for blood viscosity for middle to high shear range $^{68}$.

### 3.4.3 Geometry

Coronary arteries are tortuous. A study on the influence of fluid parameters (inlet profile, geometry, pulsatility) on the blood flow patterns in human right coronary arteries has shown the three dimensional geometry to dominate the hemodynamics $^{71}$. Experimental determination of the WSS patterns in such complex geometries is difficult to accomplish. Computational studies can be performed on *in vivo* geometries $^{72}$. Replicating these geometries *in vitro* is a great challenge. Zeng *et al.* found that right coronary artery motion has little effect on the time average wall shear stress patterns $^{73}$ and that geometry has a large effect on the temporal variation but that variations due to pulsatility are greater. Hence, geometry is an important factor influencing the WSS patterns imposed on ECs *in vivo*.

### 3.4.4 Pulsatility

Blood flow is pulsatile. Pulsatility can result in important local temporal gradients and oscillatory wall shear stress. Current *in vitro* models have shown that endothelial cells exposed to temporal shear stress gradients may present atherogenic or atheroprotective phenotype depending on the flow waveform they are exposed to $^{9,16,17}$.

### 3.5 Shear Stress Patterns in Stenotic Arteries

In arteries containing a stenosis, complex hemodynamics are present $^{12,31,32}$, characterized by fluid acceleration upstream of the stenosis, with a maximum wall shear near the peak and a deceleration zone downstream of the stenosis with possible reverse flow depending on the Reynolds number $^{32,33,74-77}$.
These complex flow profiles should not be confused with turbulent flow, which implies random fluid movement. Pulsatility is important to consider as it modifies the wall shear stress patterns. Transient flow can influence the location of the stagnation point back and forth along the wall, altering the size of the recirculation zone, as well as the particle residence time. However, others have suggested that the time average WSS represents well the stimuli provided to ECs in coronary geometries.

Hemodynamic forces are known to modify a wide range of physiological, biochemical and gene regulatory responses. Blood components are activated by high shear rates present at the throat of the stenosis and endothelial cells have been shown to express inflammatory molecules in disturbed flow regions. The environment near stenotic regions may provide favourable conditions for prolonged interactions between activated blood components and dysfunctional endothelium by potentially increasing adhesion of circulating elements, such as lipoproteins, monocytes, leukocytes and platelets to the endothelium. Hence, fluid forces can influence disease formation, atherosclerosis progression and potentially thrombosis formation.

### 3.6 Current In Vitro Models

The characterization of mechanical stresses in complex tortuous geometries, such as those seen in vivo, requires extensive experimental work and/or numerical modeling. In atheroprone regions, flow is disrupted creating high and low wall shear stress regions, which themselves vary during the cardiac cycle due to the pulsatile nature of the flow and with vessel compliance. Hence, most of the work has been performed in simplified models.

The modified cone and plate as well as parallel-plate flow chambers are the most common tools that have been used to study endothelial cell response to wall shear stress. Although much has been discovered, current in vitro techniques may be obscuring our understanding by oversimplifying the stimulus imposed as they expose cells to laminar, unidirectional levels of shear stress in simplified geometries.
Typically, once flow is fully established in an experiment, the endothelial cells are exposed to temporally and spatially uniform fluid WSS, i.e. undisturbed laminar unidirectional flow. Both temporal and spatial gradients can be generated in vitro \(^9,15,16,18,30,79,94\). Idealized in vitro geometries with relatively predictable secondary flow patterns of separation reattachment and recirculation have also been developed.

### 3.6.1 Modified Cone and Plate Viscometer

Similar to a Couette viscometer, used in microrheological studies, shear is created between a flat plate and a cone with a low angle. Controlled uniform wall shear stress is produced by rotation of the cone with respect to the stationary plate on which cells are cultured, Figure 3-3. The hemodynamic patterns in this model are easily modeled assuming Newtonian, incompressible fluid and the no slip condition at the wall. Both temporal and spatial gradients can be implemented in the modified cone and plate viscometer models.

![Figure 3-3: Cone and plate viscometer, original (A) and modified with a step (B).](image)

Davies et al. applied wall shear stresses in the physiological range (0-15 dyn/cm\(^2\)) by modifying the viscosity, the cone angle and the rotational speed. Steady, periodic and oscillating shear stresses were imposed. Rapid sinusoidal change of shear stress at 60 cycles/min (1 Hz) were produced by tilting the cone with respect to the axis of rotation resulting in a slight wobble in the cone rotation, creating pulsatile flow \(^95-97\).

Nagel et al. secured coverslips in a stainless steel ring which contained a protuberance, in the form of a rectangular bar that disturbed flow immediately downstream from the bar. Further downstream, uniform laminar shear stress was re-established. The bar on each ring was aligned in the radial direction. In this
configuration, nearly concentric flow is created so that the streamlines strike the bar at a perpendicular angle, creating complex spatial shear stress patterns.  

3.6.2 Parallel Plate Flow Chamber

In parallel plate flow chambers, a microscope slide or coverslip is used to grow endothelial cells. Culture media is perfused through the chamber, generating well-defined wall shear stress on the endothelial cell surface. The height to width ratio of the apparatus is very small and allows the assumption of infinite parallel plates for which the fluid mechanics are well characterized, Figure 3-4.

![Figure 3-4: Parallel plate chamber, original (A) and modified with a step (B).](image)

However, wall shear stress may not be constant throughout the flow chamber due to possible machining defects which have been reported to result in variations in shear stress by as much as 11% within the chamber. McCann et al. found that these changes can lead to significant variations in mRNA expression in uniform flow regions.

Parallel-plate flow chambers have been modified to study the effects of temporal and spatial gradients on endothelial cell response. A complex hemodynamic environment can be created by using a step, creating flow separation and recirculation. Tardy et al. modified a parallel plate flow chamber by placing a rectangular obstacle perpendicular to the flow field. A sudden expansion flow chamber (SEFC) has also been characterized by White et al. to expose cells to spatial and temporal wall shear stress gradients. Finally, the vertical step flow chamber was designed and resemble closely the sudden expansion flow chamber.
Converging-width flow channels have also been developed to study the effects of shear stress gradients and magnitude on endothelial cells. These models present a broader range of spatial gradients, however, lack the three dimensional geometry. LaMack and Friedman results using this model support the notion that vascular endothelial cells are able to sense shear gradient and magnitude independently \(^{30}\).

### 3.6.3 Tubular Models

The endothelium is exposed \textit{in vivo} to wall shear stress, cyclic strain and pressure forces, which act in conjunction to influence flow-induced cellular responses. To simulate the rhythmic deformation of the arterial wall associated with the systolic-diastolic pressure changes, endothelial cells have also been grown in compliant tubes and subjected to rapid stretching in specially designed cyclic strain apparatus by controlling a gear pump using a pulse generator \(^{27,104-106}\). The effects of hydrostatic pressure have also been tested in silicon distensible tubes \(^{27}\). These models are more realistic in terms of geometry and of the mechanical forces that can be implemented. Most often, temporal wall shear stress gradients and cyclic strain were studied in these models but none incorporating spatial gradients.

### 3.6.4 Existing Studies Limitations

Great advances have been made in our ability to study cellular mechanics. The impact of low and high magnitude, temporal and spatial wall shear stress gradients has been studied to a certain extent. Existing models have allowed to related regions of disturbed flow and oscillatory shear stress to the expression of markers of atherosclerosis. Unfortunately, the inability to recreate \textit{in vitro} the realistic \textit{in vivo} mechanical stimuli may be obscuring our understanding. The highly simplified \textit{in vitro} hemodynamic environments used do not accurately reproduce spatial gradients in WSS and may mask cell-cell interactions. More realistic studies are needed to assess the link between hemodynamics and atherosclerosis development and progression.
3.7 Endothelial Cells Hemodynamic Responses

Hemodynamic forces have been proposed as a factor regulating blood vessel structure and influencing the development of atherosclerosis because it remains a geometrically focal disease\textsuperscript{107}. An early hypothesis by Fry et al. proposed that high shear stress irreversibly damages the endothelial layer increasing its permeability to lipoproteins molecules which transport cholesterol\textsuperscript{108}. However, Caro et al. found that fatty streaks were located in areas which were exposed to low wall shear stress\textsuperscript{109}. Since then, \textit{in vivo} experimental studies have correlated low wall shear stress with early atherosclerosis by analyzing flow fields and correlating them with post-mortem intimal layer thickness measurements\textsuperscript{50,81,82,110-112}

The nature and influence of wall shear stress on endothelial cell activation or dysfunction leading to the development, progression and rupture of atherosclerotic plaques is still poorly understood. Indeed, endothelial cell structure and function has been the subject of many scientific studies in recent history. However, current research has not yet provided a concrete relationship due to the complexity of the biology and mechanics involved.

3.7.1 General Overview

Clinically, atherosclerotic plaques are found most frequently in areas of disturbed flow. These regions are characterized by a decrease in the endothelium-induced vasoreactivity, reduced nitric oxide, low antioxidant levels, an increase in VSMC and fibroblasts proliferation, platelet aggregation, chemotactic factors, growth promoters and vascular cell adhesion molecules expression, hence potentially accelerating plaque formation and increasing the risk of thrombosis\textsuperscript{113-115}. \textit{In vitro}, upon exposure to mechanical stimuli, endothelial cell alter their gene expression eventually leading to cellular adaptations, within hrs or days\textsuperscript{107,116,117}. High arterial wall shear stress of physiological magnitude is generally believed to result in elongated and aligned endothelial cells, which secrete vasodilators and express growth inhibitors and antithrombotic molecules as well as low levels of
growth factors and inflammatory mediators\textsuperscript{118}. In contrast, disturbed low and oscillatory wall shear stress results in the pro-coagulant, pro-thrombotic, pro-oxidant and pro-proliferative state that results in the formation of an inflamed endothelium.

Experiments are usually, for practical reasons, carried out for a short term (<24 hrs). Under these conditions, the acute response of EC to WSS is measured and may differ from \textit{in vivo} measurements. These studies help us to understand the mechanisms by which endothelial cells sense shear stress and convey these signals. However, acute changes are dramatically different then long term responses. Indeed, short term exposure at high wall shear stress can trigger expression of transcription factors which induce proinflammatory and procoagulant gene expression. This does not correlate with the long term anti-inflammatory and anticoagulant effects of high shear stress \textit{in vivo}\textsuperscript{98,119,120}. Hence, longer exposure to wall shear stress may lead to a more realistic idea of the expression patterns found \textit{in vivo}.

The following sections review what has been done to study the expression of suspected mediators of plaque development and stability. It is far from comprehensive, but rather highlights the different molecules that were investigated in the manuscripts.

\section*{3.7.2 Morphology}

\textit{In vivo}, endothelial cell morphology varies throughout the vasculature and correlates with the forces imposed on the vessels. The dysfunctional endothelium phenotype thought to be responsible for atherosclerosis is characterized by a cobblestone cell shape and a random cellular alignment. This morphology exists under pro-thrombotic and pro-inflammatory conditions, most often seen in high susceptibility regions where flow is disturbed such as bifurcations and curvatures.

\textit{In vitro} endothelial cells have a polygonal cell at rest and become gradually oriented and elongated in the direction of flow with increasing shear\textsuperscript{99,121}. Under high undisturbed laminar wall shear stress, cells maintain an atheroprotective phenotype, characterized by an elongated shape, and align their
long axis in the direction of flow whereas regions experiencing low or oscillatory wall shear stress remain more rounded and have no preferred alignment pattern. The morphological changes are dependent on the duration and magnitude of fluid wall shear stress. This response requires between 12-24 hrs and is reversible following cessation of flow. Cell elongation is preceded by changes in the architecture of the cytoskeleton. Upon flow exposure, the actin filaments, which are located at the cell periphery at rest, decrease, while thick stress fibers containing myosin and α-actinin are formed at the center of the cells.

3.7.3 Adhesion

As the vascular endothelium is located at the interface between tissue and blood, it plays a pivotal role for protecting against vascular injury. Dysfunction of the endothelium can result in the expression of adhesive molecules, procoagulant and mitogenic factors, leading to VSMC migration and proliferation and potentially atherothrombosis. Flow modifies the expression of immunoglobulins and selectins. These cell adhesion molecules (CAMs) play an important role in the adhesion and migration of platelets, leukocytes and monocytes which are involved in thrombosis, homeostasis and inflammation.

**Figure 3-5:** Rolling, tethering, firm adhesion and transmigration of leukocytes (Figures were produced using Servier Medical Art).
Leukocyte adhesion is modulated by the expression of CAMs, Figure 3-5. The initial rolling and tethering are predominantly dominated by selectins. These transient and reversible adhesive interactions bring neutrophils and monocytes close to the endothelial cells for longer time periods. They encounter activation signals which include cytokines or direct signal transduction via receptors. Activation enables the integrins to bind to ligands by altering their interaction with the cytoskeleton. Activation dependent binding of integrins to their endothelial ligand converts transient rolling to firm adhesion. Upon activation, neutrophils flatten on the endothelial cells resulting in increased contact that allows a large number of integrin bonds to form. This effect in combination with the decreased force imposed on the cells produces strong bonds. Subsequently, neutrophils and monocytes undergo dramatic shape changes allowing them to slide between the interendothelial cell junction of the vessel wall into the extravascular tissue space and migrate to inflammatory sites. Transmigration does not necessarily accompany stable adhesion to the endothelium unless a favorable chemotactic factor exists across the monolayer. In the case of monocytes, they can differentiate into macrophages and become foam cells, Figure 3-6.

**Figure 3-6:** Adhesion of monocytes to the endothelium, transmigration, migration through the vessel wall and macrophage differentiation (Figures were produced using Servier Medical Art).

Atherosclerotic lesions are usually found in localized areas within the vasculature where flow is disturbed. As adhesion molecules play a fundamental role in inflammatory processes, studies have examined the regional expression in
blood vessels in order to identify markers which may contribute to our understanding of disease development and progression. Wood et al. carried out immunohistochemical investigation of the distribution of E selectin, ICAM-1 and VCAM-1 in human atherosclerotic lesions and strongly detected ICAM-1 and E selectin in coronary artery lesions. O’Brien et al. documented the presence of VCAM-1 in advanced human atherosclerosis plaques as well as Li et al., Cybulsky and Gimbrone and Iiyama et al.

*In vitro* adhesion assays in simplified models have been used to verify the influence of WSS on blood component adhesion. In an axisymmetric stenosis model, Hinds et al. found the attachment of U-937 cells to an E-selectin layer to be negatively correlated with the magnitude of wall shear stress in the constricted region (proximal) and stenosis region. It must be noted that this was a highly idealized stenosis model consisting of a linear constriction and reverse step. Interestingly, the greatest attachment was seen in the proximal portion of their model in the region of positive wall shear stress gradient and elevated wall shear stress. Burns and DePaola examined adhesion on flow conditioned HUVECs. They reported an increased U-937 adhesion in the recirculation region. Pritchard et al. found that adhesion decreased with increased local WSS, hence an increased adhesion in the recirculation region.

### 3.7.4 Inflammatory Markers

As explained previously, cell adhesion molecules play an important role in the development and progression of atherosclerosis. Indeed, cells adhesion molecules are expressed by the endothelium in response to various mechanical and biochemical factors. The infiltration of monocytes and leukocytes, which initiates the atherosclerotic process, is mediated by adhesion molecules. Once they have entered the vascular wall, leukocytes release a variety of cytokines and other bioactive molecules which result in the proliferation and migration of VSMC and promote connective tissue deposition. Eventually, the connective matrix can degrade and render plaques more vulnerable and less stable.
3.7.4.1 NF-κB

One of the first responses to shear stress is an activation of ion channels, which eventually leads to altered gene expression through the activation of transcription factors\textsuperscript{156-171}. Exposure to altered WSS induces inflammatory and vasoactive elements, which are important in the development and progression of atherosclerosis by mediating recruitment of leukocytes and monocytes, platelet activation, vessel tone regulation, proliferation and apoptosis. Studies have examined acute changes in expression and highlighted the importance of complex integrated signalling between the different molecules involved, influencing cell function and resulting in an atheroprone or atheroprotective phenotype.

NF-κB is a transcription factor which has been demonstrated to be a key element in inflammation as it has been found to be more highly expressed in atherosclerotic lesions \textit{in vivo} than at other sites in the vasculature\textsuperscript{161,164,169,172,173}. Regulation of endothelial cell adhesion molecules \textit{in vitro} correlates with the expression of NF-κB. Indeed, upon activation, the inhibitory subunit IKBα dissociates and degrades by various mechanisms, allowing the NF-κB p65 subunit to translocate to the nucleus, binding to promoter sites in a variety of genes such as E selectin\textsuperscript{174}, VCAM-1\textsuperscript{175} and ICAM-1\textsuperscript{176} and causing their activation.

An early transient response is observed after exposure to laminar wall shear stress (10 dyn/cm\textsuperscript{2}) which causes NF-κB p65 translocation as early as 10 min after exposure\textsuperscript{167,177}. Indeed, Tsou \textit{et al.}\textsuperscript{178} observed an early (1 hr) transient response of NF-κB at WSS above 9 dyn/cm\textsuperscript{2}. Also, Nagel \textit{et al.} found that there was a slight upregulation of NF-κB expression (4 hrs) under a WSS of 10 dyn/cm\textsuperscript{2}\textsuperscript{98}. However, the long term exposure (16–24 hrs) to sustained high laminar WSS (12 dyn/cm\textsuperscript{2}) results in exclusively cytoplasmic expression of NF-κB\textsuperscript{179}.

Activation of NF-κB under disturbed flow is different than the response following uniform high WSS\textsuperscript{98,180,181}. Studies confirmed a distinct endothelial cell response with respect to different flow waveforms, with the activation of the NF-κB pathway, also altering cytoskeletal organization and expression of proinflammatory cytokines and adhesion molecules\textsuperscript{9,17}. Low and oscillatory wall shear stress increase nuclear NF-κB expression. Indeed, Mohan \textit{et al.} found that
NF-κB activation is significantly elevated in endothelial cells exposed to prolonged steady low wall shear stress and oscillatory shear stress compared to high WSS corroborating in vivo results, relating NF-κB and plaque location\textsuperscript{167}. Also, Nagel\textit{ et al.} clearly show that there is an increased translocation of NF-κB in disturbed flow regions compared to uniform laminar SS regions and to static conditions\textsuperscript{98}.

### 3.7.4.2 E Selectin

Unlike other adhesion receptors, selectins bind to carbohydrates and glycopeptides rather than protein ligands\textsuperscript{144,145,182}. The main role of selectins is the rolling and tethering of leukocytes to the endothelium, inducing relatively weak and transient adhesion and allowing the cells to roll along the vascular wall. E selectin recognizes specific proteins or glycolipids that have sialyl Lewis X or related carbohydrates that are commonly found on neutrophils\textsuperscript{183}. It is not expressed in resting endothelium but is inducible strongly and rapidly by inflammatory cytokines, being expressed maximally at 4 to 6 hrs and then rapidly declines even in the continuous presence of cytokine\textsuperscript{169}.

Mechanical forces are believed to alter E selectin expression. Bevilacqua\textit{ et al.} identified the presence of E selectin near the accumulation of leukocytes at sites of inflammation\textsuperscript{184}. However, Nagel\textit{ et al.} found that laminar shear stress (2.5-46 dyn/cm\textsuperscript{2}) did not upregulate protein expression at 4 hrs of stimulation or mRNA expression at 2, 8 or 24 hrs\textsuperscript{97}. Morigi\textit{ et al.} exposed cells to laminar WSS for 6 hrs at 8 dyn/cm\textsuperscript{2} and no change in protein expression was observed through flow cytometry\textsuperscript{185}. Other studies found a downregulation of E selectin under steady laminar wall shear stress. Indeed, Sampath\textit{ et al.} found that E selectin mRNA expression is downregulated after 1 hr of exposure to shear stress and that levels at 6 hrs did not change further\textsuperscript{186}. Tsou\textit{ et al.}\textsuperscript{178} found that E selectin was upregulated after 4 hrs of flow exposure at 2-4 dyn/cm\textsuperscript{2} and that above 8 dyn/cm\textsuperscript{2} expression was suppressed below that of untreated endothelial cells. No studies were found on the expression of E selectin in disturbed flow other than the previously stated in vivo work.
3.7.4.3 Immunoglobulins – ICAM-1 and VCAM-1

Immunoglobulin molecules such as intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are principally involved in the firm adhesion of leukocytes. ICAM-1 and VCAM-1 are expressed on activated endothelial cells. Following the rolling stage, various factors may activate leukocyte and monocyte integrins, which bind immunoglobulins and help to secure leukocytes tightly to the vascular wall. Vascular cell adhesion molecule 1 (VCAM-1) was first described as a cytokine-inducible endothelial adhesion molecule; it binds circulating monocytes and lymphocytes, through ligands (such as VLA-4) and may participate in their recruitment from the bloodstream to sites of tissue injury. Vascular expression of VCAM-1 is commonly found in atherosclerotic lesions. VCAM-1 is strongly and rapidly upregulated by cytokines but basally expressed at very low levels. ICAM-1 facilitates selective cellular interactions and binds to leukocyte integrins (such as LFA-1 and Mac-1). Endothelial cells constitutively express ICAM-1 to a certain extent, but during inflammatory responses, levels are increased.

ICAM-1

ICAM-1 has been suggested to be regulated by wall shear stress. In vivo studies by Walpola et al. revealed that ICAM-1 protein expression level increased when wall shear stress increased. Hence in vivo, ECs respond to wall shear stress magnitude and differentially express ICAM-1. In vitro, ICAM-1 has also been found to be upregulated at the protein level. Indeed, Nagel et al. found an upregulation at 24 hrs for a WSS of 3 dyn/cm². Sampath et al. found a transient upregulation at 12 hrs followed by downregulation at a WSS of 25 dyn/cm². Morigi et al. exposed cells to laminar WSS for 6 hrs at 8 dyn/cm² and found a significant change in surface protein expression, which was sustained after 15 hrs of flow. Tsuboi et al. found a significant increase at 4 hrs in ICAM-1 cell surface expression at 15 dyn/cm². Tsou et al. found that protein expression between 2 and 16 dyn/cm² constantly increased at 4 hrs after flow.
exposure. Hence, ICAM-1 protein levels were modified for different durations and magnitudes. Shear stress gradients in the model used by Tsou et al. did not alter ICAM-1 expression, neither did the gradients in the models used by LaMack and Friedman.

Hence, ICAM-1 protein expression is modulated by WSS, but gene expression is also altered. Indeed, a laminar WSS of 10 dyn/cm$^2$ upregulated ICAM-1 at the mRNA level and a maximum expression was noted at 8 hrs, with levels higher than static levels at 24 hrs but lower than at 8 hrs. Sampath et al. examined the effect of WSS from 2-25 dyn/cm$^2$ on mRNA expression. They found that ICAM-1 mRNA levels were maximum at 1 hr when compared to static controls, however, by 6 hrs the values decreased to basal values and continued exposure did not cause further induction at a WSS of 25 dyn/cm$^2$. At 2 and 10 dyn/cm$^2$, similar trends were observed. Tsuboi et al. found a time and WSS magnitude dependency of ICAM-1 mRNA expression at 15 dyn/cm$^2$. A transient increase with a maximum expression at 8 hrs was observed in this study. Levels at 24 hrs were higher than initially. Topper et al. identified that ICAM-1 mRNA levels were similarly transiently upregulated by steady laminar WSS of 10 dyn/cm$^2$.

VCAM-1

In vivo studies revealed that VCAM-1 is expressed consistently but heterogeneously in arteries. Indeed, results in flow studies are contradictory, perhaps because of the very low levels expressed basally. Tsuboi et al. found no significant difference in VCAM-1 protein expression at 15 dyn/cm$^2$ for 4 hrs. Nagel et al. found that VCAM-1 was slightly upregulated at the protein level by 24 hrs for a WSS magnitude of 10 dyn/cm$^2$. Others found that protein expression dropped at 6 hrs of WSS of 1.5 dyn/cm$^2$. Tsou et al. found that VCAM-1 protein expression was increased at 2 dyn/cm$^2$ compared to static control whereas at higher WSS magnitudes, expression decreased and no difference was found between 4 and 14 dyn/cm$^2$ at 4 hrs after flow exposure.
Levels of mRNA are also altered by shear. Nagel et al. found that at the mRNA level, no significant difference was observed for VCAM-1 at a WSS of 10 dyn/cm² and times between 2 and 24 hrs. Other investigators have demonstrated that VCAM-1 decreased after sustained wall shear stress exposure. Indeed, they presented that steady WSS decreased in a magnitude and time dependent manner VCAM-1 mRNA and protein expression. Korenaga et al. have examined the WSS magnitude dependence of VCAM-1 mRNA expression and found that at WSS higher than 5 dyn/cm² after 6 hrs and up to WSS of 20 dyn/cm² no significant change in expression was noticed. They also have shown a great difference between species in basal and flow induced expression from bovine to murine EC, which may explain the discrepancy between studies. Sampath et al. found a clear downward trend for VCAM-1 mRNA expression upon exposure to WSS of 2-25 dyn/cm². Mohan et al. reported that a low WSS of 2 dyn/cm² increased VCAM-1 mRNA expression. Bergh et al. found that VCAM-1 mRNA expression was downregulated by WSS magnitude at 24 hrs between 1 and 25 dyn/cm². Hence, reports on VCAM-1 expression vary greatly depending on the flow conditions and the cell type used.

3.7.4.4 Effect of Temporal and Spatial Wall Shear Stress Gradients

Several genes are affected by disturbed flow as opposed to laminar steady shear stress. Gene arrays are a useful tool that has been developed in the recent years in order to study this. Brooks et al. have shown that genes expressed under disturbed flow are mostly the same which respond to TNF-α, namely E selectin, ICAM-1 and VCAM-1. Other studies on disturbed flow and its effect on cell function have reported an activation of NF-κB in steady laminar flow at low shear. The effects of steady laminar shear stress on cell adhesion molecule expression is drastically different then under oscillatory flow.

VCAM-1 seems to be upregulated by disturbed flow. Indeed, Chappell et al. found that VCAM-1 was upregulated at the protein level in a time dependent manner between 6 and 24 hrs after the onset of oscillatory flow (±5 dyn/cm², 1 Hz). Resnick et al. found that VCAM-1 is downregulated by laminar
shear stress and induced by oscillatory shear stress. Brooks et al. also identified this increase at the protein and mRNA level for VCAM-1. E selectin upregulation by disturbed flow was also observed as well as for ICAM-1.

Truskey et al. suggested that endothelial cells can respond to spatial gradients of WSS by using the sudden expansion flow chamber which is a modified parallel plate design with a rectangular obstacle at the beginning of the chamber, and recreates the recirculation zone found in stenotic regions. This model was also used to study the effects of temporal gradients with respect to spatial gradients, and demonstrated that temporal gradients stimulate EC proliferation and spatial gradients do not. They showed that uniform wall shear stress affects equally proliferation than spatial WSS gradients. McKinney et al. found that ICAM-1 seemed more influenced by shear stress than shear stress gradients. Another useful model was used and lead to the hypothesis that endothelial cell can sense shear stress magnitude and gradients independently.

3.7.5 Summary

Our understanding of the role of endothelial cell response to wall shear stress in the initiation and progression of atherosclerosis is still not complete. In vitro studies have focused on analyzing the expression of known atherosclerotic markers under simplified flow regimes. The importance and sensitivity to wall shear stress of leukocyte adhesion and of the endothelial expression of ICAM-1, VCAM-1, E selectin and NF-κB all of which are markers of inflammation have been well established in literature, although never in complex models presenting spatial wall shear stress gradients. The following 5 manuscripts are my effort to improve the way we study and interpret the response of endothelial cells to wall shear stress.
CHAPTER 4: CONCENTRATION AND TEMPORAL EFFECTS OF DEXTRAN EXPOSURE ON ENDOTHelial CELL VIABILITY, ATTACHMENT AND INFLAMMATORY MARKER EXPRESSION IN VITRO

4.1 Preface

The article entitled ‘Concentration and Temporal Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression in Vitro’ by Rouleau, L., Rossi, J., Leask, R.L has been submitted for publication in Biomechanics and Modeling in Mechanobiology and describes the effects of dextran on cell attachment, viability and expression as well as leukocyte adhesion.

Dextran is commonly used to match blood rheological properties and physiological parameters. Performing in vitro flow experiments requires basic cell culture media which is much less viscous than blood. Although non-dimensionalization can be used to overcome these property differences, limitations in the maximum achievable pump flow rate and in the geometry of in vitro device make it necessary or desirable to alter the media properties. This allows matching hemodynamic parameters which characterize blood flow in coronary arteries, such as the wall shear stress, shear rate and Reynolds number. Despite its vast use reported in the literature, few studies have examined the effects of dextran on endothelial cell function. After some observations in the laboratory, we decided to examine its effects more closely.

In order to accurately measure the proliferative and adhesive modifications that occur due to dextran and analyze the response of endothelial cells in terms of protein and gene expression, protocols had to be developed. Viability and attachment of cells was quantified using a proliferation assay (L.R. in conjunction with Raffi Afeyan). Adhesion assays in straight/tubular in vitro models were performed (L.R.). After these initial promising results, cell function was further
studied under static and flow conditions. Cells were treated in a concentration and time dependent manner both in culture plates and in straight/tubular in vitro models (L.R. and J.R.). Qualitative observation of protein levels was performed using confocal microscopy (L.R.). Procedures for protein purification, quantification and western blotting analysis (L.R.) were developed as well as RNA extraction, quantification, production of cDNA and real-time PCR (J.R.). These means of analyzing cell response are vital to accurately characterize cell function. Also, the dextran modified media properties were quantified in terms of density (L.R. in conjunction with Lisa Danielczak, Stacey Meadley, Jessica Caporuscio, Jessica Van der Vooren and Carlie Piché) and viscosity (L.R. in conjunction with Andrea McGlynn).

The major finding of this work is that the effect of dextran cannot be neglected. Dextran greatly affects cell proliferation and adhesion under static conditions. Inflammatory molecule expression also varied both statically and under steady flow in a concentration and time dependent manner. However, dextran does not affect the conclusions that would be drawn from perfusion studies if the appropriate time matched dextran containing static controls are used.

Contributions
L.R. had the general idea for the publication, designed and performed the research in collaboration with J.R. and Raffi Afeyan, collected the viscosity and density data, analyzed and interpreted the proliferation, attachment, adhesion, protein (confocal and western blotting) and some mRNA expression data, performed statistical analysis and wrote the manuscript.
J.R. performed part of the research in collaboration with L.R., collected, analyzed and interpreted mRNA data, contributed to the troubleshooting, helped in the experimental design and revised the manuscript.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.
4.2 Article 1

Concentration and Temporal Effects of Dextran Exposure on Endothelial Cell Viability, Attachment and Inflammatory Marker Expression \textit{in Vitro}

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4.3 Abstract

Dextran is commonly used to alter the rheological properties of growth medium for in vitro flow experiments in order to match physiological parameters. Despite its acceptance in literature, few studies have examined the effects of dextran on cell function. In this study, we investigated changes in endothelial cell function due to dextran, under static and flow conditions, in a concentration and time dependent manner. Dextran increased endothelial cell viability, decreased their ability to attach to plates and decreased leukocyte adhesion to endothelial cells. Under static conditions, dextran increased protein and mRNA expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in a concentration and time dependent manner and caused the nuclear translocation of NF-κB. Steady laminar wall shear stress modulated the effects of dextran on ICAM-1, VCAM-1 and NF-κB expression in straight/tubular in vitro models. When the expression was normalized to their respective time matched static dextran control, it did not affect the ability to detect changes caused by shear on the mRNA expression of ICAM-1 and VCAM-1. This study demonstrates that dextran can alter endothelial cell function and therefore, caution is advised and time matched dextran controls are necessary when using dextran for dynamic cell studies.
4.4 Introduction

Hemodynamic forces have been linked to atherosclerosis and thus, many studies have examined the response of endothelial cells to flow. There is strong evidence that shear stress represents a source of endothelial injury and can contribute to the initiation and progression of atherosclerosis.

*In vitro* devices such as the parallel plate flow chamber and the cone-plate viscometer have been developed to study, in a controlled manner, the relationship between wall shear stress and endothelial cell function. Studies using these models have provided abundant information on changes in endothelial cell cytoskeleton, uptake of macromolecules, channel activations, release of pro-inflammatory molecules and vasoactive substances [1-8]. The adhesion of blood components to endothelial cells in response to mechanical stimuli has also been examined to better understand the role of inflammation in cardiovascular diseases [9;10].

Performing *in vitro* flow experiments requires the use of growth medium which is much less viscous than blood. Although non-dimensionalization can be used to overcome these property differences, limitations in pump flow rate or in device geometry make it necessary or desirable to alter the growth medium viscosity. Dextran has often been used for this as it is believed to have little side effects [1;11-14]. However, dextran has been used in other applications because of its effects on blood components [15;16]. Clinically, low molecular weight dextran (MW 40,000) is used to expand blood volume and reduce blood clotting [17;18], hence reducing risks of thrombosis as well as an anticoagulant [17;19-23]. Dextran has been shown to inhibit platelet aggregation at lower concentrations (MW 10,000 – 40,000) [16;24].

Considering the known effects of dextran on blood components, it is surprising that very few studies have examined its effects on endothelial cells and compared it to results without dextran [25]. In this study, we report the temporal and concentration effects of dextran on endothelial cell viability and attachment to
plates, leukocyte adhesion to endothelial cells and endothelial cell inflammatory marker expression under static and flow conditions.

4.5 Methods

4.5.1 Cell Culture

Human abdominal aortic endothelial cells (HAAECs) derived from a 20 year old male were purchased from ATCC (American Type Culture Collection, CRL-2472) and expanded up to passage 5. Cells were cultured in endothelial cell growth medium (Promocell, C-22010, C-39215), supplemented with 10% fetal bovine serum (Invitrogen, Gibco, 26140-079) and 1% penicillin streptomycin (Invitrogen, Gibco, 15140-122) in tissue culture flasks coated with 0.1% pig gelatin (Sigma-Aldrich, G2500). At confluency, cultures were rinsed with phosphate buffered saline solution (PBS) and harvested with 0.25% Trypsin-EDTA (Invitrogen, 25200-072).

4.5.2 Modifications of Cell Culture Medium using Dextran

The viscosity of the cell culture medium was altered with dextran (MW 135,000) (Sigma-Aldrich, D4876). Dextran powder was weighed and dissolved in cell culture medium. The solutions were allowed to degass and solubilise before filtering through a polyethersulfone membrane with a pore size of 0.22 µm (Fisher Scientific, 09-761-11). The solutions were stored at 4°C for up to one month. The viscosity was verified by rheological testing with a double gap rheometer (Bohlin, Model CVO 120 HR) at a constant temperature of 37°C. Measurements were taken over a range of shear rates to verify the Newtonian behaviour of the solutions. Density was measured using a pycnometer (Fisher Scientific, 3-247).

4.5.3 Cell Viability Assay

The effect of dextran on endothelial cell viability was measured by a MTT assay (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide, Sigma-
Aldrich, M5655). HAAECs were plated in 96-well cell culture plates at a density of $2 \times 10^5$ cells/mL and left to adhere overnight in unaltered medium. The medium was then replaced with growth medium containing dextran and allowed to incubate at 37°C. After 24 hrs, 10% v/v MTT (5 mg/mL) was added to the wells and incubated for 2 hrs. Afterwards, 100% v/v of detergent (0.01 M HCl, 10% SDS) was added to lyse the cells and solubilise the crystals. After 2 hrs incubation at 37°C, the absorbance was measured at 570 nm in a Benchmark Plus plate reader (Bio-Rad) [26].

### 4.5.4 Endothelial Cell Attachment Assay

The effect of the presence of dextran on the attachment of endothelial cells to 96-well cell culture plates was also evaluated by seeding cells in the wells in growth medium containing dextran at a concentration of $2 \times 10^5$ cells/mL. Cells were left to adhere for 24 hrs, after which an MTT assay was performed to quantify the number of viable cells that attached.

### 4.5.5 Straight/Tubular In Vitro Models

The effects of dextran on leukocyte adhesion to cultured endothelial cells and endothelial cell inflammatory marker expression in response to shear stress were tested in straight/tubular cell culture models. The models were built by pouring a non-toxic, transparent and compliant material, Sylgard184™ in an acrylic mold with a centered stainless steel rod (3.175 mm inner diameter) and connectors. Once the silicone was cured, the rod was removed to create the three dimensional *in vitro* models. The models were hydrophilized using 75% sulphuric acid for 45 minutes, boiled in sterile deionized water for 30 minutes and coated with 40 µg/mL fibronectin in deionized water (Sigma-Aldrich, F0895) overnight at 37°C on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne). Solutions were aspirated and the models washed with media once before the cells were seeded at a density of $1 \times 10^6$ cells/mL ($\pm 2 \times 10^5$ cells/mL) (Beckman Coulter, Canada). The cell suspension was left in the models and the models attached to the tube rotator for an incubation period of 48 hrs, i.e.
when a confluent monolayer had been established. Growth medium was changed the day after seeding.

4.5.6 Leukocyte Adhesion Assay

An acute promyelocytic leukaemia cell line (NB4 cells) was used to quantify the adherence of leukocytes to non-stimulated and TNF-α stimulated endothelial cells. NB4 cells were maintained in suspension culture at 2x10⁵ - 1x10⁶ cells/mL in RPMI 1640 medium with 2 mM L-glutamine (Hyclone, SH3002701), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). For differentiation into granulocytes, cells were stimulated for 48 hrs in the presence of 10⁻⁶ M all-trans-retinoic acid (ATRA) (Sigma R2625). Tumor necrosis factor alpha (TNF-α) was used for 24 hrs at 10 ng/mL (GF 023, Chemicon) in order to create an inflamed endothelium. NB4 cells were allowed to adhere in static conditions onto endothelial cells in the straight/tubular in vitro models for 1 hr. These experiments allowed the study of the adhesive properties of NB4 cells on ECs in static conditions by manually counting the number of NB4 cells adhered.

4.5.7 Flow Experiment

The three dimensional in vitro models were also used to study the response of endothelial cells to unidirectional laminar shear stress in dextran containing medium with time matched dextran static controls. The flow loop consisted of individual reservoirs, silicone tubing, flow dampeners and an 8-rollers peristaltic pump head with a programmable drive (Ismatec, ISM 404 and ISM 732) used to produce a steady flowrate. The resulting flow parameters are listed in Tables 4-1 and 4-2. Dextran concentrations were varied between 0 and 14% w/w. The entire flow loop was located in at 37°C incubator with 5% CO₂. After the perfusion, the cells were gathered from the models with 0.25% Trypsin/EDTA for Western blotting or quantitative RT-PCR or fixed in 1% paraformaldehyde and stored in glycerol:PBS (1:1, v/v) at 4°C for immunofluorescent staining.
Table 4-1: Hemodynamic data concerning RNA expression analysis (10 dyn/cm²).

<table>
<thead>
<tr>
<th>Dextran concentration (%)</th>
<th>0</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (kg/m³)</td>
<td>998.2</td>
<td>1021.4</td>
<td>1046.3</td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>0.975</td>
<td>3.778</td>
<td>10.692</td>
</tr>
<tr>
<td>Flowrate (mL/min)</td>
<td>193.36</td>
<td>49.90</td>
<td>17.63</td>
</tr>
<tr>
<td>Re</td>
<td>1323</td>
<td>90</td>
<td>12</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>1025.6</td>
<td>264.7</td>
<td>93.5</td>
</tr>
<tr>
<td>Shear stress (dyne/cm²)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4-2: Hemodynamic data concerning protein expression analysis for a dextran concentration of 6.7% (5 and 10 dyn/cm²).

<table>
<thead>
<tr>
<th>Dextran concentration (%)</th>
<th>6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (kg/m³)</td>
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</tr>
<tr>
<td>Viscosity (cP)</td>
<td>3.438</td>
</tr>
<tr>
<td>Flowrate (mL/min)</td>
<td>27.42</td>
</tr>
<tr>
<td>Re</td>
<td>55</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>145.4</td>
</tr>
<tr>
<td>Shear stress (dyne/cm²)</td>
<td>5</td>
</tr>
</tbody>
</table>

4.5.8 Quantitative Real-time PCR

Cells were gathered with 0.25% Trypsin/EDTA (Invitrogen) and total RNA was extracted using RNeasy spin columns (Qiagen) with DNase I (Qiagen) on column digestion. Total RNA was quantified by absorbance measurements at 260 nm. First-strand complementary DNA (cDNA) was synthesized with 0.5 µg total RNA, random hexamers (Applied Biosystems) and MultiScribe™ reverse transcriptase (Applied Biosystems) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time PCR reactions were carried out in Power SYBR® Green PCR Master Mix (Applied Biosystems) with QuantiTect Primer Assays (Qiagen) and performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The housekeeping gene was β-Actin and the ΔΔCt method was used for relative quantification of gene expression.
4.5.9 Protein Analysis and Western Blotting

Proteins were gathered and lysed in RIPA Lysis Buffer (50mM Tris-HCl (pH 6.8), 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Sigma P8340), using cycles of freezing and thawing. Cell debris were discarded from proteins by centrifugation at 16,000g for 15 minutes at 4°C. After quantification using the Bicinchoninic acid assay, proteins were resuspended in loading buffer (12mM Tris-HCl, 10% glycerol, 0.4% SDS and 0.02% Bromophenol blue) and boiled for 5 minutes. Proteins (2 µg) were loaded on a 7% polyacrylamide gel (30% w/v, 1:29.2) in a Mini Protean III apparatus. The proteins were transferred on Polyvinylidene Fluoride (PVDF) membranes in a Transblot apparatus. The membranes were incubated for 30 minutes at room temperature in phosphate buffer saline (PBS) containing 5% nonfat dry milk in 0.1% Tween-20 to block nonspecific protein binding and incubated overnight at 4°C in primary antibody (Santa Cruz biotechnology) specific for β-actin (1:200, sc-47778, used as an internal control), ICAM-1 (1:100, sc-8439) and VCAM-1 (1:100, sc-8304) in antibody dilution buffer (0.05% Tween-20, 2% Milk in PBS). After three washes in PBST (0.1% Tween-20 in PBS), secondary antibodies were incubated for 1.5 hr at room temperature (1:10,000). The membranes were washed and detection accomplished with the enhanced chemiluminescence method (Pierce) and X-Ray films (X-Omat, Kodak).

4.5.10 Immunofluorescent Staining and Confocal Imaging

Endothelial cells fixed in 1% paraformaldehyde were stained for ICAM-1 and VCAM-1 expression as well as NF-κB translocation. Cells were washed with PBS and blocked for 30 minutes at room temperature in PBS containing 2% normal donkey serum (NDS, Jackson Immunoresearch, 017-000-121) and 0.2% Triton X-100 (Sigma, T8787). Primary antibodies (Santa Cruz biotechnology) against ICAM-1 (1:100, sc-8439), VCAM-1 (1:100, sc-20069) and NF-κB (1:500, sc-372) diluted in 1% NDS and 0.05% Triton-100 were incubated overnight at 4°C. After three washes in PBS, secondary antibodies were incubated for 1 hr at
room temperature (Invitrogen, donkey Alexa Fluor 488 anti-rabbit IgG (A21206) and Alexa Fluor 555 anti-mouse IgG (A31570), dilution 1:600). Models were then mounted using 0.2% Dabco/Glycerol (Sigma D2522, R6513, 1:5). Cells were examined under a laser scanning confocal microscope (LSM 510, Zeiss), using Argon (488 nm) and HeNe1 (543 nm) lasers for excitation of the fluorochromes and a 32x/0.4 A-Plan objective (Zeiss). Maximum intensity projections were produced from Z series.

### 4.5.11  Statistical Analysis

Statistical analysis was performed by nonparametric tests using GraphPad Prism™ software. Mean values were compared using one-way and two-way analysis of variance (ANOVA) followed by a Bonferroni post tests with a 95% confidence interval. P values less than 0.05 were considered statistically significant.

### 4.6 Results

#### 4.6.1  Cell Culture Medium Properties

The changes in growth medium viscosity and density as a function of dextran concentration were measured for a dextran molecular weight of 135,000. Viscosity increases rapidly with an increase in concentration whereas density increases linearly, Figure 4-1. In all cases, the growth medium was Newtonian over the measured shear rates (25-1000 s⁻¹) (data not shown).

![Figure 4-1: Growth media viscosity (A) and density (B) for different dextran concentrations varying between 0 and 17.5% w/w (MW 135,000) (mean ± standard deviation, n=6).](image)
4.6.2 Endothelial Cell Viability

The effect of dextran on cell viability was quantified. Dextran appeared to be non-toxic after 24 hrs. High dextran concentrations significantly increased cell viability (10.5%, 14% and 17.5% w/w, P<0.01, P<0.001, P<0.001 respectively, one-way ANOVA, Bonferroni post tests) when compared to cells grown in unaltered growth medium, Figure 4-2A.

Figure 4-2: Effect of dextran on endothelial cell viability (A) and attachment to culture plates (B) and adhesion of NB4 cells to TNF-α and non-stimulated endothelial cells (C) at different concentrations. Significant difference indicated with respect to the unaltered medium, 0% w/w dextran (** P<0.01, *** P<0.001) (mean ± standard deviation, n=4).

4.6.3 Attachment

Endothelial cell attachment to plates was significantly altered by the presence of dextran in the growth medium, Figure 4-2B. A significant reduction in cell attachment was observed at higher dextran concentrations (14% and 17.5% w/w, P<0.001, one-way ANOVA, Bonferroni post tests) as measured by the MTT assay.

4.6.4 Leukocyte Adhesion

Leukocyte adhesion to endothelial cells under static conditions was quantified. Stimulation of endothelial cells with TNF-α significantly increased leukocyte adhesion in the presence or absence of dextran with respect to non-stimulated cells (P<0.001, one-way ANOVA, Bonferroni posts tests). Very few
NB4 cells adhered to non-stimulated endothelial cells and no statistical difference was seen with the addition of dextran, Figure 4-2C. However, dextran (6.7% w/w) significantly decreased leukocyte adhesion to TNF-α stimulated endothelial cells (P<0.001, one-way ANOVA, Bonferroni posts tests).

4.6.5 Inflammatory Marker Expression

Changes in cell adhesion molecule expression after 24 hrs of dextran exposure were observed qualitatively by immunofluorescent staining and Western blotting as well as quantitatively by mRNA analysis using RT-PCR. Confocal imaging showed noticeable ICAM-1 and VCAM-1 upregulation as well as translocation of transcription factor NF-κB to the nucleus at high (≥ 7% w/w) dextran concentrations, Figure 4-3A. Increasing amounts of ICAM-1 and VCAM-1 protein were observed visually by Western blotting with increased dextran concentration, Figure 4-3B. In addition, mRNA analysis showed significant upregulation of VCAM-1 and ICAM-1 with higher dextran concentrations (≥ 10.5% w/w). The greatest increase was observed at 17% w/w dextran, whereby VCAM-1 and ICAM-1 mRNA levels were 20- and 15-fold greater than unstimulated cells respectively (P<0.001, one-way ANOVA, Bonferroni posts tests), Figure 4-3C.

Figure 4-3: Effect of dextran concentration on the expression of cell adhesion molecules (ICAM-1 and VCAM-1) and the translocation of transcription factor NF-κB observed by confocal microscopy (A). Protein and mRNA expression as
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determined by Western blotting (B) and quantitative RT-PCR (C) (mean ± standard error, n=3). Experiments were performed under static conditions for 24 hrs (*** P<0.001, with respect to the 0% w/w dextran control).

4.6.6 Time Dependent Effects of Dextran under Static Conditions

The temporal difference in the inflammatory marker expression due to the presence of dextran was also investigated. Cells were incubated for 0, 0.5, 2, 6, 12 or 24 hrs in straight/tubular in vitro models. The endothelial cell expression of VCAM-1 and ICAM-1 after exposure to dextran (6.7% w/w) over a 24 hrs time period is presented in Figure 4-4. Significant increases in VCAM-1 and ICAM-1 mRNA levels were found at times greater than 6 hrs compared to the initial baseline levels (P<0.05, two-way ANOVA, Bonferroni posts tests). ICAM-1 mRNA levels continued to be significantly elevated after 24 hrs of exposure to dextran, whereas the upregulation in VCAM-1 dropped at this time point. No significant differences in VCAM-1 and ICAM-1 expression were detected in unaltered medium (0% w/w dextran) over the 24 hrs time frame.

Figure 4-4: Time dependent effects of dextran (6.7% w/w) on mRNA expression of cell adhesion molecules (ICAM-1 (A) and VCAM-1(B)) as determined by quantitative RT-PCR (mean ± standard error, n=3). Experiments were performed under static conditions for various time periods between 0 and 24 hrs. Statistically significant differences between the dextran stimulated and the initial (0 hrs time point) cells are indicated (*P<0.05, **P<0.001).
4.6.7 Combined Dextran and Hemodynamic Effects

The effect of dextran on inflammatory marker expression was evaluated after 24 hrs of steady flow ($\tau = 5$ and 10 dyn/cm$^2$). Confocal analysis showed a greater expression and nuclear translocation of transcription factor NF-κB in endothelial cells exposed to dextran than to unaltered growth medium under both static and flow conditions (10 dyn/cm$^2$), Figure 4-5A. As seen with western blotting and confocal microscopy, without dextran, cell adhesion molecules are basally expressed at low levels. ICAM-1 is visibly upregulated with increasing wall shear stress magnitude after 24 hrs of flow, whereas results concerning VCAM-1 showed no clear upregulation with wall shear stress magnitude. In the presence of dextran (6.7% w/w, $\mu=3.5$cP) their expression followed the same trends, Figure 4-5B&C.

**Figure 4-5:** Combined dextran and hemodynamics effects. Translocation of transcription factor NF-κB determined by confocal microscopy, at dextran concentrations of 0, 7 and 14% w/w at a mean wall shear stress of 10 dyn/cm$^2$ after 24 hrs (A). Protein expression of cell adhesion molecules measured by western blotting (B) and by confocal microscopy (C) under mean entrance wall shear stresses of 5 and 10 dyn/cm$^2$ after 24 hrs of exposure to 6.7% w/w dextran.
Analysis of VCAM-1 and ICAM-1 mRNA confirmed that increasing concentration of dextran significantly upregulated VCAM-1 and ICAM-1 (P<0.001 and P<0.001, two-way ANOVA) after exposure to a wall shear stress of 10 dyn/cm², Figure 4-6A. However, by 24 hrs of steady flow, the expression of both VCAM-1 and ICAM-1 mRNA are significantly lowered by shear compared to the static controls (P<0.001 and P<0.001, two-way ANOVA) despite the increase seen at the protein level. When the mRNA levels under flow are normalized to the dextran static controls, the concentration of dextran does not appear to affect the fold change in either VCAM-1 or ICAM-1 (P=0.9956 and P=0.7404, two-way ANOVA), Figure 4-6B.

Figure 4-6: Dextran concentration effects under perfusion at 10 dyn/cm² after 24 hrs. Expression of cell adhesion molecules analyzed by quantitative RT-PCR (mean ± standard error, n=3). The data was either normalized to the static control containing no dextran (A) or to the respective dextran containing static control (B). (* P<0.05, *** P<0.001, with respect to the 0% control, † P<0.001 with respect to the static controls).
4.6.8 Time Dependent Effect of Shear and Dextran

Time matched controls were used to normalize the data obtained in perfusion studies. Endothelial cells in straight/tubular in vitro models were extracted and normalized either to the unaltered static control or to the time matched dextran containing static control, Figure 4-7. Expression levels did not vary in time in the static control containing no dextran for both VCAM-1 and ICAM-1 (two-way ANOVA, P>0.05) (data not shown). When the unaltered medium is used to normalize the mRNA expression, a significant increase in VCAM-1 and ICAM-1 is observed after 6 hrs of flow exposure (P<0.05, two-way ANOVA). This increase is followed by a decrease in the mRNA of these genes at 12 and 24 hrs. On the other hand, markedly different results are obtained if the time matched static dextran controls are used, Figure 7B. When time matched dextran static controls are used no significant increase is observed due to shear stress and mRNA levels are lower after 24 hrs of flow exposure than initially.

![Figure 4-7](image)

Figure 4-7: Temporal effects of dextran under perfusion at 18 dyn/cm² on VCAM-1 and ICAM-1 mRNA expression analyzed by quantitative RT-PCR (mean ± standard error, n=3). The data was normalized either to the initial static control containing no dextran (A) or to the respective time matched dextran static control (B) (* P<0.05).

4.7 Discussion

Much of our knowledge of atherosclerosis and thrombosis has come from in vitro endothelial cell culture studies in defined geometries and many have used dextran to alter the rheologic properties of the growth medium [1;3;5-7;11-13;27-29]. In endothelial cell hemodynamic studies, dextran is often used to help match
values of blood viscosity and density. Despite the known effects of dextran on cells and its use in the isolation of blood components, it is often assumed in endothelial cell hemodynamic studies that the effects of neutral dextran are negligible [1;3;6;7]. Indeed, very few studies have examined the specific effects of dextran in a concentration and time dependent manner, under static and flow conditions and presented results obtained with time matched dextran containing static controls.

Dextran not only increases medium viscosity and density [10;30] but also osmolality. In this work, we chose a dextran molecular weight in the range used in perfusion dynamic cell studies (MW 135,000) [8;11-13;31;32] and concentrations varying between 1% w/w [3;33] and 9.5% w/w [11-13]. Molecular weights up to 2 million and concentration up to 30% w/w have been shown to have a Newtonian behaviour [34-37]. The viscosity values we obtained compare well with literature data, Figure 4-1. We did not measure the osmolality of the dextran containing growth medium. However, since no decrease in cell viability or noticeable morphological changes were observed, it is likely that any increase in osmolality was minimal. Indeed, concentrations up to 10% have been reported to minimally alter osmolality [3;38], dextran concentrations of 5% changing osmolality less than 10% [9].

Once the rheological properties were quantified, we examined cell viability and observed a significant increase with increasing dextran concentration, Figure 4-2A. An increase in cell viability has not been noted in other studies. Very few studies have examined the concentration effects of dextran on viability. Wechezak et al. [25] found no significant difference in static culture, indicating that there might not be mitogenic or cytotoxic effects on endothelial cells. However, they showed that dextran increased subconfluent endothelial cell survival under shear, with less severe cell loss on when they were exposed to shear stress. It has also been documented that adding 5% w/w uncharged dextran to growth medium does not affect cell viability [39], integrity or endothelial cell detachment [40].
Dextran decreased endothelial cell attachment at concentrations $\geq 14\%$ (Figure 4-2B). At these concentrations, the solution was difficult to transfer. Consequently, the observed decrease in cell attachment could result from limitations in the ability of cells to disperse and settle in the wells. We also found that neutrophil adhesion to TNF-$\alpha$ stimulated endothelial cells was significantly reduced in growth medium containing 6.7% dextran, Figure 4-2C. It is not believed that this reduction is a result of altered endothelial cell adhesion molecule and/or leukocyte ligand expression since static mRNA and protein levels of ICAM-1 and VCAM-1 progressively increased in time with increasing dextran concentration, Figures 4-3 and 4-4. In agreement with our results, Termeer et al. found that lymphocyte (T cells, TK-1) adhesion to endothelial cells (eEnd.2) decreased with increasing dextran concentration for both non-stimulated and TNF-$\alpha$ stimulated cells [41]. It is possible that the reduction in adhesion is a physical effect whereby dextran interferes with the ability of cells to settle because of changes in growth medium property, as cell density is function of osmolality [16;42]. Dextran is used in the isolation of blood components [15;43], and a similar phenomena could explain the reduction seen in endothelial cell attachment to plates and the results obtained for NB4 cell adhesion to endothelial cells.

Dynamic endothelial cell studies are often used to evaluate the link between hemodynamics and inflammation. We investigated the effect of dextran on transcription factor NF-$\kappa$B and cell adhesion molecules, VCAM-1 and ICAM-1. Transcription factor NF-$\kappa$B (p65 subunit) is involved in inflammation. It is typically translocated from the cytoplasm to the nucleus upon activation by cytokines or following the onset of flow. It is associated with the subsequent upregulation of VCAM-1 and ICAM-1 in endothelial cells, which are involved in the attachment of leukocytes to the vessel wall [7;28;29;44;45]. Under static conditions, we found that dextran, in a concentration dependent manner, translocated NF-$\kappa$B to the nucleus and increased VCAM-1 and ICAM-1 expression at the protein and mRNA levels after 24 hrs of exposure, Figure 4-3. This was followed by an evaluation of the time dependent increase in VCAM-1
and ICAM-1 mRNA levels due to 6.7% w/w dextran exposure, Figure 4-4. A significant increase in VCAM-1 and ICAM-1 mRNA was detected after 6 hrs. The increase in ICAM-1 was maintained up to the 24 hr time point, whereas the upregulation in VCAM-1 decreased after 12 hrs. These results suggest that dextran can produce an inflammatory endothelial phenotype. Very few studies have thoroughly examined the effects of dextran on endothelial cell inflammatory marker expression under static conditions and to our knowledge none in a time dependent manner.

After obtaining these results, we thought it was important to evaluate the effects of dextran under flow, as it is only used to increase growth medium viscosity in perfusion experiments. This work would allow us to know whether dextran could confound the effects of shear stress on endothelial cell expression. Under steady laminar flow, NF-κB showed increased expression as well as nuclear translocation in the dextran treated cells when compared to the untreated cells (0% dextran), Figure 4-5A. Under increased wall shear stress levels (5 and 10 dyn/cm²), protein expression, as observed using western blotting and confocal imaging, was increased for ICAM-1 in the presence and absence of dextran after 24 hrs of perfusion in a wall shear stress magnitude dependent manner. However, results showed no clear difference for VCAM-1, Figure 4-5B&C.

Indeed, Tsuboi et al. examined ICAM-1 at the protein level at 4 hrs of exposure and found a WSS magnitude dependence [8]. Nagel et al. [7] and Morigi et al. [46] observed an increased protein expression in a time-dependent manner, at a wall shear stress at 10 dyn/cm² and 8 dyn/cm² respectively, with a sustained expression at 48 hrs. Hence, the upregulation of ICAM-1 noticed at the protein level in a wall shear stress magnitude dependent manner above 5 dyn/cm² is in agreement with previous studies that have used dextran. VCAM-1 protein levels were less affected. Ando et al. [47] found that the decrease in VCAM-1 protein expression was wall shear stress magnitude dependent at 6 hrs [48]. Nagel et al. [7] found that VCAM-1 was not stimulated at 10 dyn/cm² for 4 hrs, however, their results show a slight increase of cell surface expression at 24 hrs.
However, the medium composition, the experiment design and the cell line used in these studies differ, hence responding differently to wall shear stress.

We also observed that dextran increased VCAM-1 and ICAM-1 mRNA in a concentration dependent manner under flow and that shear stress decreased their expression, Figure 4-6A. Consequently, VCAM-1 and ICAM-1 mRNA levels were lower under flow than under static conditions at 24 hrs after the onset of flow. When normalized to the matched dextran containing static control, dextran does not affect the conclusions that would be drawn on the wall shear stress effects, Figure 4-6B. These results suggest that time matched static containing controls are necessary to separate wall shear stress and dextran effects on the expression of cell adhesion molecules in endothelial cells.

In our time dependent study, different conclusions would be drawn when evaluating the mRNA levels of VCAM-1 and ICAM-1 as a result of wall shear stress exposure depending on the controls used. Normalization of mRNA data with respect to the initial static control containing no dextran would suggest a significant increase in mRNA expression at 6 hrs, for both VCAM-1 and ICAM-1, Figure 4-7A, comparable to other studies [7;8;47;48]. However when compared to the time matched dextran containing static control (6.7% w/w), there is no significant difference, Figure 4-7B. Nagel et al. [7] observed mRNA levels of ICAM-1 and VCAM-1, using 1% w/w dextran (MW ~500 000, 1.74cP). They found that shear stress induced in a time dependent increase in ICAM-1, independent of wall shear stress magnitude. No significant differences were observed for VCAM-1 in this study. In another study, Ando et al. [47] observed that VCAM-1 mRNA levels decreased in response to low wall shear stress levels (1.5 dyn/cm$^2$). They determined that this response was shear stress specific and not affected by the shear rate. Similarly, another study found that increasing levels of shear stress further decreased VCAM-1 expression [48]. However, it is not clear, whether the above mentioned studies used time matched dextran static controls. As a result, it is difficult to separate the changes due to dextran from effects of flow and to relate their work to our own study.
In conclusion, we found that dextran has a non negligible effect on endothelial cell viability, attachment and inflammatory marker expression, as well as leukocyte adhesion. Dextran concentration and duration of exposure had a significant effect on inflammatory marker expression both under static and flow conditions. This work suggests that it is prudent to use time matched dextran containing static controls when evaluating wall shear stress effects in dynamic endothelial cell experiments in order to limit confounding conclusions by the response of cells to dextran.

4.8 Acknowledgements

We thank A. McGlynn, J. Caporuscio, J. van der Vooren, C. Piché and S. Meadley for their help in the determination of the medium properties. Technical assistance from L. R. Villeneuve in confocal imaging was also greatly appreciated.

4.9 References


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CHAPTER 5: THE DEVELOPMENT OF 3-D \textit{In Vitro}, ENDOTHELIAL CULTURE MODELS FOR THE STUDY OF CORONARY ARTERY DISEASE

5.1 Preface

The article presented in this Chapter has been resubmitted (L.R.) for publication in BioMedical Engineering OnLine with the title of ‘The Development of 3-D, \textit{In Vitro}, Endothelial Culture Models for the Study of Coronary Artery Disease’ by Farca, M.A., Rouleau, L., Fraser, R., Leask, R.L. This manuscript describes the morphological response in a geometrically realistic three dimensional models of a human right coronary artery.

The response of the endothelium to wall shear stress is believed to play a central role in the development and progression of atherosclerosis. Current studies have used idealized \textit{in vitro} flow chambers which cannot accurately replicate the complex \textit{in vivo} wall shear stress patterns arising from anatomical geometries. To better understand the link between proximal coronary plaques, we created both straight/tubular and anatomically realistic \textit{in vitro} models of the human right coronary artery. A post-mortem vascular cast of the human left ventricular outflow tract was used to create geometrically accurate silicone elastomer models (M.A.F.). Straight/tubular models were created using a custom made mould (L.R. and M.A.F.). Following the culture of human abdominal aortic endothelial cells within the inner lumen, cells were exposed to steady flow (Re=233) for varying time periods (M.A.F.). The resulting cell morphology was analyzed in terms of the shape index and the angle relative to the flow direction (M.A.F.).

The main objective of this study was to prove the feasibility of using anatomically realistic models to study the progressive elongation and alignment of endothelial cell in the flow direction after 8, 12, and 24 hrs of exposition to steady flow. Findings include significantly less pronounced changes in the anatomical model when compared to the simplified/tubular models. Also, differences were
observed between the inner and outer walls in the disease-prone proximal 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.

**Contributions**
M.A.F. designed and performed the research, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.
L.R. helped editing the manuscript, gathered part of the data, resubmitted the manuscript and completed the response to reviewers.
R.F. provided access to human tissue.
R.L.L. helped editing the manuscript and interpreting the data, funded the research and contributed to the troubleshooting and the experimental design.

**Conference Proceedings**

5.2 Article 2

The development of 3-D, in vitro, endothelial culture models for the study of coronary artery disease

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5.3 Abstract

The response of the vascular endothelium to wall shear stress plays a central role in the development and progression of atherosclerosis. Current studies have investigated endothelial response using idealized \textit{in vitro} flow chambers. Such cell culture models are unable to accurately replicate the complex \textit{in vivo} wall shear stress patterns arising from anatomical geometries. To better understand this implication, we have created both simplified/tubular and anatomically realistic \textit{in vitro} endothelial flow models of the human right coronary artery. A post-mortem vascular cast of the human left ventricular outflow tract was used to create geometrically accurate silicone elastomer models. Straight, tubular models were created using a custom made mould. Following the culture of human abdominal aortic endothelial cells within the inner lumen, cells were exposed to steady flow (Re=233) for varying time periods. The resulting cell morphology was analyzed in terms of shape index and angle of orientation relative to the flow direction. In both models a progressive elongation and alignment of the endothelium in the flow direction was observed following 8, 12, and 24 hrs. 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 proximal 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.
5.4 Introduction

Cardiovascular disease is a leading cause of mortality and hospitalization in North America [1]. Atherosclerosis, a disease characterized by arterial wall fibrosis and lipid accumulation, occurs in elastic and large to medium size muscular arteries throughout the human vasculature, and is particularly evident in regions of curvature and bifurcation [2]. This focal predilection cannot be explained by lifestyle or genetic risk factors alone and has been linked to the response of endothelial cells (ECs) lining the luminal surface of blood vessels. It has been hypothesized that dysfunction of the endothelium leading to atherogenesis is stimulated by complex hemodynamic forces, such as wall shear stress [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 hemodynamics over a cultured monolayer of ECs in a controlled environment. These models include: parallel plate chambers [4-9]; cone and plate viscometer systems [10-14]; and three-dimensional, straight, tubular flow models [15-18]. Much has been discovered about the way in which endothelial cells respond to wall shear stress in such idealized models [19]. Most of these devices assume uniform shear stress and uniform cell response across the flow surface, allowing for easy quantification of average cell response.

Blood flow is characteristically three-dimensional, defined principally by the tortuous geometry of the vessel. The preservation of arterial geometry when studying wall shear stress has been shown to be of primary importance [20]. The complex wall shear stress patterns created in vivo are impossible to recreate in existing endothelial cell culture models, and their omission may be obscuring our understanding of shear induced EC dysfunction.

No model has accurately replicated the geometry of an artery for the creation of an anatomically accurate cell culture model. Such a 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 idealized straight tubular model was also developed for comparison.

Anatomical models, such as the one presented in this study, will be important in further studies of the activation of endothelial cell biochemical pathways involved in atherosclerosis, and in the testing of vascular devices and treatment strategies.

5.5 Materials and Methods

Ethical approval was granted by McGill Institutional Review Board (A06-M62-04B) in accordance with Canada’s tri-council policy on ethical conduct for research involving humans. 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 a mildly atherosclerotic post-mortem human heart was cast at physiologic pressure (100 mmHg) using Batson’s No. 17 anatomical casting (Polysciences Inc., PA) [21]. The same procedure has been previously used to develop anatomically correct models for flow analysis [22]. The most successful cast was used to create Sylgard™ 184 silicone elastomer (Dow Corning, Canada) models for cell culture, using a low melting point alloy as an intermediate negative mould (Figure 5-1). Sylgard™ straight tubular models, 3.2 mm in diameter, were created using a custom made mould for comparison of cell response to steady one-dimensional laminar flow (Figure 5-1). The diameter of the straight tubular model was chosen to correspond to the average diameter of the inlet region of the anatomical model.
**Figure 5-1:** (A) Simplified straight tubular model (B) Anatomically accurate model of a 57 year male with no significant coronary artery disease who died of complications arising from colorectal surgery.

Preparation of the Sylgard™ models for cell culture was based on the procedure originally described by Qiu et al. [18]. The models were hydrophilized in 70% sulphuric acid, followed by sterilization, and coating with fibronectin (40 μg/ml) (F0895, Sigma-Aldrich, Canada). Human abdominal aortic endothelial cells (HAAECs) (HIAE-101, ATCC, VA), passage five, expanded within T-75 flasks, were cultured within the models using the following method. The cell suspension (5.0 ± 0.9x10⁵ cells/mL) was pipetted into each model and allowed to settle for 2 minutes. The model was rotated and the process repeated until all sides were seeded. The model was then placed in the incubator strapped to a rotator (Labquake Rotor, Series 1104, Barnstead/Thermolyne) and slowly (8 rev/min) rotated for 4 hrs prior to flow experiments. The cell density (4.5 ± 0.8x10⁴ cells/cm²) was verified using light microscopy. For the flow experiments, the models were then connected in parallel into a perfusion loop composed of individual vented media reservoirs and a low-pulsatility peristaltic pump (Ismatec A-78002-34, Canada), as seen in Figure 5-2A. The use of a peristaltic pump can introduce unwanted pressure fluctuations in the flow loop, however, these were minimal. The use of flow dampeners did not influence significantly cell shape in straight models (data not shown). The reservoir contained low-serum EC culture media (C-22210 PromoCell, Germany) containing 0.4% endothelial cell growth

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supplement, 0.1 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone, 1 ng/mL basic fibroblast growth factor, 50 ng/mL amphotericin B, 50 µg/mL gentamicin (Promocell, C-39215), supplemented with 10% fetal bovine serum (Invitrogen, 26140-079), 1% penicillin streptomycin (Invitrogen, 15140-122) and supplemented with 6.7% dextran by weight (D4876, Sigma-Aldrich, Canada), to increase its viscosity to 3.5 cP and density to 1030 kg/m³. These values are in the physiological range for blood [23] and were used in order to match as closely as possible both a mean physiological Reynolds number of 233 [24] and an entrance wall shear stress of 20 dynes/cm² [25]. 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 hrs. At the end of each experiment cells were fixed in 4% paraformaldehyde (P6148, Sigma-Aldrich, Canada) and stained with crystal violet (212525, BD Biosciences, Canada) for visualization under a light microscope.

Figure 5-2: (A) Schematic representation of the experimental flow system. The closed-loop flow system used in this study consisted of the cell culture model(s), individual vented reservoirs, and a low-pulsatility 8-roller peristaltic pump (Ismatec, A-78002-34) coupled using biocompatible peroxide cured silicone tubing. All fittings and tubing were sterilized by autoclaving. (B) Illustration of the morphometric parameters calculated for each endothelial cell.

High resolution images captured at 100X total magnification were acquired using an inverted microscope and camera (DC300, Leica Microsystems, Canada) and processed for image analysis. The transparent nature of the models allowed us to directly visualize the cell surface. Due to the curvature of the model, only
the center of the field of view could be used for analysis. Approximately 280-790 cells per experiment were analyzed in each location. For the anatomical model, two regions of the proximal RCA on both the inner (myocardial) and outer (pericardial) walls were analyzed. The first region (Region 1), approximately 5 mm in length, was selected in the relatively straight region near the ostium to the beginning of the first curvature (Figure 5-3). A second region (Region 2), approximately 7 mm in length, in the curved region of the proximal RCA was also analyzed.

![Figure 5-3](image)

Figure 5-3: The approximate location of the proximal RCA regions under study (Region 1 and Region 2) indicated on the anatomical cast; (A) ventral view (C) dorsal view.

To select cells within each image, an interactive Matlab™ 7.0 (Mathworks, CA) program was created. This software displays a filtered image and allows the user to select individual cells. Only cells in focus were taken for analysis. This type of semi-automated cell-picking is performed by allowing the user to manually select a pixel on the boundary of a deeply stained cell region. The algorithm then selects all pixels 1) with intensities higher than and 2) in contact with the chosen pixel which define the cell region. Information about each particular cell (x,y of all the pixels) is stored, and the process is repeated for all discernable cells within each image.

A second algorithm processed the resulting cell information using Matlab™ image processing routines to compute the morphological parameters of each cell, including the angle of orientation (θ), the perimeter (P) and the area (A), as defined by Nerem et al. [26]. These are used to calculate the cell shape index (SI)
as defined in Figure 5-2B. 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 with the GraphPad statistical package (GraphPad, CA). Mean values were compared using a one-way analysis of variance (1-way ANOVA). 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.

5.6 Results

HAAECs were successfully cultured in both models. Confluence was achieved in the three-dimensional models after 24 hrs of static culture. Under static (no flow) conditions, the morphology of the cells in the three-dimensional models was similar to that seen in cell culture flasks.

5.6.1 Tubular Model

Within the simplified tubular model, significant differences in the cell elongation (mean shape index) were found between the static and the 8, 12, and 24 hr perfusion experiments (p<0.001 for all pair-wise comparisons, 1-way ANOVA, Bonferroni post-test) (Figure 5-4 and Figure 5-5). Under perfusion, progressive evidence of cell alignment in the axial flow direction was seen starting at 8 hrs (Figure 5-4). This was followed by further cell elongation and alignment at 12 and 24 hrs.
CHAPTER 5: ANATOMICALLY REALISTIC MODEL

Figure 5-4: Light microscope images of EC morphological changes in the tubular Sylgard™ model. HAAECs were subjected to a steady laminar shear stress of magnitude 22 dynes/cm² for 8, 12, and 24 hrs, (B-D) respectively. (A) Represents the no flow control. (Bar = 100 µm, Magnification = 100X). The arrow points in the direction of net flow.

Figure 5-5: Cell shape index time history for tubular model experiments; SI = 1 corresponds to a perfect circle, while SI = 0 corresponds to a line; all values are expressed as mean ± standard deviation.

The orientation (alignment angle) of the cells was normally distributed around 0º, the direction of flow (Figure 5-6). In the static control (no flow) a random distribution was observed. A significant narrowing (p<0.05, F-test) of the distributions around 0º occurred as experimental flow time increased.
5.6.2 Anatomical Model

The EC morphology in the relatively straight Region 1 was compared with the tubular model. There was no statistical difference between the mean shape indices of the static control models (anatomical vs. tubular, p>0.05) (Figure 5-7 and Table 5-1). When subjected to flow, the pattern of HAAEC elongation and alignment in the direction of flow in the relatively straight Region 1 and curvature of Region 2 was less pronounced (Figure 5-8 and Table 5-1). In both regions, at all matched time points, the anatomical model displayed significantly less elongation than the tubular models (p<0.01, ANOVA Bonferroni post-test). Interestingly, the mean cell shape in Region 1 of the anatomical model following 24 hrs of flow was not statistically different from the 12 hr tubular model experiment (p>0.05, ANOVA Bonferroni post-test). Similarly, the anatomical 12 hr experiment was also not different from the 8 hr simplified model experiment (p>0.05, ANOVA Bonferroni post-test).
Figure 5-7: Bar graph illustrating cell shape index time history for both the tubular and anatomical models. The symbol (*) denotes a significant difference in the means.

Figure 5-8: Light microscope images of ECs in anatomical Sylgard™ models following 8, 12, and 24 hrs, (B-D) respectively. (A) represents the static (no flow) 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.

Region 1 alignment angle histograms for anatomical model experiments were observed to be more widely distributed around 0° (Figure 5-9). In general, there was a significant decrease in the angle variability with flow; however, for the anatomical model this was true only following 8 hrs of flow. At all time points, significantly less alignment was seen in the anatomical model (Table 1).
Figure 5-9: Histograms illustrating the distribution of cell angles of orientation for the static, 8 hr, 12 hr, and 24 hr anatomical model flow experiments.

Table 5-1: Significance test results for comparisons between the anatomical (A) and tubular (T) models

<table>
<thead>
<tr>
<th>Experiment</th>
<th>SI: Bonferroni test</th>
<th>Angle:F-ratio test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static (T) vs. Static (A)</td>
<td>P &gt; 0.05</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Static (T) vs. 8 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Static (T) vs. 12 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Static (T) vs. 24 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>8 hrs. (T) vs. Control (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>8 hrs. (T) vs. 8 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>8 hrs. (T) vs. 12 hrs. (A)</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>8 hrs. (T) vs. 24 hrs. (A)</td>
<td>P &gt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>12 hrs. (T) vs. Static (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>12 hrs. (T) vs. 8 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
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<tr>
<td>12 hrs. (T) vs. 12 hrs. (A)</td>
<td>P &lt; 0.001</td>
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<tr>
<td>12 hrs. (T) vs. 24 hrs. (A)</td>
<td>P &gt; 0.05</td>
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<tr>
<td>24 hrs. (T) vs. Static (A)</td>
<td>P &lt; 0.001</td>
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<td>24 hrs. (T) vs. 8 hrs. (A)</td>
<td>P &lt; 0.001</td>
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<tr>
<td>24 hrs. (T) vs. 12 hrs. (A)</td>
<td>P &lt; 0.001</td>
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</tr>
<tr>
<td>24 hrs. (T) vs. 24 hrs. (A)</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>
5.6.3 Regional Variation

Region 1 and 2 of the proximal RCA in the anatomical model were further analysed to investigate possible differences in cell morphology between the inner (myocardial) wall and the outer (pericardial) wall at the 24 hr time point. A significant difference in shape index was found in the relatively straight section (Region 1), with cells on the inner wall displaying a more elongated morphology (p<0.0001, ANOVA Bonferroni post-test) (Figure 5-10). A similar trend was observed with the angle of orientation, with a significant difference between the variance of the two walls (P<0.0001, F-test). In contrast, in the highly curved region (Region 2), no significant difference was observed between the inner and outer wall cell shape index. The coefficient of variation for all regions was similar.

![Bar graph illustrating cell shape index variation between the inner and outer walls of the anatomical model in Region 1 and 2. The symbol (*) denotes a significant difference in the means.](image)

**Figure 5-10:** Bar graph illustrating cell shape index variation between the inner and outer walls of the anatomical model in Region 1 and 2. The symbol (*) denotes a significant difference in the means.

5.7 Discussion

In this study, we developed an anatomical three-dimensional flow model of a human right coronary artery which was able to support cultured human abdominal aortic endothelial cells. We have shown that these cells remain adherent under a
physiologic magnitude of wall shear stress during steady flow and alter their
morphology in response to flow differently in uniform cell culture flow chambers
(Figures 5-4 and 5-8). To our knowledge, the anatomical model developed in this
study is the first endothelial cell culture model with realistic arterial geometry.

Flow studies have shown that the preservation of arterial geometry is
fundamental in reproducing in vivo wall shear stress patterns [20, 27]. Even the
most accurate flow models of human coronary blood flow have been incapable of
capturing the flow through the ostium [22, 28]. Our model accurately replicates
the geometry driven flow through the ostium and the proximal RCA. The
morphological adaptation of endothelial cells to wall shear stress in this region
was analyzed because the proximal region of human coronary arteries is a high
risk area for eccentric intimal thickening and atherosclerosis [29, 30].

EC response to shear has been widely documented in simplified in vitro
models [4-14]. Studies involving such models have shown that low [31], high [32]
and oscillatory [33] wall shear stress can cause cultured ECs to respond
differentially by changing their structure and function [11, 34-36]. Much of this
work has been conducted in parallel flow chambers because of their relative
simplicity and commercial availability. These devices assume uniform shear
stress and uniform cell response across the flow surface, allowing for easy
quantification of average cell response. Unfortunately, such simplified models fail
to mimic the diversity of 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 in vivo cell-cell signalling interactions [37] and
therefore may not be representative of the endothelium in general.

Spatial gradients in wall shear stress have been shown to create a
heterogeneous cell response in culture. 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). Morphological and functional differences have been
identified in the three regions [9, 33]. However, the physiological significance of
these changes is difficult to interpret, since this is an unnatural geometry. Despite such limitations, these studies have provided support for the endothelial heterogeneity hypothesis of focal atherosclerosis \[38\].

The model we have developed will allow us to directly determine the response of endothelial cells to realistic spatial and temporal gradients in wall shear stress present in human coronary arteries. The realistic spatial flow pattern produced in our model causes significant regional differences in cell morphology in the disease-prone proximal region (Figure 5-10). When compared to a straight tubular model, we observed significantly less cell elongation and cell alignment in the flow direction in the anatomical models at all time points (Figure 5-8). These results suggest that the dramatic endothelial elongation seen in simplified models may not be representative of true EC behaviour to realistic spatial wall shear stress patterns. Local heterogeneity in endothelial cell response was evident in Region 1, where a significant difference between the outer and inner wall morphology was demonstrated. Moreover, there is little consistency in endothelial cell morphology in the proximal region, suggesting possible phenotypical differences. This preliminary work has highlighted the local response of ECs to realistic shear stress and is the first step in evaluating the role of realistic spatial gradients in wall shear stress in the development of focal atherosclerosis. We believe it is important to preserve the \textit{in vivo} anatomy to properly investigate the mechanism of shear induced endothelial cell dysfunction in atherosclerosis, since geometrical variation has implications in cell to cell signalling, upstream release of cytokines and blood component/EC interaction.

Few three dimensional \textit{in vitro} and \textit{in vivo} morphological studies are available to compare our results with others. The shape index values obtained for the straight/tubular models are lower than those reported by Helmlinger \textit{et al.} \[39\] for bovine aortic endothelial cells exposed to 25 dynes/cm$^2$ in a parallel plate chamber, namely 0.65 at 8 hrs and 0.45 at 24 hrs. This may be due to differences in cell type or differences in the hemodynamic stimuli created by a tubular model. The area targeted for morphometric analysis (the nuclear region, rather than the cell boundary) and staining technique may also contribute to this difference. Our
simplified model is in closer agreement with the findings of Nerem et al. [26] who analyzed cell shape directly from silver stained arterial tissue and observed shape index of 0.35±0.02. Ziegler et al. [16] employed similar Sylgard™ tubular models with a greater diameter of 6 mm and analysed endothelial cell shape. However, in this study, the cell monolayer was only exposed to very low shear stress, with a maximum shear stress of 6 dynes/cm², resulting in a reported shape index similar to our static models.

Protein and gene expression studies performed in simplified in vitro geometries have greatly contributed to the understanding of atherosclerotic development in vivo. Our work provides evidence that a late step (morphological adaptation) in the cascade of events that occurs after the onset of flow is influenced by the presence of realistic spatial wall shear stress gradients. Once, validation of the flow patterns have been fully performed in these anatomically realistic models, analysis of the cell phenotype can be performed by extraction of cells (proteins and RNA) from the models [16]. Due to the transparent nature of the model, local observations on the expression through confocal imaging are also possible.

A number of assumptions were made for the development of our model that are similar to existing cell culture models. We have used a monolayer of ECs to approximate the arterial wall, ignoring interactions with other cells of the artery wall. We have also used cell culture media as a blood substitute, and have not considered the effects of blood components. Other mechanical forces, such as transmural pressure and cyclic strain, are known to affect endothelial cells. Although the model we have created is made from a material with distensible properties, we have not attempted to quantify or replicate these. It is important to note that endothelial cell response to pulsatile flow can be significantly different [11, 39]. Temporal average and spatial wall shear stress gradients are important parameters influencing endothelial cells. In this model, steady flow was assumed; however, simulations of both steady and pulsatile flow in numerical models of human RCAs [22] have shown that the time averaged wall shear stress is well represented by steady flow results [28].
It is also important to remember that there are significant variations in the anatomical structure of human coronary arteries [22]. 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 needs to be repeated with several other casts.

In the right coronary artery, it has been estimated that over 60% of flow occurs during diastole, and this can rise to 80% in the presence of aortic valve disease [24]. Hence, as a first approximation, we have assumed steady retrograde flow in the ascending aorta to capture diastolic coronary flow. We have not considered the time varying characteristics of blood flow, geometrical changes due to the motion of the RCA 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.

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. 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 devices and treatment strategies.

5.8 Acknowledgements

Funding for this work was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), the McGill University Eugenie-Ulmer Lamothe Fund, and the Canada Foundation for Innovation (CFI).
5.9 References


CHAPTER 6: ENDOTHELIAL CELL MORPHOLOGIC RESPONSE TO ASYMMETRIC STENOSIS HEMODYNAMICS: EFFECTS OF SPATIAL WALL SHEAR STRESS GRADIENTS

6.1 Preface

The article presented in this Chapter has been submitted for publication in the Journal of Biomechanical Engineering with the title of ‘Endothelial Cell Morphologic Response to Asymmetric Stenosis Hemodynamics: Effects of Spatial Wall Shear Stress Gradients’ by Rouleau, L., Farcas, M.A., Tardif J.-C., Mongrain, R., Leask, R.L. This section describes the morphological response of endothelial cells to the hemodynamic patterns created in an asymmetric stenosis model presenting a 50% occlusion. Flow in stenotic vessels creates complex spatial wall shear stress gradients. Endothelial cells respond to wall shear stress and this response may be linked to the initiation and stability of coronary plaques. Morphological changes are one of the last steps to occur upon flow exposure and are the easiest and cheapest to measure.

This worked aimed at creating a new in vitro model to evaluate the endothelial cell morphological response in a more realistic environment, compared to conventional in vitro models. Three dimensional asymmetric stenosis models were created to represent in vivo atherosclerotic regions (L.R.). Human abdominal aortic endothelial cells were cultured within the models and a seeding procedure was modified and optimized (L.R.) from work done by Monica A. Farcas. This is the current in vitro cell culture procedure used in the laboratory. In order to quantify cell morphology, a staining procedure was developed, as well as a semi-automated Matlab ™ analysis program (L.R. & M.A.F.). A fixation protocol was optimized in order to image cells using immunofluorescent staining and confocal imaging (L.R.).

They key findings of this work include the importance of spatial wall shear stress gradients on endothelial cell morphology. Cells were more randomly
oriented and of cobblestone shape compared to uniform one-dimensional flow regions in the proximal and distal regions to the stenosis. Overall, the results demonstrate the need to use more realistic hemodynamic environments when studying endothelial cell response. Further study of endothelial cells to spatial wall shear stress gradients will improve our understanding of focal remodeling.

**Contributions**

L.R. designed and performed the research, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.

M.A.F. contributed to the development of the procedure used to grow the cells in the models and the making of the moulds.

J.C.T. provided funding and contributed to the initial troubleshooting of the experimental set-up.

R.M. designed the stenosis model geometry and provided funding.

R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

**Conference Proceedings**


9. Rouleau, L., Farcas, M.A., Mongrain, R., Tardif, J.-C., Thorin, E., Leask, R.L. Endothelial Cell Response to Shear Stress in an Asymmetric Stenosis Model. 9th Journée de la Recherche Institut de Cardiologie de Montréal, Montréal, QC, Canada, June 1st 2006. (1st prize 1000$)


11. Rouleau, L., Farcas, M.A., Zigras, T., Tardif, J.-C., Thorin, E., Mongrain, R., Leask, R.L., Development of an asymmetric stenosis model to study endothelial...


6.2 Article 3

**Endothelial Cell Morphologic Response to Asymmetric Stenosis Hemodynamics: Effects of Spatial Wall Shear Stress Gradients**

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6.3 Abstract

Endothelial cells are known to respond to hemodynamic forces. Their phenotype has been suggested to differ between atheroprone and atheroprotective regions of the vasculature which are characterized by the local hemodynamic environment. Once an atherosclerotic plaque has formed in a vessel, the obstruction creates complex spatial gradients in wall shear stress. Endothelial cell response to wall shear stress may be linked to the stability of coronary plaques. Unfortunately, in vitro studies of the endothelial cell involvement in plaque stability have been limited by unrealistic and simplified geometries, which cannot reproduce accurately the hemodynamics created by a coronary stenosis. Hence, in an attempt to better replicate the spatial wall shear stress gradient patterns in an atherosclerotic region, a three dimensional asymmetric stenosis model was created. Human abdominal aortic endothelial cells were exposed to steady flow (Re = 50, 100, 200 and $\tau = 4.5, 9, 18$ dyn/cm$^2$) in idealized 50% asymmetric stenosis and straight/tubular in vitro models. Local morphological changes that occur due to magnitude, duration and spatial gradients were quantified to identify differences in cell response. In the one dimensional flow regions, where flow is fully developed and uniform wall shear stress is observed, cells aligned with flow direction and had a spindle-like shape when compared to static controls. Morphological changes were progressive and a function of time and magnitude in these regions. Cells were more randomly oriented and had a more cobblestone shape in regions of spatial wall shear stress gradients. These regions are present both proximal and distal to the stenosis and on the wall opposite to the stenosis. This study shows the need to incorporate more realistic geometrical features when studying endothelial cell response to hemodynamics. The response of endothelial cells to spatial wall shear stress gradients both in regions of acceleration and deceleration and without flow recirculation has not been previously reported. These results may help explain plaque stability.
6.4 Introduction

Coronary artery disease is one of the leading causes of death and hospitalization in North America (1). Atherosclerotic plaques are known to develop in regions where blood flow is disturbed, such as bifurcations and curvatures (2, 3). It has been hypothesized that forces such as wall shear stress may account for the non-random distribution of atherosclerosis (4). In addition, it is clear that once an obstruction has developed, blood flow is further disturbed and hemodynamic forces continue to play an important role as the stenosis progresses (5).

Endothelial cells (EC) are known to be involved in the development of coronary artery disease as they regulate vascular tone, inflammation, thrombosis, and vascular remodeling (4, 6). Their structure and function have been shown to change in response to wall shear stress (WSS). It is now believed that wall shear stress alone can alter endothelial cell phenotype to one that promotes atherosclerosis and potentially thrombus formation (7). In vitro studies have been developed to replicate in vivo hemodynamics. Most often, parallel plate flow chambers and rotational viscometers have been used (8-11). Few in vitro endothelial cell flow experiments have been performed in realistic three dimensional compliant geometries (12-14).

Blood flow in stenotic vessels creates a complex pattern of hemodynamics in that shear stress increases dramatically in the throat of the stenosis and then decreases very rapidly in the post stenotic recirculation region (15). The response of endothelial cells in this complex hemodynamic environment is believed to play a role in plaque progression and stability. Although studies have been performed on tubular models (12-14, 16) these devices expose cells to uniform wall shear stress and may mask cell-cell signalling interactions present in a complex geometry with the presence of wall shear stress gradients. Temporal gradients have been examined and are important regulators of endothelial cell function (6, 17, 18). Few models presenting spatial gradients have been investigated, but have also shown a differential response of endothelial cells to spatial wall shear stress
gradients (17-19). Unfortunately, current in vitro models do not accurately reproduce the forces present in stenotic regions. Hence, we designed a three dimensional in vitro asymmetric stenosis model to study the regional morphological response of human aortic abdominal endothelial cells to the wall shear stress patterns created near an eccentric atherosclerotic plaque.

6.5 Methods

6.5.1 Wall Shear Stress Determination

An idealized stenosis model with a 50% area reduction was custom built, Figure 6-1A. The model has an inlet diameter of 3.175 mm and a length of 10 cm. The velocity and wall shear stress profiles were evaluated using photochromic molecular flow visualization (PMFV) (20-23). In these experiments, a flow model was perfused with a solution of odorless mineral spirits (Shell-Sol 715) and trace amounts of colourless dye (1',3',3'-trimethylindoline-6-nitro-benzospiropyran), which exposed to UV light, turns opaque and forms a photochromic trace. A CCD camera (Dalsa Pantera TF IM60, Dalsa, ON) and an image-processing board (National Instruments PCI 1428, Laval Quebec) were used to track the molecular displacement and calculate the wall shear rate. To account for differences in fluid properties, the mean entrance Reynolds number were matched between the PMFV experiments and the endothelial cell perfusion assays.
Figure 6-1: (A) Regions of the three dimensional in vitro 50% asymmetric stenosis model, including the inlet, acceleration, deceleration and outlet regions on the side of the stenosis and positive and negative gradient regions on the opposite side. Flow profiles for a mean entrance Reynolds number of 200 and wall shear stress of 18 dyn/cm² are shown which includes a recirculation zone. (B) Schematic of the perfusion flow-loop, including individual vented reservoirs and custom built dampeners, a low pulsatility peristaltic pump and the in vitro models.

6.5.2 Endothelial Cell Culture

Human abdominal aortic endothelial cells derived from a 20 year old male were purchased (American Type Culture Collection, CRL-2472). Cells were cultured in endothelial cell growth medium (Promocell, C-22010) containing supplements (Promocell, C-39215), 10% fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). The cell line was subsequently expanded to further passes using 0.25% (w/v) trypsin-53 mM EDTA solution (Invitrogen, 25200-072). Cells at passage 5 were used in all perfusion experiments.

6.5.3 Model Preparation

Casts to create the lumen of the artery were made with a low melting point alloy (Cerrolow117™, Cerro Metal Products Co. 4470-2, melting point 47ºC, Bellefonte, PA, USA). Transparent Sylgard184™ (Dow Corning, PA, USA) silicone elastomer was poured around the lumen casts and allowed to cure. The cast was then removed from the model. The models were hydrophilized using
75% sulphuric acid for 45 minutes, boiled in sterile deionized water for 30 minutes and coated with 40 µg/mL fibronectin in deionized water (Sigma-Aldrich, F0895) overnight at 37°C on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne). Solutions were aspirated and the models washed with media once before the cells were seeded, at a seeding density of 1 x 10^6 cells/mL (± 2 x 10^5 cells/mL) (Beckman Coulter, Canada). The cell suspension was left in the models and the models attached to the tube rotator for an incubation period of 24 hrs, i.e. when a confluent monolayer had been established.

### 6.5.4 Perfusion Experiments

The flow loop consisted of a reservoir, tubing, dampeners and an 8-rollers peristaltic pump head with a programmable drive (Ismatec, ISM 404 and ISM 732) used to produce a steady flowrate of 25, 50 and 100 mL/min, the resulting flow parameters are listed in Table 6-1. The sterilized system was assembled and installed in an incubator, Figure 6-1B. Medium was modified with 6.7% w/w dextran (Sigma-Aldrich, D4876) to match blood viscosity (3.44 cP ± 0.2 cP) measured using a double gap rheometer (Bohlin, Model CVO 120 HR) and a density of 1027 kg/m^3 measured using a pycnometer (Fisher Scientific, 3-247).

<table>
<thead>
<tr>
<th>Flowrate (mL/min)</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>132.6</td>
<td>265.2</td>
<td>530.4</td>
</tr>
<tr>
<td>Shear stress (dyne/cm²)</td>
<td>4.5</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

### 6.5.5 Morphological Analysis

Endothelial cell confluence in the models was monitored using an inverted light microscope (Leica DM IL, Leica Microsystems). The transparent nature of the models allowed direct visualization of the monolayer using an inverted microscope. At the end of the perfusion, the models were disconnected from the flow loop, the medium drained, the tubes were rinsed twice with growth medium and the cells fixed with freshly made 2% paraformaldehyde (Sigma-Aldrich,
P6148) in phosphate buffered saline (PBS). Cells were stained using crystal violet 4% solution (BD Biosciences, 212525). Images (magnification 100x) were captured using a CCD camera (Leica DC300 DC Camera, Leica Microsystems). A Matlab™ code was developed to allow the quantification of the shape index, as first described by Nerem et al. (24). The shape index characterizes the degree of cell elongation and is equal to one for a circle and zero for a straight line. Cell morphology was analyzed in the different wall shear stress gradient regions of the models (inlet, acceleration, deceleration, outlet, positive and negative gradient).

Immunofluorescence staining and confocal imaging

Local cytoskeletal rearrangement of F-actin fibers was observed using a fluorescent stain, phalloidin-ALEXA 647 (1:300, Molecular Probes). For visual observation of local cell expression, the models were cut open and laid face down in glass bottom petri dishes (MatTek, P35G-1.0-14-C). Samples were analyzed using a Zeiss LSM 510 inverted laser scanning confocal microscope (Oberkochen, Germany). Images were collected with a LD A-Plan 32X/0.4 objective. A HeNe2 (633 nm) laser was used for excitation of the Alexa Fluor™ 647. Z stacks were acquired with images taken every 2 µm to respect the Nyquist criteria in Z sampling. Maximum projections (view of the lumen) were applied to each Z stack using the projection tool of the LSM 510 software (Zeiss).

6.5.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism™ software. Results are shown as mean ± standard deviation. Mean values of shape index were compared using one-way and two-way analysis of variance (ANOVA) followed by a Bonferroni post-processing test with a 95% confidence interval. Coefficients of variation were used as an indication of consistent data variability. Differences between means were considered significant at $P<0.05$. 
6.6 Results

6.6.1 Wall Shear Stress Patterns

The wall shear stress values were measured by the photochromic technique and these values were used to identify the location of the different flow regions. At the entrance and the exit of the model, the flow is steady and one dimensional. A parabolic fully developed velocity profile is observed, Figure 6-1A. As the area is restricted in the model, the flow accelerates and the peak velocity moves away from the centerline of the flow channel towards the wall opposite the stenosis, resulting in a skewed profile. Increased wall shear stress is created along the wall as the model constricts and reaches approximately 3.5 times the inlet WSS just upstream of the stenosis peak. The flow profile eventually recovers a symmetric form in the outlet region and the wall shear stress value approaches the one of the inlet.

Figure 6-2: Photochromic molecular flow visualization results for the stenosis side (A) and the opposite side (B) of the model for different Reynolds numbers ranging from 50 to 200 and resulting in a mean entrance wall shear stresses from 4.5 to 18 dyn/cm². The normalized shear plots are presented. On the side of the stenosis, a recirculation zone with flow reversal is created at Re=200 and similar but slightly smoother gradients are present on the opposite side of the stenosis.

The normalized wall shear stress patterns at the Reynolds number used in this study are shown in Figure 6-2. Lower Reynolds numbers (50, 100) showed
no noticeable flow reversal in the deceleration region. At \( \text{Re} = 200 \), the flow along the stenosis clearly separates after the peak and reattaches further downstream from the shoulder of the stenosis (Figure 6-2A). The area between the separation and reattachment points is called the recirculation zone which is characterized by low, negative velocities, indicating flow reversal. The location of maximum wall shear on the stenosis side wall shifts upstream at higher Reynolds numbers due to a developing adverse pressure gradient distal to the stenosis peak which eventually leads to the appearance of a recirculation zone. There are also significant gradients in the WSS on the opposite side of the stenosis (Figure 6-2B). Peak shear on this side appeared to remain at the same axial position with varying Reynolds number.

### 6.6.2 Cell Morphology: One Dimensional Flow Regions

Perfusion experiments were performed to observe endothelial cell response to wall shear stress. Different conditions were tested, varying in time and magnitude. Morphometric analysis was performed on the cells present in each region, in the wall shear stress gradient and the one dimensional fully developed flow regions in order to investigate changes in cell shape following exposure to complex shear stress patterns. Morphological changes are one of the last events in the signalling cascade of endothelial cell response upon flow exposure.

Cells grown within the models under static conditions were randomly oriented and of cobblestone shape. They progressively aligned with flow direction in the one dimensional flow regions when exposed to steady wall shear stress. Upon flow cessation, cells recovered their polygonal shape and became randomly oriented (data not shown). Morphological changes were dependent on wall shear stress exposure duration and magnitude. Increased wall shear stress and longer perfusion times produced a more elongated and aligned cell morphology (Figure 6-3A).

Endothelial cells grown in straight/tubular models showed no significant difference in their morphological response when compared to the inlet and outlet sections of the asymmetric stenosis model, at any time point or for any Reynolds
number. Figure 6-3B shows a comparison of the straight model to the inlet and outlet sections of the stenosis model at 12 and 24 hrs for a Reynolds number of 200 (P=0.1368 and P=0.6404 respectively, One-Way ANOVA).

Morphometric analysis confirms the visual observations of cell shape elongation with time in the one dimensional fully developed flow regions. Perfusion duration and magnitude had a significant effect in the one dimensional flow regions (P<0.0001 and P<0.0001 respectively, Two-Way ANOVA), Figure 6-3C. Changes were most pronounced at higher mean entrance wall shear stress, for which by 12 hrs the cell shape index had achieved a minimal value.

![Image of Figures 6-3A, 6-3B, and 6-3C showing cell morphology and wall shear stress effects.](image)

**Figure 6-3:** Qualitative observation of microscopic images of the cells showed changes in shape and alignment after 24 hrs upon flow exposure (direction indicated by the arrow) at different wall shear stress magnitude (A). Comparison of the straight tubular model with the inlet and outlet regions of the stenosis model for Re=200 at 12 and 24 hrs (B). The wall shear stress magnitude and duration effects on the shape index in the fully developed regions (inlet and outlet) are shown (C).

### 6.6.3 Cell Morphology: Wall Shear Stress Gradient Regions

Four distinct regions with spatial gradients in wall shear stress are found within the models. On the side of the stenosis, an acceleration region, with a positive gradient, and a deceleration region, with a recirculation zone at high Reynolds number, are present. On the opposite wall, similar positive and negative
gradient regions are observed but with slightly smoother transition in WSS and no flow reversal.

Representative images of cells on the side of the stenosis exposed to one dimensional fully developed flow in the inlet and outlet regions, positive and negative gradients in the acceleration and deceleration zones respectively are presented in Figure 6-4A. Morphological changes were evident everywhere but to a lesser extent in the spatial wall shear stress gradient regions. In the acceleration and deceleration regions, cells were more randomly oriented with a cobblestone shape similar to that found in static models.

The morphological differences due to flow exposure between these regions and the one dimensional flow regions were progressive and more pronounced at higher mean entrance WSS values, hence at greater Reynolds number. The shape index values at a Reynolds number of 200 are presented in Figure 6-4B. The shape index values were compared on the stenosis side between the inlet, acceleration, deceleration and outlet regions. A regional variation in morphology is evident by 12 hrs. Cells in the recirculation region (deceleration) were significantly more rounded than in all other regions at both 12 and 24 hrs. Interestingly, cells in the acceleration region were also more rounded and significantly different that the inlet and outlet regions at 12 hrs (P<0.001 and P<0.01 respectively, Bonferroni post tests) and at 24 hrs (P<0.001 and P<0.001 respectively, Bonferroni post tests).
CHAPTER 6: MORPHOLOGY IN STENOTIC MODEL

Figure 6-4: Representative images of crystal violet stained endothelial cells on the stenosis side of the model in the inlet, acceleration, deceleration (recirculation) and outlet regions after 24 hrs of flow exposure at Re=200 (A). Shape index values for each region at Re=200 and different times (B).

On the side opposite to the stenosis, in the positive and negative WSS gradient regions, morphological differences were also observed as shown in Figure 6-5A. Similar to the stenosis side, cells in the negative WSS gradient region were most rounded and the shape index was significantly different than all other regions by 12 hrs at a Reynolds number of 200, Figure 6-5B. By 24 hrs, the cells exposed to the positive WSS gradient were significantly different than the inlet and outlet regions (P<0.001 and P<0.001 respectively, Bonferroni post tests). Hence, the shape index in the gradient regions was greater than in the one dimensional flow sections and significantly different in these regions compared to static controls, indicating that cells aligned in these regions as well, and suggesting adaptation to flow.
Figure 6-5: Microscopic images of endothelial cells stained with crystal violet on the opposite side of the stenosis in the inlet, positive and negative wall shear stress gradient and outlet regions after 24 hrs of flow exposure at Re=200 (A). Corresponding shape index values at Re=200 and different times (B).

6.6.4 Immunofluorescence Staining and Confocal Imaging

Immunofluorescence staining and confocal imaging techniques were developed to examine the cytoskeletal structure. Representative images of F-actin filaments are shown in Figure 6-6. It can be seen that fibers rearrange with time and WSS magnitude. The images presented demonstrate the reorganization of cell microfilaments with flow exposure, with more aligned fibers as time and flow increased. With these images we demonstrate that examination using confocal microscopy is possible in the models designed, hence making them useful in the localized observation of cell expression in realistic stenotic geometries.
Figure 6-6: F-actin filament distribution at different times and wall shear stress magnitudes as imaged by confocal microscopy (magnification 20x). The changes were progressive and magnitude dependent.

### 6.7 Discussion

Hemodynamic forces are believed to play a key role in the development and stability of coronary plaques. To our knowledge, this is the first *in vitro* endothelial cell culture model replicating an eccentric atherosclerotic plaque. The hemodynamic forces present in those regions can cause a heterogeneous morphological adaptation of the endothelium when compared to fully developed flow regions. The effects of WSS on endothelial cell function have been most often studied *in vitro* by exposing cells to uniform hemodynamic patterns (8, 11, 13, 25-27). Few studies have employed spatial gradients (17, 18) in wall shear stress and none in three dimensional geometries.

The idealized 50% asymmetric stenosis model developed represents a mildly diseased coronary artery, Figure 6-1. Very few tubular geometry studies can be found in the literature, likely because they are a challenge to build and to analyse. To our knowledge, this is the first three dimensional *in vitro* model
presenting the spatial wall shear stress gradient regions present near an atherosclerotic plaque. In this study, we were able to achieve confluence in three dimensional asymmetric stenosis models and to expose endothelial cells to steady physiologic WSS levels at varying magnitudes and for different durations. The spatial WSS gradients created by this geometry were evaluated, Figure 6-2.

Endothelial cell response to flow has been widely documented, indicating that they differentially change their structure and function in the presence of low (28), high (29) and oscillatory flow (8). Current in vitro models compare well to the study we performed in the one dimensional flow regions. Static cells were more rounded than cells under flow, Figure 6-3A. The shape index was used to quantify changes in endothelial cell morphology (24). As expected, it was significantly lower in the one dimensional flow regions (inlet and outlet) under high WSS magnitude and long duration (~0.35) than in the static models (~0.55), as shown in Figure 6-3B. The same response was seen in straight/tubular models. The shape index is known to decrease under steady uniform WSS and to be dependent on its magnitude (24, 30-34).

Existing in vitro models expose endothelial cells to relatively simple hemodynamic patterns. The majority of these models impose uniform WSS which may mask cell-cell signalling and fail to mimic the arterial in vivo geometry. Spatial wall shear stress gradients have been shown to create a heterogeneous cell response in culture (17, 18). Modified cone and plate and parallel plate chambers have been used to generate gradients, presenting flow separation, reversal and reattachment regions, hence presenting spatial gradients (8, 35). However, the significance of these changes is difficult to interpret, due to the unrealistic geometry of these models. Some studies have also examined the effects of temporal gradients (17-19, 36).

Our model includes, within the same model; one dimensional flow regions (inlet and outlet), a large increase in wall shear stress near the peak of the stenosis (acceleration) and a drastic reduction in wall shear stress (deceleration) distal to the stenosis. On the opposite wall, a similar but slightly smoother spatial wall shear stress gradient with the presence of both positive and negative gradients is
created. Over time, in these WSS gradients regions, the endothelial cell shape progressively changed, becoming aligned but to a lesser extent than in the inlet and outlet regions and with a distinctly more rounded morphology (Figures 6-4 & 6-5). The most significant difference was noted at higher Reynolds number and longer duration possibly because of the increased effect of flow in the one dimensional flow regions.

As flow approaches the stenosis, it accelerates through the throat creating a large positive wall shear stress gradient on the proximal face. Inferring from the one dimensional flow morphology results, the increase in wall shear stress would be expected to cause further cell alignment and elongation if morphology is function of WSS magnitude only. However, we found something different. In the acceleration regions, cells were significantly more rounded than in the inlet. This was most pronounced at higher inlet WSS magnitude and longer duration. To our knowledge, shape index values have not been previously reported in vitro in a positive wall shear stress gradient. Downstream of the reattachment point in the idealized step model, a positive wall shear stress gradient region exists, however, little interest has been given to this region. Tardy et al. and Depaola et al. noted a reduction in cell density within this region (8, 35). Qualitatively from their images, the cells in this region appear more rounded. Much more interest has been placed on the cells in the recirculation region and at the reattachment point. A reduction in confluency under uniform shear produces more rapid elongation (37), however, we did not observe a reduction in cell density in the regions of spatial wall shear stress gradients.

In the deceleration region, where a recirculation zone is present at the higher Reynolds numbers, there was a significant difference in shape index when compared with the one dimensional flow regions. The cells were overall more rounded in the deceleration region at all times and WSS magnitudes. Previous in vitro studies have carefully investigated the effect of flow recirculation distal to a step in cone and plate viscometers and parallel plate flow chambers. The majority of studies have qualitatively described cell morphology, showing more rounded morphology in the recirculation zone. Chiu et al. (38) using a parallel plate with a
step, similar to Nagel *et al.* (39), exposed endothelial cells to a steady 24 dyn/cm$^2$ wall shear stress. They found shape index values of 0.56 for static controls, 0.34 downstream of the reattachment point, 0.62 near the step, 0.44 in the recirculation zone between the step and 0.59 at the reattachment point (38). Decreased cell density and altered proatherogenic cell expression has also been documented in this region (8, 10, 35, 39-44). These studies have attributed the rounded morphology in the recirculation region to a reduced wall shear stress magnitude. Owing to the similarity with the morphology in the acceleration region leads us to hypothesize that this adaptation is a response to the gradient and not the magnitude in wall shear stress alone.

Rounded cells, similar to those in static models, were also found on the opposite wall of the stenosis models. On this side, a slightly smoother WSS gradient is created. The cells showed a progressive decrease in shape index with time and mean entrance WSS magnitude. The difference between the spatial gradient and the one dimensional flow regions was greatest at longer time and higher WSS. Although cells in the deceleration/negative gradient regions were the most rounded, the acceleration/positive gradient regions did vary significantly from the one dimensional fully developed flow regions. This response of ECs to this unique flow pattern has never been reproduced *in vitro* and is therefore difficult to compare to other studies.

Similar to others, we found that the time course of the cytoskeletal rearrangement is influenced by the magnitude and duration of the applied wall shear stress and this alignment is reversible upon flow cessation (30, 37, 43, 45, 46). Due to the transparent nature of the model, local observations on the expression through confocal imaging were possible. Morphological changes are the endpoint of a complex network of events in endothelial cells, from the sensing of the force to the intracellular transduction within the cell to the subsequent upregulation and downregulation of genes resulting in the expression of inflammatory molecules (10, 40, 44, 47-53). Cell-cell interactions and signalling between endothelial cells through cell junctions are also affected (54-57). Future
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studies will investigate the local response of inflammatory markers to the spatial gradients in shear stress created by the 50% asymmetric stenosis model.

This novel model and the techniques developed to study endothelial cells within it, can lead the way to study complex interactions and signalling between endothelial cells present near atherosclerotic plaques. Our results suggest that cells respond to the gradients in WSS and not only the WSS magnitude. Overall, this work demonstrates the need to use more realistic hemodynamic environments when studying endothelial cell response. Spatial WSS gradients produce a different response than uniform shear and this is true in regions of acceleration and deceleration. Further study of endothelial cells to local shear gradients will improve our understanding of focal remodeling and plaque stability.

6.8 Acknowledgements

We thank J. Rossi and L. Danielczak for their technical assistance in the experiments and C. Piche and S. Meadley for her help in the determination of the medium properties. This work was supported by grants from the Canadian Foundation for Innovation (CFI), the National Sciences and Engineering Research Council (NSERC), the Canadian Institutes of Health Research (CIHR), the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) and the Eugenie Ulmer Lamothe Fund (EUL). L.R. holds scholarships from the National Sciences and Engineering Research Council (NSERC), the McGill Engineering Doctoral Award, the Vadasz Family Foundation and the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT).
6.9 References


CHAPTER 6: MORPHOLOGY IN STENOTIC MODEL


CHAPTER 7: Neutrophil Adhesion on Endothelial Cells in a Novel Asymmetric Stenosis Model: Effect of Wall Shear Stress Gradients

7.1 Preface

This article has been submitted for publication in the American Journal of Physiology – Heart and Circulatory Physiology with the title of ‘Neutrophil Adhesion on Endothelial Cells in a Novel Asymmetric Stenosis Model: Effects of Wall Shear Stress Gradients’ by Rouleau, L. Copland, I.B., Tardif J.-C., Mongrain, R., Leask, R.L. Atherosclerosis is a complex disease involving a variety of cell types. Leukocyte adhesion is key in the progression of atherosclerosis. In this study, we investigated the adhesive properties of neutrophils on endothelial cells in three dimensional stenosis models. As morphology differed in all regions of spatial wall shear stress gradients, it was interesting to observe the localized adhesion of neutrophils within a complex geometry, as conventional models expose both blood components and endothelial cells to simplified shear fields.

Cytokines, such as TNF-α, are known to create an inflamed or dysfunctional endothelium characterized by the upregulation of cell adhesion molecules, partly responsible for the adhesion of neutrophils on the endothelium. This was observed by confocal microscopy and by western blotting (L.R.). The previously designed models were used and endothelial cells were cultured within them (L.R.). Adhesion was quantified by manually counting the cells on acquired microscopic images (L.R.).

Neutrophils adhered to a greater extent on stimulated endothelial cells and less with increasing wall shear stress magnitude. Regionally, significantly more adhesion was observed in the wall shear stress gradient region distal to the stenosis than in the inlet, and slightly more proximal to the stenosis. Cells were presheared in order to determine the potential protective effect of shear. Laminar
shear stress decreased the expression of cell adhesion molecules in stimulated cells and significantly decreased adhesion. However, the ratio of adhesion between the recirculation zone and the inlet increased, hence exhibiting an increased regional difference. In the recirculation zone, neutrophils were entrapped, resulting in enhanced contact time and possible activation with endothelial cell contact. Endothelial cells within this region may also respond differentially to shear forces, creating a pro-inflammatory endothelium.

This work highlights the importance of local shear forces in neutrophil adhesion. This suggests a role for neutrophil-endothelial cell interactions in the atherosclerotic process, especially in wall shear stress gradient regions and demonstrates the influence of hemodynamics in the local recruitment of leukocytes to an established coronary plaque.

Contributions
L.R. designed and performed the research, collected, analyzed and interpreted the adhesion data, performed statistical analysis and wrote the manuscript.
I.B.C. provided the NB4 cells and the general idea for the publication.
J.C.T. provided resources at the Montreal Heart Institute and contributed to the initial troubleshooting of the experimental set-up.
R.M. designed the stenosis model geometry and provided funding.
R.L.L. revised the manuscript, helped to interpret the data, funded the research and contributed to the troubleshooting and the experimental design.

Conference Proceedings
2. Rouleau, L., Farcas, M.A., Copland, I., Tardif, J.-C., Mongrain, R., Leask, R.L. In Vitro Assessment of Endothelial Cell Inflammatory Response and Leukocyte


7.2 Article 4

Neutrophil Adhesion on Endothelial Cells in a Novel Asymmetric Stenosis Model: Effect of Wall Shear Stress Gradients

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7.3 Abstract

Leukocytes play a pivotal role in the progression of atherosclerosis. The adhesion of leukocytes to the endothelium is believed to be mediated by biochemical and mechanical factors. A novel three dimensional in vitro asymmetric stenosis model was used to better investigate the role of local hemodynamics in the adhesion of leukocytes to an established plaque. The adhesion of a human promyelocytic cell line (NB4) on a human abdominal aortic endothelial cell (EC) monolayer was quantified. NB4 cells were circulated over TNF-α stimulated and non-stimulated ECs for 1 or 6 hrs at 1.25 or 6.25 dyne/cm² and compared to static conditions. Cytokine stimulation increased significantly endothelial cell expression of intercellular adhesion molecule and vascular cell adhesion molecule as observed by confocal microscopy and by western blotting. Under static conditions, neutrophils adhered overall more than under flow, with decreased adhesion with increasing shear. Significantly more adhesion was observed in the wall shear stress gradient region distal to the stenosis (recirculation) than in the inlet, and slightly more proximal to the stenosis. Preshearing the endothelial cells decreased the expression of cell adhesion molecules in inflamed endothelium and significantly decreased adhesion. However, the ratio of adhesion between the recirculation zone and the inlet increased, hence exhibiting an increased regional difference. This work suggests an important role for neutrophil-endothelial cell interactions in the atherosclerotic process, especially in wall shear gradient regions. This is important clinically, potentially helping to explain plaque stability.
7.4 Introduction

Inflammation plays a pivotal role in the initiation and progression of atherosclerotic lesions. There is substantial evidence that shows the involvement of inflammatory pathways in the pathogenesis of atherosclerosis (25; 43; 44). In established plaques, inflammatory cell recruitment produces various enzymes, activators and inhibitors which can destabilize the fibrous cap (26; 40). Post mortem studies have shown an accumulation of inflammatory cells in the shoulder regions of ruptured coronary plaques (22; 37).

The local response of endothelial cells (ECs) to hemodynamic forces is hypothesized to cause the predilection of atherosclerosis in regions of complex blood flow, such as bifurcations, branches and curvature (11; 23). In vitro studies have confirmed the ability of ECs to alter their phenotype in response to fluid shear stress into a pro-inflammatory or pro-atherogenic phenotype (16; 31). The recruitment and attachment of leukocyte to the endothelium is dependent on the cell phenotype and the local fluid forces acting on the circulating leukocyte (5; 42). The local response of the endothelial cells and the complex hemodynamics created by the geometry of a stenosis likely plays a role in the regional attachment of leukocytes. Incorporation of leukocytes into the vessel wall follows a complex sequence of events which includes the tethering and rolling of the leukocytes along the endothelial cell surface, attachment to the endothelial cells and transmigration through the endothelial layer into the vessel wall (30; 36). This is a very dynamic process and one that needs to be understood in the context of coronary stenosis and plaque stability.

To better understand the role of hemodynamics in the local recruitment of leukocytes to an established coronary plaque, we have developed a novel three dimensional in vitro asymmetric stenosis cell culture model. The results show local increases in cell adhesion in regions of spatial wall shear stress gradients and a dependency on the mean entrance wall shear stress, adhesion assay duration and endothelial cell phenotype.
CHAPTER 7: NEUTROPHIL ADHESION

7.5 Methods

7.5.1 Cell Cultures

The interaction of an acute promyelocytic leukaemia cell line (NB4 cells) with human abdominal aortic endothelial cells (HAAECs) was investigated in a three dimensional cell culture model. NB4 cells were maintained in suspension culture at 2x10^5 - 1x10^6 cells/mL in RPMI 1640 medium with 2 mM L-glutamine (Hyclone, SH3002701), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). For differentiation into granulocytes, cells were stimulated for 48 hrs in the presence of 10^-6 M all-trans-retinoic acid (ATRA) (Sigma R2625), diluted in cell culture media from a stock of 10^-4 M in DMSO, prior to the adhesion assays. Cell viability was assessed using the trypan blue dye exclusion test (Invitrogen, 15250-061).

Human abdominal aortic endothelial cells (HAAECs) derived from a 20 year old male were purchased from ATCC (American Type Culture Collection, CRL-2472) and expanded up to fifth passages. Flasks were coated with 0.1% pig gelatin (Sigma-Aldrich, G2500), and cells cultured in endothelial cell growth media (Promocell, C-22010, C-39215), 10% fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). At confluency, cultures were rinsed with phosphate buffered saline (PBS) solution and harvested with Trypsin-EDTA (Invitrogen, 25200-072). Endothelial cells were stimulated with Tumor Necrosis Factor alpha (TNF-α) for 24 hrs 10 ng/mL (Chemicon, GF 023).

7.5.2 Model Preparation

The geometric model used is an idealized model of a coronary artery presenting an eccentric stenosis with a 50% area reduction. Casts to create the lumen of the artery were made with a low melting point alloy (Cerrolow117™, Cerro Metal Products). Transparent Sylgard184™ (Dow Corning) silicone elastomer was poured around the lumen casts and allowed to cure. The casts were
then removed from the models. The resulting model geometry is shown in Figure 7-1A. The models were hydrophilized using 75% sulphuric acid for 45 minutes, boiled in deionized water for 30 minutes and coated with 40 μg/mL fibronectin in deionized water (Sigma-Aldrich, F0895) overnight at 37°C. Solutions were aspirated and the models washed with media once before the cells were seeded, at a seeding density of $1 \times 10^6$ cells/mL ($\pm 2 \times 10^5$ cells/mL) (Beckman Coulter).

![Figure 7-1: Asymmetric stenosis model. (A) Model flow regions. (B) Perfusion flow loop diagram. (C) Representative images of flow profiles in the recirculation region at a Reynold number of 165.](image)

7.5.3 Perfusion Flow Loop

The flow loop (Figure 7-1B) consisted of a reservoir, tubing, dampeners and an 8-rollers peristaltic pump head with a programmable drive (Ismatec, ISM 404 and ISM 732) producing a steady flow rate at the entrance of the models to match a mean entrance wall shear stress (WSS) of 1.25 and 6.25 dynes/cm². A water bath was used to heat the media in the reservoir which was supplemented with 25 mM HEPES (Invitrogen, 15630-080). The sterilized system was assembled and installed on an inverted microscope. In the section upstream of the model, a length of straight tubing was used to enable the flow to develop before entering the model. The viscosity (0.78 cP) and density (994.3 kg/m³) of the media were measured using a double gap rheometer (Bohlin, Model CVO 120 HR) and a pycnometer (Fisher Scientific, 3-247).
7.5.4 Wall Shear Stress Determination

The photochromic dye tracer technique was used to verify the wall shear stress patterns within the models and to determine the points of separation and reattachment. In these experiments, an identical cast model to the cell culture model was perfused with a solution of odorless mineral spirits (Shell-Sol 715) and trace amounts of colourless dye (1',3',3'-trimethylindoline-6-nitrobenzospiropyran). When exposed to UV light from a laser, the solution turns opaque and forms a photochromic trace (9; 24; 32; 33). A CCD camera (Dalsa Pantera TF IM60, Dalsa) and an image-processing board (National Instruments PCI 1428) were used to track the molecular displacement and calculate the wall shear rate. To account for differences in fluid properties, the mean entrance Reynolds number was matched (Re=165 and Re=827) corresponding to the 1.25 and 6.25 dynes/cm² mean entrance WSS of the cell culture experiments.

7.5.5 Adhesion Assays

Adhesion assays were performed under static, low shear (1.25 dyne/cm²) and high shear (6.25 dynes/cm²) conditions. The NB4 cells were circulated over either a TNF-α stimulated or a non-stimulated endothelium for 1 or 6 hrs. In a parallel set of experiments, the endothelial cells were pre-treated by shearing for 24 hrs prior to the adhesion assay. The presheared models were washed twice with fresh media before the adhesion assay was performed. After washing, the models were inserted back in the flow loop and the pump was started, circulating fresh media.

In all experiments, the ATRA differentiated NB4 cells were resuspended in fresh media and the final concentration flowing over the endothelial cells was adjusted to 5x10⁵ cells/mL (Beckman Coulter) and injected in the media reservoir. After incubation, non-adherent cells were removed by flowing fresh media over the endothelial cells. The pump was stopped and the models were taken out of the loop. The models were washed with PBS twice and fixed for 20 minutes with 1% fresh paraformaldehyde (Sigma, P6148). The models were kept at 4°C in a solution (1:1) of PBS and glycerol (Sigma, G5516). For morphological
observations and adhesion quantification, an inverted light microscope with a 
CCD camera was used (Leica, DMIL). Representative images (n=3) of each 
region were taken and NB4 cells present in each field of view were counted in 
order to quantify cell adhesion. Videos were acquired using a high speed camera 
(Sony, DFW-X710) which allowed for the visualization of the tethering, rolling 
and attachment of the cells within each flow region.

7.5.6 Analysis of Cell Surface Adhesion Molecules by Confocal 
Microscopy

Fixed endothelial cells within the models were analyzed for ICAM-1 and 
VCAM-1 protein expression by immunofluorescence. Cells were washed with 
PBS, and blocked in 2% normal donkey serum (NDS, Jackson Immunoresearch, 
017-000-121), 0.2% Triton X-100 (Sigma, T8787) in PBS for 30 minutes at room 
temperature. Primary antibodies were diluted in 1% NDS, 0.05% Triton-X100 
and incubated overnight at 4°C in mouse monoclonal anti-VCAM-1 (sc- 
20069,1:100, Santa Cruz Biotechnology) and anti-ICAM-1 (sc-8439, 1:100, Santa 
Cruz Biotechnology). After three PBS washes, secondary antibodies were 
incubated for 1 hr at room temperature (Alexa Fluor 555 anti-mouse IgG 
(A31570), 1:600, Molecular Probes). TOPRO-3 nuclear counter stain was used 
for 1 hr at room temperature (T3605, 1:300, Molecular Probes) after an RNase 
treatment of 30 minutes (Sigma R6513, 1 mg/mL). Models were mounted using 
0.2% Dabco/Glycerol (Sigma D2522, 1:5). Cells were examined under a laser 
scanning confocal microscope (LSM 510, Zeiss), using HeNe1 (543 nm) and 
HeNe2 (633 nm) lasers for excitation of the fluorochromes and a 32x/0.4 A-Plan 
objective (Zeiss). Maximum intensity projections were produced from Z series 
using the LSM software (Zeiss).

7.5.7 Analysis of Protein Expression using Western Blotting

Control and stimulated straight models were assayed in parallel to quantify 
protein expression after TNF-α stimulation by western blot. Cells were collected
from the models and lysed in RIPA lysis buffer (50 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Sigma, P8340). The cells were lysed using three cycles of freezing and thawing, with vortexing and a final sonication step of 30 minutes on ice. Cell debris were separated from proteins by centrifugation at 16,000 g for 15 minutes at 4°C. After quantification using the bicinchoninic acid assay (Pierce), proteins were resuspended in 12 mM Tris-HCl, 10% glycerol, 0.4% SDS and 0.02% bromophenol blue and boiled for 5 minutes. The proteins (2 µg) were then loaded on 7% polyacrylamide gels (30% w/v, 1:29.2) and run in a Mini Protean III apparatus (Biorad). The proteins were transferred on polyvinylidene fluoride (PVDF) membranes in a Transblot apparatus (Biorad). The membranes were incubated for 30 minutes at room temperature in 5% nonfat dry milk in 0.1% Tween-20 in PBS to block nonspecific protein binding and incubated overnight in primary antibody in 0.05% Tween-20, 2% Milk in PBS. Antibodies for ICAM-1 (sc-8439, 1:100, Santa Cruz Biotechnology), β-actin (sc-47778, 1:200, Santa Cruz Biotechnology) and VCAM-1 (sc-8304, 1:100, Santa Cruz Biotechnology) were used. After washing in a solution of PBS and Tween-20, secondary antibodies were incubated for 1.5 hrs at room temperature (goat anti-rabbit and anti-mouse IgG, 1:10,000, Biorad). The membranes were washed and signal detected using enhanced chemiluminescence method (Pierce) and X-Ray films (X-Omat, Kodak) were exposed.

### 7.5.8 Statistical Analysis

Results are expressed as mean ± standard deviation. All experiments were performed at least in triplicates. Graphpad Prism 5 (Graphpad Software, La Jolla, CA) was used to analyze the results. Comparisons were made with One-way and Two-way ANOVAs when appropriate followed by multiple comparisons test (Bonferroni post-tests). Differences between means were considered significant at P < 0.05.
7.6 Results

7.6.1 Asymmetric Stenosis Model and Hemodynamic Regions

The asymmetric geometry of the 50% area stenosis creates a complex flow field distal to the stenosis at a Reynolds number of 165 and 827, Figure 7-1C (Re = 165). The wall shear stress patterns and location of the separation and reattachment points, defining the recirculation region, were measured using the photochromic dye tracer method. At the entrance of the model, flow was steady and had a parabolic velocity profile. Wall shear stress increases along the stenosis wall as the model constricts and reaches a peak of approximately 3.5 times the inlet wall shear stress just upstream of the stenosis peak. The near wall fluid velocity slows downstream of the stenosis and separates 2.5 mm downstream of the peak. A recirculation zone is created in this region of adverse pressure gradient which extends to the reattachment point approximately 7.5 mm downstream of the stenosis peak. With increasing Reynolds number, the separation point remained more or less at the same location, however, the reattachment point moved further downstream.

7.6.2 Endothelial Cell Expression of ICAM-1 and VCAM-1: Effect of Stimulation and Shear Magnitude

Endothelial cell response to stimulation and shear was analyzed in terms of ICAM-1 and VCAM-1 expression in the inlet region of the models. Stimulation of endothelial cells with TNF-α for 24 hrs upregulated ICAM-1 and VCAM-1 expression, Figure 7-2A. The total protein levels of ICAM-1 and VCAM-1 were determined by western blotting and were visibly upregulated with stimulation time, Figure 7-2B.
**CHAPTER 7: NEUTROPHIL ADHESION**

**Figure 7-2:** Endothelial cell adhesion molecules expression. (A) Confocal images showing endothelial cell upregulation of ICAM-1 and VCAM-1 upon stimulation with TNF-α. (B) Western blotting results showing the dependency in time for ICAM-1 and VCAM-1 upregulation using β-actin as a loading control.

Upregulation of ICAM-1 and VCAM-1 using TNF-α was time and shear dependent. The effect of TNF-α on CAM expression was noticeably reduced by 1 hr under static conditions, Figure 7-3. Flow prolonged and/or increased the expression of ICAM-1 and VCAM-1. At low shear (1.25 dyn/cm²) the expression of ICAM-1 and VCAM-1 was visually increased after 1 hr when compared to the static controls, Figure 7-3. At high shear (6.25 dyn/cm²) cell adhesion molecules were expressed at a lower level than at the lower shear, however still increased in comparison to the static controls. EC not exposed to TNF-α had very low basal expression of ICAM-1 and VCAM-1 and no noticeable differences were evident with time or shear.

**Figure 7-3:** Expression of cell adhesion molecules ICAM-1 and VCAM-1 following exposure to TNF-α as observed using confocal microscopy. Levels after removal of stimulation for 1 hr, under static conditions, low and high shear stresses.
7.6.3 Neutrophil Adhesion: Effect of Shear Magnitude and Assay Duration

Stimulation of NB4 cells with ATRA increased their adhesion to ECs significantly, Figure 7-4A, and all subsequent adhesion experiments were performed with ATRA stimulated NB4 cells. NB4 cells tended to preferentially adhere at the endothelial cell-endothelial cell junctions instead of at the nuclear area. This was observed both statically (Figure 7-4B) and under flow (Figure 7-4C). It was also observed on video that cells appeared to adhere in a more rapid and firm manner in the regions of lower wall shear stress.

![Image](image_url)

**Figure 7-4:** (A) Representative microscopic images showing the effect of ATRA differentiation and TNF-α stimulation, (B) microscopic and (C) confocal images showing adhesion location.

In the one dimensional flow regions of the models (inlet and distal), the number of adhered cells were compared in order to verify the influence of shear magnitude and assay duration on the total adhesion of NB4 cells to the endothelial cells. Static adhesion tests were performed to validate that stimulating ECs with TNF-α increases leukocyte adhesion. Static models showed uniform coverage through the models. TNF-α stimulation and assay duration significantly increased adhesion (P<0.0001 and P<0.0001, Two-way ANOVA). Significantly more NB4 cells adhered on TNF-α stimulated cells at 1 and 6 hrs when compared to control endothelial cells, Figure 7-5 A&D (P<0.001, Bonferroni post-test). There was no difference in adhesion between the 1 and 6 hrs static experiments for the non-stimulated ECs.
Figure 7-5: Flow magnitude and time effect on adhesion. Representative microscopic images (A-C) and quantified NB4 cells adhesion on HAECs data (D-F) for static (A,D), 1.25 dyn/cm$^2$ (B,E) and 6.25 dyn/cm$^2$ (C,F) conditions. Comparison with respect to the time and shear magnitude for TNF-α stimulated (G) and non stimulated cells (H).

Both time and the level of shear effected cell adhesion under flow (P<0.0001, P<0.0001, Two-way ANOVA), Figure 7-5 G&H. Flow reduced the overall adhesion to the TNF-α stimulated and non-stimulated endothelial cells, Figure 7-5 A-C. Overall, low shear (1.25 dyne/cm$^2$) reduced the adhesion by about 3 fold and high shear (6.25 dynes/cm$^2$) by 15 fold when compared to the static control models, Figure 7-5 D-F. More cells adhered with increased perfusion time at low and high shear, with the most noticeable difference seen in the non-stimulated cells. At the higher shear, there was no difference between the stimulated and non-stimulated cells by 6 hrs, however, in all other conditions, stimulation had a significant impact on cell adhesion (P<0.0001, Bonferroni post-tests). At even higher shear values (12.5 dynes/cm$^2$, data not shown) very few cells adhered to either the stimulated or non-stimulated endothelial cells.
7.6.4 Regional Endothelial Cell Expression of ICAM-1 and VCAM-1

Regionally, there appeared to be higher levels of ICAM-1 and VCAM-1 near the stenosis peak for the 1.25 dyne/cm² models, Figure 7-6. At 6.25 dynes/cm², there was less ICAM-1 signal and no noticeable difference in expression (data not shown).

Figure 7-6: Regional expression of cell adhesion molecules ICAM-1 and VCAM-1 following exposure to TNF-α for cells grown in static conditions before the adhesion assays, which was performed at low shear (1.25 dyn/cm²) for 1 hr, as observed using confocal microscopy.

7.6.5 Regional Adhesion of Neutrophils

Adhesion in the models was quantified systematically in the regions presented in Figure 7-1. Videos of the adhesion assay clearly showed the region of flow recirculation downstream of the stenosis. A distinct line of attachment was seen near the separation point distal to the stenosis and the recirculation point showed neutrophils oscillating and being split on either sides.

When quantifying the regional attachment an average over the region was used. Figure 7-7 shows the regional adhesion variation in the perfused models. There appears to be a distinct regional pattern of attachment. In general, the recirculation zone had the most attached NB4 cells. Activation of endothelial cells with TNF-α increased significantly the overall attachment everywhere except at
the peak of the stenosis, most probably due to the high shear present in this region. The adhesion was not only dependent on the location but also on time and shear magnitude for all cases (Two-way ANOVA). Without stimulation, no noticeable regional differences occur until after 6 hrs of adhesion. At low shear (1.25 dyne/cm²) attachment to the non-stimulated EC was significantly greater in the recirculation and distal regions by 6 hrs. Stimulating the cells increased the attachment at all locations with the exception of the peak of the stenosis. With a stimulated EC layer at low shear, both time and location had a significant effect on adhesion (P<0.0001 for both time and location, Two-way ANOVA). The peak and recirculation regions showed the most difference with few cells attaching to the peak and the most cells attaching in the recirculation region, Figure 7-7. The same pattern of adhesion is seen at 1 and 6 hrs, however, time appears to amplify this pattern. At 1 hr, the recirculation region has significantly more cells than all other locations (Bonferroni post-tests). By 6 hrs, the accumulation in the proximal and distal regions is similar to the recirculation region, and these two regions had more cells than the inlet (P<0.01 for both, Bonferroni post-tests). There were significantly fewer cells at the peak than at all other regions at 6 hrs. At the higher shear (6.25 dynes/cm²), there were significantly less cells attached. Only the peak region could be easily distinguished as different. For both the stimulated and non-stimulated endothelium, there was significantly less attachment in the peak region than all other regions.

**Figure 7-7:** Regional adhesion. NB4 cells adhesion to non stimulated HAECs (A) and TNF-α stimulated HAECs (B).
7.6.6 Effect of Endothelial Cell Preshearing

Preshearing the ECs for 24 hrs prior to the adhesion assay was conducted to evaluate the adaptive effect of shear on the ECs. It lowered their cell adhesion molecule expression, as shown in Figure 7-8. This was clearly visible in the TNF-α stimulated cells. Preshearing seemed to have a greater effect on ICAM-1 than VCAM-1.

![Figure 7-8: Preshearing effect on endothelial cell expression of cell adhesion molecules. (A) ICAM-1 and (B) VCAM-1.](image)

Adhesion experiments lasting 1 hr were conducted at low shear (1.25 dyne/cm²) following preshearing (1.25 dyne/cm²) for 24 hrs. Preshearing significantly decreased the adhesion of NB4 cells to non stimulated and TNF-α stimulated endothelial cells (P=0.007 and P<0.0001 respectively, Two-way ANOVA), Figure 7-9A. This effect was seen in all regions, Figure 7-9B. Shearing had less effect on adhesion on the non-stimulated endothelial cells. At the inlet, a decrease in adhesion of approximately 2.5 fold is noticed in the presheared models. To better separate the global and local response of the ECs to preshearing, the fold increase with respect to the inlet was calculated (Figure 7-9C). With the quiescent endothelial cells prior to adhesion assay, the recirculation region showed a 2-3 fold increase in cell attachment. Preshearing the cells significantly increased the normalized attachment in the recirculation zone to 6-8 fold in both the stimulated and non-stimulated models (P<0.001 and P<0.05,
Bonferroni post-tests respectively). This suggests a differential response in the regional adhesion in the recirculation zone due to the preshearing of the cells, suggesting a local change in phenotype.

**Figure 7-9:** Effect on NB4 adhesion of preshearing HAECs. (A) Representative microscopic images, (B) adhesion quantification and (C) regional fold increase with respect to the inlet.

Regionally expression of ICAM-1 and VCAM-1 was assessed through confocal microscopy. Presheared cells appeared to express higher levels of ICAM-1 and VCAM-1 in the recirculation region for the 1.25 dyne/cm² models than in other regions, Figure 7-10. In all regions, the expression of ICAM-1 and VCAM-1 appear lower than in cells that were not presheared.
**Figure 7-10:** Regional expression of cell adhesion molecules ICAM-1 and VCAM-1 following exposure to TNF-α for presheared cells, and adhesion assay at low shear (1.25 dyn/cm²) for 1 hr, as observed using confocal microscopy.

### 7.7 Discussion

Inflammation and adhesion of leukocytes to the endothelium is a critical step in the initiation and progression of atherosclerosis. In this study, we show for the first time that the flow patterns created by a 50% stenosis can locally increase the attachment of leukocytes when compared to uniform flow regions. The overall number of cell adhered depended on the phenotype of the endothelial cells, the magnitude of entrance shear stress and the duration of the adhesion assay. Taken together, this work may help to explain the presence and location of leukocytes in unstable plaques.

Most of our understanding of leukocyte adhesion and endothelial cell dysfunction has come from parallel plate and rotational viscometer cell culture experiments (17; 39). These studies have demonstrated that leukocyte attachment is dependent on the magnitude of the hydrodynamic forces, the contact frequency and duration, and the cellular response to shear stress (3; 6; 13; 14; 41). These studies have mainly been limited to exposure with steady flow and hence constant shear stresses. Few studies have incorporated more complex flow fields (3; 8) and none have investigated the attachment around an idealized stenosis, Figure 7-1.
In this study, both a non-stimulated and an inflamed (TNF-α stimulated) endothelium were used. TNF-α stimulation upregulates the expression of cell adhesion molecules (Figure 7-2), which are expressed in atheroprone regions and regions presenting plaque (10; 15). It thus creates a dysfunctional endothelium, characterized by the expression of pro-inflammatory molecules and other ligands supporting leukocyte adhesion (4). Adhesion molecules, specifically intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), bind integrins expressed on leukocytes and direct their adhesion, eventually leading to transmigration across the endothelium (12; 28; 35). The expression of ICAM-1 and VCAM-1 was upregulated in HAECs treated with TNF-α, as detected by confocal microscopy and western blotting, thus behaving similarly to other endothelial cell lines, Figure 7-2. ATRA-induced NB4 cells, by the transition from the promyelocyte to the neutrophilic myelocyte stage, have been proven to show a number of markers of the granulocytic lineage and increased expression of α4 (VLA4) and β2 (LFA-1, Mac-1), therefore, increasing adhesion to the endothelium statically (12; 20; 21; 29). Neutrophils have been identified at sites of plaque rupture, infiltrating in culprit lesions in acute coronary syndromes and have been found to accumulate at sites of denudation, despite being the first appearing phagocytes cells in acute inflammatory responses to tissue injury (27). In this study, we show the complexity of the adhesion of neutrophil like cells in a stenotic region, which was not previously appreciated.

As previously observed (18), the attachment of NB4 cells occurred at the junction between endothelial cells, Figure 7-3. Removal of TNF-α stimulation caused a decrease in the expression of cell adhesion molecules, Figure 7-4, however, under low shear, higher expression was observed. Increasing adhesion time significantly influenced the total adhesion at both shear magnitudes. This effect was most evident in the non-stimulated cells where overall fewer cells adhered, Figure 7-5. Under flow, adhesion is significantly influenced by wall shear stress magnitude, with increased wall shear stress resulting in an overall reduction in adhesion. The magnitude of wall shear stress determines many parameters that affect leukocyte adhesion, most directly the hydrodynamic forces.
that drive cell motion (39). In our experiments, there seems to be a limit to the attachment to non-stimulated endothelial cells under static adhesion, as a maximum was reached after 1 hr. This might be explained by a limited number of ligands available or a limitation of the neutrophil density in suspension. Under flow, the overall attachment values were similar to the static values at 6 hrs for both low (1.25 dyne/cm²) and high (6.25 dynes/cm²) shear experiments. The TNF-α stimulated endothelial cells showed increased NB4 adhesion at 6 hrs and adhesion under flow was significantly less at both shear levels, never reaching levels of static adhesion.

Very few studies have looked at the regional attachment of leukocytes in regions of complex flow dynamics. In an idealized model of a bifurcation, Cicha et al. found more THP-1 monocytes adhered to HUVECs in the region of non-uniform shear stress created by the expansion at the bifurcation (7; 8). Burns and DePaola used a reverse step to evaluate the attachment of U-937 cells to HUVECs. Under their flow conditions, attachment was only found in the recirculation zone and none was seen in the recovery region distal to the reattachment point (3). In the same reverse step model, Chen et al. showed that neutrophils, lymphocytes and monocytes adhered and transmigrated more in the reattachment region than in the recirculation and did not note any real difference between the recirculation and recovery (distal) regional attachment (5). They hypothesize a longer residence time and a higher near wall concentration as cells roll more slowly in the reattachment point compared to other areas possibly causing this preferential attachment. In an axisymmetric stenosis model, Hinds et al. found the attachment of U-934 cells to an E-selectin layer to be negatively correlated with the magnitude of wall shear stress in the constricted region (proximal) and stenosis region (19). It must be noted that this was a highly idealized stenosis model consisting of a linear constriction and reverse step, and the shear stress applied was an order of magnitude less than in our experiments. Interestingly, the greatest attachment was seen in the proximal portion of their model in the region of positive shear stress gradient and elevated shear stress.
When comparing these previous finding to ours, it must be noted that there are significant differences in the magnitude, duration and cell lines used. In addition, our model does not have any sharp corners and therefore presents more realistic local hemodynamics. Under the flow conditions evaluated, (Re=167 and 827) a stable recirculation zone appears downstream of the stenosis. At low shear stress (1.25 dyne/cm²) at 1 hr, the recirculation zone contained the most adherent NB4 cells in the presence of a TNF-α stimulated endothelium, Figure 7-5. Unlike Chen et al. we did not find leukocyte clustering at the reattachment point (5). This may be due to a well documented instability of the reattachment point, creating high temporal shear stress gradients (34). We did note a distinct line of attachment at the separation point (data not shown). This very focal adhesion could be due to the minimum shear stress and lack of momentum of the cells as they flow in reverse along the recirculation region to this point.

At peak stenosis, where the shear stress is highest, we saw significantly less attachment in the TNF-α stimulated cells for all conditions and in the non-stimulated cells at high shear stress and longer time. This is likely explained by the dominance of hydrodynamic forces at this location. Furthermore, at higher shear, this force was enough to strip some of the endothelial cells. With the exception of the 1 hr time point at low shear on the TNF-α stimulated endothelium, the attachment in the recovery or distal region was similar to the recirculation zone, Figure 7-7. The probability of adhesion may be increased in this region owing to the increased contact time and the potential cell activation due to cells interactions (39). Chen et al. also found little difference between the recirculation and recovery regions at higher wall shear stresses in a reverse step model. Finally, it is interesting to note that although not significant, there was a tendency for more cells to adhere in the proximal region than the inlet. This is an area of increased shear stress as the geometry creates a positive shear gradient. Hinds et al. found a similar increased adhesion of U-937 cells to the proximal part of the constriction in their idealized E-selectin coated axisymmetric stenosis (19). Taken together, our results and previous studies suggest that there is more firm
attachment of leukocytes to an endothelium in the presence of complex shear gradients (14; 17; 39).

To investigate the role of local adaptation of endothelial cells to flow, we also conducted preshearing prior to some of the adhesion assays. Preshearing the endothelial cells decreased their expression of CAMs in the one dimensional flow regions (Figure 7-8); this may explain the drastic drop in adhesion throughout the models. Sheikh et al. found preshearing to reduce the response of ECs to TNF-α and the subsequent adhesion (38). Ando et al. also found preshearing to decrease adhesion (1).

Although preshearing decreased the overall and localized cell adhesion, preshearing also appeared to have a localized effect. In both the TNF-α stimulated and non-stimulated cells, preshearing noticeably increased the adhesion difference between the inlet and recirculation regions, Figure 7-9. Local differences in cell adhesion molecule expression between the presheared and quiescent models were observed through confocal microscopy, Figure 7-10. The sensitivity of the confocal analysis was limited and qualitative. However, an interesting greater difference between the inlet and the recirculation regions was noted in presheared when compared to quiescent cells. This suggests that preshearing has a differential effect in all the regions of the models. In this sense, traditional models are adequate to detect homogeneous changes and response of endothelium to shear forces, but cannot be used to reveal the more subtle heterogeneous response caused by adaptation of the endothelial cells to local hemodynamics.

Many assumptions were made in this study to make it feasible. In vitro dynamic cell culture models were used to simulate in vivo response. The wall shear stress values used are in the low physiological range but within the range for leukocyte adhesion assays. Although limiting assumptions were made, this work is a significant improvement from previous studies as it subjects endothelial cells and blood components to realistic hemodynamic gradients in a controlled environment. As with any experimental model, the pathophysiological plausibility
of the findings can only be established when carefully analyzed in the context of other experimental and clinical data.

Facilitating inflammation is a hallmark of endothelial cell dysfunction. Inflammatory processes are important in all stages of atherosclerosis development including the initiation, progression and plaque disruption (2). The asymmetric stenosis model presented creates a complex hemodynamic environment. In established plaques in vivo, inflammatory cell recruitment produces various enzymes and pro-coagulation factors (40). Enzymatic activity is believed to destabilize the fibrous cap and lead to plaque rupture. A more realistic model like this one, can improve our understanding of the possible location for plaque rupture and the progression once a plaque has formed, with one of the first step being leukocyte adhesion. The videos acquired provided clues on the possible reasons of the increased adhesion in the recirculation zone distal to the stenosis. Adhesion was higher in wall shear stress gradient regions, both proximal and distal to the stenosis. Neutrophils were entrapped in the recirculation zone, with resulting enhanced contact time and possible activation with endothelial cell contact. This is of great clinical interest as it is believed that plaque rupture occurs preferentially at the shoulders of the plaque.

7.8 Grants

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7.9 References


CHAPTER 8: INFLAMMATORY RESPONSE OF HUMAN AORTIC ENDOTHELIAL CELLS IN A STENOTIC HEMODYNAMIC ENVIRONMENT

8.1 Preface

The article presented in this Chapter has yet to be submitted for publication with the title of ‘Inflammatory Response of Human Aortic Endothelial Cells in Stenotic Hemodynamics Environment’ by Rouleau, L., Rossi, J., Leask, R.L. This section describes the differential endothelial cell inflammatory marker expression in a time and wall shear stress dependent manner. Regional analysis through confocal imaging was performed in the wall shear stress gradient regions of the asymmetric stenosis model developed. The local expression of the endothelial cells is currently being quantified in order to add it to the manuscript before its submission.

Atherosclerotic plaques most frequently form an eccentric stenosis, hence modifying the fluid dynamic environment. This change in local hemodynamics is capable of influencing endothelial expression of pro-inflammatory and pro-thrombotic molecules. One of the first steps in the initiation of atherosclerosis is the attachment of mononuclear cells. The presence of a stenosis alters hemodynamics, possibly creating conditions which could enhance endothelial inflammation marker expression and blood component adhesion. Selectins and cell adhesion molecules are particularly important in the rolling/tethering, firm adhesion and transmigration of white blood cells to a dysfunctional endothelium and are highly expressed in athero-prone regions of the vasculature.

This work aimed at examining, in more detail, the expression of endothelial cells present in the in vitro models designed previously along with the procedure developed to expose cells to steady flow (L.R.). A modified protocol allowed the extraction of the cells from the models (L.R.). Western blotting (L.R.) and real-time PCR (L.R.) were used to analyze cell expression as well as confocal
Regional expression was analyzed using immunostaining and confocal microscopy as it is one of the few tools in molecular biology which allows examining the protein expression of very few cells without having to collect and pool cells together (In progress L.R.).

The main findings of this work include the importance of examining the early and long term expression of inflammatory molecules in order to conclude on the effects of steady wall shear stress on endothelial cell expression. Regional analysis showed that inflammatory markers (ICAM-1, VCAM-1, E selectin, NF-κB) are differently expressed within both positive and negative wall shear stress gradient regions. This is important clinically, as these gradients are present in plaque shoulders, where rupture is more likely to occur.

**Contributions**

L.R. designed and performed the research, collected, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript.

J.R. developed the RNA analysis procedure.

R.L.L. revised the manuscript, interpreted the data, funded the research and contributed to the troubleshooting and the experimental design.

**Conference Proceedings**


8.2 Article 5

Inflammatory Response of Human Aortic Endothelial Cells in a Stenotic Hemodynamic Environment

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8.3 Abstract

Inflammation plays a key role in the development and stability of coronary plaques. Endothelial cells are known to regulate inflammation by altering the expression of adhesion molecules. Wall shear stress is believed to be able to create a pro-inflammatory endothelial cell phenotype. The duration (0 – 24 hrs) and magnitude (4.5 – 18 dyn/cm²) effect of steady wall shear stress exposure of human aortic endothelial cells was analyzed in straight/tubular *in vitro* models. The mRNA expression of ICAM-1, VCAM-1 and E selectin was quantified by RT-PCR and the translocation of NF-κB by immunostaining. The effects of spatial wall shear stress gradients created by an eccentric plaque were locally investigated by immunofluorescence and confocal microscopy. Endothelial cell exhibit a transient increase in transcription factor NF-κB which was WSS magnitude dependent. Intercellular adhesion molecules (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E selectin exhibited a sustained increase in protein expression in time. The mRNA levels of these molecules were transiently upregulated and followed by a decrease in expression lower than static controls. Regionally, increased inflammatory marker expression was observed in the wall shear stress gradient regions proximal and distal to the stenosis. Endothelial cells in regions of wall shear stress gradients displayed increased inflammatory molecule expression and both time and WSS magnitude had a significant effect. The results from the straight/tubular model cannot explain the regional variation seen in the stenosis models, hence spatial wall shear stress gradients are believed to alter endothelial cell inflammatory marker expression.
8.4 Introduction

Atherosclerosis is recognized as a focal disease occurring mainly in areas of disturbed blood flow \(^1-^9\). Indeed, hemodynamic forces are believed to play a key role in the development coronary plaques \(^10-^12\). In stenotic regions, once an obstruction has developed, blood flow is disturbed, potentially affecting disease progression \(^13,^14\). Geometry in stenotic regions creates complex fluid and tissue stresses which may cause plaque rupture \(^15,^16\). Great advances have been made in our ability to study the response of cells to mechanical forces. The impact of low and high wall shear stress (WSS) as well as temporal and spatial gradients in WSS have been studied in simple geometries \(^11,^17-^26\). Unfortunately, the inability to recreate \textit{in vitro} the complex \textit{in vivo} mechanical stimuli may be obscuring our understanding. Currently, the models that best replicates the hemodynamics present near a stenosis use one dimensional flow over a step or a sudden expansion \(^20,^27\). This geometry recreates the recirculation downstream of the stenosis but fails to mimic the fully three dimensional \textit{in vivo} environment and neglects the positive gradient region proximal to the stenosis.

Eccentric coronary stenoses are prone to atherothrombosis which is believed to be a major cause of coronary artery disease mortality \(^28\). Inflammation plays a pivotal role in plaque stability and thrombosis, as cells may infiltrate the cap and weaken the plaque, leading to rupture \(^29,^30\). Indeed, leukocytes have been shown to localize in the shoulder regions of plaques \(^31,^32\). Endothelial cell (EC) expression of inflammatory markers, such as transcription factor NF-\(\kappa\)B which can regulate inflammatory proteins such as intercellular adhesion molecules (ICAM-1) \(^3,^33\), vascular cell adhesion molecule (VCAM-1) \(^34-^36\) and E selectin \(^3\), have been demonstrated to be key in the recruitment and transendothelial migration of leukocytes and monocytes. These molecules are regulated, at least in part, by WSS and expressed in atherosclerotic lesions \textit{in vivo} \(^37-^39\). In this study, we examined the effects of steady WSS over time on the expression of these key inflammatory molecules. Furthermore, we hypothesized that the spatial gradients
created by an asymmetric stenosis would cause regional differences in inflammatory marker expression.

8.5 Methods

8.5.1 Wall Shear Stress Determination

Experiments were performed in straight/tubular and asymmetric stenosis models as described in the previous chapters. An idealized model of a coronary artery presenting an eccentric stenosis with a 50% area reduction was used, Figure 8-1A. The model has an inlet diameter of 3.175 mm and a length of 10 cm. The velocity and wall shear stress profiles were evaluated using photochromic molecular flow visualization (PMFV) as described previously. In these experiments, an identical cast model to the cell culture model was perfused with a solution of odorless mineral spirits (Shell-Sol 715) and trace amounts of colourless dye (1',3',3'-trimethylindoline-6-nitro-benzospiropyran). When exposed to UV light from a laser, the solution turns opaque and forms a photochromic trace. A CCD camera (Dalsa Pantera TF IM60, Dalsa) and an image-processing board (National Instruments PCI 1428) were used to track the molecular displacement and calculate the wall shear rate. The Reynolds number and the corresponding fully developed entrance wall shear stresses are found in Table 8-1.

Table 8-1: Hemodynamic parameters used to subject endothelial cells to different wall shear stress values (viscosity 3.44 cP, density 1027 kg/m³).

<table>
<thead>
<tr>
<th>Flowrate (mL/min)</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re</td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>132.6</td>
<td>265.2</td>
<td>530.4</td>
</tr>
<tr>
<td>Shear stress (dyne/cm²)</td>
<td>4.5</td>
<td>9</td>
<td>18</td>
</tr>
</tbody>
</table>

8.5.2 Endothelial Cell Culture

Human abdominal aortic endothelial cells (HAAEC) derived from a 20 year old male were purchased from ATCC (American Type Culture Collection, CRL-2472). Cells were grown with endothelial cell growth media (Promocell, C-
22010) containing supplements (Promocell, C-39215), 10% fetal bovine serum (Invitrogen, 26140-079) and 1% penicillin streptomycin (Invitrogen, 15140-122). Cells at passage 5 were used in all experiments.

**Figure 8-1:** Asymmetric stenosis model. (A) Model flow regions. (B) Perfusion flow loop diagram. (C) Normalized wall shear stress on the stenosis side for different Reynolds numbers.

### 8.5.3 Model Preparation

Casts to create the lumen of the artery were made either using straight stainless steel rod or with a low melting point alloy (Cerrolow117™, Cerro Metal Products Co. 4470-2, melting point 47ºC, Bellefonte, PA, USA) poured in a cast of a 50% asymmetric stenosis, Figure 8-1A. Transparent Sylgard184™ (Dow Corning, PA, USA) silicone elastomer was poured in acrylic molds around the lumen geometry and allowed to cure 47, after which the cast/rod was removed from the model. Sulphuric acid treatment for 45 minutes (70%), followed by sterilization in boiling deionized water for 30 minutes and coating with fibronectin (Sigma-Aldrich, F0895, 40 µg/mL) overnight at 37ºC on a rotator at 8 rpm (Labquake Rotor, Series 1104, Barnstead/Thermolyne) allowed cell culture within the models. Models were washed with media before the cells were seeded in the models at a concentration of 1 x 10⁶ cells/mL (± 2 x 10⁵ cells/mL) (Z2 Coulter Counter, Beckman Coulter, Canada). The cell suspension was left in the
models and the models attached to the tube rotator for an incubation period of 48 hrs and the media changed after 24 hrs, leaving a monolayer to be established in the models.

8.5.4 Perfusion Experiments

The flow loop (Figure 8-1B) consisted of a reservoir, tubing, dampeners and an 8-rollers peristaltic pump head with a programmable drive (Ismatec, ISM 404 and ISM 732) producing a steady flowrate of 25, 50 and 100 mL/min at the entrance of the models to match a mean entrance wall shear stress (WSS) of 4.5, 9 and 18 dynes/cm². Hemodynamic parameters are shown in Table 1. Individual reservoirs containing 40 mL of modified growth medium were inserted in the loop as well as custom built dampeners to reduce flow pulsatility. This allowed perfusing steady flow over the endothelial cell monolayer in the models. The sterilized system was assembled and installed in an incubator at 37°C and 5% CO₂. Growth medium contained 6.7% w/w dextran (Sigma-Aldrich, D4876, MW 135 000) to match blood viscosity (3.44 cP ± 0.2 cP) and was verified using a double gap rheometer (Bohlin, Model CVO 120 HR). The density (1027 kg/m³) was measured using a pycnometer (Fisher Scientific, 3-247). Concurrent time matched dextran containing controls allowed for appropriate comparison. These were repeated at least three times under different perfusion times (0, 0.5, 2, 6, 12 and 24 hrs) and WSS magnitudes (4.5, 9 and 18 dyn/cm²). Straight/tubular in vitro models were used to verify the temporal and magnitude effects of steady laminar wall shear stress. Collection from the entire tube provided a sufficient amount of cells to perform quantitative real-time polymerase chain reaction analysis. The regional expression in the asymmetric stenosis model was evaluated by immunostaining and confocal microscopy.

8.5.5 Quantitative Real-time PCR

Cells were gathered from straight/tubular in vitro models with 0.25% Trypsin/EDTA (Invitrogen) and total RNA was extracted using RNeasy spin columns (Qiagen) with DNase I (Qiagen) digestion. Total RNA was quantified by
absorbance measurements at 260 nm. First-strand complementary DNA (cDNA) was synthesized with 0.5 μg total RNA, random hexamers (Applied Biosystems) and MultiScribe™ reverse transcriptase (Applied Biosystems) under the following conditions: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Real-time PCR reactions were carried out in Power SYBR® Green PCR Master Mix (Applied Biosystems) with QuantiTect Primer Assays (Qiagen) and performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) under the following conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. The housekeeping gene was β-Actin and the ΔΔCt method was used for relative quantification of gene expression.

8.5.6 Immunofluorescent Staining and Confocal Imaging

Endothelial cells fixed in 1% paraformaldehyde for 20 minutes were analyzed regionally for NF-κB, ICAM-1, VCAM-1 and E selectin expression. After fixation, cells were washed with phosphate buffered saline (PBS) and blocked for 30 minutes at room temperature in PBS containing 2% normal donkey serum (NDS, Jackson Immunoresearch, 017-000-121), 0.2% Triton X-100 (Sigma, T8787). Primary antibodies (Santa Cruz biotechnology and R&D systems) against NF-κB (1:500, sc-372), ICAM-1 (1:100, sc-8439, BBA3), VCAM-1 (1:100, sc-20069, BBA5) and E selectin (1:100, BBA2) were diluted in 1% NDS, 0.05% Triton-100 and incubated overnight at 4°C. After three washes in PBS, secondary antibodies were incubated for 1 hr at room temperature (Invitrogen, donkey Alexa Fluor 488 anti-rabbit IgG (A21206) and Alexa Fluor 555 anti-mouse IgG (A31570), dilution 1:600). Models were then mounted using 0.2% Dabco/Glycerol (Sigma D2522, R6513, 1:5). Cells were examined under a laser scanning confocal microscope (LSM 510, Zeiss), using Argon (488 nm) and HeNe1 (543 nm) lasers for excitation of the fluorochromes and a 32x/0.4 A-Plan objective (Zeiss). Maximum intensity projections were produced from Z series.
8.5.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism™ 5.0 software. Results are shown as mean ± standard error mean (SEM) unless otherwise specified. Mean ΔΔCt mRNA expression values were compared using one-way and two-way analysis of variance (ANOVA) using repeated measures followed by a Bonferroni post-test. A P<0.05 was considered statistically significant.

8.6 Results

Photochromic molecular flow visualization verified the presence of fully developed flow throughout the straight/tubular models and the inlet/outlet of the asymmetric stenosis models. It also allowed the characterization of the complex hemodynamic environment within the different regions of the asymmetric stenosis, Figure 8-1A. Proximal to the stenosis peak, an acceleration zone is found with a maximum wall shear stress (~3.5 times the inlet value) slightly proximal to the throat, Figure 8-1C. Distal to the stenosis, at moderate Reynolds number, flow reversal is observed with the formation of a recirculation zone. Alternatively, a deceleration region is observed at lower Reynolds number with no flow separation. We have previously shown a regional correlation in endothelial cell morphology and leukocyte attachment in these spatial wall shear stress gradient regions

8.6.1 Early Activation of Transcription Factor NF-κB

The location of transcription factor NF-κB within the cell was influenced by time and WSS magnitude in regions of fully developed flow, Figure 8-2. At high WSS (18 dyn/cm²), no translocation was observed at 2 hrs, however, at 0.5 hr some translocation was observed (data not shown). At lower WSS magnitude (4.5 and 9 dyn/cm²), the maximum translocation seemed to occur at 2 hrs after the onset of flow. By 6 hrs, NF-κB expression was mainly cytoplasmic, with some cells expressing the transcription factor in the nucleus area, and compared to the onset of flow, an increase in the expression level was noted. After 12 hrs of flow,
lower levels of cytoplasmic NF-κB fluorescence were observed and fewer cells showed NF-κB in the nucleus, very similar to the 24 hrs time point.

![Figure 8-2: Effect of duration and magnitude of wall shear stress on transcription factor NF-κB expression as observed using confocal microscopy.](image)

### 8.6.2 Transient Upregulation of Cell Adhesion Molecules: ICAM-1, VCAM-1 and E selectin

Immunofluorescence intensity of ICAM-1 suggests an upregulation over time up to 24 hrs after the onset of flow exposure. The expression at 24 hrs is increased at greater WSS magnitude. Hence, there is a magnitude and time dependent expression of the protein levels of ICAM-1 in the one dimensional flow regions of the model, Figure 8-3A. Although protein expression increased until 24 hrs of exposure, a decrease in mRNA expression is observed with time at the higher WSS magnitudes tested (9 and 18 dyn/cm²). By 24 hrs, ICAM-1 mRNA levels are significantly less than the initial levels (P<0.05, and P<0.001 respectively, One-way ANOVA, Bonferroni post-test), Figure 8-3B. At the lower WSS (4.5 dyn/cm²) a statistically significant increase in mRNA expression is noticed at 6 hrs of flow over initial levels (P <0.01, One-way ANOVA, Bonferroni post-test), with a decline at later times. Also, wall shear stress significantly decreased mRNA expression at 9 and 18 dyn/cm² after 24 hrs of flow exposure (P<0.05, P<0.01 respectively, Bonferroni post-test), Figure 8-3C.
Figure 8-3: Wall shear stress magnitude and duration effect on ICAM-1 expression as assessed by confocal microscopy (A) and Real-Time PCR (B, C). Temporal variations (B) and long term (24 hrs) magnitude effect (C) (n=3, mean ± SEM) (* P<0.05, ** P<0.01, *** P<0.001, with respect to the initial levels, ⬂ P<0.05, ⬂⬀ P<0.01, with respect to the 6hrs levels).

Representative images of the expression of VCAM-1 are presented, Figure 8-4A. At all WSS magnitudes, VCAM-1 expression increased in time and was the greatest at high WSS magnitude (18 dyn/cm²). The mRNA levels of VCAM-1 show a similar trend as ICAM-1 expression, Figure 8-4B. A wall shear stress of 4.5 dyn/cm² increased significantly VCAM-1 mRNA levels after 6 hrs of exposure (P<0.05, One-way ANOVA, Bonferroni post-test). The decreased expression was greater for VCAM-1 then for ICAM-1 and wall shear stress magnitude had a significant effect on expression at 24 hrs (P<0.05, Bonferroni post-test), Figure 8-4C.
CHAPTER 8: INFLAMMATORY RESPONSE

Figure 8-4: Effect of wall shear stress magnitude and duration of exposure on VCAM-1 expression as assessed by confocal microscopy (A) and Real-Time PCR (B, C). Temporal variations (B) and long term (24 hrs) magnitude effect (C) (n=3, mean ± SEM) (* P<0.05, *** P<0.001, with respect to the initial levels, † P<0.05, †† P<0.01, with respect to the 6hrs levels).

The expression of E selectin was altered with time and WSS magnitude, Figure 8-5A. Protein expression increased and then decreased by 24 hrs. This was also found in the mRNA expression analysis, Figure 8-5B. A significant increase above the static controls was observed at the lower WSS magnitude after 6 and 12 hrs of perfusion (P<0.01, P<0.001, respectively, One-way ANOVA, Bonferroni post-test). This temporal variation was reduced and non-significant at higher WSS magnitudes. These results make it clear that cell expression varies greatly in terms of the time of exposure and the magnitude of the WSS imposed. Indeed, a significant difference was found between the lower WSS magnitude and higher values at the 24 hrs time point (P<0.05, Bonferroni post-test), Figure 8-5C.
8.6.3 Regional Expression of Inflammatory Markers

To properly evaluate the regional expression in situ, immunofluorescence staining was used. Images were acquired in the different regions of the models as well as various times and WSS magnitudes. Regional variations were observed in NF-κB expression. As observed previously, after 4 hrs flow exposure, fewer cells showed NF-κB in the nucleus in the one dimensional flow regions. Hence, this time was chosen to observe regional variations in the stenosis region. Cytoplasmic expression appeared maximum at the peak of the stenosis. A slight difference was observed for cells within the acceleration and deceleration zones, where spatial...
gradient regions are present. More cells in these regions expressed NF-κB in the nucleus when compared to both the inlet and outlet regions.

Expression of inflammatory markers was variable along the stenosis wall. A representative set of pictures of the regional expression of ICAM-1, VCAM-1 and E selectin is shown in Figure 8-6. For ICAM-1, the images shown are for a mean WSS of 18 dyn/cm² at 24 hrs after the onset of flow exposure. Maximum intensity was found at the peak. This trend was consistent over the range of WSS tested in this study. Cells in the acceleration and deceleration regions expressed more consistently ICAM-1 than in the inlet and outlet regions. VCAM-1 expression at 12 hrs and 9 dyn/cm² is shown. Visually, intensity was greater in the spatial wall shear stress gradient regions proximal and distal to the stenosis than the one dimensional flow regions. E-selectin was also more highly expressed in these regions. In this case, an earlier time (4hrs) was examined as maximum E selectin expression in the inlet was upregulated at earlier times.

Figure 8-6: Regional inflammatory markers expression at different times and inlet wall shear stress magnitudes.
8.7 Discussion

The response of endothelial cells to steady laminar WSS has largely been studied in highly simplified *in vitro* models. Protein and gene expression studies performed in these geometries have greatly contributed to the understanding of atherosclerosis. However, few studies have examined the time course of the effect of low, high and disturbed flow in the same model, hence allowing the distinction between the early cell response and the results of long term exposure to shear forces. Most studies choose one time point which could be misleading as WSS regulates cell expression vary significantly over time and for different magnitudes, Figure 8-2 to 8-5. Our work confirms the temporal effect of WSS on inflammatory genes, as well as the influence of WSS magnitude. In addition, we have been able to evaluate the regional variation of endothelial cell expression under controlled conditions.

8.7.1 Steady Fully Developed Flow

The straight/tubular *in vitro* models were used to carefully and thoroughly study the transient response of ECs to WSS. The models allowed quantification of mRNA samples, as large amounts of cells could be gathered from the whole models (~10 cm²). Confocal images for NF-κB, ICAM-1, VCAM-1 and E selectin revealed changes in protein levels in a time and WSS magnitude dependent manner. Transcription factor NF-κB has been reported to be involved in the control of several important physiological processes, including inflammation^{48-50}. Upon nuclear translocation, NF-κB binds to sites of a variety of genes, including E selectin^{51}, VCAM-1^{52} and ICAM-1^{53}. Indeed, regulation of endothelial adhesion molecules with WSS was found to correlate with NF-κB expression *in vitro*^{54-56}.

The intensity and location of NF-κB varied in time and with WSS magnitude within the fully developed flow regions. Low WSS magnitude caused the transient translocation of NF-κB. At higher arterial WSS levels, however, an early acute response was observed followed by a lower expression of NF-κB
suggesting an atheroprotective phenotype at longer exposure times and higher WSS magnitudes, Figure 8-2. Our results compare well with evidence from short and long term exposures in literature \(^{24,57-59}\). Nagel et al. found at 0.5 hr a slight upregulation of NF-κB expression under a WSS of 10 dyn/cm\(^2\), with increased nuclear expression at lower wall shear stress \(^{24}\), whereas Partridge et al. examined the long term effects (16–24 hrs) of laminar wall shear stress (12 dyn/cm\(^2\)) and found an exclusive cytoplasmic expression \(^{58}\).

ICAM-1 and VCAM-1 protein and mRNA expression levels exhibit the same pattern. Protein seemed to increase with time and WSS magnitude, whereas mRNA levels varied with a transient increase at 6 hrs and a subsequent decrease with time until 24 hrs, to levels lower than the static control. Previous studies observed quite consistent results concerning ICAM-1 expression, whereas reports on VCAM-1 expression are less consistent. Indeed, several \textit{in vitro} studies concerning the protein expression of ICAM-1 are in close agreement with ours concerning an upregulation which is shear (between 2-25 dyn/cm\(^2\)) and time dependent (4 to 24 hrs) \(^{57,60-63}\). Maximum expression of ICAM-1 mRNA has been previously reported at 8 hrs followed by a transient decrease until 24 hrs at a WSS of 10 dyn/cm\(^2\) \(^{60,63,64}\).

VCAM-1 expression was more variable than ICAM-1 levels, perhaps because basal levels are very low. Nagel et al. \(^{60}\) found that VCAM-1 was slightly upregulated at the protein level by 24 hrs for a WSS magnitude of 10 dyn/cm\(^2\), whereas Tsuboi et al. \(^{63}\) found no significant difference at 4 hrs. At the mRNA level, Nagel et al. found no significant difference between 2 and 24 hrs for a WSS of 10 dyn/cm\(^2\) \(^{60}\). However, similar to our results, Bergh et al. \(^{65}\) found that VCAM-1 mRNA expression was downregulated by WSS at 24 hrs between 1 and 25 dyn/cm\(^2\). Other investigators have demonstrated that VCAM-1 expression decreased after sustained shear stress exposure \(^{61,66}\). However, studies have also shown a great difference between species basal and flow induced expression \(^{67}\).

E selectin protein expression increased with a maximum at 8 hrs and then decreased until 24 hrs. It was magnitude dependent, with increased WSS resulting in increased expression at earlier times. At the mRNA level, similar trends to
ICAM-1 and VCAM-1 were observed, with a transient increase and decrease at low wall shear stress magnitudes. Our results compare with previous reports and contradict others. Indeed, Nagel et al. found that laminar shear stress (2.5-46 dyn/cm²) did not upregulate protein expression at 4 hrs or mRNA expression at 2, 8 or 24 hrs ⁶⁰ and Morigi et al. found no change in protein expression when cells were exposed to laminar WSS for 6 hrs at 8 dyn/cm² ⁶². However, Sampath et al. found an early transient E selectin mRNA downregulation after 1 hr ⁶¹ and Tsou et al. ⁵⁷ found that E selectin was upregulated after 4 hrs of low flow exposure (2-4 dyn/cm²) and that higher WSS (>8 dyn/cm²) suppressed expression below that of untreated cells, similar to results from our study.

8.7.2 Regional Inflammatory Marker Expression

Inflammatory markers are differentially expressed in vivo in disturbed flow regions, such as those created by an eccentric stenosis ³³,³⁸. These regional variations may help explain plaque stability ³¹,³². Few in vitro studies have examined the effect of the complex wall shear stress patterns created by atherosclerotic plaques. Most studies have focused on the post-step region, where flow reversal is observed and on the reattachment point, where flow is disturbed. In our model, separation and flow reversal only occurs at higher Reynolds number (WSS = 18 dyn/cm²). Also, an acceleration region usually absent in conventional in vitro devices is present in our model.

The wall shear stress gradients present in the asymmetric stenosis model caused a heterogeneous endothelial cell inflammatory marker expression in regions of WSS gradients compared to fully developed flow as analyzed by confocal microscopy. This model was used previously to study the morphological changes due to spatial wall shear stress gradients ⁴¹, as it is one of the last events in the signalling cascade of endothelial cell response upon flow exposure.

Activation of NF-κB under disturbed flow is known to differ from laminar uniform wall shear stress ²⁴,³⁷,³⁹,⁶⁸. Nagel et al. ²⁴ showed NF-κB upregulation in the reattachment and recirculation regions after a step at 4 hrs after flow. Indeed, we found that downstream of the stenosis, the nuclear NF-κB expression was
greater than in the inlet and outlet regions, Figure 8-6. This was not dependent on flow reversal and occurred at all Reynolds number studied. In the acceleration region proximal to the stenosis, an increased expression was also found. This is the first report of increased nuclear expression in an acceleration zone, as most in vitro models observed cell expression distal to a step. The maximal NF-κB expression was found at the peak of the stenosis. Comparing these results to the straight/tubular results suggests more than a magnitude effect. Extrapolating from the straight/tubular models, the higher wall shear stress at the acceleration and peak regions should result in decreased NF-κB expression, which is not the case.

Passerini et al.\textsuperscript{69} found an upregulation of several inflammatory molecules in disturbed flow regions, in addition to NF-κB which is consistent with a proinflammatory phenotype. Indeed, we found that ICAM-1, VCAM-1 and E selectin were selectively upregulated by spatial wall shear stress gradients. At a mean WSS of 18 dyn/cm\textsuperscript{2} at 24 hrs after the onset of flow exposure, ICAM-1 was increased at the peak, Figure 8-6. Cells downstream of the stenosis expressed ICAM-1 more highly and more consistently than in the inlet and outlet regions. VCAM-1 and E selectin expression was also altered by WSS gradients and visually a maximum intensity was observed upstream and downstream of the stenosis, Figure 8-6. In the positive gradient region, we report for the first time an increased expression of all inflammatory markers, when compared to the inlet. Again these results cannot be explained by a magnitude effect as seen in the one dimensional flow model.

Our findings agree with previous results shown in the region of flow reversal distal to a step. Resnick et al. found that VCAM-1 is downregulated by laminar shear stress\textsuperscript{70}. Brooks et al.\textsuperscript{71} identified that protein and mRNA levels increase for VCAM-1 in disturbed flow. ICAM-1 and E selectin upregulation was also reported. Truskey et al.\textsuperscript{27} using the sudden expansion flow chamber suggested that endothelial cells can respond to spatial WSS gradients. McKinney et al. found that ICAM-1 seemed influenced by shear stress gradients\textsuperscript{72}. They also found a decreased expression of ICAM-1 at 8 hrs in the reattachment point which does not agree with our results. Converging width flow channels were used.
by LaMack and Friedman\textsuperscript{73} to determine the effect on mRNA expression of wall shear stress magnitude and gradient. They found that VCAM-1 was independent of WSS magnitude or gradient, whereas ICAM-1 increased with shear stress magnitude. However, confirming our results, Cicha \textit{et al.}\textsuperscript{74} perfused bifurcation models at 10 dyn/cm\textsuperscript{2} and found that protein expression of VCAM-1, ICAM-1 and E selectin was increased in the disturbed flow regions compared to laminar wall shear stress of comparable levels. Hence, disturbed flow including the presence of wall shear stress gradients can alter endothelial cell inflammatory expression.

Although pulsatility and other factors such as pressure and cyclic strain were ignored, the asymmetric stenosis model is a significant improvement on conventional \textit{in vitro} models. No other study has looked at the response of endothelial cells to flow over an entire stenosis. Our results show that flow reversal is not required to cause regional changes in inflammatory markers previously noted distal to a step expansion. Furthermore, the WSS gradients upstream of a stenosis cause a similar response. This may help explain the localization of inflammatory cells to the shoulders of the plaques noted \textit{in vivo}.

### 8.8 Acknowledgements

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CHAPTER 8: INFLAMMATORY RESPONSE

8.9 References


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CHAPTER 9: NOVEL CONTRIBUTIONS

All five papers presented have contributed significantly to the study of endothelial cell response to wall shear stress. For example, although dextran is commonly used in perfusion studies in vitro, our study is the first to evaluate the effects it may have on endothelial cell response. Indeed, we found that its presence alters in a concentration and time dependent manner inflammatory molecule expression, hence highlighting the importance of using appropriate time matched dextran containing static controls.

My most important contribution has been the design of straight/tubular, asymmetric stenosis and realistic in vitro models along with repeatable methods to perform cell culture and to analyze cell expression using these models. Means of analysis, such as expression analysis through confocal imaging as well as protein and mRNA measurements through cell extraction procedures, were modified and adapted for the novel in vitro cell culture models. To our knowledge this is the first study in an eccentric stenosis model. Indeed, a report of invention has been filed (ROI #10021) at McGill University for the production of three dimensional in vitro cell culture models which will be made available to other researchers and to companies.

Additionally, a throughout study was performed on the temporal effects of wall shear stress exposure of different magnitudes in the straight/tubular in vitro models, documenting the time course of endothelial cell expression of key atherogenic molecules. Morphology changes were quantified in the in vitro models as well as the regional expression of inflammatory markers. The changes in morphology have not been previously reported in accelerating flow regions or regions of smooth spatial wall shear stress gradients. Neutrophil adhesion was also studied for the first time in a three dimensional realistic model representing an atherosclerotic plaque.
CHAPTER 10: CONCLUSIONS

The response of endothelial cells to wall shear stress is a complex problem that needs to be better understood. To aid in this better understanding, in vitro models are needed. This thesis details the efforts I have made to improve endothelial cell dynamic stimulation.

Dextran has a non-negligible effect on endothelial cell response to wall shear stress. The effects of dextran were studied in a time and concentration dependent manner, under static and flow conditions. We show that appropriate static controls containing dextran are required when using dextran in perfusion in vitro experiments as it alters inflammatory marker expression.

We demonstrated our ability to form a three dimensional flow responsive confluent endothelial cell monolayer and developed the tools to analyze the cells within the in vitro models, in terms of confocal microscopy, as well as protein and gene expression. The shape index was used to quantify cell morphology, one of the last events in the adaptation of endothelial cells to flow. Hence, this long term response was analyzed and the results showed that ECs respond to spatial wall shear stress gradients both in the anatomically realistic and the asymmetric stenosis model. Within all disturbed flow regions of the model, positive and negative as well as the smoother wall shear stress gradient opposite to the stenosis, cells were more rounded, exhibiting an atheroprone phenotype. In the one dimensional steady flow regions, cells aligned in a time and wall shear stress magnitude dependent manner.

As the hemodynamic forces were well described in the asymmetric stenosis model using photochromic flow visualization and computational simulations, adhesion assays and expression analysis were performed in this geometry. Neutrophils adhered more in the distal region of the stenosis, where flow reversal occurs and slightly more proximal to the stenosis. Preshearing of endothelial cells differentially affected the adhesion with an increased adhesion in the low wall shear stress recirculation zone. In order to better understand the role of fluid wall shear stress induced inflammation, an analysis of temporal and magnitude effects was conducted. The regional expression within the asymmetric
steno}\text{sis model also indicates a role for spatial wall shear stress gradients in the progression of atherosclerosis.

Overall, this work suggests that spatial gradients in wall shear stress can alter endothelial cell response. This is true not only downstream of a stenosis but also in the acceleration zone proximal to a stenosis. The results may help to explain the tendency of coronary plaques to fail in the shoulder regions.
CHAPTER 11: RECOMMENDATIONS

The *in vitro* environment used in these studies has limitations in terms of respecting *in vivo* geometry and some recommendations could render the system even more physiological. Mechanical forces such as cyclic strain and pressure fluctuations were absent in our study as well as flow pulsatility. Despite these limitations, the model presented here is a vast improvement on current technology.

### 11.1 Geometry

A casting procedure could be used to recreate realistic *in vivo* geometry \(^{196-199}\). However, this can be quite tedious and challenging in terms of producing and analyzing the models as hemodynamic experimental flow determination is difficult to accomplish in complex geometries. The geometry was simplified, but still incorporates spatial wall shear stress gradients present in atherosclerotic plaque regions.

### 11.2 Cyclic Strain and Pressure Forces

In order to incorporate other mechanical forces present *in vivo*, the compliance of Sylgard184\(^{\text{TM}}\) should be characterized and wall thickness adjusted to match blood vessel compliance \(^{197}\). Subsequently, numerical simulations can be performed and experimental studies made to quantify the mechanical forces resulting from the movement of the walls with steady and pulsatile flow. This will allow endothelial cell exposure to cyclic strain, pressure as well as shear stress, all important mechanical forces present *in vivo*.

### 11.3 Pulsatility

Continuous steady flow was applied to a cultured endothelial cell monolayer. The flow loop could be modified to incorporate pulsatility. The implementation of Labview\(^{\text{TM}}\) to control the pump and to mimic *in vivo* flow
patterns could be set-up in order to expose cells to temporal wall shear stress gradients.

11.4 Rheological Properties

Blood is a non Newtonian fluid. However, most studies use a Newtonian fluid to mimic blood properties. Indeed, it was found by others that the Newtonian model of blood viscosity is a good approximation in regions of mid-range to high shear.\textsuperscript{68}

11.5 Cell Line

Endothelial cells from different vascular beds respond differently to shear stress as well as to cytokines, inflammatory molecules and drugs. Established cell lines were used in this study; however primary cells could have been used. NB4 cell were used as they exhibit most neutrophil factors, however if possible freshly isolated blood components should be perfused through the models instead.

Substrate, growth factors and extracellular matrix are known to alter cell phenotype.\textsuperscript{200} Indeed, growth supplements were used and models were coated with fibronectin. It would be possible to reduce the fibronectin concentration and coating time as reports in literature mention that attachment does not vary much after 30 minute for concentrations greater than 10 $\mu$g/mL.\textsuperscript{201}

11.6 Surrounding Environment

This model does not take into account the role of inflammation, the influence of vascular cells surrounding endothelial cells and in the blood stream, as well as the role of matrix changes and of remodeling occurring within the tissue. Other vascular cells, such as smooth muscle cells, fibroblasts, platelets and neutrophils, can be added to the model and seeded onto the models or passed through it to even better recreate blood vessel biology. However, this would greatly complicate the experiment design.
BIOHAZARDOUS MATERIALS APPLICATION

McGill University
University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Richard Leask
   ADDRESS: 3610 University St., H3A 2B2
   TELEPHONE: x4270
   FAX NUMBER: x6678
   DEPARTMENT: Chemical Engineering
   E-MAIL: richard.leask@mcgill.ca
   PROJECT TITLE: Aortic endothelial cell seeding of a silicone rubber

2. FUNDING SOURCE: CIHR  NSERC  NIH  FQRNT  FRSQ  INTERNAL  OTHER (specify) Dawson Chair Funds
   Grant No.: TBA
   Beginning date ASAP
   End date Sept 2007

3. Indicate if this is
   □ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
   Approval End Date - Sept 2003
   □ New funding source: project previously reviewed and approved under an application to another agency.
   Agency Approval End Date
   □ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 □ 2 □ 3 □ 4 □
Principal Investigator or course director: [Signature] date: 02 02 2005 day month year
Chairperson, Biohazards Committee: [Signature] date: 03 02 05 day month year
Approved period: beginning: day month year ending: day month year

* as defined in the "McGill Laboratory Biosafety manual"
CHAPTER 12: REFERENCES


149. Butcher EC. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991 Dec;67(6):1033-6.


CHAPTER 12 : REFERENCES


