VASCULAR ENDOTHELIAL GROWTH FACTOR-INDUCED PERMEABILITY IN THE PATHOGENESIS OF CARDIAC ALLOGRAFT VASCULOPATHY

by

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B.M.L.Sc., The University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

July 2011

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Abstract

Rationale: Endothelial dysfunction can lead to increased permeability, and this may contribute to the pathogenesis of cardiac allograft vasculopathy (CAV). This dissertation focuses on vascular endothelial growth factor (VEGF), a protein that can mediate angiogenesis and is a potent inducer of vascular permeability. It was my goal to characterize the expression and localization of VEGF in CAV and to elucidate the mechanisms which may relate to its role in the pathogenesis of CAV.

Central hypothesis: VEGF plays a significant role in the pathogenesis of CAV by inducing endothelial cell hyperpermeability to low-density lipoproteins (LDL).

Methods: Immunohistochemistry and in situ hybridization for VEGF were performed on coronary artery segments from patients with native atherosclerosis (NA), diabetes mellitus with atherosclerosis (DM) and CAV. Human coronary artery endothelial cell (HCAEC) and human cardiac microvascular endothelial cell (HCMEC) primary cultures were used to investigate VEGF-induced permeability using transendothelial electrical resistance (TER) measurements, immunocytochemistry for tight junction proteins and LDL permeability. A mouse model of heterotopic cardiac transplantation was used to assess the therapeutic potential of abrogation of VEGF function on CAV using soluble VEGF receptor-1 (sVEGFR1) administration.

Key results: There was significant upregulation of VEGF-A in the intima and media of coronary arteries in CAV, NA and DM. As well, there was significant upregulation of VEGF-D in the media of coronary arteries in CAV and in the intima and media of coronary arteries in DM. Treatment with VEGF-A_{121}, VEGF-A_{165} and VEGF-D significantly decreased TER, significantly increased LDL permeability, and induced the formation of intercellular gaps and decreased immunoreactivity
of the tight junctional protein zonula occludens-1 (ZO-1) along adjacent endothelial membranes in confluent monolayers. Co-incubation with the mitogen-activated protein kinase kinase (MAPKK/MEK1) inhibitor U0126 prevented the formation of intercellular gaps and maintained regularity of ZO-1 immunoreactivity along endothelial membranes. Administration of sVEGFR1 in a mouse model of heterotopic cardiac transplantation resulted in a significant decrease in luminal narrowing in transplanted hearts at 21 days post-transplantation.

**Conclusion:** Taken together, this body of work clearly demonstrates that VEGF plays a significant role in the pathogenesis of CAV.
Preface

The following dissertation includes chapters which are based on published manuscripts.

Portions of Chapter 1 are based on a review article published in the journal Cardiovascular Pathology [Wong BW, Rahmani M, Rezai N, McManus BM. Progress in heart transplantation. Cardiovasc Pathol. 2005; 14: 176-80]. I wrote this review article and the co-authors assisted in critical review and revision.

Portions of Chapter 5 are based on a manuscript submitted to the journal Circulation Research which is currently in revision [Wong BW, Williams SJ, Tao K, West LJ, Luo H, Bernatchez PN, McManus BM. Soluble vascular endothelial growth factor receptor-1 reduces luminal narrowing in mouse cardiac allograft vasculopathy]. I performed all of the writing on the manuscripts and assisted in care of the animals post-transplantation, administration of the drugs, sacrifice and tissue harvesting. As well, I performed the morphometric assessment of luminal narrowing and the in vitro aortic ring angiogenesis assays co-cultured with bone marrow-derived cells. Dr. Sarah Williams assisted in care of the animals, sacrifice and tissue harvesting, blood chemistry analyses, immunohistochemistry for VEGF and some image analysis of aortic ring outgrowth. Dr. Kesheng Tao was the skilled microsurgeon who performed all of the heterotopic heart transplants. Drs. Lori West, Honglin Luo, Pascal Bernatchez and Bruce McManus made intellectual contributions to the design and final editing of the manuscript. The procedures detailed in Chapter 5 were reviewed and approved by the University of British Columbia Animal Care Committee (Protocol #A08-0509).
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List of symbols

$\alpha$ – alpha
$\beta$ – beta
$\gamma$ – gamma
$\delta$ – delta
$\epsilon$ – epsilon
$\zeta$ – zeta
$\eta$ – eta
$\theta$ – theta
$\lambda$ – lambda
$\mu$ – micron [mu]
List of abbreviations

ABC/AP – streptavidin-biotin complex conjugated with alkaline phosphatase

ACE – angiotensin converting enzyme

acLDL – acetylated low-density lipoprotein

AgNO₃ – silver nitrate

ANOVA – analysis of variance

AP-2 – activating protein-2

apo – apolipoprotein

ATP – adenosine triphosphate

AV – allograft vasculopathy

β-catenin – beta-catenin

Ca²⁺ – calcium

CaM – calmodulin

cAMP – cyclic adenosine monophosphate

CAS – Crk-associated substrate

CAV – cardiac allograft vasculopathy

cGMP – cyclic guanosine monophosphate

CRAM – cysteine-rich acidic transmembrane protein

CVB3 – coxsackievirus B3

DAG – 1, 2-diacylglycerol

DI – diabetes insipidus

dil – 1’,1’-dioctadecyl-3, 3’, 3’-tetramethylindocarbocyanine perchlorate
DM – diabetes mellitus
E-cadherin – epithelial-cadherin
EC – endothelial cell
ECM – extracellular matrix
EGF – epithelial growth factor
EGFR – epithelial growth factor receptor
eNOS – endothelial nitric oxide synthase
ER – endoplasmic reticulum
ERK – extracellular signal-regulated kinase
FAK – focal adhesion kinase
FITC – fluorescein isothiocyanate
flk-1 – fetal liver kinase-1
flt-1 – fms-like tyrosine kinase-1
flt-4 – fms-like tyrosine kinase-4
GEF – guanine nucleotide exchange factor
GRAF – GTPase regulator associated with FAK
GSK3β – glycogen synthase kinase-3 beta
GTPase – guanosine triphosphate hydrolase enzyme
H₂O₂ – hydrogen peroxidase
HCAEC – human coronary artery endothelial cell
HCASMC – human coronary artery smooth muscle cell
HCMEC – human cardiac microvascular endothelial cell
HLA – human leukocyte antigen

HRP – horseradish peroxidase

HSPG – heparan sulphate proteoglycans

HUVEC – human umbilical vein endothelial cell

IFN – interferon

iNOS – inducible nitric oxide synthase

IP₃ – inositol 1, 4, 5-triphosphate

IP₃R – inositol triphosphate receptor

ISH – in situ hybridization

ISHLT – International Society for Heart and Lung Transplantation

IVUS – intravascular ultrasound

JAM – junctional adhesion molecule

kDa – kilodalton

KDR – kinase domain receptor

LDL – low-density lipoprotein

MDCK – Madin-Darby canine kidney [cells]

MI – myocardial infarction

MLC – myosin light chain

MLCK – myosin light chain kinase

MMP – matrix metalloproteinase

NA – native atherosclerosis

NBT/BCIP – nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate
N-cadherin – neural-cadherin
oxLDL – oxidized low-density lipoprotein
PAF – platelet-activating factor
PBS – phosphate-buffered saline
PDAY – *Pathobiological Determinants of Atherosclerosis in Youth* [study]
PDE – phosphodiesterase
PDGF – platelet-derived growth factor
PDGFR – platelet-derived growth factor receptor
PECAM-1 – platelet and endothelial cell adhesion molecule-1; CD31
PI3K – phosphotyidylinositol-3 kinase
PIP2 – phosphotyidylinositol 4, 5-bisphosphate
PKA – protein kinase A
PKB – protein kinase B; Akt
PKC – protein kinase C
PL – phospholipase
PLA2 – phospholipase A2
PLC – phospholipase C
PLD – phospholipase D
PIGF – placental growth factor
PMA – phorbol 12-myristate 13-acetate
PMNL – polymorphonuclear leukocyte
Rap1 – Ras-proximate-1; Ras-related protein-1
SE – standard error [of the mean]
SEM – scanning electron microscopy
SMC – smooth muscle cell
SM-α actin – smooth muscle-alpha actin
SOS – Son of sevenless
SPSS – Statistical Package for the Social Sciences [software]
SSC – saline sodium citrate [buffer]
TEM – transmission electron microscopy
TER – transendothelial electrical resistance
TGF-β – transforming growth factor-beta
TNF-α – tumor necrosis factor-alpha
U0126 – 1, 4-diamino-2, 3-dicyano-1, 4-bis[2-aminophenylthio] butadiene
VE-cadherin – vascular endothelial-cadherin
VEGF / VPF – vascular endothelial growth factor / vascular permeability factor
VEGFR1 / flt-1 – vascular endothelial growth factor receptor-1 / fms-like tyrosine kinase-1
VEGFR2 / flk-1 / KDR – vascular endothelial growth factor receptor-2 / fetal liver kinase-1 /
kinase domain receptor
VEGFR3 / flt-4 – vascular endothelial growth factor receptor-3 / fms-like tyrosine kinase-4
VLDL – very low-density lipoprotein
VVO – vesiculo-vacuolar organelle
ZO – zonula occludens
Acknowledgements

My research career began under the direct tutelage of Dr. Donald Wong, a post-doctoral fellow in the laboratory of my supervisor, Dr. Bruce McManus. Through the many personal and professional conversations we have engaged in, Donald has instilled solid academic foundations of diligence, persistence and honesty. As well, Donald was instrumental in establishing the basis for the grant-funded VEGF research program in the laboratory.

I first met Dr. Honglin Luo when she began as a research associate under the supervision of Dr. McManus. Honglin has always provided unbiased and straightforward assessment of my work and her serendipitous rise to associate professor is a constant reminder that strong scientific bases and productivity in publication prevails. It is of particular satisfaction that I was able to collaborate with her and through that interaction, expand my scientific basis.

Ms. Zongshu Luo has been one of my closest friends and colleagues within the laboratory. Of particular note, after unforeseen interruptions in my studies, Zongshu went above and beyond in ensuring that I ‘kept at it’ despite my personal setbacks.

My interactions with Ms. Huifang (Mary) Zhang and Dr. Decheng Yang were one of my first collaborative interactions in research and re-enforced the central idea that the most important thing in science is the science. Through numerous collaborations and publications, I have been afforded the opportunity to elevate my level of scientific writing and technique while assisting in their research focuses.

I would like to acknowledge the personal and professional contributions of Dr. Maziar Rahmani, Dr. Bobby Yanagawa, Dr. Hubert Walinski, and Dr. Jonathan Choy – through our many
conversations, debates and heated arguments, you have always pushed me to do more and be better.

There have been many dedicated and skilled individuals who have helped me with various portions of my graduate work and training, including Ms. Agripina Suarez (Pining), Ms. Sylvia Loo, Mr. Nathanael Kuipers, Dr. Thomas Podor, Mr. Albert Lee, Mr. Dean English, Ms. Elizabeth Walker, Ms. Elaine Humphreys, Ms. Kris Gillespe, Dr. Kesheng Tao, Ms. Tatjana Bozin, and Ms. Claire Smits. I would like to also like to specifically acknowledge the tutelage, support and friendship of Mr. Stuart Greene and Dr. Alexandra (Sasha) Kerjner.

During my studies I have been fortunate to be supported by awards from the Heart and Stroke Foundation, the Canadian Institutes of Health Research and the Michael Smith Foundation for Health Research. As well, the research program has been generously supported by grants from the Heart and Stroke Foundation and the Canadian Institutes of Health Research.

The guidance and direction provided by my doctoral thesis committee members, Dr. Edward Pryzdial (chair), Dr. Urs Steinbrecher and Dr. Wan Lam, have helped to refine and develop the scientific structure and foundation of my doctoral thesis project.

Finally, I would like to acknowledge the patience, support and mentorship provided by my doctoral thesis supervisor, Dr. Bruce McManus. It is impossible to summarize everything I have learned in my time under his tutelage but his unwavering enthusiasm and dedication towards research and science reminds me to always “Read More” and “Go for the Gold.”
I would like to dedicate this thesis to the numerous friends and family who have provided innumerable support during my graduate studies.

Of particular note is the unwavering support of my parents, Mr. David Tai Kong Wong and Mrs. Cecilia Man Kwan Wong.

From an early age, you have allowed me to pursue my dreams and desires with reckless abandon and for that I am eternally grateful.

I love you both very much and hope you will see when I get there it was worth it all.
Chapter 1 – Background

1.1 – Heart failure

Heart failure is characterized as an abnormality of the structure or function of the heart which impairs its ability to provide sufficient blood flow to the body. Heart failure is a highly prevalent condition with significant economic, social and personal costs. In Western society, approximately 2% of adults suffer from heart failure.\textsuperscript{1,2} The lifetime risk of developing heart failure is approximately 20% for individuals over 40 years of age.\textsuperscript{3,4} This percentage increases by 6-10% in those over the age of 65.\textsuperscript{2,5} About 30-40% of patients die from heart failure within one year after receiving the diagnosis.\textsuperscript{2} Over time, prognosis of heart failure generally worsens, and this progressive disease is associated with an overall annual mortality rate of approximately 10%.\textsuperscript{6} As well, heart failure is associated with significant reductions in physical and mental health, resulting in a striking decrease in quality of life.\textsuperscript{2}

1.1.1 – Primary causes of heart failure

Heart failure results from abnormalities in cardiac structure, function, rhythm or conduction. It can be caused by a wide variety of conditions including myocardial infarction (MI), hypertension and cardiomyopathy. As well, degenerative valvular diseases are becoming more common. Over time, increased cardiac demand results in reduced contractility due to ventricular overload, reduced stroke volume, reduced reserve capacity, increased heart rate, hypertrophy and enlargement or dilation of the ventricles.\textsuperscript{2}
Native atherosclerosis is a condition where a combination of environmental, genetic and lifestyle factors lead to the gradual change in architecture of arteries and accumulation of lipids and lipoproteins.\textsuperscript{7,8} Factors such as oxidative stress,\textsuperscript{9,10} hypercholesterolemia,\textsuperscript{11,12} diabetes mellitus (DM)\textsuperscript{13} and others contribute to intimal hyperplasia – the proliferation and expansion of the intimal layer of arteries. As a result of thickening of the intimal layer of the vessel wall, the area of the lumen of the blood vessel is reduced as the intima encroaches on the lumen resulting in a reduction in blood flow. This process results in blood vessel dilation to compensate for this reduction in luminal area, and ultimately triggers a compensatory thinning or dissolution of the medial layer of smooth muscle cells (SMC) when the vessel is no longer able to dilate further to increase blood flow. Changes in the intima and media of the blood vessel also result in an increased propensity for the insudation and retention of lipids and lipoproteins within the deep intimo-medial layer.\textsuperscript{8,14}

Progressive occlusion of the coronary arteries within the heart may result in either complete blockage or obstruction, resulting in downstream myocardial infarction (MI) or an increased propensity for thrombolytic events, where, in the face of a thinning fibrous cap overlying the pro-thrombotic atherosclerotic lesion may result in plaque rupture and intravascular thrombosis, which may eventually lead to downstream embolism and ischemia. Atherosclerosis can lead to heart failure through the reduction of blood flow in the coronary arteries, resulting in increased strain on the heart to maintain cardiac output and perfusion. Progressive advancement of atherosclerosis can ultimately result in MI, with ischemic damage to the myocardium, thereby further reducing the ability of the heart to function.\textsuperscript{8}
1.1.2 – Current treatment strategies

Treatment for heart failure consists of lifestyle changes (such as decreased dietary intake of salt; increased physical activity) and medications such as beta-blockers,\textsuperscript{15} angiotensin converting enzyme (ACE) inhibitors\textsuperscript{16,17} or angiotensin receptor blockers,\textsuperscript{18,19} vasodilators, and in cases of severe cardiomyopathy, aldosterone receptor antagonists.\textsuperscript{17} Treatment for heart failure focuses on improving symptoms and preventing progression of the disease. Despite all of these methodologies for intervention, heart failure is still associated with an annual mortality rate of 10\%.\textsuperscript{20}

1.1.3 – End-stage heart failure

At maturity, adult cardiomyocytes are largely terminally differentiated, and as such, the heart is unable to replace damaged or dead myocytes. Instead, compensatory changes such as cardiomyocyte hypertrophy and polyploidy and cardiac fibrosis results, and dilation of the ventricular chamber initially helps to maintain cardiac output. Despite significant advances in our ability to treat heart disease and heart failure, currently, there are only two long-term solutions for end-stage heart failure, the main one being organ transplantation. The advent of ventricular assist devices and other supportive technologies may allow for the maintenance and extension of the length of time a patient may live with end-stage heart failure; however, ultimately the only long-term solution is heart transplantation.
1.2 – Overview of heart transplantation

To appreciate the significance of heart transplantation as both a life-saving medical procedure and also the accumulative understanding of the efforts to modify and manipulate the cardiovascular and immune systems, one must appreciate the many milestone advancements which have been cornerstones for transplantation medical science and heart transplantation in particular.

1.2.1 – Understanding allo-immunity

In 1901, Karl Landsteiner published a paper demonstrating that clumping of the donor’s red blood cells was responsible for the clinical manifestations of the transfusion reaction.21 This paper revealed that the clumping was due to the presence of three different types of iso-agglutinins, which formed the basis for his blood group classification known initially as A, B and C. His suggestions received little attention until 1909, when he classified the human blood into the A, B, AB and O groups and showed that catastrophic reactions could occur when a person receives blood from a different group.21 Compatibility was later found to be not only a requirement for transfusion, but also for transplantation.

Peter Medawar was the first to demonstrate that the immune system was responsible for the rejection of transplanted organs, and later went on to show that it could be “tricked” into tolerating transplanted tissues. Medawar started his pioneering work in Glasgow on skin grafting burns. He found that skin grafted from a donor lasted about 10 days, but a second graft was rejected immediately. It was as though the immune system remembered what the intruder looked like, and promptly rejected it.22 Medawar suggested that the rejection was an
immunological process. In the early 1950s, Medawar inoculated mouse embryos with the cells of mice from another strain. After their birth they were grafted with skin taken from the strain of mice to which they had been exposed \textit{in utero}. Remarkably, these grafts were not rejected, introducing the concept of acquired immunological tolerance.\textsuperscript{23}

Frank Burnet suggested that the body’s immune cells learn very early on to accept whatever tissues are there as part of the body and only attack and reject material that shows up later. This theory later developed the notion of clonal selection and the recognition of self and non-self by vertebrate immune systems.\textsuperscript{24} In 1958, Jean Dausset described the first leukocyte antigen, MAC (now known as human leukocyte antigen-A2 (HLA-A2)).\textsuperscript{25,26} The discovery allowed for tissue matching beyond blood types.

\textbf{1.2.2 – Surgical milestones}

Vascular surgery, preceding solid organ transplantation, did not emerge as a specialty until the end of the 19th century. At that time, Alexis Carrel introduced a “leak-proof” technique for the anastomosis of blood vessels without constricting the lumen or causing thrombosis. Anastomosis of blood vessels was a crucial advance required for the field of solid organ transplantation to become tenable. Carrel demonstrated the feasibility of grafting veins to arteries, and arteries to arteries using his innovative anastomotic approach.\textsuperscript{27-29} His work detailed the refinement and perfection of vascular anastomotic techniques, the usage of vein grafts in the arterial system, the development of tissue preservation techniques, and organ and limb transplantation.\textsuperscript{29-31} Alexis Carrel performed the first heterotopic canine heart transplant with Charles Guthrie in 1905.\textsuperscript{29} At the time, he clearly recognized the difference in the survival
times between autografts and allografts in experimental animals, but he did not conceptualize rejection as distinct from other graft-destroying processes. Twenty years later, the concept of cardiac allograft rejection was proposed by Frank Mann at the Mayo Clinic to explain the eventual failure of heterotopic canine allografts. He described the rejection process as a “biologic incompatibility between donor and recipient” manifested by an impressive leukocytic infiltration of the rejecting myocardium.\textsuperscript{32}

\textbf{1.2.3 – Successful heart transplants}

On January 23\textsuperscript{rd}, 1964, the first heart transplant of a non-human primate into a human was performed by James Hardy at the University of Mississippi Medical Center in Jackson. The heart of a chimpanzee was transplanted into the 68-year-old, Boyd Rush; however, the heart was too small to maintain independent circulation and functioned only for 90 minutes before failing.\textsuperscript{33} On December 3\textsuperscript{rd}, 1967, Christiaan Barnard successfully transplanted the heart of Denise Darvell, a young woman who had died in a car crash, into 54-year-old Louis Washkansky at the Groote Schuur Hospital in Cape Town, South Africa. He died of pneumonia 18 days later.\textsuperscript{34} On January 6\textsuperscript{th}, 1968, Norman Shumway performed the first adult human-to-human heart transplant in North America at the Stanford University School of Medicine in Palo Alto, California.\textsuperscript{35} In 1969, Denton Cooley implanted the first total artificial heart (the Liotta Total Artificial Heart) at the Texas Heart Institute in Houston. The heart was implanted into the 47-year-old Haskell Karp, but was not intended to be permanent. It was used as a bridge to transplant until he could receive a donor heart, which he did 64 hours later.\textsuperscript{36}
1.2.4 – Pathological diagnosis

The detection of allograft rejection is one of the most important yet unsettled areas of cardiac transplantation. The investigation of the transvascular endomyocardial bioptome by Sakakibara and Konno in 1963, and the introduction of transvenous endomyocardial biopsy by Philip Caves in 1973 finally provided a reliable means for monitoring allograft rejection. Throughout the 1980s, various grading scales emerged from different centers, causing much confusion. The International Society for Heart & Lung Transplantation (ISHLT) commissioned the development of a common grading scale in 1990, in an attempt to develop uniform description and grading criteria of various transplant histologies to refine communication and comparison of treatment regimens and outcomes between transplant centers. Due to the insight and diligence of cardiac pathologists such as Margaret Billingham, the ISHLT grading system for cellular rejection was developed in 1990. In 2004, a new grading scale was commissioned by the ISHLT to address the challenges and inconsistencies in the use of the old grading system. Although the current grading system has allowed for better consistency in assessment of rejection severity, there are inherent limitations to its usage: variability in assessment of rejection severity, particularly regarding grade 2 lesions, the presence of Quilty lesions (endocardial infiltrates) tends to cause overestimation of rejection severity, and humoral (antibody-mediated) rejection is just beginning to be addressed.

Hemodynamic change in the absence of acute cellular rejection is termed biopsy-negative rejection, and occurs in 10 to 20% of cardiac allograft recipients. In the pre-cyclosporine era, biopsy-negative rejection was not apparently an important phenomenon. It is suggested that immunologic pathways other than lymphocytic infiltration are important in
mediating cardiac allograft dysfunction and injury, and humoral rejection may be the primary mediator.\textsuperscript{42,43} Humoral rejection is associated with increased graft loss, accelerated coronary allograft vasculopathy and increased mortality.\textsuperscript{42,43}

1.2.5 – \textit{Past and present immunosuppressive regimens}

With advances in immunosuppression and surgical techniques, the pathologies seen post-transplantation have changed. Over the last 20 years, the rates of acute rejection and infection leading to graft failure have greatly declined owing to refined immunosuppressive drug regimens, better diagnosis of ischemic injury, and improved monitoring of immune status. As such, chronic rejection and cardiac allograft vasculopathy (CAV) have become more prevalent as major expressions of transplant rejection. CAV is an accelerated form of atherosclerosis which occurs in 30-60\% of transplant recipients within the first 5 years post-transplantation.\textsuperscript{44} Studies using intravascular coronary ultrasound (IVUS) techniques have demonstrated intimal thickening in 75\% of cardiac allograft recipients by the end of the first year post-transplantation.\textsuperscript{45}

1.2.6 – \textit{Etiology of cardiac allograft vasculopathy}

CAV is a rapidly progressing form of atherosclerosis, whereby the heightened allogeneic immune response between donor and recipient, along with classical risk factors for atherosclerosis and ischemic factor contribute to the rapid narrowing of blood vessels. Although there are many similarities between CAV and native atherosclerosis, they are clinically and pathologically separate entities (\textbf{Figure 1}).
Figure 1 – Representative micrographs of coronary arteries with native atherosclerosis and cardiac allograft vasculopathy.
Cardiac allograft vasculopathy is characterized by a largely immune-driven etiology, with concentric matrix and lipid deposition and rapid progression, whereas native atherosclerotic lesions are generally focal, eccentric, proliferative and degenerative lesions in the intima of proximal coronary vessels, mostly fibro-inflammatory, fatty plaques with ultimate necrotic cores and progressively thinned fibrous caps.
Scale bars represent 1mm.

L – lumen     i – intima     m – media     * – atheromatous core
As acute rejection is better controlled by tailored immunosuppressive regimens, the central role of the immune system in the pathogenesis of CAV is also complemented by other factors which may augment its etiology. These factors include peri-transplant injury, generally resulting in epicardial fibrosis and myocardial infarction, graft denervation, and infection.

It is important to note that allograft vasculopathy (AV) occurs to a significant degree in all solid organ transplants.

1.2.7 – Current treatment strategies

Over the last 20 years, rates of acute rejection and infection leading to graft failure have greatly declined owing to refined immunosuppressive drug regimens, better diagnosis of ischemic injury and improved monitoring of immune status.\textsuperscript{45-49} Chronic rejection and AV have become a major focus. As noted, studies employing IVUS have revealed intimal thickening in 75\% of cardiac allograft recipients by one year post-transplantation.\textsuperscript{45,50} Current treatment strategies primarily focus on immunosuppression. Targeted therapies against CAV have been investigated, primarily as adjuncts to existing therapies used in native atherosclerotic disease, such as “statins”, low-dose acetylsalicylic acid (Aspirin\textsuperscript{®})\textsuperscript{51} and others.\textsuperscript{52} Currently, there is no effective treatment for CAV and AV in other solid organ transplants, and it remains the primary cause of graft loss beyond one year post-transplantation.
1.3 – Current concept of the pathogenesis of CAV

My current concept of the pathogenesis of CAV is fundamentally rooted to structural observations of histopathological sections of CAV (Figure 2), coupled with specific observations from the literature, these guide my experimental direction.

Endothelial insults, which may arise from oxidative stress, hemodynamic changes to blood flow, direct physical or mechanical injury, are coupled with an ongoing allogeneic immune response against the donor vasculature. These insults and injuries result in both an endothelial and immune response. Vascular endothelial cells (EC) produce pro-inflammatory cytokines and chemokines, as well as protective anti-apoptotic and pro-survival growth factors. Growth factors and pro-inflammatory mediators facilitate the preservation and repair of the endothelium. Congruent to their effects on the endothelium, these growth factors and pro-inflammatory cytokines also act on the vascular SMC underlying the basal lamina. This milieu results in excess intimal SMC proliferation and extracellular matrix (ECM) remodeling, termed neointimal formation or intimal hyperplasia.

Concomitant with inflammation and re-endothelialization is an increase in endothelial permeability, and coupled with the remodeling of the ECM, results in the insudation and retention of circulating lipids and lipoproteins from the blood stream. An important publication established the relationship between hyperlipidemia and post-transplant obesity with luminal narrowing in human heart allografts. Subsequent investigations demonstrated profound lipid accumulation in coronary arteries of many grafts begins very early post-transplant and appears to contribute substantially to intimal thickening.
Figure 2 – Structural progression of cardiac allograft vasculopathy.
Human vessels were obtained from the Cardiac Registry at the Institute for Heart + Lung Health and slides were stained with Movat’s pentachrome. In a normal coronary artery from a 20-year-old male, the intimal layer (I) is minimal, and consists primarily of the endothelium and its supporting basal lamina and is bounded by the internal elastic lamina (IEL); there is an intimo-medial layer; and the media (M) itself, which is bounded by the IEL and the external elastic lamina (EEL). In normal vessels the media is thick, smooth muscle- and elastin-rich, and exhibits normal vascular function. In mild CAV, represented here by a coronary artery from a 60-year-old male transplant recipient whose graft failed 102 days post-transplantation, there is already a notable thickening of the intimal layer. This thickening is largely comprised of smooth muscle cells and matrix. Conversely, the medial layer is thinned. In severe CAV, represented here by a coronary artery from a 67-year-old female transplant recipient whose graft failed 360 days post-implantation, there is marked thickening of the intimal layer. As well, at the intimo-medial junction there is abundant accumulation of lipids, lipoproteins, and infiltrating macrophages (*). The medial layer in this vessel with advanced AV is further diminished, damaged by intimal processes as well as adventitial inflammatory processes. Scale bars represent 50 microns.
This accumulation of lipids, in concert with inflammatory mediators, results in the chemoattraction of monocytes, which differentiate into macrophages within the vessel wall, as well as the chemoattraction of other inflammatory cells. Concurrently, oxidative and cytotoxic mediators released by macrophages and other inflammatory cells result in the modification of low-density lipoproteins (LDL) to the more pernicious oxidized low-density lipoproteins (oxLDL) within the vessel wall.\textsuperscript{70-72} Macrophages and SMC are able to uptake oxLDL but are unable to extravasate or breakdown the lipid. This leads to excess lipid accumulation within the cells and resultant lipid overload.\textsuperscript{73,74} Pro-survival effects from oxLDL itself, as well as other growth factors and cytokines in the extracellular milieu of the evolving lesion lead to the transformation into lipid-laden foam cells.\textsuperscript{72,75-77} Death of these foam cells results in an acellular, necrotic lipid-rich core. SMC apoptosis and ECM breakdown in the superficial intima covering the atheromatous core is mediated by infiltrating and now-resident inflammatory cells, resulting in the thinning of the fibrous cap containing the atheromatous lesion. A thin fibrous cap in native atherosclerosis increases the propensity for plaque rupture and thrombotic events, leading to acute MI or death.\textsuperscript{78-80} Less is known about the precipitants of acute coronary occlusion in CAV.
1.4 – Dysregulation of endothelial permeability in vascular disease

Alterations in endothelial permeability in vascular disease are often concomitants of inflammation. Inflammation is characterized in part by edema due to increased endothelial permeability to fluid and other blood components, and such leakage plays a key role in the pathogenesis of many diseases. Inflammatory mediators increase vascular permeability by inducing the retraction or contraction of endothelial cells and the formation of gaps between adjacent endothelial cells in post-capillary venules. Since the 1980s, it has been generally held that diverse classes of inflammatory mediators produce this type of gap formation exclusively at the post-capillary venules, allowing fluids and macromolecules to enter the tissue and cause potentially massive edema, affecting vascular beds proximate or remote to the inflammatory process. Capillary leak subsequent to prolonged inflammation can be achieved. The modeling in vitro using endothelial cell cultures treated with pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) has been visualized using light microscopy and silver nitrate (AgNO₃) staining or transmission electron microscopy (TEM) and horseradish peroxidase (HRP) tracers (Figure 3; panels b,d from Dr. Donald Wong). A number of clinical disorders are associated with aberrant vascular permeability.
Figure 3 – Assessment of endothelial permeability under transmission electron microscopy (TEM) using horseradish peroxidase (HRP) as a tracer. Human endothelial cells were cultured and received either 100ng/mL tumor necrosis factor-alpha (TNF-α; c,d) or no treatment (a,b). Silver nitrate staining (AgNO3; a,c), and captured by light microscopy allowing for the visualization of intercellular gaps at the structural level [arrow]. Use of HRP tracer molecules in conjunction with visualization by TEM allows not only for the ultrastructural examination of junctional integrity, but also for demonstrating the permeability of small molecule tracers and their deposition in the junctions and beneath the monolayer (b,d).

1.4.1 – Endothelial dysfunction in native atherosclerosis

Inflammation has been clearly associated with the pathogenesis of human atherosclerosis as illustrated by the presence of infiltrating monocytes-macrophage and T-lymphocytes in the subendothelial tissue and in the shoulders of lipid rich plaques.8,86,87 Atherosclerosis was initially viewed as a disease of lipid accumulation in the vessel wall, with the endothelium being affected secondary to the underlying cell death within the atheroma. It is now clear that the endothelium is intimately involved in the initiating processes, with early endothelial dysfunction being a hallmark in the pathogenesis of atherosclerosis. As well, associated vascular hyperpermeability results in the insudation of lipids, lipoproteins, and other...
Increased permeability of the arterial wall to lipoproteins such as LDL leads to lipid accumulation or retention, atheromatous plaque formation and subsequent progression of atherosclerosis.\textsuperscript{92}

Many factors contribute to increased arterial wall permeability, including changes in flow dynamics and shear stress (i.e., at areas of bifurcation or luminal narrowing),\textsuperscript{93,94} and expression of vasoactive factors such as TNF-$\alpha$,\textsuperscript{95-98} histamine\textsuperscript{99-101} and vascular endothelial growth factor (VEGF).\textsuperscript{77,102}

\textbf{1.4.2 – Altered endothelial permeability in cardiac allograft vasculopathy}

Although CAV and native atherosclerosis are clinically and pathologically distinct entities, these diseases share certain pathogenic mechanisms. Both processes may be regarded as responses to injury, including participation of the immune system in the injury and the response, early endothelial dysfunction and early atheromatous plaque formation are cornerstones in both conditions.\textsuperscript{103}

The major mechanisms of injury in organ transplantation rest with the immune reactions involving allogeneic lymphocytes and infiltrating macrophages. The endothelial antigens of the transplanted organ are among the first to be recognized by the host’s immune system and thus plays a pivotal role in both acute and chronic responses to injury that lead to vasculopathy following solid organ transplantation. Interactions between donor endothelial cells and the recipient’s immune system initiate a series of inflammatory responses with repetitive injury to the endothelium, resulting in endothelial dysfunction and destruction, increased permeability with enhanced influx of blood components into the sub-endothelial
space, exposure of vascular ECM, and subsequent intimal hyperplasia. This pathobiological series of events leads to rapidly accumulating, concentric lipid deposits as prominent features within the transplanted vessel.

Using a rat model of heterotopic cardiac transplantation, we have observed insults to the donor endothelium, both functional and structural. The overexpression of endothelial nitric oxide synthase (eNOS) early and inducible nitric oxide synthase (iNOS) late within intramural allograft coronary vessels was associated with a progressive and profound loss of endothelial agonist responses and myogenic tone. The loss of normal tone in resistance vessels may convey an increase in hydrostatic force favoring the development of interstitial edema in the transplanted heart.

In related studies, we observed intercellular gap formation and transcellular disruptions of endothelial integrity in allograft aorta and epicardial coronary arteries. These changes clearly result in increased endothelial permeability (Figure 4; data from Mr. John Lai; complementary to). It has been suggested that intercellular gap formation may begin at tricellular corners in the endothelium. In addition, endothelial exfoliation and denudation in allografts, as a result of cell death by apoptosis and/or necrosis, may also result in increased endothelial permeability.

Endothelial disruptions may allow macromolecules, particularly larger lipoproteins such as very low-density lipoprotein (VLDL) particles, and inflammatory cells to readily enter the sub-endothelial intimal compartment, where entrapment can occur in the ECM. In the pathogenesis of native atherosclerosis, LDL is the predominant lipoprotein particle, particularly small and dense LDL particles – VLDL particles do not play a crucial role, in part due to their large size. The
endothelial damage and increased vascular permeability in the allograft setting, perhaps being more profound and progressive than in native atherosclerosis, may allow larger numbers of lipoprotein particles (both LDL and VLDL) to enter the sub-endothelial compartment at a higher influx rate. Indeed, we have observed marked, early accumulation of apolipoprotein (apo) B-, apo (a)- and apo E-containing lipoproteins in human allograft arteries.\textsuperscript{112} We also observed increased permeability using \textsuperscript{131}I-radiolabelled sucrose, albumin, and LDL in allograft rat hearts as compared to syngraft hearts as early as four days post-transplantation (unpublished data). These entrapped lipoproteins may then undergo oxidative modification and be taken up by macrophages through scavenger receptors.\textsuperscript{71,113} These stages of vasculopathy are further accelerated due to increased oxidative stress and the dyslipidemia associated with transplantation.\textsuperscript{114-116}
Figure 4 – Examination of endothelial perturbations in a rat model of heterotopic cardiac transplantation. Right coronary arteries from explanted donor rat hearts were perfused with glutaraldehyde and prepared en face for silver nitrate staining (AgNO₃; a, b) or scanning electron microscopy (SEM; c-f). At the structural level, coronaries from non-transplanted control rats demonstrate a classic cobblestone arrangement without apparent intercellular gaps or endothelial cell loss (a). In comparison, non-immunosuppressed allografts as early as one day post-transplantation demonstrate abundant cell loss or presence of intercellular gaps, as demonstrated by the black AgNO₃ precipitation as a result of interactions with the exposed basal lamina (b). SEM of a segment of native recipient aorta from a rat syngraft 42 days post-transplantation (c) demonstrates an intact, largely unaltered endothelium, mirroring the cobblestone pattern seen in the AgNO₃-stained controls. In stark comparison, SEM of a rat aorta (d) or septal artery (e) from a non-immunosuppressed allograft four days post-transplantation visualizes endothelial inflammation and activation, as well as the presence of interendothelial gaps (arrow). At 42 days post-transplantation, SEM of a rat aorta from a cyclosporine-treated allograft (f) demonstrates preserved endothelial morphology; however, there are still numerous intercellular gaps (arrows).
1.5 – Endothelial barrier function and permeability

The endothelium separates the blood from underlying tissue, and is well situated to modulate the physiology of both compartments. The classical view of the endothelium as a static barrier has been replaced in face of the identification of a large number of inducible endothelial functions that reflect adaptation to changing conditions and pathophysiological processes. The following section focuses on the cellular mechanisms that regulate endothelial permeability and particularly govern its modulation in vascular disease.

1.5.1 – Pathways through the endothelium

Two basic routes exist across the endothelium: intracellular (through the endothelium) and intercellular. The former can be mediated by the process of endocytosis; the latter, by opening of the intercellular junctions. The first endothelial structure at the luminal endothelial surface is the glycocalyx, a layer made up of glycated proteins. The composition and thickness of the glycocalyx varies with the location of the vessel and treatments/stimuli given. A thicker glycocalyx is associated with lower permeability of the endothelium. Removal, in part, of the sugar or protein components by gentle enzymatic digestion with heparanase, hyaluronidase or pronase invariably increases permeability.

1.5.1.1 – Intracellular passage

Vesicular transport via receptor-mediated endocytosis and transcytosis is important for the movement of substances such as lipoproteins, albumin, insulin and transferrin across the endothelium. Albumin binds to receptors in vesicles of a limited number of capillaries, including
heart, lung, skeletal muscle and adipose tissue.\textsuperscript{123} In other types of endothelium (artery, arteriole, venule, vein, endocardial, fenestrated and sinusoidal), it is taken up by fluid phase endocytosis, while the endothelium of macrovessels (aorta, large arteries, veins) utilize both pathways.\textsuperscript{124}

\textit{1.5.1.1.1 – Endocytosis}

The uptake of macromolecules usually occurs by receptor-mediated endocytosis in clathrin-coated vesicles. After binding the appropriate receptor on the cell surface, macromolecules are collected into a localized area where the plasma membrane becomes coated on the cytoplasmic side by the protein clathrin. The membrane invaginates and fuses to form a vesicle containing the ligand-receptor complex. This vehicle is subsequently moved to another compartment for processing (i.e., the lysosomal compartment) or to the opposite end of the cell where the contents are released into the extracellular milieu. A short list of substances utilizing this pathway includes iron,\textsuperscript{125,126} insulin\textsuperscript{127,128} and lipoproteins.\textsuperscript{129,130} Uncoated vesicles may non-specifically take up a portion of the extracellular milieu in fluid phase endocytosis. Actin and microtubules appear to be involved in the initiation of endocytosis and the movement of endocytotic vesicles in the cytoplasm, respectively.\textsuperscript{131,132}

Endocytosis is coupled to a number of signal transduction pathways (\textit{Figure 5}). Cell surface receptors, such as epithelial growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor-2 (VEGFR2/flk-1/KDR), possess intrinsic tyrosine kinase activity, which regulates the internalization and downregulation of the receptors themselves. Tyrosine phosphorylation of the regulatory
regions in the cytoplasmic domain of these receptors recruits the receptors into clathrin-coated pits by binding to the clathrin-associated adaptor complex activating protein-2 (AP-2).\textsuperscript{133,134,135} Inhibition of phosphatidylinositol-3 kinase (PI3K) prevents transport of PDGF-R from early to late endosomes and their translocation to the nucleus but the initial internalization of the receptors is unaffected.\textsuperscript{136,137} Further, inhibition also extends to non-clathrin-dependent fluid-phase endocytosis but not clathrin-dependent endocytosis.\textsuperscript{138} Other signal transduction pathways implicated in endocytosis include protein kinase C (PKC)\textsuperscript{139} and protein kinase A (PKA),\textsuperscript{140} depending on the cell type.

![Diagram](image_url)

**Figure 5 – Regulation of endocytosis by signal transduction pathways.**

Ligand-receptor interactions such as vascular endothelial growth factor (VEGF) interaction with vascular endothelial growth factor receptor-2 (VEGFR2), a tyrosine kinase receptor, can modify the endocytosis of other molecules, such as low density lipoproteins, through signal transduction pathways downstream of VEGFR2. Signaling through inositol triphosphate (IP$_3$) receptors in the endoplasmic reticulum (ER) can facilitate calcium (Ca$^{2+}$) release, which may directly or indirectly (for example through 1,2-diacylglycerol (DAG) and protein kinase C (PKC) affect other endocytic processes.)
1.5.1.1.2 – Transcytosis

Caveolae at the cell membrane and vesicles in the cytoplasm with a diameter of less than 70nm can potentially take up substances at one side of the endothelium by endocytosis and shuttle them to the opposite side, a process called transcytosis. Like endocytotic vesicles, they are also coated invaginations of the cell membrane, but their coating is distinct from those mentioned above with the presence of receptors, signal transduction molecules, other integral membrane proteins and lipids such as caveolin-1, eNOS, Ras, PDGF-R, interferon (IFN)-α/β receptor, IFN-γ receptor, glycosylphosphatidylinositol-anchored membrane proteins, cholesterol and sphingomyelin. These vesicles are made at the Golgi apparatus, shipped to specific areas of the cell membrane and do not interact with other vesicles. They appear to open and close but do not leave their general locale. These caveolae can open to receive extracellular material such as folate and simian virus 40, then close to process, concentrate or store these substances that can subsequently be transported into the cell by endocytosis, or by carriers or channels, the latter process called potocytosis. In contrast to endocytosis, potocytosis is inhibited by PKC. It has also been shown that caveolae can be induced to swell, fuse together and form transcellular channels called vesiculo-vacuolar organelles (VVO), which allow the passage of molecules across the endothelium. The most prominent molecule able to induce this function is VEGF.
1.5.1.2 – **Intercellular permeability**

The cytoskeleton, especially actin and myosin, plays a dynamic role in the regulation of vascular permeability. Intercellular gap formation is dependent on the rearrangement of F-actin and myosin, resulting in a reversible loss of the peripheral actin band and increased F-actin stress fiber density.\(^{149-151}\) As noted above, structural components of the tight junction and adherens junction are linked to the cytoskeleton; individual actin fibers terminate at the plasma membrane.\(^{152}\) Pharmacological studies using agents that disrupt actin such as cytochalasin B, cytochalasin D, and latrunculin B increase tracer flux, decrease resistance and decrease the complexity of tight junctions.\(^{153-155}\)

It has been observed that agents that increase the contractility of the cytoskeleton, especially affecting the dense peripheral band consisting of actin and myosin adjacent to junctions, lead to an increase in permeability. Thus, the tension generated by the perijunctional cytoskeleton may regulate permeability.\(^{156}\) In addition to actin, myosin is a major component of the endothelial cytoskeleton. The mechanism of contraction in the peripheral band is comparable to that of the SMC. It is regulated by the phosphorylation of the myosin light chains (MLC) by the myosin light chain kinase (MLCK), which activates myosin to slide along actin. In addition, a decrease in the action of MLC phosphatase has the same effect.\(^{157}\) A large number of studies have demonstrated that inflammatory mediators induce myosin phosphorylation mainly through MLCK, thereby leading to endothelial cell retraction and hyperpermeability.\(^{158-160}\) Tyrosine phosphatases can, in turn, regulate the activity of MLCK.\(^{161}\)
1.5.1.2.1 – Tight junctions

Tight junctions are regions where the outer leaflets of the cell membrane of adjacent cells are fused together. They are formed by the phosphorylation-dependent assembly of zonula occludens (ZO)-1, ZO-2, and ZO-3 proteins to the plasma membrane, where they interact with transmembrane occludin and claudin proteins, and attach the tight junction to the cellular cytoskeleton (Figure 6). Immunohistochemical staining has localized occludin to tight junction strands in EC and epithelial cells,\(^{162-164}\) and its expression may be modulated by the ECM composition.\(^{165}\) It has been shown that occludin is involved in conferring low permeability at the tight junction. This concept was supported by an observed decrease in permeability due to overexpression of occludin in cultured Madin-Darby canine kidney (MDCK) cells.\(^{166}\) Reciprocally, a reduction in occludin expression is correlated with an increase in endothelial permeability.\(^{167}\)

![Figure 6 – Regulation of tight junctions by signal transduction pathways.](image)

Ligand-receptor interactions such as vascular endothelial growth factor (VEGF) interaction with vascular endothelial growth factor receptor-2 (VEGFR2) can also impact tight junctional integrity and organization. Signaling downstream of tyrosine kinase receptors can modify the phosphorylation status of zonula occludens (ZO), such as ZO-1, which serve to bind the transmembrane tight junctional proteins such as occludin and claudin to the actin cytoskeleton.
Occludin is also involved in the recruitment of a number of proteins in the family of membrane-associated guanylate kinase homologues, ZO-1, ZO-2 and ZO-3, to the cytoplasmic side of the tight junction. ZO-1 is a ~200kDa protein. It binds via its amino terminus to the carboxy terminus of occludin and claudin.\textsuperscript{168-170} Actin and \(\beta\)-catenin binding sites are present at their carboxy termini.\textsuperscript{171,172} Partly because of these specific binding sites, ZO-1 is present at the tight junction, but not at the adherens junction of epithelial cells. ZO-2 is a 160kDa protein with occludin, claudin, \(\beta\)-catenin and actin binding sites. It also binds ZO-1 to form a heterodimer.\textsuperscript{170,172,173} ZO-3 is a 130kDa protein that binds to occludin, claudin, actin and ZO-1, but not to ZO-2.\textsuperscript{170,172,174} ZO-3 is also targeted to tight junctions in epithelial cells. ZO-1, ZO-2 and ZO-3 can cross-link the cytoskeleton to the tight junction and adherens junction, potentially regulating their involvement in activities such as vascular permeability.\textsuperscript{171,175}

Claudins are 22kDa proteins with four transmembrane domains.\textsuperscript{176} When fibroblasts were transfected with these genes, they formed tight junctional strands in extensive networks.\textsuperscript{177,178} This is in contrast to cells transfected with occludin, which formed only a small number of short strands.\textsuperscript{179} Claudins appear to form the backbone of tight junctions. Different sets of these proteins seem to make up the tight junction in a tissue dependent fashion, perhaps related to the level of permeability of the junctions.\textsuperscript{180} It has been proposed that the number, type and ratio of the different species of claudin in the tight junction may determine permeability. Some combinations are able to form heterodimers and thus a tight seal in the junction, while other combinations cannot adhere to each other. Thus, when they are present in the junction, a “pore” is formed through which material can pass across the tight junction.\textsuperscript{181} The current view holds that tight junction strands are composed of several species of claudin
polymerized to form a backbone with occludin co-polymerized into the strands. Tight junctions can be seen as dynamic structures that control vascular permeability in response to extracellular and intracellular stimuli, thereby regulating vascular integrity and function.

1.5.1.2.2 — Adherens junctions

Another junctional complex that is present between adjacent endothelial cells is the adherens junction (Figure 7). This structure does not form barriers to molecular movement, but rather forms areas where adjacent cells attach to each other and are held together through the homophilic calcium-dependent interactions of transmembrane proteins called cadherins. Adherens junctions regulate the integrity of the tight junctions. Endothelial cells express at least two types of cadherins. N-cadherins are distributed diffusely, while VE-cadherins are located at the junctions. VE-cadherin, also called cadherin-5, is a 150kDa protein specific for endothelial cells, with β-catenin and plakoglobin-binding domains at its carboxy-terminal region. The tyrosine phosphorylation status of adherens junction proteins has been implicated in the regulation of vascular permeability. Overexpression of VE-cadherin increases the recruitment of β-catenin into adherens junctions and subsequently reduces its nuclear translocation for transactivation. Transfection of VE-cadherin promotes adhesion and contact inhibition of growth. The use of a truncated form of the protein abolishes the latter effects as well as control of paracellular permeability and stabilization of junction. Subconfluent and migrating cells exhibit high levels of tyrosine phosphorylation of this protein. Phosphorylation is correlated with binding to β-catenin and a low level of adhesiveness as compared to other members of the cadherin family, such as E- and N-cadherin in other cell types. The dynamic nature of this
interaction is illustrated by a reduction in the level of phosphorylation when cells become confluent and the junctions mature and stabilize. Binding to β-catenin is replaced by plakoglobin and actin at this time.\textsuperscript{186}

**Figure 7 – Regulation of adherens junctions by signal transduction pathways.**
Similar to their modification of tight junctions, signal transduction molecules such as protein kinase C (PKC) downstream of tyrosine kinase receptors can affect barrier properties maintained by adherens junctions. Typically, this may occur through calcium (Ca\textsuperscript{2+}) and/or calmodulin (CaM)-dependent modulation of myosin light chain kinases, which can affect cell contraction through myosin light chains. This may also occur through modifications in phosphorylation status of adherens junction molecules by tyrosine phosphatases or kinases.

Thrombin, histamine, VEGF, TNF-α, IFN-γ and polymorphonuclear leukocytes (PMNL) are all known to increase endothelial permeability and have been shown to affect the adherens junction, especially VE-cadherin. VEGF induces tyrosine phosphorylation of VE-cadherin 15 minutes to one hour after treatment.\textsuperscript{187} TNF-α and IFN-γ cause disorganization of VE-cadherin in focal areas.\textsuperscript{188} Thrombin reduces plakoglobin binding to VE-cadherin, while PMNL reduce β-catenin binding.\textsuperscript{184,186,189,190} Anti-VE-cadherin antibodies induce a redistribution of VE-cadherin and reorganization of the cytoskeleton along with an increase vascular permeability, as
illustrated by interstitial edema, inflammatory cell accumulation, endothelial gap formation and exposure of basement membrane to the circulation. At the same time, a rapid production of VEGF and re-synthesis of VE-cadherin occurs to restore cell-cell contact. The importance of the cytoskeletal interaction (especially actin) for the function of VE-cadherin is illustrated by the redistribution of the latter after treatment with isoproterenol or an inhibitor of small GTPase, p21 Rho.

N-cadherin, the other major cadherin in endothelial cells, is a 140kDa protein that is cytoplasmically distributed rather than localized to the adherens junction. It has been proposed that whereas VE-cadherin promotes homotypic interactions between adjacent endothelial cells, N-cadherin anchors endothelial cells to surrounding N-cadherin-expressing cells such as SMC and pericytes.

Cadherins are connected to actin through the linking proteins catenins, vinculin, vimentin, talin, α-actin, zyxin and moesin. The contractile tension generated by the cytoskeleton modulates the barrier function. Members of the catenin family include α-catenin, β-catenin and plakoglobin/γ-catenin. β-catenin, plakoglobin and p120, members of the Armadillo family, bind to VE-cadherin. Plakoglobin and β-catenin also binds α-catenin, which is homologous to vinculin. In turn, α-catenin bind to α-actin, actin, vinculin and talin. This association strengthens cell-cell binding via VE-cadherin to withstand shear stress. Overexpression of vinculin decreases cell motility, while disruption of vinculin expression results in decreased adhesion and enhanced motility. Binding of actin to vinculin induces the dimeric conformation of the latter different from that formed by phosphotidylinositol 4, 5-bisphosphate (PIP_{2}). It has been proposed that actin may thus function directly in signal
transduction.\textsuperscript{201} Vinculin phosphorylation by PKC and tyrosine kinases has been correlated to hyperpermeability during energy depletion in coronary endothelial cells (Figure 7).\textsuperscript{202} Since p120 does not bind α-catenin, its presence leads to relatively weaker adhesive strength and a more dynamic junction. In recently confluent cells, VE-cadherin is bound to p120 and the adherens junction is weak and immature. After 48-72 hours, p120 is replaced by plakoglobin and the adherens junction stabilizes.\textsuperscript{183,186} VEGF induces the tyrosine phosphorylation of β-catenin, plakoglobin and p120 15 minutes to one hour after treatment.\textsuperscript{187} A number of other growth factors such as PDGF, epithelial growth factor (EGF) and transforming growth factor-beta (TGF-β) also cause phosphorylation of the catenins, either directly through tyrosine kinase receptors or indirectly through Src tyrosine kinases. Phosphorylation of β-catenin and plakoglobin leads to their dissociation from cadherin and disrupts cell-cell adhesion. Conversely, a number of protein tyrosine phosphatases associated with the adherens junction dephosphorylate catenins and increase adhesion.\textsuperscript{203,204}

1.5.1.3 – Focal adhesion complexes and the extracellular matrix

Complex structures called focal adhesions link the cytoskeleton of the cell to the ECM. They are composed of a cytoplasmic focal adhesion plaque, transmembrane integrins and ECM molecules. The focal adhesion plaque is a complex structure composed of α-actinin, vinculin, talin, paxillin and tyrosine kinases (Figure 8), most notably focal adhesion kinase (FAK). Similar to the adherens junction, α-actinin links vinculin and actin. This is linked to integrins by the binding of talin to vinculin and the β subunit of integrins.\textsuperscript{205} Alternatively, α-actinin can directly link actin to the β subunit of integrin.\textsuperscript{206} A number of key enzymes localized to the focal
adhesion plaque participate in the regulation of permeability through this structure. The major regulators include FAK, c-Src, PKC, and Ca\textsuperscript{2+}-dependent proteases. Vinculin and talin are substrates for these enzymes (Figure 8). FAK itself is tyrosine phosphorylated in the presence of inflammatory mediators\textsuperscript{209} and in subsequent vascular hyperpermeability.\textsuperscript{210} However, the function of these kinases varies between different cell types. In endothelial cells, vinculin and talin are redistributed and the cytoskeleton is rearranged by thrombin treatment, but the phosphorylation state of these proteins remains unchanged.\textsuperscript{211} Other focal adhesion proteins that are substrates for these kinases include paxillin,\textsuperscript{212,213} GTPase regulator associated with FAK (Graf) and Crk-associated substrate (Cas).\textsuperscript{214}

**Figure 8 – Regulation of focal adhesion complexes by signal transduction pathways.**

The integrity of focal adhesion complexes can be modulated through signal transduction pathways such as protein kinase C (PKC) or tyrosine kinases downstream of tyrosine kinase receptors, such as vascular endothelial growth factor receptor-2 (VEGFR2). These may act on focal adhesion complex molecules such as vinculin or talin, respectively, which facilitate the binding of integrin complexes joined to the extracellular matrix at the abluminal side of the cell to the actin cytoskeleton through intermediates such as α-actinin.
Talin is a homodimer of 230kDa polypeptides.\textsuperscript{215} It cross-links actin and potentiates the function of $\alpha$-actinin.\textsuperscript{216,217} Paxillin is a 68kDa protein that binds vinculin, FAK and Src. It is phosphorylated by inflammatory mediators and growth factors\textsuperscript{212} as well as during subsequent increases in vascular permeability.\textsuperscript{210} Graf is a regulatory protein that can stimulate the intrinsic GTPase activity of RhoA and cdc42. It may serve as an effector or negative regulator of these signals.\textsuperscript{218} Cas regulates the Ras family of GTP binding proteins by binding the adaptor protein Crk and CrkL which then binds the guanidine nucleotide exchange factors (GEF) Son of sevenless (SOS) and C3G, which in turn regulate the activity of Ras and Ras-proximate-1 (Rap1).\textsuperscript{219} Inhibition of RhoA activity reduces endothelial permeability and stress fiber linkage to focal adhesions.\textsuperscript{220} FAK itself can be regulated by tyrosine phosphorylation at six sites and serine phosphorylation in multiple sites. These events appear to be associated with cell attachment, recruitment of kinases and activation of Ras.\textsuperscript{214}

Integrins are heterodimeric transmembrane proteins. Their $\beta$-subunit non-covalently binds to the $\alpha$-subunit, which confers the specificity of binding. Of the eight $\beta$-subunits, $\beta$1, $\beta$2, $\beta$3, $\beta$4 and $\beta$5 are present in endothelial cells, along with $\alpha$1, $\alpha$2, $\alpha$3, $\alpha$5, $\alpha$6 and $\alpha$v from the 16 $\alpha$ subunits in different combinations.\textsuperscript{205,206,221,222} Inhibition of their function by antibodies leads to altered permeability as well as migration and attachment.\textsuperscript{223} Increased permeability in diseased states also involves integrins. TNF-$\alpha$ induces internalization of the $\alpha$5$\beta$1 integrin\textsuperscript{224} and reduces its co-localization with fibronectin.\textsuperscript{225} The ECM provides points for cell binding and components include fibronectin, laminin, vitronectin, collagen and proteoglycans.

The basement membrane and ECM also affect permeability. The diffusion of albumin is decreased by the presence of matrix. Components of the vessel wall – elastin, collagen and
proteoglycans – in isolation can variably retain molecules such as LDL.\textsuperscript{226} It is not known how this translates to the blood vessel wall. Interactions between endothelial cells and ECM are required to maintain low permeability. Endothelial cells bind to the ECM via integrins on their abluminal surface, which in turn, bind talin, vinculin, α-actinin and actin (Figure 8). Digestion of the extracellular matrix by protease treatment or endogenous metalloproteinases significantly increases permeability. The mode of action for this proteolytic effect is uncertain. It may involve changes in force transduction between the extracellular matrix and the cytoskeleton or of “outside in” signal transduction through the integrins.\textsuperscript{227}

1.5.2 - Modulation of permeability

The transport pathways used across different endothelium vary, even within the same organ. The permeability of different vascular beds can vary over several orders of magnitude under normal conditions.\textsuperscript{228} As well, numerous substances, both exogenous and endogenous, affect the permeability of the endothelium. As early as the 1960s, Majno \textit{et al} had shown that inflammatory mediators act on venular endothelium and increase vascular permeability to colloidal carbon, thus establishing the endothelium as an important functional component of the vasculature.\textsuperscript{82,83} Both histamine and serotonin induce the formation of gaps 0.1-0.8μm in width in venules, visible under the electron microscope. It was believed that these are not holes through the endothelial cells but rather intercellular gaps. Later, these investigators hypothesized that such gaps formed due to histamine, serotonin and bradykinin are a results of focal disruption of endothelial cell junctions following endothelial contraction.\textsuperscript{81,84} This view is supported by the demonstration of contractile elements (actin, myosin, and microfilaments) in
endothelial cells. The gaps produced permit fluid and macromolecules to flood the tissue – one of the cardinal signs of inflammation. The dynamics of these processes were studied in the hamster cheek pouch model using intra-vital light microscopy and direct measurements of tracer (dextran-conjugated fluorescein isothiocyanate (FITC)) movement into the tissue. Using this model with a combination of electron and light microscopy, Arfors et al showed that sites of leakage correspond to gaps in the endothelium. The number of gaps increases with the dose of the inflammatory agent and, importantly, this event is reversible.

The number of known permeability-altering agents has grown steadily. In addition to histamine, serotonin and bradykinin discussed above, there are prostaglandins, thromboxane, leukotrienes, thrombin, platelet-activating factor (PAF), cytokines, complement, substance P, vascular permeability factor (VPF), free radicals, endotoxin, various inorganic and organic salts, hypoxia and many others. Permeability changes were historically demonstrated mainly in venules. However, due to the polymorphic structural and functional nature of the endothelium in different categories of vessels and different organ systems, the action of these agents can vary considerably in different vascular beds. In this regard, it has also been important to compare studies utilizing different vessels in vivo and in vitro.

Thrombin and histamine cause an increase in cytoplasmic calcium. In the presence of adenosine triphosphate (ATP) and calcium, myosin light chain kinase MLCK rapidly and transiently phosphorylate myosin light chains, allowing actin to interact with myosin, leading to contraction. The nature and identity of endothelial (MLCK) is not fully known. A number of candidates have been found in different vascular beds. In addition to contraction, some authors have also described retraction. Such retraction may be a passive process independent
of MLCK, but requiring phosphorylation of cytoskeleton linking proteins such as vinculin, talin, caldesmon and vimentin, by PKC and/or tyrosine kinases, thus leading to reduced cell-cell and cell-extracellular matrix contact and increased gap formation. Vinculin connects the cadherins and catenins to α-actinin and is involved in retraction of endothelial cells from adjacent cells. Talin bridges the integrins, vinculin and α-actinin at the abluminal cell membrane and is involved in retraction from the extracellular matrix. Endothelial cells from different vascular beds possess different abilities to contract. Pulmonary arterial endothelial cells do not develop enough tension to deform silicon supports when stimulated, whereas microvascular endothelial cells readily cause deformation.

Thrombin and histamine has also been shown to cause the redistribution of VE-cadherin at the adherens junction. This is associated with a reduction in intracellular free calcium and an increase in permeability, but with no detectable gap formation. It has been demonstrated that neutrophil adhesion to rat brain endothelial cells, leading to blood-brain barrier breakdown, causes an increase in phosphotyrosine, loss of occludin and ZO-1, and induces vinculin redistribution. With human umbilical vein endothelial cells, neutrophils disrupt adherens junctions and reduce the amount of VE-cadherin and β-catenin.

1.5.3 – Second messengers and signal transduction pathways

The signal transduction pathway involved in the regulation of endothelial permeability has been the focus of many studies. Second messengers implicated include calcium, G proteins, phospholipases, PKC, tyrosine kinases, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Many of the inflammatory mediators noted above utilize
these second messengers. Thrombin increases intracellular calcium and activates PKC. Hypoxia decreases cAMP levels. Prostaglandins increase cAMP. However, the same second messenger can also have diverse actions on endothelial cells of different origins. Cyclic AMP can increase the permeability of bovine pulmonary artery endothelial monolayers, but prevent the permeability increases due to thrombin in bovine aortic endothelial cells. \textsuperscript{231} The targets of these second messengers and how these different pathways interact with each other are less well characterized.

1.5.3.1 – Calcium

Calcium is one of the most studied messengers involved in permeability changes. Histamine treatment causes intracellular free calcium to increase to a peak level within 15-30 seconds, and then fall to 30-50\% of this level within 1-2 minutes. This calcium first originates from intracellular pools, then by way of influx from the extracellular milieu. These changes are consistent between intact microvessels and cultured cells, and upon treatment with different inflammatory agents such as thrombin and bradykinin. \textsuperscript{237} The increased level of free calcium can activate calcium-calmodulin dependent kinases, as well as calcium and phospholipid-dependent PKC, leading to phosphorylation of actin-binding proteins and myosin light chains, eventually leading to endothelial contraction. \textsuperscript{238} However, it has been reported that increases in intracellular calcium alone do not increase the permeability of microvessels, in contrast to effects in larger vessels such as arteries. \textsuperscript{239}
1.5.3.2 – Phospholipases

Another early step in signal cascades is the activation of the phospholipases. Shear stress and oxygen radicals can directly activate phospholipases. On the other hand, thrombin, histamine and other agents first bind to and activate their receptors, leading to G protein activation, which in turn activates the phospholipases. There are four classes of G proteins (Gs, Gi, Gq and G12) each formed from one α, β and γ subunit and coupled to specific receptors. Each class of G protein can be activated by the binding of a ligand to a number of different receptors, and each receptor can activate a number of classes of G proteins. These proteins regulate the activity of phospholipase C (PLC), phospholipase A2 (PLA2), adenylyl cyclase, phosphodiesterases and ion channels. Ligand binding to the H2 histamine receptor causes the level of intracellular cAMP to rise, probably via Gs, which activates adenylyl cyclase. Binding to the H1 receptor causes phosphoinositide turnover and calcium mobilization via the activation of PLC by Gi and Gq.227

There are five types of phospholipases (A1, A2, B, C and D). The most important one relative to permeability is PLC. It is a membrane-bound enzyme that mediates the hydrolysis of phosphotidylinositol 4, 5-bisphosphate (PIP2) to inositol 1, 4, 5-trisphosphate (IP3) and 1, 2-diacylglycerol (DAG). IP3 binds its receptor on the endoplasmic reticulum to induce mobilization of calcium. This event allows the initial rapid and maximal increase in concentration of internal calcium discussed above. Thrombin increases IP3 levels in pulmonary artery endothelial cells within 10 seconds and intracellular calcium levels within 17 seconds. This is followed by cytoskeletal reorganization and an increase in permeability at about two minutes.240 Both the increase in intracellular calcium and DAG activates PKC.241 The rise in intracellular calcium can
also induce the formation of DAG by PLC and arachidonic acid by PLA2 in most systems. Arachidonic acid can go on to activate PKC and ion channels, including calcium channels. Phospholipase D (PLD) may also be involved. It also generates DAG, but does not require a rise in intracellular calcium. Bradykinin induces an increase in permeability dependent on PKC, but not on increased intracellular calcium. This pathway has been suggested as a mechanism for persistent increases in permeability. These pathways appear to converge on PKC.

There are ten PKC isoenzymes, all of which phosphorylate serine and threonine residues. The isoenzymes α, βI, βII and γ are activated by calcium, phosphatidylserine and DAG, while ε, δ, θ and η do not require calcium, and ζ and λ do not require either DAG or calcium. Although it is not clear which isoenzyme is involved in permeability changes, it is known that PLC and DAG are involved. PKC-α and PKC-β are the prime candidates since they are most abundant in endothelial cells. Moreover, overexpression of transfected PKC-β1 in endothelial cells increases the permeability due to phorbol 12-myristate 13-acetate (PMA) treatment. However, PKC activation by different agents can have contradictory effects on permeability. Depletion of PKC, treatment with PKC inhibitors or inhibitors of PKC activation all prevent the increase in permeability due to thrombin and H2O2 in pulmonary microvascular endothelial cells. Although activation of PKC increases PAF-induced permeability, PKC activation by phorbol esters reduces permeability. Moreover, MLC were not phosphorylated. Indeed, thrombin causes MLC phosphorylation by a mechanism independent of PKC. In addition to the cytoskeleton, PKC also affects components of the adherens junctions. Thrombin-mediated disruption of the VE-cadherin-catenin complex is inhibited by PKC and tyrosine kinase inhibitors. Other PKC targets proposed include MLCK, vinculin and vimentin. How PKC is
involved in the regulation of the tight junction components and endocytosis/transcytosis is less clear. One study indicates that an increase in endocytosis due to angiotensin II is also mediated by PLC and PLD production of IP$_3$ and a rise in intracellular calcium levels, but not PKC.$^{247}$

1.5.3.3 – Tyrosine kinases

Tyrosine kinases are another family of kinases implicated in the regulation of permeability. Tyrosine kinases are located at the adherens junctions.$^{248}$ The components of these junctions such as vinculin, talin and β-catenin are substrates for these kinases. Tyrosine phosphorylation of β-catenin is associated with an increase in permeability.$^{249}$ These enzymes have also been implicated in endocytosis in one study.$^{250}$ gp60 is a cell surface albumin binding protein present in caveolae. Activation of this receptor by albumin or by cross-linking leads to the phosphorylation of its tyrosine residues, which in turn phosphorylates the Src family tyrosine kinases. Tyrosine kinase inhibitors prevent the uptake of albumin and gp60 activation.

Increases in permeability due to inflammatory mediators are reversible. Permeability returns to basal levels 15-60 minutes after the removal of the agent.$^{227}$ Many negative feedback mechanisms have been proposed, including receptor desensitization by proteolysis, internalization or phosphorylation, downregulation of G proteins, uncoupling of PLC and G proteins by PKC, activation of phosphatases, or inhibition of protein kinases.$^{227}$ For example, thrombin causes the degradation of its receptor after binding.$^{251}$ Bradykinin causes its receptor to be internalized.$^{252}$ PKC uncouples PLC from its G protein, but not from PLD.$^{253,254}$ Dephosphorylation of myosin returns permeability to basal levels.$^{255}$
**1.5.3.4 – cAMP and cGMP**

The cyclic nucleotides cAMP and cGMP affect the permeability of the endothelium and modulate the effects of inflammatory mediators. Increases in cAMP levels by activation of adenylate cyclase, inhibition of phosphodiesterase or direct increases of intracellular cAMP by incubation with membrane permeable analogs of cAMP not only decrease the basal permeability of the endothelium,\(^{238}\) but also prevent the increase in permeability due to thrombin, histamine and other agents in the micro- and macrovasculature.\(^{233}\) The mechanism involves cAMP dependent protein kinases, reduction in MLC phosphorylation, loss of actin-myosin interactions, and redistribution of F-actin. In addition, the restoration of junctional complexes and inhibition of PL and PKC have also been proposed.\(^{233,238}\) Cyclic GMP can be increased by similar mechanisms and this can produce similar effects on permeability in many cases, including reduction of thrombin and \(\text{H}_2\text{O}_2\)-induced permeability and basal permeability.\(^{233}\) This effect seems to involve the reduction of intracellular calcium by cGMP dependent protein kinases.\(^{256}\) However, differing results have also been reported. Elevation of cGMP levels does not affect the basal permeability of aortic endothelial cells.\(^{257}\) In stark contrast, one group working with animals *in vivo* has reported that cAMP and cGMP increase albumin penetration of brain microvessel consistently.\(^{258}\) Other groups working with human material *in vitro* has found the opposite.\(^{259}\) One possible explanation is that different vascular beds and species may have different amounts of phosphodiesterases (PDE) that break down these cyclic nucleotides.\(^{233}\) In human umbilical vein endothelial cells (HUVEC), cGMP reduces the effect of thrombin by inhibiting a cGMP-inhibited PDE III. A PDE III inhibitor has the same effect on HUVEC, but not on human aorta or pulmonary artery endothelial cells.\(^{256}\) Another
PDE, cGMP stimulated PDE II, may also be involved in permeability. Activation of this PDE by elevated cGMP levels can reduce both the concentration of cAMP and cGMP. Unfortunately, the level of PDE in different vascular beds has rarely been studied.

Another cGMP dependent mechanism of interest is its reduction of intracellular calcium concentrations. Increases in calcium activate eNOS to produce nitric oxide (NO), which in turn increases cGMP production by guanylyl cyclase. This increased level of cGMP then reverses the change in calcium levels and the increase in permeability. Indeed, NO generated by thrombin counters the increase in aortic and pulmonary artery endothelial cell permeability. Through this mechanism, NO also modulates its own calcium-dependent formation.

1.6 – Vascular endothelial growth factor

1.6.1 – Search for a tumor angiogenic factor

More than a century ago, increased vascularization was observed alongside tumor growth. In 1939, Ide et al suggested the existence of a tumor-derived blood vessel growth stimulating factor. In 1945, Algire et al suggested that the rapid growth of tumor transplants is dependent on the development of a rich vascular supply after observing that the growth of tumor xenografts was preceded by local increases in vascular density. In 1968, Greenblatt and Shubick provided early evidence that tumor angiogenesis may be mediated by diffusible molecules. In 1971, Folkman proposed that anti-angiogenesis might be a strategy to treat human cancer. This key hypothesis fueled particular interest in the field of angiogenesis research and began the search for regulators of blood vessel growth. However, the identification and isolation of such factors proved elusive.
In 1983, Senger et al identified a protein which induces vascular leakage in the supernatant of a guinea pig tumor cell line and named it vascular permeability factor (VPF).\textsuperscript{264} It was proposed that VPF may be a mediator of the increased leakiness or permeability in tumor blood vessels. In 1989, VEGF was sequenced and described as an angiogenesis-inducing factor.\textsuperscript{265} Another group at Monsanto Company led by Daniel Connolly had also reported the cloning of VPF at approximately the same time and sequence comparison revealed that these two groups had independently identified the protein.\textsuperscript{266} This report described a human clone which encoded a protein identical to vascular endothelial growth factor (VEGF)-A\textsubscript{189}.\textsuperscript{266}

Inactivation of the VEGF gene in mice provided definitive evidence for a key role in angiogenesis. In 1996, groups led by Carmeliet and Ferrara reported that VEGF is required for normal embryonic vasculogenesis and angiogenesis.\textsuperscript{267,268} Inactivation of even a single VEGF allele in mice resulted in developmental abnormalities and early embryonic lethality.\textsuperscript{267,268}

### 1.6.2 – Vascular endothelial growth factor family members and receptors

VEGF belongs to a gene family that also includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF).\textsuperscript{269} Multiple isoforms of VEGF-A (121, 145, 165, 189, 206 amino acids) can be generated by alternative exon splicing. These isoforms differ in their ability to bind heparan sulphate, which determines its bioavailability and may play distinct roles in angiogenesis during development.\textsuperscript{270} In addition, extracellular proteolysis can regulate VEGF activity. Early studies showed that plasmin is able to cleave heparan sulphate-binding VEGF isoforms at the COOH-terminus to generate bioactive and diffusible fragments.\textsuperscript{271,272} More recently, it has been reported that matrix metalloproteinase-3 (MMP-3) is able to generate
VEGF proteolytic fragments which are biologically and biochemically similar to those resulting from plasmin cleavage. All VEGF isoforms can bind to VEGF receptor-1 (VEGFR1; fms-like tyrosine kinase-1, flt-1) and VEGF receptor-2 (VEGFR2; kinase insert domain receptor, KDR; fetal liver kinase-1, flk-1) (Figure 9). Despite the finding that VEGF binds to VEGFR1 with an approximately 10-fold higher affinity than VEGFR2, VEGFR2 primarily mediates VEGF signaling in endothelial cells. It is believed that VEGFR1 may act as a decoy receptor in some respects, preventing interaction with VEGFR2.

1.6.3 – The role of vascular endothelial growth factor

Research conducted over the last two decades has established that VEGF plays an essential role in the regulation of embryonic, postnatal physiological angiogenesis processes, including normal development. A variety of animal models have generated much information on the biology of VEGF and the therapeutic potential of VEGF or VEGF inhibitors. VEGF has many effects on endothelial cells – it is a potent endothelial growth factor, inducer of vascular permeability, and vasodilator. VEGF can cause the migration of endothelial cells, new vessel formation, can affect pericytes and vessel maturation, and can impact the matrix metalloproteinases secreted by endothelial cells.

1.6.4 – Vascular endothelial growth factor in disease

VEGF has been reported to play a role in a variety of diseases, including cancer, diabetic retinopathy, age-related macular degeneration, renal failure, and native atherosclerosis, among others.
Figure 9 – Vascular endothelial growth factor family and their receptors.

There are numerous members of the VEGF family and a number of receptors, including VEGF-A (VPF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF). There are 5 splice variants of VEGF-A (121, 145, 165, 189, 206 in humans) – the lowest molecular weight form is free in the circulation while the others are immobilized by binding to heparan sulphate proteoglycans. There are also 2 splice variants of VEGF-B (167, 186). VEGF-A binds VEGFR1 and VEGFR2, also called flt-1 and flk-1, respectively. VEGF-B binds VEGFR1. VEGF-C and VEGF-D bind VEGFR2 and VEGFR3. VEGF-E is from the Orf (pox) virus and binds to VEGFR2. Both PlGF-1 and PlGF-2 bind to VEGFR1. Neuropilins are also co-receptors for splice variants of VEGF-A and PlGF.
Pathological VEGF expression is driven by hypoxia, and in cancer, is one of the primary regulators of tumor angiogenesis. VEGF produced in this pathological state drives not only angiogenesis, but also increases vascular permeability, resulting in edema. In several models, inhibition of VEGF in ovarian carcinoma,\textsuperscript{314} brain tumors,\textsuperscript{315} and vestibular Schwannomas\textsuperscript{316} result in decreased edema around the tumors, resulting in reduced morbidity.

Similarly, increased VEGF expression results in the neovascularization of the choroidal vasculature underlying the retina.\textsuperscript{278} When these choroidal vessels invade the retina, increased vascular permeability results in sub-retinal edema, causing loss of sight.\textsuperscript{317} In diabetes, there is systemic endothelial dysfunction, resulting in loss of endothelial cells from the retina and localized areas of ischemia.\textsuperscript{318} As a result, hypoxic induction of VEGF acts on the vasculature surrounding the periphery of the ischemic area, resulting in proliferation and increased leakage of vessels, ultimately leading to hemorrhage, edema and neovascularization.\textsuperscript{319}

Since the first characterization of VEGF expression in human coronary atherosclerotic lesions,\textsuperscript{311} the possible pathophysiological significance of VEGF in the progression of atherosclerosis was introduced. Key concepts that have developed along these lines, including the discovery of VEGF-induced chemotactic response in monocytes mediated by VEGFR1 (flt-1),\textsuperscript{320,321} suggesting a new role for VEGF in monocyte/macrophage chemotaxis, a crucial inflammatory process in wound repair. As well, the interplay of VEGF gene expression and its induction by hypoxia,\textsuperscript{322-324} transforming growth factor-\(\beta\) (TGF-\(\beta\)),\textsuperscript{325} angiotensin II,\textsuperscript{326} basic fibroblast growth factor\textsuperscript{327} and interleukin-1,\textsuperscript{328,329} among others, present a collection of factors that are all known to be expressed in atherosclerotic lesions.
Another primary role for VEGF in atherogenesis is the neovascularization of plaque tissue, often driven by the development of a hypoxic region within the developing neointima and fueled by the emerging lipid-rich core.\textsuperscript{8,330-332} In addition, VEGF plays a significant role in the induction of vascular permeability. Within the context of atheromatous disease and CAV, this role has been one that has been less focused upon. In contrast to VEGF-mediated effects in physiological angiogenesis where excess edema is uncommon or physiological VEGF-induced increases in permeability where angiogenesis is rarely seen, VEGF expression and actions in pathological states often results in aberrant angiogenesis coupled with excess permeability.
Chapter 2 – Hypothesis and specific aims

2.1 – Rationale

Cardiac allograft vasculopathy (CAV) is the leading expression of chronic rejection and the major cause of heart transplant failure beyond one year post-transplantation. This chronic vascular disease results in the partial or complete obstruction of blood vessels, particularly macrovessels in transplanted organs, and results in tissue ischemia and heart failure. As such, CAV invokes substantial personal, social and financial costs in our society. In physiological and pathological settings, the endothelium serves as a key structural and functional regulator of vascular health, guiding leukocyte traffic, modulating transport of micro- and macromolecules and ions, and regulating homeostatic vascular function. Our laboratory has shown through a long-running series of investigations that pathogenesis of CAV in transplanted hearts involves endothelial injury and dysfunction, smooth muscle perturbations, inflammation, accumulation of extracellular matrix, and insudation of lipids and lipoproteins within affected vessels. As well, the abundance of lipids, lipoproteins and proteoglycans within the coronary arteries of human heart allografts occurs with an apparent lack of endothelial damage, death or denudation, suggesting the possibility of a permeability-inducing agent facilitating the insudation of plasma lipids and lipoproteins into the vessel wall post-transplantation. Dysfunction of the endothelium results in hyperpermeability to these factors and may contribute to the pathogenesis of CAV. Particularly relevant is the mechanism of increased low density lipoprotein (LDL) insudation and deposition in CAV arteries.
Among all growth factors known to date, vascular endothelial growth factor (VEGF) is the only one capable of inducing inflammation. VEGF increases vascular permeability, leukocyte adhesion and transmigration, and platelet aggregation via the synthesis of various paracellular signaling molecules such as platelet-activating factor and tissue factor, and may be potentially deleterious to grafts. On the other hand, VEGF induces endothelial cell proliferation, migration, and angiogenesis as well as bone marrow-derived cell mobilization and re-endothelialization, events that may be potentially beneficial to grafts.

This doctoral dissertation focuses on the characterization of VEGF expression in CAV, the investigation of VEGF-induced endothelial hyperpermeability to LDL, and whether VEGF abrogation in vivo can reduce the number or severity of CAV lesions. These concepts are summarized in Figure 10.

2.2 – Central hypothesis

Vascular endothelial growth factor plays a significant role in the pathogenesis of CAV by inducing endothelial cell hyperpermeability to low-density lipoproteins.

2.3 – Specific aims

1. To characterize the expression and localization of VEGF in CAV.
2. To elucidate the mechanisms of VEGF-induced endothelial hyperpermeability to LDL.
3. To examine the effect of abrogation of VEGF on the pathogenesis of CAV.
1. To characterize the expression and localization of VEGF in CAV, immunohistochemistry was performed for VEGF. Digital micrographs were analyzed using ImagePro Plus® image analysis software to allow for direct comparison of VEGF immunoreactivity in the intima and media of coronary arteries from human heart allografts as compared to age-matched and sex-matched normal, non-atherosclerotic controls from the Pathobiological Determinants of Atherosclerosis in Youth study.

2. To elucidate the mechanisms of VEGF-induced endothelial hyperpermeability to LDL, in vitro investigation using human coronary artery endothelial cells (HCAEC) and human cardiac microvessel endothelial cells (HCMEC) was performed. Transendothelial electrical resistance (TER) was used as an indirect measure of endothelial barrier properties in confluent endothelial monolayers. Immunocytochemistry was performed to examine the localization of tight junction protein components. A modified transwell system and fluorescently conjugated low-density lipoprotein (LDL) were used to determine whether VEGF can increase LDL passage through the endothelium.

3. To examine the effect of abrogation of VEGF on the pathogenesis of CAV, a minor histocompatibility complex-mismatched mouse model of heterotopic cardiac transplantation was used to assess the role of VEGF in the pathogenesis of CAV in a hypercholesterolemic environment. Mice received intraperitoneal injections of either soluble VEGF receptor-1 (sVEGFR1) or vehicle control (PBS) every two days for 21 days, alongside an immunosuppressive regimen of FK506 (tacrolimus). The primary endpoint of this investigation was frequency and severity of CAV lesions.
Chapter 3 – The role of vascular endothelial growth factor in cardiac allograft vasculopathy

3.1 – Rationale

The major cause of morbidity and mortality in human cardiac allograft recipients is the development of an accelerated form of atherosclerosis termed cardiac allograft vasculopathy (CAV). CAV is characterized by diffuse, intimal hyperplasia which occurs soon after transplantation and affects the entire arterial vasculature. Our laboratory and others have shown that lipid accumulation is an important early and persistent phenomenon in the development of CAV.

Several growth factors are believed to be involved in various aspects of vessel growth, remodeling, and physiology. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is a multifunctional angiogenic cytokine first reported to increase the permeability of tumor microvessels to plasma and plasma proteins. VEGF is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis, enhancing vascular permeability, and modulating thrombogenicity. VEGF is expressed by smooth muscle cells (SMC), macrophages and endothelial cells. VEGF-A has been previously been associated with animal models and human CAV.

Native atherosclerosis (NA) is a chronic vascular disease which results in the hardening of the arteries. It is a multifactorial and multifaceted disease where fibro-fatty deposits accumulate in the inner lining of elastic and medium to large muscular arteries. These plaques are comprised of a lipid-rich core containing a heterogeneous milieu of fibrous tissue, vascular and inflammatory cells and an abundant and dynamic matrix composition. Congruently, diabetes mellitus (DM) is an independent risk factor for the development of atherosclerosis. It
is believed that a combination of hypertension, impaired vascular function, and increased glucose levels, among other factors; contribute to the development of atherosclerosis. Patients with DM often develop atherosclerosis at a more rapid pace than those with atherosclerosis but without DM.

In this chapter, I characterize the differential immunoreactivity and localization of VEGF-A and VEGF-D within coronary arteries from patients with either NA, DM, CAV, or “normal” patients consisting of individuals under the age of 35 who died of acute trauma unrelated to the heart, with less than 25% vessel occlusion. The purpose of investigating VEGF immunoreactivity in NA and DM alongside CAV was to gain insight into the localization with respects to etiology and atherogenesis. The results from this study characterize the differential expression of VEGF isoforms and suggest possible roles in the pathogenesis of CAV.

3.2 – Materials and methods

3.2.1 – Case materials

Normal, non-atherosclerotic coronary artery tissues were obtained from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. The vessels were from male and female individuals between the ages of 15-34 years and who died as the result of acute trauma. These cases from the PDAY study provided a baseline for investigation of VEGF expression in coronary arteries which did not have significant atherosclerosis (<25% luminal narrowing). PDAY cases were age- and sex-matched with the donors from the CAV cases. All of the pathological materials for study of CAV were obtained from the Cardiovascular Registry of the Institute for Heart + Lung Health (Vancouver, BC). Patients with native atherosclerosis were
selected on the criterion of greater than 25% luminal narrowing, as examined at the time of autopsy. Patients in the diabetic group were selected based on diagnosis of both diabetes mellitus and presence of atherosclerosis. All of the diabetic patients were diagnosed with non-insulin-dependent diabetes mellitus with a duration ranging from 3 to 23 years and all had their glucose levels under control by a combination of diet, anti-hypoglycemic drugs, or subclinical doses of insulin. Cases of native atherosclerosis and diabetes mellitus with atherosclerosis were also age-matched and sex-matched with each other. Refer to Table 1 for a summary of case details. A full listing of all of the cases used for immunohistochemical profiling can be found in the Appendix in Tables 3-6.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Males</th>
<th>Females</th>
<th>Mean Age +/- SD (years)</th>
<th>Range of Ages (years)</th>
<th>Mean Implant Duration +/- SD (days)</th>
<th>Implant Duration (days)</th>
<th>Mean Age of Donor +/- SD (years)</th>
<th>Range of Donor Ages (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>22.0 +/- 5.9</td>
<td>17 - 34</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Native Atherosclerosis</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td>65.2 +/- 17.0</td>
<td>24 - 87</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Diabetes Mellitus with Atherosclerosis</td>
<td>15</td>
<td>8</td>
<td>6</td>
<td>64.6 +/- 9.6</td>
<td>39 - 85</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cardiac Allograft Vasculopathy</td>
<td>21</td>
<td>12</td>
<td>8</td>
<td>41.7 +/- 15.4</td>
<td>16 - 67</td>
<td>355.1 +/- 329.8</td>
<td>13 - 1432</td>
<td>26.8 +/- 10.8</td>
<td>16 - 47</td>
</tr>
</tbody>
</table>

Table 1 – Summary of cases used for immunohistochemical profiling of VEGF in atheromatous disease.

3.2.2 – Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections for VEGF-A, and VEGF-D using streptavidin-biotin amplification. Polyclonal rabbit anti-human antibodies against VEGF-A<sub>463</sub> (a kind gift from Dr. Harold F. Dvorak, Harvard Medical School; Boston, MA) and VEGF-D (Santa Cruz) were used. Antibodies used in immunohistochemical profiling of VEGF-A and VEGF-D were verified by Western blot using
recombinant VEGF-A and VEGF-D (R&D Systems), and found to be specific, recognizing the correct molecular weight bands with no observed non-specific reactivity. Briefly, sections were dewaxed and rehydrated in xylene and graded ethanol, then incubated with primary antibody overnight. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc.; Burlingame, CA) and StreptABComplex/AP (Dako; Mississauga, ON) were incubated sequentially at room temperature. Antibodies were localized using the chromagen Vector Red (Vector), followed by counterstaining with hematoxylin. The positive control tissues consisted of placenta and normal-appearing kidney tissue adjacent to a kidney carcinoma. Negative controls included isotype-matched rabbit IgG and omission of primary antibody.

3.2.3 – In situ hybridization

In situ hybridization (ISH) was performed as previously described.349 Formalin-fixed, paraffin-embedded tissue sections were dewaxed, rehydrated and permeabilized with proteinase K (Sigma-Aldrich Canada Ltd.; Oakville, ON). Tissues were subsequently acetylated, dehydrated, air-dried then hybridized overnight at 55°C using digoxigenin-labeled VEGF-A and VEGF-D antisense riboprobes or irrelevant probe (CVB3 antisense riboprobe used as a negative control). Placental tissue was used as a positive control. The VEGF-A and VEGF-D riboprobes were prepared from VEGF-A and VEGF-D cDNA, respectively (a kind gift from Dr. Steve Charnock-Jones, University of Cambridge, UK) by in vitro transcription according to manufacturer’s (Promega-Fisher; Nepean, ON) instructions. Following stringent washing in 50% formamide in 2x SSC, detection of hybridization was performed by incubating the tissue in alkaline phosphatase-conjugated anti-digoxigenin antibody followed by an overnight incubation
in the color substrate 5-bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT).

### 3.2.4 – Color segmentation and image analysis

The area and intensity of positive staining were quantified using the *ImagePro Plus*® software. Briefly, an image of the tissue was digitally captured and an “area of interest” (AOI) was traced (Figure 11a). The AOI separated areas in the structure for assessment of immunoreactivity intensity. Adjacent sections stained with hematoxylin & eosin (H&E) and Movat’s pentachrome were used to determine the location of the internal and external elastic laminae, which were used to define the intima, media and adventitia within the vessel segment. A segmentation file was then created using positive and negative control slides and stains to define hue, saturation and intensity ranges considered positive immunoreactivity (Figure 11b). The segmentation file was then applied to the image, allowing the software program to report the percentage area of the staining intensities within the AOI (Figure 11c). The numerical data was exported to *Microsoft Excel*® for statistical analysis and further confirmed using *SPSS* statistical software (Figure 11d).
3.2.5 – Statistical analysis

Analysis of variance (ANOVA) was first performed between all the groups for each structure of interest. Where a significant difference was found between the groups, Student’s t-test was performed to further determine the significance of particular differences between specific disease groups and the normal group, with p < 0.05 considered statistically significant.
3.3 – Results

3.3.1 – Aberrant VEGF-A expression in human cardiac allograft vasculopathy

Staining in control cases (PDAY) was diffuse and had minimal immunoreactivity (Figure 12a), while the positive controls of these antibodies differentially stain the decidual trophoblasts of the placenta [inset]. Computer-assisted image analysis demonstrated a significant increase in VEGF immunoreactivity in the intima and media of coronary arteries from cardiac allografts when compared to healthy arteries (Figure 12b). The average percentage VEGF-A positive area in the intima of CAV vessels was significantly increased as compared to age- and sex-matched controls from the PDAY study (12.18±5.84% vs 3.98±0.69%, p = 0.0036). As well, the average percentage VEGF-A positive area in the media of CAV was also significantly increased compared with controls (27.13±13.78% vs 5.21±1.13%).

ISH in adjacent sections demonstrated increased VEGF transcript within cells of the superficial intima and in smooth muscle cells in the intimo-medial layer, as well as within the media of diseased vessels (Figure 12c).
Figure 12 – Immunohistochemical profiling of VEGF-A in human CAV.
(a) Healthy arteries had weak, diffuse immunoreactivity for VEGF, while the positive controls of these antibodies differentially stain the decidual trophoblast cells of the placenta [inset]. The representative micrograph of a coronary artery from a cardiac allograft clearly illustrates VEGF immunoreactivity (red), largely localized to the superficial intima, the intimo-medial region, and strikingly within smooth muscle cells in the media. (b) Color segmentation analysis of the IHC reaction product was performed using ImagePro Plus® imaging software as described in the Materials and Methods. Computer-assisted image analysis demonstrated a significant increase in VEGF immunoreactivity in the intima and media of coronary arteries from cardiac allografts when compared to healthy arteries. Data are represented as mean ± SE; *, p < 0.01. (c) In situ hybridization in adjacent sections demonstrated increased VEGF transcript within cells of the superficial intima and in smooth muscle cells in the intimo-medial layer, as well as within the media of diseased vessels.
3.3.2 – Aberrant VEGF-A expression in native atherosclerosis and diabetes mellitus

Staining was moderate in NA cases and further increased in DM (Figure 13). The percentage of VEGF-A positive area in the intima of coronary arteries is significantly higher in DM as compared to normal controls (9.75±4.61% vs 3.98±0.69%, p = 0.0085) and also significantly increased in NA (6.54±2.61% vs 3.98±0.69%, p = 0.0347). There was also a significant increase in VEGF immunoreactivity in the media of coronary arteries from both NA and DM cases compared with controls (25.23±6.16% and 32.83±8.90%, respectively, vs 5.21±1.13%).
Figure 13 – Immunohistochemical profiling of VEGF-A in native atherosclerosis (NA) and diabetes mellitus with atherosclerosis (DM).

VEGF protein localization was characterized using immunohistochemical staining. (a) Healthy arteries had weak, diffuse immunoreactivity for VEGF. The representative micrograph of a coronary artery from a patient with native atherosclerosis illustrates VEGF immunoreactivity primarily localized to the media, as well as the superficial and deep intima. Comparatively, the representative micrograph of a coronary artery from a patient with diabetes mellitus as well as atherosclerosis demonstrates a largely similar localization pattern to that of native atherosclerosis; however, with an apparent increase in immunoreactivity or staining intensity. (b) Color segmentation analysis of the IHC reaction product was performed using ImagePro Plus® imaging software as described in the Materials and Methods. Computer-assisted image analysis demonstrated a significant increase in VEGF immunoreactivity in the intima and media of coronary arteries in both native atherosclerosis and diabetes mellitus with native atherosclerosis as compared to healthy arteries. Data are represented as mean ± SE; *, p < 0.01.
3.3.3 – VEGF-D in cardiac allograft vasculopathy

VEGF-D immunoreactivity was also significantly increased in coronary arteries from patients with CAV when compared with controls (Figure 14). Pseudocolored images generated by ImagePro Plus® imaging software help to visually highlight the lower intensity immunoreactivity detected. There was no significant difference in VEGF-D immunoreactivity in the intima of CAV vessels (9% vs 4%). The percentage VEGF-D positive area was, however, significantly increased in the media of CAV vessels as compared to controls from the PDAY study (15% vs 1%).

3.3.4 – VEGF-D in native atherosclerosis and diabetes mellitus

The percentage of VEGF-D positive area in the intima of coronary arteries is significantly higher in DM compared with controls (Figure 15). Interestingly, there was an observed significant decrease in VEGF-D immunoreactivity in the intima of coronaries from patients with NA. Within the medial layer of coronary arteries measured, there was a significant increase in VEGF-D immunoreactivity only in cases of DM.

VEGF-D was significantly increased in the intima of DM vessels (19% vs 4%). Interestingly, in NA vessels, the percentage VEGF-D positive area was significantly decreased as compared to controls (<1% vs 4%). Within the media, VEGF-D was significantly increased in DM vessels compared with normal cases from the PDAY study (22% vs 1%). There was no observed significant difference in VEGF-D immunoreactivity between NA and control vessels (2% vs 1%).
Figure 14 – Immunohistochemical profiling of VEGF-D in human CAV.

(a) Healthy arteries had weak, diffuse immunoreactivity for VEGF-D as compared to CAV cases, which had abundant, albeit low intensity immunoreactivity for VEGF-D. Using pseudocolor overlay derived from color segmentation analysis of VEGF-D immunoreactivity intensity helps to highlight lower intensity immunoreactivity, as seen by the yellowish-green color over the black pseudocolored tissue. (b) Computer-assisted image analysis demonstrated a significant increase in VEGF-D immunoreactivity only in the media of coronary arteries from cardiac allografts when compared to healthy arteries. Data are represented as mean ± SE; *, p < 0.01.
Figure 15 – Immunohistochemical profiling of VEGF-D in human native atherosclerosis (NA) and diabetes mellitus with atherosclerosis (DM).

(a) Healthy arteries had weak, diffuse immunoreactivity for VEGF-D. In NA cases, there was no apparent difference as compared to normal controls; however, in DM cases there is an abundant increase in lower intensity immunoreactivity throughout the vessel wall. (b) Computer-assisted image analysis demonstrated a significant increase in VEGF-D immunoreactivity in the intima and media of coronary arteries from patients with DM, compared with normal controls. Interestingly, patients with NA actually had significantly less VEGF-D within the intimal layer of the vessel wall. Data are represented as mean ± SE; *, p < 0.01.
3.4 – Discussion

My immunohistochemical profiling and subsequent computer-assisted image analysis clearly illustrates the specific overexpression of VEGF-A within both the intima and media of coronary arteries from patients with NA, DM, and CAV (Figures 12 and 13). These observations are commensurate with studies examining VEGF-A polymorphisms which confer high or low VEGF-A protein expression. Although genotypes which conferred high VEGF-A expression did not impact the risk of repeated or late rejection in cardiac transplantation, when data was combined with high IL-6 (pro-inflammatory) and low IL-10 (regulatory cytokine) phenotypes, there was a significant increase in risk of late rejection irrespective of age and race/ethnicity.\textsuperscript{350} As well, phenotypes leading to decreased VEGF-A expression has been shown to have a protective effect in atherogenesis.\textsuperscript{351} Conversely, polymorphisms which confer high VEGF-A expression are significantly associated with improved graft survival in multiple transplantation models, as compared to low expression genotypes.\textsuperscript{352-356}

Complementary to the above observations, IHC on adjacent sections for smooth muscle-alpha (SM-α) actin and CD68, a human macrophage surface marker confirmed my histological observations of apparent immunoreactivity of SMC and macrophages within the deep intimo-media layer and SMC within the media of affected vessels. Revisiting my current concepts of the pathogenesis of CAV overviewed in Subsection 1.3, these results implicate infiltrating cells within the intima and SMC within the media as the primary producers of VEGF-A in atheromatous disease.
A role for VEGF-A in heart transplantation and rejection was initially sought in biopsies from transplanted human hearts and this report suggested VEGF-A may impact the cardiac microvasculature during myocardial damage.\textsuperscript{347} VEGF-A was observed in cardiomyocytes and the ECM, and rarely on endothelial cells and vascular SMC.\textsuperscript{347} A follow-up study by the same group also demonstrated the relationship between fibrin deposition and VEGF-A immunoreactivity in biopsies from cardiac allografts, suggesting that cardiomyocyte-derived VEGF-A production following microvascular fibrin deposition may act in a paracrine manner to promote changes in the microvasculature that provide a survival advantage for heart allografts.\textsuperscript{357}

As well, a relationship between increased VEGF-A expression in rejecting human cardiac allografts and the development of CAV has been suggested.\textsuperscript{358} Reinders \textit{et al} demonstrated the relationship between VEGF-A expression in human cardiac allografts and mononuclear cell infiltrates and acute rejection, and suggested that chronic overexpression of VEGF during the first year post-transplantation identifies patients likely to develop CAV.\textsuperscript{358}

My work complements these reports by demonstrating aberrant VEGF-A overexpression in the intima and media of coronary arteries from patients with CAV, as well as in NA and DM. Consolidating these other results with my own, I believe that early, high VEGF-A expression may reduce the risk of acute rejection episodes through pro-survival/proliferation signaling in endothelial cells and inducing progenitor cell differentiation to endothelial cells.\textsuperscript{359,360} Conversely, chronic, high VEGF-A expression in CAV is significantly associated with increased risk of repeated or late rejection, suggesting that this overexpression may lead to lipid and lipoprotein insudation, driving atherogenesis in CAV. Thus, promotion of early VEGF-A
expression with inhibition of late VEGF-A expression may provide the best therapeutic approach in the evolving allogeneic environment of transplantation as it progresses from one of mainly acute rejection to one plagued by CAV.

I also demonstrated significant, lower-intensity immunoreactivity for VEGF-D within the media of coronary arteries from patients with CAV and significant overexpression of VEGF-D within both the intima and media of coronaries from patients with DM with atherosclerosis (Figures 14 and 15). Interestingly, quantitative analysis of coronary arteries from patients with NA demonstrated a significant decrease in VEGF-D immunoreactivity within the intimal layer, and no significant difference when compared with controls within the medial layer of the vessel (Figure 15). This result correlates with previous reports from Rutanen et al, who demonstrated a reduction in VEGF-D staining in intima of complicated human atherosclerotic lesions. My immunohistochemical profiling and computer-assisted image analysis clearly illustrates the specific overexpression of VEGF-D in the media of coronary arteries in CAV and in both the intima and media of coronary arteries in DM. This result differs from our observations, and those of others, in NA, and this may be due to the relative stage within the time-course of atherogenesis between the diseases. Both DM and CAV are accelerated forms of atherosclerosis, and the overexpression we observed in these disease settings may reflect an earlier time-point in atherogenesis. This is the first characterization of aberrant overexpression of VEGF-D in CAV and diabetic atherosclerosis in humans.

In the literature, VEGF-D has been primarily been characterized as a member of the VEGF family which is able to induce lymphangiogenesis. The pathobiological implications of this finding require more specific experiments tailored to elucidate a role related to this function;
however, VEGF-D has also been reported in the literature to be able to induce vascular EC permeability, and as such, I chose to include the investigation of this isoforms alongside VEGF-A in their ability to induce endothelial hyperpermeability to LDL in Chapter 4.

Concurrent with my profiling of VEGF-A and VEGF-D in atheromatous disease, other relevant VEGF family members were investigated in the same cases using IHC, including VEGF-B, PI GF, VEGFR1 and VEGFR2. Semi-quantitative assessment of immunoreactivity for these proteins using a 0-4 scale did not demonstrate significant increases in graded immunoreactivity. As such, the subsequent in vitro verification studies detailed in Chapter 4 focuses on the investigation of the effects of VEGF-A and VEGF-D on endothelial permeability.

Taken together, it is likely that not only is VEGF-A aberrantly regulated in CAV, but also VEGF-D and possibly other VEGF family members and receptors. Synthesizing my work with the literature, it appears that multiple VEGF family members and receptors may be dysregulated in atherogenesis in the vessel wall in light of numerous pathogenic insults. In continuing this line of investigation, it is necessary to consider the regulation of the entire VEGF family and related receptors and co-receptors to determine the balance of physiological and pathological stimuli dictating vascular permeability, angiogenesis, lymphangiogenesis and ultimately atherogenesis.

The work from this chapter has clearly validated my original hypothesis by verifying that, indeed, VEGF is overexpressed in the coronary arteries of not only CAV vessels, but also those from NA and DM vessels. The implications of the key findings of this chapter are discussed in Figure 16.
Injury
eg. alloimmune, ischemia/reperfusion, hypoxia

Response to injury

Cytokines
eg. TNF-α, IL-1

↑VEGF-A

↑VEGF-A

↑VEGF-D

Cytokines
eg. IFN-γ

Migration
Proliferation

Figure 16 – Diagrammatic representation of the key findings from Chapter 3.

There are numerous injurious stimuli and factors which contribute to endothelial injury in transplantation, including alloimmune injury, mediated through Fas and granzyme B, ischemia/reperfusion injury, and hypoxia, among others. In response to injury, and other stimulatory factors such as cytokines induced by the alloimmune response against the graft, vascular endothelial growth factor (VEGF)-A and VEGF-D are expressed by smooth muscle cells in the media. I believe this response to injury occurs initially as a physiological response to repair the endothelium and maintain endothelial integrity; however, chronic overexpression of VEGF within the vessel wall results in endothelial hyperpermeability, to factors such as low-density lipoproteins (LDL), and also contributes to vascular remodeling.

The next chapter in this thesis examines one aspect of this response. I believe that VEGF overexpression within the vessel wall increases endothelial permeability to LDL, and this may, in part, contribute to increased LDL insudation within the vessel wall post-transplantation. Other factors such as increased proteoglycan expression and reorganization of the extracellular matrix may increase lipid and LDL retention within the vessel wall, resulting in continued stimuli for vascular remodeling and increased propensity of oxidative modification of LDL.
Chapter 4 – Vascular endothelial growth factor induces endothelial hyperpermeability to low-density lipoproteins \textit{in vitro}

4.1 – Rationale

Tight junctions form an impermeable seal between adjacent endothelial cells, and prevent the lateral migration of substances through the endothelium. They are formed by the phosphorylation-dependent assembly of a tight junctional complex, with the transmembrane occludin and claudin proteins associating with the cytoplasmic zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3, which attach the tight junctional complex to the cellular cytoskeleton.$^{104,169}$

Vascular endothelial growth factor (VEGF) was first discovered in tumor cells as a potent inducer of vascular permeability.$^{264,337}$ It is a potent and specific mitogen for vascular endothelial cells that is capable of stimulating angiogenesis and is one of the most potent inducers of vascular permeability known.$^{264-266,337}$ Previous reports have suggested that VEGF may enhance vascular permeability by affecting tight junction protein expression and assembly via various signaling pathways such as protein kinase B (PKB/Akt),$^{362,363}$ endothelial nitric oxide synthase (eNOS),$^{363-365}$ Src kinase$^{366-370}$ and protein kinase C (PKC),$^{304,338,371}$ among others. Our laboratory and others have characterized the aberrant expression of VEGF-A$^{311,372}$ and VEGF-D$^{361}$ in the coronary arteries of patients with native atherosclerosis, as well in human heart allografts with transplant-associated atherosclerosis.$^{347}$

The goal of my experiments was to determine whether VEGF-A$_{121}$, VEGF-A$_{165}$ or VEGF-D disrupts tight junctions in primary human cardiac microvessel endothelial cell cultures. I hypothesized that VEGF induced the disassembly of tight junctions between adjacent
endothelial cells, resulting in the formation of intercellular gaps. It is likely the presence of these intercellular gaps will result in a loss of endothelial integrity and create a hyperpermeable state, facilitating increased low-density lipoprotein (LDL) permeability through confluent endothelial monolayers.

4.2 – Materials and methods

4.2.1 – Reagents and antibodies

Endothelial basal medium (EBM) and endothelial growth medium - microvascular (EGM-MV) SingleQuots® (BBE 3mg/mL, 2mL; hEGF, 0.5mL; hydrocortisone, 0.5mL; FBS, 25mL; GA-1000, 0.5mL) were obtained from Clonetics (San Diego, CA). Cellagen® solution (0.5% type I collagen, pH 3.0) used to coat various cultureware was obtained from ICN Biomedicals (Costa Mesa, CA). BioCoat® Collagen I coated flasks and plates used to expand cell cultures, and BioCoat® Collagen I coated 8-well chamber slides used for immunocytochemical staining experiments were obtained from Becton Dickinson (Mississauga, ON). BD Falcon™ HTS FluoroBlok™ 1.0µm inserts and 1.0µm transparent cell culture inserts for 24-well plates used for LDL permeability assays were also purchased from Becton Dickinson. 1, 1’-dioctadecyl-3, 3, 3’, 3’-tetramethylindocarbocyanine perchlorate (diI)-labeled LDL and diI-labeled acetylated low-density lipoprotein (acLDL) used for permeability assays were obtained from Intracel Corp. (Frederick, MD). Recombinant human VEGF-A121, VEGF-A165 and VEGF-D protein was obtained from R&D Systems Inc. (Minneapolis, MN). Mouse anti-von Willebrand Factor and anti-smooth muscle-α actin antibodies were obtained from Dako Canada, Inc. (Burlington, ON). Mouse anti-platelet and endothelial cell adhesion molecule-1 (PECAM-1; CD31) and mouse anti-β-actin
antibodies were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Rabbit anti-ZO-1 antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA). Rabbit anti-phospho extracellular signal-regulated kinase (ERK) 1/2, p38, glycogen synthase-3 beta (GSK3β) and pan-protein kinase C (PKC) antibodies were obtained from New England Biolabs (Pickering, ON).

The mitogen-activated protein kinase kinase (MAPKK/MEK1) inhibitor 1, 4-diamino-2, 3-dicyano-1, 4-bis[2-aminophenylthio] butadiene (U0126) was obtained from Promega-Fisher (Nepean, ON). U0126 acts as a selective, non-competitive inhibitor of MEK1 and MEK2, two MAPK upstream of ERK1/2 which function in signal transduction pathways involved in cell proliferation and differentiation. MEK1 and MEK2 are activated by Raf-like molecules through the specific phosphorylation at serine residues 217 and 221, which are located in the activation loop of subdomain VIII.

4.2.2 – Cell cultures

Primary human coronary artery endothelial cells (HCAEC) and human cardiac microvessel endothelial cells (HCMEC) were obtained from Clonetics. HCAEC and HCMEC were used for in vitro investigations of VEGF-induced effects as they represent site- and tissue-specific endothelial cells relevant to the heart and CAV. HCAEC represent macrovascular endothelial cells that line the coronary arteries affected by CAV, whereas HCMEC represent cardiac-specific endothelial cells which populate the cardiac capillary network, and also may be representative of the vasa vasora, and possibly the microvasculature that supports the neovascularization of CAV and atherosclerotic lesions.
Cells were grown in EGM-MV at 37°C under 5% CO₂-95% air. Cells were passaged by trypsinization with 0.25% trypsin-EDTA and were seeded onto various type I collagen coated cultureware. All experiments used cells between the fourth and sixth passage, and cell phenotype was routinely monitored by phase contrast microscopy, as well as occasionally by positive immunocytochemical staining for von Willebrand Factor (vWF) and negative immunoreactivity for smooth muscle-α actin.

4.2.3 – Transendothelial electrical resistance experiments

The barrier properties of tight junctions can be directly measured by transendothelial electrical resistance (TER). Briefly, TER measurements are the gold standard for endothelial and epithelial barrier integrity, and TER values have shown to be indirectly correlated with the number of tight junction strands within a confluent monolayer. Endothelial cells used for the TER experiments were grown on Cellagen® discs (ICN Biomedicals). TER measurements were performed using a System EVOM voltohmmeter and Endohm-12 tissue resistance measurement chamber obtained from World Precision Instruments, Inc. (Sarasota, FL). Briefly, cells were grown to 100% confluence as verified using phase contrast microscopy and TER measurements. Once confluent, cells were serum starved for 24 hours before being treated with 0.01, 1 or 100pg/mL of VEGF-A₁₂₁, VEGF-A₁₆₅, or VEGF-D. TER measurements were recorded at 2, 4 and 20 hours post-treatment. Six independent experiments from three donors were performed, with four replicates per treatment group.
4.2.4 – Immunocytochemistry

HCMEC used for immunocytochemical staining were grown to confluence on type I collagen coated BioCoat® 8-well chamber slides, as verified by phase contrast microscopy. Cells were serum starved for 24 hours before treatment with 100pg/mL of VEGF-A_{121}, VEGF-A_{165} or VEGF-D. Cells were fixed with Clark’s solution (90% ethanol, 10% acetic acid) at 30, 60, and 120 minutes post-treatment. Briefly, cells were permeabilized using 0.1% Triton X-100 (Sigma), blocked using normal goat serum, then incubated with primary antibody overnight. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and StreptABComplex/AP (Dako) were incubated sequentially at room temperature. Antibodies were visualized using the chromagen Vector Red (Vector), followed by counterstaining with hematoxylin. Negative controls included isotype-matched IgG and omission of primary antibody.

4.2.5 – Immunofluorescent microscopy

HCMEC used for confocal microscopy were grown to confluence on type I collagen coated BioCoat® 8-well chamber slides. Cells were fixed with Clark’s solution (90% ethanol, 10% acetic acid) at 120 minutes post-treatment. Briefly, cells were permeabilized using 0.1% Triton X-100 (Sigma), blocked for 1 hour using normal goat serum, then incubated with rabbit anti-ZO-1 and mouse anti-PECAM-1 antibodies overnight. AlexaFluor® 594-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) were used to detect rabbit anti-ZO-1 antibodies. AlexaFluor® 488-conjugated goat anti-mouse IgG (Molecular Probes) was used to detect mouse anti-PECAM-1 antibody. Hoechst 33342 (Molecular Probes) was used to stain nuclei. Negative controls included isotype-matched IgG and omission of primary antibody. Slides were mounted
using Prolong® antifade reagent (Molecular Probes) and visualized using a Leica TCS SP2 AOBS confocal microscope (Leica Microsystems (Canada), Inc., Richmond Hill, ON).

4.2.6 – Cell lysates, electrophoresis, and Western blotting

Cells were washed twice in cold PBS then suspended in 1mL of cold lysis buffer (20mM Tris pH 8; 137mM NaCl; 10% glycerol; 1% Nonidet P-40; 1mM phenylmethylsulfonyl fluoride; 10μg/mL aprotinin) per 100-mm² culture area. After 15 minutes on ice, supernatant was collected, followed by centrifugation at 10,000g at 4°C. Cell lysate protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, IL). Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were transferred to nitrocellulose (Hybond ECL; Amersham Pharmacia Biotech, Uppsala, Sweden). Following blocking with 1% skim milk and incubation with primary and secondary antibodies, horseradish peroxidase conjugated secondary immunoglobulins were detected using the enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech) and exposed to Hyperfilm (Amersham Pharmacia Biotech). Densitometry results were obtained using NIH Image J software.

4.2.7 – Low-density lipoprotein permeability experiments

HCMEC used for the LDL permeability experiments were grown to confluence on Cellagen® coated HTS Fluoroblok® inserts, as determined by TER measurements using a System EVOM voltohmeter and Endohm-6 tissue resistance measurement chamber (World Precision Instruments). As well, cells were subcultured onto one transparent cell culture insert per 24-
well plate to allow for direct visualization using phase contrast microscopy and correlation to TER values. At confluence, cells were serum starved for 24 hours before treatment with 100pg/mL of VEGF-A121, VEGF-A165 or VEGF-D. Concomitant with treatment, 25μg/mL of diI-LDL or diI-acLDL were added to the insert. Cells were incubated at 37°C in the cell culture incubator and serial fluorometric measurements were taken at 5, 15, 30, 60, 120, 180, 240, 300 and 360 minutes post-treatment using a GENios Plus multi-label reader (Tecan Instruments, Inc., Durham, NC). Fluorometric measurements were normalized to an empty Fluoroblok® insert containing only medium and standardized to a standard curve of fluorescent substrate alone for each independent experiment.

4.2.8 – Statistical analysis

Analysis of variance (ANOVA) was performed on all treatment groups at each time point. Student’s t-test (p < 0.05) was used to make individual comparisons between untreated and VEGF-treated groups at each time point for TER measurements and fluorometric measurements to determine significance.
4.3 – Results

4.3.1 – Effect of VEGF in HCAEC and HCMEC on TER

To optimize culture and treatment conditions for my in vitro investigations on the effects of VEGF on endothelial permeability to LDL, numerous factors were tested, including variations in culture media and growth supplements, composition of basal matrix coating on cultureware, the effects of various VEGF isoforms at a range of concentrations and the duration of treatment.

It was determined that HCAEC and HCMEC grew most uniformly when cultured in EBM supplemented by EGM-MV Singlequot® supplements (which contain BBE, hydrocortisone and FBS). All cells were cultured on either BioCoat® Collagen I coated cultureware, or cultureware custom-coated with a type I collagen solution (Cellagen®). Endothelial phenotype was regularly verified by a combination of microscopic examination of cell morphology, TER measurements, vWF-positive immunocytochemical staining and smooth muscle-α actin-negative immunocytochemical staining. It was observed that cells maintained these endothelial phenotypes until at least 7th passage, and as such, all experiments were performed between passages 4-6. As well, serum-reduction using EBM supplemented with 0.5% FBS was used as an overnight pre-treatment to synchronize cells before treatment. This pre-treatment was chosen as it best-maintained TER, while allowing to synchronize cells in serum-reduced conditions before experimental treatment with VEGF, without changing cell number as verified by microscopic examination of cell morphology and cell number, and MTS assay.
4.3.1.1 – The effect of VEGF-A₁₆₅ on TER

To begin with, I wanted to confirm and extend the basis of the hypothesis from observations from Chapter 3, so, I examined the effects of VEGF-A₁₆₅ on TER over time in HCAEC and HCMEC. HCAEC primary cultures were performed in quadruplicate from cells originating from two independent donors. At 2 hours post-treatment, there was a significant decrease in TER in HCAEC treated with 0.01, 1 or 100pg/mL VEGF-A₁₆₅ compared with untreated controls (-26.73±2.82%, -35.30±3.97% and -41.55±3.45%, respectively). This significant decrease was also observed at 4 hours post-treatment in HCAEC treated with either 0.01, 1, or 100pg/mL VEGF-A₁₆₅ (-26.73±2.82%, -46.55±5.64% and -41.55±3.45%, respectively, compared with untreated controls) and this significant decrease persisted at 20 hours post-treatment with 0.01, 1 or 100pg/mL VEGF-A₁₆₅ (-35.30±3.97%, -54.29±2.54% and -46.55±5.64%, respectively) (Figure 17a).

HCMEC primary cultures were performed from cells originating from three independent donors. Treatment with 0.01, 1 or 100pg/mL VEGF-A₁₆₅ significantly reduced TER, compared with controls, at 2 hours (-39.48±4.83%, -46.48±3.34% and -45.56±6.48%, respectively) and at 4 hours (-39.90±3.56%, -49.07±2.74% and -50.00±2.58%, respectively). Of note, the untreated HCMEC controls at 20 hours post-treatment had significantly decreased resistance as compared to untreated HCMEC controls at 2 and 4 hours; however, despite this decrease, samples treated with either 0.01, 1 or 100pg/mL VEGF-A₁₆₅ were still significantly decreased compared with untreated controls at 20 hours post-treatment (-43.96±6.90%, -52.59±1.74% and -51.67±3.07%, respectively) (Figure 17b). Overall, all doses of VEGF-A₁₆₅ tested in both HCAEC and HCMEC significantly decreased TER at 2, 4 and 20 hours post-treatment.
Figure 17 – VEGF-A_{165} significantly decreases TER in HCAEC and HCMEC.

HCAEC and HCMEC were grown to confluence on Cellagen® inserts, and then treated with 0.01, 1, or 100pg/mL VEGF-A_{165}. (a) Treatment of HCAEC with VEGF-A_{165} significantly decreased TER at 2, 4 and 20 hours post-treatment. (b) Similarly, VEGF-A_{165} significantly decreased TER in HCMEC at 2, 4 and 20 hours post-treatment. Data are represented as mean±SE; *, p < 0.0005.
4.3.1.2 – The effect of VEGF-A\textsubscript{121} on TER

After observing the ability of VEGF-A\textsubscript{165} to decrease TER in HCAEC and HCMEC, I then examined the effects of VEGF-A\textsubscript{121} on TER over time in HCAEC and HCMEC. VEGF-A\textsubscript{121} differs from VEGF-A\textsubscript{165} in that it lacks the heparan sulphate binding regions found on VEGF-A\textsubscript{165} through alternative exon splicing. In HCAEC at 2 hours post-treatment, there was a significant decrease in TER only in HCAEC treated with 1pg/mL VEGF-A\textsubscript{121}, compared with untreated control (-26.88±10.87%; p < 0.05). In samples treated with either 0.01 or 100pg/mL VEGF-A\textsubscript{121} at 2 hours post-treatment, there was a trend towards decreased TER; however, this was not significantly different (-21.07±10.52%; p = 0.0920 and -29.58±12.37%; p = 0.0539, respectively). At 4 hours post-treatment, only samples treated with 100pg/mL VEGF-A\textsubscript{121} resulted in a significant decrease in TER (-29.58±12.37%; p < 0.05). At 20 hours post-treatment, 0.01, 1 and 100pg/mL VEGF-A\textsubscript{121} all significantly decreased TER in HCAEC (-32.98±6.31%, -31.04±7.80% and -28.75±5.06%, respectively; p < 0.005) (Figure 18a).

Treating HCMEC with doses of 1 or 100pg/mL VEGF-A\textsubscript{121} significantly decreased TER at 2 hours post-treatment (-31.98±7.05% and -31.77±4.11%, respectively; p < 0.001). At 4 hours post-treatment, 0.01, 1 and 100pg/mL VEGF-A\textsubscript{121} all significantly decreased TER (-12.96±2.75%, -15.60±3.53%, and -22.28±3.34%, respectively; p < 0.01). This significant decrease was only maintained in HCMEC treated with 100pg/mL VEGF-A\textsubscript{121} at 20 hours (-32.56±5.13%; p < 0.05) (Figure 18b).
Overall, VEGF-A121 significantly decreased TER at 20 hours post-treatment at all doses tested in HCAEC. As well, 1pg/mL VEGF-A121 at 2 hours and 100pg/mL at 4 hours also significantly decreased TER, compared with control. In HCMEC, VEGF-A121 significantly decreased TER at all doses at 4 hours post-treatment. Additionally, both 1 and 100pg/mL VEGF-A121 at 2 hours and 100pg/mL at 20 hours resulted in significant decreases in TER in HCMEC. The high degree of variability (larger standard deviation/error values) may account for the dissimilar results to VEGF-A165, and this may be due, at least in part, to the fact that VEGF-A121 is “more soluble,” lacking heparan sulphate binding regions.
Figure 18 – VEGF-A121 significantly decreases TER in HCAEC and HCMEC.

HCAEC and HCMEC were grown to confluence on Cellagen® inserts, and then treated with 0.01, 1, or 100pg/mL VEGF-A121. (a) Treatment of HCAEC with VEGF-A121 significantly decreased TER at 2 hours with a 1pg/mL dose, at 4 hours with a 100pg/mL dose, and at 20h at 0.01, 1, and 100pg/mL doses. (b) In HCMEC, VEGF-A121 significantly decreased TER in HCMEC at 2h at 1 and 100pg/mL doses, at 4 hours at 0.01, 1, and 100pg/mL doses, and at 20 hours only at the 100pg/mL dose. Data are represented as mean±SE; *, p < 0.05.
4.3.1.3 – The effect of VEGF-D on TER

Treatment of HCAEC using 0.01, 1, or 100pg/mL of VEGF-D significantly decreased TER at 2 hours post-treatment (-35.00±8.66%, -25.83±8.09% and -29.58±4.43%, respectively; p < 0.05). At 4 hours post-treatment, this significant decrease was maintained when treated with 0.01, 1, or 100pg/mL VEGF-D (-47.50±15.88%, -36.25±13.41% and -34.58±8.91%, respectively; p < 0.05). At 20 hours post-treatment, 0.01, 1 and 100pg/mL VEGF-D maintained significant decreases in TER in HCAEC (-41.25±13.29%, -30.00±7.58% and -35.83±6.29% respectively; p < 0.05) (Figure 19a).

When 0.01, 1 or 100pg/mL VEGF-D were used to treat HCMEC cultures, there was a significant decrease in TER at 2 hours (-21.94±2.60%, -28.97±6.25% and -38.26±7.00%, respectively; p < 0.001). At 4 hours, treatment of HCMEC with 0.01, 1 or 100pg/mL all significantly decreased TER (-26.34±5.47%, -28.04±9.24% and -34.46±6.08%, respectively; p < 0.005). At 20 hours post-treatment, only the 100pg/mL dose VEGF-D significantly decreased TER in HCMEC (-40.77±7.40%; p < 0.05) (Figure 19b).

Overall, in HCAEC, all doses of VEGF-D tested significantly decreased TER at 2, 4 and 20h. In HCMEC, all doses significantly decreased TER at 2 and 4 hours post-treatment; however, only 100pg/mL VEGF-D significantly decreased TER at 20 hours post-treatment.
Figure 19 – VEGF-D significantly decreases TER in HCAEC and HCMEC.
HCAEC and HCMEC were grown to confluence on Cellagen® inserts, and then treated with 0.01, 1, or 100pg/mL VEGF-D. (a) Treatment of HCAEC with VEGF-D significantly decreased TER at 2, 4 and 20 hours with 0.01, 1, or 100pg/mL VEGF-D. (b) In HCMEC, VEGF-D significantly decreased TER in HCMEC at 2 and 4 hours with 0.01, 1, and 100pg/mL VEGF-D. At 20 hours, only 100pg/mL VEGF-D significantly decreased TER in HCMEC. Data are represented as mean±SE; *, p < 0.05.
4.3.2 – VEGF increases LDL permeability through confluent HCMEC monolayers in vitro

Based on my experiments from 4.3.1, I decided to perform subsequent experiments using the 100pg/mL dose for VEGF-A_{121}, VEGF-A_{165} and VEGF-D, as it provided consistent TER decreases in all VEGFs tested. It is important to note that this 100pg/mL dose is much lower than the standard 10-100ng/mL doses routinely used in the literature to investigate VEGF-induced effects. As peak TER decreases were observed at 2 hours post-treatment, the following time points were chosen for investigation: 5, 15, 30, 60, 120, 180, 240, 300 and 360 minutes. The lipophilic fluorescent dye 1, 1'-dioctadecyl-3, 3, 3’, 3’-tetramethylindocarbocyanine perchlorate (diI) conjugated to either LDL or acLDL was co-incubated with VEGF on the luminal side of HCMEC grown to 100% confluence on Fluoroblok® transwell inserts. Passage of LDL and acLDL was measured using a fluorometer over the time-course of the experiment.

After stimulation with VEGF-A_{165}, I observed significant increases in LDL permeability as early as 5 minutes post-treatment (30% increase as compared with control). This increased permeability peaked at 60 minutes post-treatment at around 40%, as compared with control) and remained significantly increased up to 6 hours post-treatment. When confluent HCMEC grown on Fluoroblok® inserts were co-incubated with VEGF-A_{121} and LDL, significant increases in permeability were measured beginning at 60 minutes post-treatment (25%, as compared with control) and was sustained to 6 hours post-treatment. Treatment with VEGF-D significantly increased LDL permeability, peaking at 60 minutes post-treatment (48%, as compared to control) (Figure 20a).
Figure 20 – VEGF significantly increases LDL permeability through HCEMC monolayers.
(a) All treatments significantly increased LDL permeability through endothelial monolayers as early as 5 minutes post-treatment. VEGF-A121 showed modest (10-20%) increases in LDL passage, whereas, VEGF-A165 and VEGF-D showed greater increases, which peaked at 60 minutes post-treatment (as compared with control). These increases in LDL were sustained for up to 6 hours. (b) Interestingly, only VEGF-A165 and VEGF-D significantly increased acLDL permeability (~40% and 20%, respectively). VEGF-A121 actually induced significant decreases in acLDL permeability, as compared with control, at some time points. Experiments were performed in triplicate, with four replicates per treatment group per independent experiment. Data are represented as mean±SE; *, p < 0.05.
When cells were treated with 100pg/mL VEGF-A<sub>165</sub> and co-incubated with dil-acLDL, peak increases in acLDL permeability were observed at 15 minutes post-treatment (52%, as compared to control) and was maintained to 6 hours post-treatment. When VEGF-A<sub>121</sub> was co-incubated with acLDL, permeability was actually decreased (as compared with control (approximately 15% decrease through the 6 hour time-course of the experiment). When co-incubated with acLDL, VEGF-D significantly increased permeability, peaking at 15 minutes (23%), and maintained to 6 hours post-treatment (Figure 20b).

It is important to note that Figure 20 represents experimental data as a percentage of control through the time-course of the experiment. When calculating total protein permeability (in μg) based on relative fluorescence readings from a standard curve of LDL or acLDL substrate only, total quantity of LDL permeability is clearly greater than acLDL permeability for untreated samples and also those treated with VEGF-A<sub>121</sub>, VEGF-A<sub>165</sub> or VEGF-D (Figure 21a).

When re-plotting the sum of LDL and acLDL protein permeability as a percentage of control, this derivation more clearly illustrates the early VEGF-A<sub>165</sub>-induced increase in permeability at 5 minutes. At 60 minutes post-treatment, a common peak of increased permeability induced by VEGF-A<sub>121</sub>, VEGF-A<sub>165</sub> and VEGF-D, to varying degrees, was also evident (Figure 21b). Of note, and consistent with previous derivations, it is apparent that VEGF-A<sub>165</sub> and VEGF-D induced the greater changes in lipoprotein permeability measured.
Figure 21 – LDL permeability is greater than acLDL permeability.
(a) Plot of quantity of lipoprotein permeability (μg) measured, as derived from standard curve of substrate only. (b) Plot of LDL + acLDL totals as a percentage of control. Data are represented as mean±SE.
4.3.3 – VEGF-induced alterations to endothelial tight junctions

After demonstrating the ability of VEGF to significantly increase LDL permeability in endothelial cells, I performed immunocytochemistry for junctional proteins to observe changes in endothelial cell morphology and tight junctional integrity and protein localization.

4.3.3.1 – Treatment with VEGF results in the formation of intercellular gaps

To determine whether the disassembly of tight junctions in confluent endothelial monolayers would result in the formation of intercellular gaps, as we have observed in vivo, we used immunocytochemistry for platelet and endothelial cell adhesion molecule-1 (PECAM-1/CD31) to highlight the membranes of adjacent endothelial cells. In untreated cells, CD31 immunoreactivity highlighted adjacent endothelial borders, with no apparent disruptions in endothelial cell contact (Figure 22a,e). Two hours after treatment with 100pg/mL of VEGF-A$_{165}$ (Figure 22c,g) or VEGF-D (Figure 22d,h), I observed the appearance of interendothelial cell gaps; whereas the presence of these gaps was less apparent in VEGF-A$_{121}$-treated monolayers (Figure 22b,f). Isotype matched IgG and primary antibody omission negative controls showed no non-specific immunoreactivity (Figure 22 insets).
Figure 22 – VEGF induces intercellular gap formation. HCMEC (a-d) and HCAEC (e-h) were stained using immunocytochemistry for CD31. In untreated monolayers, CD31 was localized to cell membranes of adjacent untreated ECs (a,e). An increase in the number of intercellular gaps (arrows), as well as an decrease in the continuity and increase in immunoreactivity was shown as a result of VEGF-A_{121} (b,f), VEGF-A_{165} (c,g), and VEGF-D (d,h) at 2 hours post-treatment. Isotype-matched mouse IgG was used as a negative control [insets]. Pictures taken at 400x.

4.3.3.2 – VEGF increases cytoplasmic immunoreactivity of ZO-1

To further examine the effect of VEGF on the structure of the tight junctions, I performed immunocytochemistry for the tight junction protein ZO-1. I observed diffuse ZO-1 immunoreactivity in the cytosol as well as along adjacent cell membranes (Figure 23a,e). As early as 30 minutes post-treatment with VEGF-A_{121} (Figure 23b,f), VEGF-A_{165} (Figure 23c,g) and VEGF-D (Figure 23d,h). I observed an increase in both cytoplasmic and membrane ZO-1 immunoreactivity, with maximal increases observed at 2 hours post-treatment. Isotype-matched IgG and primary antibody omission negative controls had no immunoreactivity (Figure...
This result suggests that the formation of intercellular gaps in confluent monolayers may be due to tight junction disruption.

Figure 23 – VEGF induces changes in ZO-1 immunoreactivity.
HCMEC (a-d) and HCAEC (e-h) were stained using immunocytochemistry for ZO-1. ZO-1 was localized to cell membranes, with some diffuse cytoplasmic staining in untreated ECs (a,e). An increase cytoplasmic immunoreactivity as well as decrease in the continuity of ZO-1 immunoreactivity along the cellular membrane was seen as a result of VEGF-A$_{121}$ (b,f), VEGF-A$_{165}$ (c,g), and VEGF-D (d,h) at 2 hours post-treatment. Isotype-matched rabbit IgG was used as a negative control [insets]. Pictures taken at 400x.

4.3.3.3 – Non-conventional localization of occludin in HCAEC and HCMEC

Occludin has previously been characterized to be a transmembrane protein which associates with ZO-1 in the tight junctions of human epithelial cells and human umbilical vein endothelial cells.$^{163,169,179,379,380}$ My profiling for occludin expression using immunocytochemistry revealed perinuclear occludin immunoreactivity in HCAEC and HCMEC. I
performed Western blot analysis and confirmed that the antibody recognized both α and β forms of occludin, at 62kDa and 60kDa, respectively (data not shown). Interestingly, VEGF-A_{121}, VEGF-A_{165} and VEGF-D all increased occludin protein levels in comparison to untreated cells, which correlated with an apparent increase in occludin immunoreactivity. However, occludin localization was not altered by VEGF stimulation (Figure 24).

Figure 24 – Non-conventional localization of occludin in HCAEC and HCMEC. HCMEC (a-d) and HCAEC (e-h) were stained using immunocytochemistry for occludin. Occludin was unconventionally localized exclusively to the cytosol in untreated ECs (a,e). A slight increase in perinuclear, cytoplasmic immunoreactivity was observed in samples treated with VEGF-A_{121} (b,f), VEGF-A_{165} (c,g), and VEGF-D (d,h); however, occludin localization was not altered by VEGF treatment. Isotype-matched rabbit IgG was used as a negative control [insets]. Pictures taken at 400x.
Immunocytochemical staining for occludin revealed an atypical localization in untreated, confluent HCAEC and HCMEC monolayers. Occludin immunoreactivity was exclusively perinuclear, with increased intensity of immunoreactivity upon treatment with VEGF-A_{121}, VEGF-A_{165} or VEGF-D; however, the localization of occludin immunoreactivity did not change upon treatment. To verify the atypical localization of occludin in my endothelial cell cultures, I performed double-immunofluorescence staining for PECAM-1 and occludin (Figure 25a-d), as well as staining for F-actin and occludin (Figure 25e), simultaneously. Congruent with my immunocytochemistry using colorimetric substrate and brightfield microscopy, dual immunofluorescent labeling and visualization with confocal microscopy confirmed the lack of membrane localization of occludin immunoreactivity in cultured endothelial cells.
Figure 25 – Occludin does not localize to cultured endothelial cell membranes.
Dual immunofluorescent labeling and visualization with confocal microscopy demonstrate the exclusively cytoplasmic localization of occludin. In the top panel, cells were stained for nuclei (a; blue), CD31 (b; green) and occludin (c; red). (d) Color overlay of the three color channels demonstrates the lack of co-localization between the membrane-localized CD31 and occludin. (e) In the bottom panel, cells were stained for nuclei (blue), F-actin (green) and occludin (red). Orthogonal reconstruction from confocal stacks helps to illustrate the XZ and YZ planes, clearly demonstrating the lack of membrane localization of occludin.
4.3.4 – Profiling of the VEGF-induced signaling pathways in endothelial cells.

Previous reports have suggested that VEGF may enhance vascular permeability by affecting tight junction protein expression and assembly via various signaling pathways such as PKB/Akt, eNOS, Src, and PKC, among others. I sought to profile for signal transduction molecule expression and phosphorylation after VEGF-treatment. Initial profiling for a variety of signal transduction pathways, including ERK1/2, p38, GSK3β and PKC revealed that only the ERK1/2 pathway was markedly activated by VEGF-A_{121}, VEGF-A_{165} and VEGF-D in my model system at the time points investigated (Figure 26a,b).

As the apparent disruption of endothelial tight junctions was observed as early as 30 minutes post-treatment with VEGF, I sought to determine the relevant signal transduction pathways that may be mediating this effect. Initially, I treated endothelial cells with 100pg/mL VEGF-A_{165} and profiled signal transduction pathway activation at 0, 5, 10, 15 and 30 minutes post-treatment. VEGF-A_{165} treatment resulted in the phosphorylation of ERK1/2, peaking at 10 minutes post-treatment (Figure 27a).

After establishing the 10 minute time-point as the peak ERK1/2 phosphorylation post-treatment with VEGF-A_{165}, I examined ERK1/2 activation in response to VEGF-A_{121}, VEGF-A_{165} and VEGF-D. VEGF-A_{165} and VEGF-D resulted in an approximately 2-fold increase in ERK1/2 phosphorylation; VEGF-A_{121} also increased ERK1/2 phosphorylation, albeit to a lesser extent (Figure 27b).
Figure 26 – Profiling of signal transduction molecules activated by VEGF.
(a) Western blot analysis was performed for phosphorylation of ERK1/2, p38 and GSK3β in endothelial cells treated with VEGF-A121, VEGF-A165 and VEGF-D at 30 minutes post-treatment. Only ERK1/2 appeared to be activated by treatment with VEGF. TNF-α was used as a positive control for signal transduction pathway activation. (b) As well, a pan PKC phosphorylation marker was used to profile VEGF-induced activation at 15 and 60 minutes, and no increase was observed over baseline.
Figure 27 – Treatment with VEGF induces ERK1/2 phosphorylation in endothelial cells.

(a) Western blot analysis was performed for endothelial cells treated with VEGF-A165 at 0, 5, 10, 15 and 30 minutes post-treatment. Peak ERK1/2 phosphorylation was seen at 10 minutes. (b) Western blot analysis was then performed on lysates from endothelial cells treated with VEGF-A121, VEGF-A165 and VEGF-D for 10 minutes. All treatments with VEGF resulted in ERK1/2 activation at 10 minutes post-treatment, although VEGF-A121 induction of ERK1/2 phosphorylation occurred to a lesser extent. Blots from this figure are representative of three independent experiments.
To determine whether the ERK1/2 activation observed in Figure 2 was responsible for changes in tight junction organization and the formation of intercellular gaps, I used the MEK1 inhibitor U0126 and performed immunocytochemistry for ZO-1. Treatment of endothelial cells with either DMSO or U0126 compound alone without the addition of VEGF had no apparent effect on ZO-1 immunoreactivity or localization (Figure 28a,e). The addition of U0126 appeared to preserve tight junctional integrity 30 minutes after treatment with 100pg/mL of VEGF-A_{121}, VEGF-A_{165} or VEGF-D. At this 30 minute time point, the presence of intercellular gaps was less noticeable compared to the 2 hour time point from Figure 22. Of note, this earlier time point illustrates the early disruption of endothelial tight junctions induced by VEGF, as evidenced by the ZO-1 immunoreactivity highlighting the increased irregularity along adjacent endothelial cell membranes (Figure 28b-d). The disruption of tight junction regularity appeared to be restored in samples treated with VEGF and co-incubated with U0126 (Figure 28f-h).
Figure 28 – Inhibition of ERK1/2 prevents VEGF-induced intercellular gap formation and changes in ZO-1 immunoreactivity.

Endothelial cells treated with DMSO (control; a-d) or the MEK1 inhibitor U0126 (e-h) were stained using immunocytochemistry for ZO-1. As before, ZO-1 was localized to cell membranes in cells treated with DMSO or U0126 alone (a,e). Cells treated with DMSO as well as either VEGF-A_{121} (b), VEGF-A_{165} (c) or VEGF-D (d) resulted in the formation of intercellular gaps (blue arrows), as well as an increased irregularity of ZO-1 immunoreactivity along adjacent endothelial cell membranes (white triangles). When cells treated with U0126 as well as either VEGF-A_{121} (f), VEGF-A_{165} (g) or VEGF-D (h), there were no observable intercellular gaps, and the regularity of ZO-1 immunoreactivity along endothelial cell membranes was restored.
4.4 – Discussion

Taken together, the results from this aim demonstrate the ability of VEGF to disrupt HCAEC and HCMEC tight junctions, as determined by TER measurements and immunocytochemical staining for tight junctional proteins. This tight junctional disruption resulted in the formation of intercellular gaps, which is correlated to the increase in LDL permeability through endothelial monolayers. However, there is also a possible contribution of endocytotic transport induced by VEGF treatment.

Previous work has suggested a role for VEGF in the induction of endothelial cell hyperpermeability. These reports have been primarily completed in human umbilical vein endothelial cells or bovine aortic endothelial cells and make no distinction between the different splice variants of VEGF-A or whether other VEGF family members may have similar effects. My initial profiling attempts in native and transplant-associated atherosclerotic coronary arteries reveal aberrant VEGF expression within the intima and media of diseased vessels. As VEGF was first described as a permeability-inducing agent in tumor cells, I sought to determine whether aberrant VEGF expression in atherosclerotic arteries resulted in a hyperpermeable endothelium.

I first hypothesized that VEGF-A$_{121}$, VEGF-A$_{165}$ and VEGF-D disrupt tight junctions between HCAEC and HCMEC. I sought to determine the relative efficacy of VEGF-A$_{121}$, VEGF-A$_{165}$ and VEGF-D in human coronary-derived endothelial cells. TER measurements were used as an indirect measure of the number of tight junction strands between adjacent endothelial cells in a confluent monolayer, which is an important factor in determining the barrier properties of tight junctions. I have demonstrated that VEGF-A$_{121}$, VEGF-A$_{165}$ and VEGF-D can all induce significant
decreases in TER (Figures 17-19). Comparing the TER changes upon VEGF-A_{121}, VEGF-A_{165} or VEGF-D treatment, VEGF-A_{165} caused the greatest reduction of TER at all doses tested. As well, the effects of VEGF-A_{165} were sustained up to 20 hours post-treatment, and extended time points from these experiments demonstrate VEGF-A_{165}-induced TER changes remain significantly decreased as long as 72 hours post-treatment (data not shown). VEGF-A_{121} and VEGF-A_{165} have been reported to interact with VEGFR1 and VEGFR2. Comparing the TER reducing effects of VEGF-A_{165} to VEGF-A_{121}, VEGF-A_{165} appears more potent across all doses and time points, especially in HCAEC monolayers. This may be due to the difference in the splice variants, as VEGF-A_{121} lacks a heparan sulphate binding region, rendering it “soluble”, while its presence may allow for retention of VEGF-A_{165} protein on heparan sulphate proteoglycans present on the endothelial glycocalyx or extracellular matrix produced in culture.\textsuperscript{381,382}

Treatment of endothelial monolayers with VEGF-D resulted in decreases in TER of similar magnitude to VEGF-A_{165}. VEGF-D has been reported to interact with VEGFR2 and VEGFR3.\textsuperscript{383} It has previously been reported that VEGFR2 is responsible for VEGF-induced permeability effects and it seems that the permeability-inducing actions of VEGF-A_{121}, VEGF-A_{165} and VEGF-D in this model are acting primarily through this receptor, as it is the common receptor between the different VEGF family members tested.

The hyperpermeability-inducing effects of VEGF on endothelial monolayers has been well described in the literature using tracers such as horseradish peroxidase,\textsuperscript{384,385} FITC-dextran,\textsuperscript{362,386-390} and other small molecular weight compounds.\textsuperscript{385,391,392} It has been suggested that these tracers cross the endothelium through intercellular gaps or possibly through transcytosis or vesiculo-vacuolar organelles (VVO),\textsuperscript{147,345,393,394} however, the effect of VEGF on
LDL permeability through endothelial monolayers has not been examined. I demonstrated that VEGF-A_{121}, VEGF-A_{165} and VEGF-D are all able to significantly increase dil-conjugated LDL permeability through confluent endothelial monolayers (Figure 20a). This effect was observed as early as 5 minutes post-treatment, peaked around one hour post-treatment, and was sustained for up to 6 hours post-treatment. Correlated with my observations of decreased TER and the formation of intercellular gaps, it may be likely that VEGF-induced LDL permeability may be through an intercellular route.

When the lysine residues of the LDL apoprotein have been acetylated, the LDL complex no longer binds to the LDL receptor, but instead, is taken up by scavenger receptors specific for modified LDL. I used dil-conjugated acLDL to determine whether the VEGF-induced LDL hyperpermeability observed may be mediated through specific LDL receptor interactions, or whether it may simply be due to the size of the large intercellular gaps formed between endothelial cells. Surprisingly, when examining dil-conjugated acLDL permeability through endothelial monolayers, only VEGF-A_{165} and VEGF-D significantly increased acLDL permeability. The pattern of response to treatment did not appear to be time-dependent, with a consistent increase of approximately 40 and 20%, respectively, for VEGF-A_{165} and VEGF-D (as compared to control) (Figure 20b). VEGF-A_{121} may not have induced significant increases in acLDL permeability because once the acLDL complexes accumulate within the cells; dil-acLDL conjugates are covalently bound to the modified apoprotein portion of the LDL complex, and are not extracted during subsequent manipulations of the cells. As a result, the majority of dil-acLDL may be retained within endothelial cell, and thus, would not be detected through the opaque Fluoroblok® inserts by the fluorometer. The increase in acLDL permeability observed in
VEGF-A_{165} and VEGF-D treated cells may be a result from acLDL passage through the intercellular gaps formed through VEGF-induced transcytosis of acLDL. When comparing absolute amounts of lipoprotein passage through endothelial monolayers, there is significantly more LDL passage as compared to acLDL passage, suggesting that both pathways may be involved in VEGF-induced endothelial cell hyperpermeability to lipoproteins; however, possibly through different routes of passage.

After verifying that VEGF-A_{121}, VEGF-A_{165} and VEGF-D all significantly decrease TER in HCAEC and HCMC, I wanted to determine whether this decrease in TER and number of tight junction strands resulted in the formation of intercellular gaps whether the localization of the tight junctional proteins was affected by VEGF treatment. Using immunocytochemistry for PECAM-1, I visualized intact cell contacts between adjacent endothelial cells in confluent, untreated monolayers, and did not observe the presence of any intercellular gaps (Figure 22). However, upon stimulation with VEGF-A_{121}, VEGF-A_{165} or VEGF-D, I observed an increase in the number of intercellular gaps between adjacent endothelial cells. As well, in some cases, there was an apparent increase in PECAM-1 immunoreactivity, which may be attributed to increased epitope availability when gaps are formed in endothelial cell junctions. As evident in the micrographs, VEGF-treated monolayers displayed heterogeneity in cell shape and size, however, cell number between treatment groups within 2h was not significantly different (as determined by MTS assay and manual cell counting; data not shown). Increased vascular permeability has been linked to angiogenesis as early as 1935, where Clark and Clark demonstrated that dyes could leak out of growing capillaries. It is recognized that a cardinal feature of pathological angiogenesis is increased vascular permeability, and growing evidences
demonstrate that a regulated increase in vascular permeability to both solute and water can occur as capillaries grow and form new vessels,\(^{396,397}\) a phenomenon which may be happening during plaque neovascularization. It has been demonstrated that exposure to tumor necrosis factor-alpha (TNF-\(\alpha\)) led to PECAM-1 surface redistribution and disruption of cytoskeletal contacts, accompanied by increased permeability to macromolecules.\(^ {398}\) Indeed, my own observations of TNF-\(\alpha\)-induced changes to PECAM-1 in HCAEC and HCMEC mirrored those of previous reports (data not shown).\(^ {398}\)

Immunocytochemical staining for ZO-1 in untreated HCAEC and HCMEC monolayers demonstrated localization along adjacent endothelial membranes, with faint cytoplasmic immunoreactivity. Upon treatment with VEGF-A\(_{121}\), VEGF-A\(_{165}\) or VEGF-D, there was an increase in the cytoplasmic localization of ZO-1, suggesting dissociation of tight junctional complexes at the membrane. Interestingly, ZO-1 immunoreactivity in treated groups appeared strongest in the perinuclear region of the cytosol. To confirm the localization of ZO-1 and occludin immunoreactivity observed using immunocytochemistry in my HCAEC and HCMEC cultures, I performed double-immunofluorescent staining for PECAM-1 and ZO-1 and verified the membrane localization of ZO-1 (Figure 23).

Immunocytochemical staining for occludin revealed an atypical localization of occludin in untreated, confluent HCAEC and HCMEC monolayer. Occludin immunoreactivity was exclusively perinuclear, with increased intensity of immunoreactivity upon treatment with VEGF-A\(_{121}\), VEGF-A\(_{165}\) or VEGF-D; however, the localization of occludin immunoreactivity did not change upon treatment (Figure 24). These results are different from previous reports in the literature, which have demonstrated exclusive membrane localization of occludin in different
endothelial cultures such as HUVEC and BAEC. My results suggest that occludin may not be an important protein in the formation and regulation of tight junctional complexes in HCAEC and HCMEC in culture.

To verify the atypical localization of occludin in my HCAEC and HCMEC cultures, I performed double immunofluorescent staining for PECAM-1 and occludin, as well as staining for F-actin and occludin (Figure 25). These two approaches verified that occludin immunoreactivity was cytoplasmic and not localized along the membrane. This observed difference of occludin localization may be as a result of cell-specific expression and localization in HCAEC and HCMEC, as other reports utilized endothelial cells from other tissue sites or other species. Another possibility may be due to the specific culture conditions in my experimental investigations; however, the basal media and growth supplements used in these studies was certified and purchased from Cambrex Corporation and TER measurements indirectly demonstrated the formation of tight junction strands, despite the lack of membrane localization of occludin.

Many signaling molecules have been implicated in VEGF-induced permeability effects in endothelial cells, including PKB/Akt, eNOS, Src kinase, and PKC, among others. I demonstrated ERK1/2 activation 30 minutes post-treatment with 100pg/mL of VEGF-A121, VEGF-A165 and VEGF-D (Figure 27b). Inhibition using the MEK1 inhibitor U0126 abrogated VEGF-induced tight junctional disruption 30 minutes post-treatment (Figure 28). At 30 minutes post-stimulation with VEGF, I did not observe activation of p38, GSK3β (downstream of PKB) or PKC in my model system (Figure 26). The differences between my results and others may be as a result of the lower dosage used for my experiments, the time
points examined, or as a result of species- or tissue-specific differences in endothelial cells. These results suggest that in my model system, VEGF-induced disruption of endothelial tight junctions occurs through an ERK1/2-dependent pathway, and inhibition of ERK1/2 signaling using the MEK1 inhibitor U0126 can preserve tight junctional protein organization and thus tight junction and endothelial cell morphology.

In conclusion, these studies have demonstrated that VEGF-A\textsubscript{121}, VEGF-A\textsubscript{165} and VEGF-D can all induce significant decreases in TER, resulting in increased ZO-1 immunoreactivity within the cytosol, and the formation of intercellular gaps. As well, treatment with VEGF-A\textsubscript{121}, VEGF-A\textsubscript{165} or VEGF-D significantly increases LDL permeability through confluent endothelial monolayers. Interestingly, only VEGF-A\textsubscript{165} and VEGF-D significantly increased acLDL permeability through endothelial monolayers, suggesting that VEGF may be inducing transcytosis of internalized acLDL, although to a lesser extent than intercellular passage of LDL. Inhibition of ERK1/2 signaling using the MEK1 inhibitor U0126 prevented VEGF-induced disruption of endothelial tight junctions. These key concepts are represented in Figure 29.
Figure 29 – Diagrammatic representation of the key findings from Chapter 4.

(a) Vascular endothelial growth factor (VEGF) disrupts endothelial barrier function at the level of the tight junction. (b) This tight junctional disruption is ERK1/2-dependent, and results in the formation of intercellular gaps and relocation of zonula occludens-1 (ZO-1) protein from the tight junctions at the cell membrane to the cytoplasm. (c) Concurrently, this endothelial barrier disruption and intercellular gap formation results in endothelial hyperpermeability to low-density lipoproteins (LDL).

This chapter affirms my original hypothesis that VEGF can increase endothelial permeability to LDL and supports the notion that this may contribute to the prominent lipid and lipoprotein accumulation observed in CAV. The next chapter in this thesis investigates the role of VEGF in the pathogenesis of CAV using a proof-of-principle study focusing on the abrogation of VEGF in a hypercholesterolemic mouse model of heterotopic cardiac transplantation.
Chapter 5 – Administration of soluble vascular endothelial growth factor receptor-1 in a mouse model of heterotopic cardiac transplantation

5.1 – Rationale

Cardiac allograft vasculopathy (CAV) is an occlusive vascular disease which occurs in approximately 70% of transplant patients and is the leading cause of organ rejection/failure one year after solid organ transplantation. The concentric vascular atherogenesis characteristic of CAV occurs, and progresses rapidly, within almost all solid organ transplants. Although the etiology is poorly defined, it is widely accepted that the pathology is initiated by a combination of allogeneic and ischemia/reperfusion injury to the graft, which result in endothelial damage post-revascularization. Progression of CAV involves a multifactorial process, strongly related to increased adhesiveness and permeability of the endothelium of blood vessels. Plasma components including fibrinogen, lipids and apolipoproteins B, (a) and E enter and accumulate in vessel walls, causing further injury. This results in diffuse, concentric intimal thickening which progressively occludes blood flow.

CAV is the leading expression of chronic organ rejection and the major cause of graft failure beyond one year post-transplantation. This chronic vascular disease results in the partial or complete obstruction of blood vessels, particularly macrovessels in transplanted organs, resulting in tissue ischemia and organ failure. In physiological and pathological settings, the endothelium serves as a key structural and functional regulator of vascular health, guiding leukocyte traffic, modulating transport of micro- and macromolecules and ions, and regulating homeostatic smooth muscle function. My work, and that of our laboratory, has shown through a long-running series of investigations that pathogenesis of CAV in transplanted hearts involves
endothelial injury, smooth muscle perturbations, aberrant expression of inflammatory cytokines, immune-mediated cell death, accumulation of extracellular matrix, and insudation of lipids and lipoproteins within affected vessels. 

One intriguing observation from my work is the documentation of endogenous overexpression of vascular endothelial growth factor (VEGF) within coronary arteries from human heart allografts. Among all growth factors known to date, VEGF is the only one capable of inducing inflammation. VEGF increases vascular permeability, leukocyte adhesion and transmigration, and platelet aggregation via the synthesis of various paracellular signaling molecules such as platelet-activating factor and tissue factor which could potentially be deleterious to grafts. On the other hand VEGF induces endothelial cell proliferation, migration, and angiogenesis as well as bone marrow-derived cell mobilization and re-endothelialization. In light of these possible roles, coupled with my findings in Chapter 4, demonstrating the ability of VEGF to increase endothelial permeability to LDL in coronary artery and cardiac-specific endothelial cells, determining the role of VEGF in the pathogenesis of CAV is an interesting prospect.

Endothelial cell injury, which can arise through a variety of stimuli during transplantation such as mechanical damage, hypoxia, ischemia and reperfusion, contributes to the initiation and progression of CAV. Moreover, alloimmune injury to the graft endothelium also contributes to endothelial dysfunction. While current immunosuppressive regimens are typically effective in managing acute rejection, they have not diminished the prevalence of CAV accordingly. Once activated, the endothelial response can promote atherogenesis via numerous mechanisms, including platelet adhesion, release of growth factors
and donor antigens, major histocompatibility complex (MHC) class I and II expression, adhesion molecule expression and promotion of vascular smooth muscle cell proliferation. Endothelial damage can promote CAV development by increasing vascular permeability, and increasing intimal smooth muscle cell proliferation. Concurrently, some factors produced by the damaged endothelium initiate the physiological process of re-endothelialization, or repair of the endothelium, including via bone marrow-derived cell recruitment. It is thought that a specific subpopulation of bone marrow-derived cells may be endothelial progenitor cells, whose role is to specifically repair the endothelium.

With these concepts in mind, it is likely that the observed overexpression of VEGF in injured arteries within the allograft, produces both beneficial and adverse effects, and their respective contribution to the overall outcome is dependent on timing and related pathogenic factors. Since VEGF is expressed endogenously in CAV, it is therapeutically relevant to assess methods of inhibiting its pathological activity, while leaving its healing potential intact. It is my intent to investigate the biological or pathological functions of endogenous VEGF overexpression in CAV by abrogation of the VEGF axis using treatment with soluble VEGF receptor-1 (sVEGFR1; soluble fms-like tyrosine kinase-1, sFlt-1).

Soluble VEGFR1 contains the extracellular ligand-binding domain of the full-length, membrane-bound VEGFR1, and is generated physiologically by alternative splicing of the same pre-mRNA that encodes the VEGFR1 gene. Soluble VEGFR1 can bind VEGF, preventing its interaction with VEGFR2, and thus inhibiting its downstream signaling and action. It has been used in several contexts and has been shown that usage of it can inhibit intraplaque angiogenesis and suppress the development of atherosclerotic plaque. As well, Onoue et al
demonstrated that administration of sVEGFR1 reduced atherosclerotic plaque formation while significantly reducing infiltration of macrophages into aortic tissues. Thus, the investigation of the use of sVEGFR1 in the context of interfering with VEGF-mediated actions in the pathogenesis of CAV is an attractive prospect and the focus of this chapter.

5.2 – Materials and methods

5.2.1 – Animals

The C57Bl/6 (C57; Jackson Laboratories, Bar Harbor, ME) mouse was chosen as the recipient because it is the most common background for transgenic mice. The 129X1/SvJ (129J; Jackson Laboratories) mouse was chosen as the donor because its antigenic profile creates a minor histocompatibility antigen-mismatch between donor and recipient mice species. Mice received Western diet (TD.88137; Harlan Teklad, Madison, WI) with water ab libitum and were acclimatized for a minimum of one week before surgery. Hearts from 12 female 129J mice were used as donors into 12 C57 male recipients (6 transplants per group). The number of animals per treatment group was determined using power calculations based on our previous experience with the mouse model of heterotopic cardiac transplantation, the expected variability of our primary experimental endpoint, percentage luminal narrowing, and accounted for expected morbidity and mortality. All procedures were reviewed and approved by the University of British Columbia Animal Care Committee (Protocol #A08-0509).
5.2.2 – Heterotopic cardiac transplant model

Cardiac transplantation was performed as previously described.\textsuperscript{359,360,425,426} Hearts from female 129J donors were implanted into the abdomen of 5- to 7-week old male C57 mice (six transplants were performed for each group). Animals were anesthetized with 4% halothane and anesthesia maintained with 1-2% halothane (Halocarbon Laboratories, River Edge, NJ). Donor mice were infused with heparinized saline and their hearts excised. The recipient’s abdominal aorta and inferior vena cava were located and clamped. The donor’s aorta and pulmonary artery were anastomosed to the recipient’s abdominal aorta and inferior vena cava, respectively, in an end-to-side manner. Transplantation was performed within 30 to 40 minutes of removal of the donor heart. One dose of 0.01mg/kg buprenorphine (Buprenex Injectable; Reckitt and Colman Pharmaceuticals, Richmond, VA) was administered sub-cutaneously after surgery. Mice were given intraperitoneal injections of 6mg/kg/day FK506 (Prograf®, tacrolimus; Fujisawa Canada, Markham, ON) as the primary immunosuppressive agent in this study. FK506 was chosen as the primary immunosuppressive agent in this study after pilot investigation in the murine model of heterotopic cardiac transplantation revealed unexpected bleeding complications leading to significant mortality in apolipoprotein E-deficient mouse recipients treated with cyclosporine at multiple doses. Multiple doses of FK506, and oral and injectable forms of sirolimus were also tested, and the 6mg/kg/day dose of FK506 was found to completely prevent acute rejection while allowing the development of CAV within 30 days post-transplantation. Gross examination of all organs, including kidney, liver, lung, pancreas and spleen, as well as histological examination were used in all animals to ensure no non-specific drug toxicity.
5.2.3 – Administration of soluble VEGFR1 and monitoring of mice

Mice were monitored regularly by the animal care staff in the Genetically Engineered Models (GEM) facility in the UBC James Hogg Research Centre. In conjunction, at 10am daily, I weighed each mouse individually to determine the exact dosing for daily injections of FK506 and bi-daily injections of either 5µg/kg soluble VEGFR1 (R&D Systems, Minneapolis, MN) or vehicle (PBS). As well, during this time, observations of mouse health and well-being were noted and abdominal palpations were performed and recorded for assessment of heterotopic heart function according to Table 2.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Strong, regular heart beat</td>
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<tr>
<td>B</td>
<td>Regular heart beat</td>
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<tr>
<td>C</td>
<td>Weak and/or irregular heart beat</td>
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<tr>
<td>D</td>
<td>No heart beat detectable</td>
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Table 2 – Grading criteria for monitoring heterotopic heart beat function by abdominal palpation.

The following criteria were followed to determine premature sacrifice of animals in the experiment: a) no detectable heart beat (D grade) which persisted for greater than two consecutive days; b) >10% weight loss (excluding the first three days); c) bi-lateral hind limb paralysis persisting >3 days or if in conjunction with significant body weight changes; and/or d) steady decline in general signs of animal well-being, including grooming, socialization, nesting,
appearance of stool and urine, eating and drinking, among others, in accordance with GEM facility and UBC Animal Care Committee guidelines.

For mice that did not reach experimental endpoint, autopsy or necropsy was performed to assess gross organ pathology and all tissue were harvested according to regular specified parameters for this study for archiving, and in some cases, histopathological review by cardiovascular pathologist experienced in transplantation pathology.

5.2.4 – Tissue harvesting and histopathological examination

Mice were fasted at least 12 hours prior to euthanization for tissue and blood collection. At 21 days post-transplantation, mice were anesthetized by injection with ketamine/xylazine. After anaesthetization, the abdominal cavity was opened for visual verification of transplant heart function and gross examination. The native and transplanted hearts were perfused with sterile saline followed by 10% formalin. Subsequent to perfusion-fixation, hearts were rapidly removed, photographed, weighed and dissected, then immersion-fixed in 10% formalin overnight before tissue processing. Ventricular transverse sections were embedded in paraffin. As well, liver, lung, kidney, spleen and pancreas were harvested for tissue processing and archival.

Paraffin-embedded sections were cut serially (4µm) and stained with hematoxylin and eosin (H&E) and Movat’s pentachrome. Blinded assessment of vasculitis, rejection and CAV were performed in H&E section by trained cardiovascular pathologist (0-4+ scale). Luminal narrowing in all observed medium to large-size coronary arteries was also evaluated in Movat’s
pentachrome-stained sections. These histopathological assessments by cardiovascular pathologist guided my further morphometric evaluation of CAV.

5.2.5 – Blood chemistry

Mice were injected with 100µL Hepalean (heparin sodium solution; Eli Lily and Company, Indianapolis, IN) to prevent coagulation 10 minutes before sacrifice. Blood was aspirated by direct cardiac puncture of the native heart and immediately placed on ice. Subsequently, the whole blood was centrifuged at 10,000 rpm for 10 minutes at 4°C. Plasma was aspirated and stored at -80°C. High-density lipoprotein (HDL), LDL, and triglyceride levels were assayed using BioVision quantification kits (Mountain View, CA). ELISA was also performed to quantify mouse plasma VEGF-A levels (R&D Systems, Minneapolis, MN) as per manufacturer’s instructions.

5.2.6 – Morphometry

To evaluate CAV, all visible medium to large arteries from both native and donor hearts were photographed at 400x magnification using a Spot digital camera. Using ImagePro Plus® software, “areas of interest” (AOI) were created by tracing the endothelium, internal elastic lamina and external elastic lamina in digital micrographs of Movat’s pentachrome-stained sections. The area bounded by the endothelium was defined as the lumen. The area bounded by the internal elastic lamina and endothelium was defined as the intima. The area bounded by the external elastic lamina and internal elastic lamina was defined as the media. Luminal narrowing was the primary measure of CAV used in this study (Figure 30).
% luminal narrowing = \frac{\text{intima}}{\text{lumen + intima}}

<table>
<thead>
<tr>
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<td>12.26%</td>
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<table>
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<tr>
<th>Transplant</th>
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<td>85.56%</td>
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**Figure 30 – Determination of luminal narrowing in a mouse model of heterotopic cardiac transplantation.**

Digital micrographs were captured of all visible arteries and arterioles in the cross-section of native and transplant hearts. Using *ImagePro Plus*® software, “areas of interest” (AOI) are defined by tracing the endothelium, internal elastic lamina (IEL) and external elastic lamina (EEL). The areas bounded by these AOI define the lumen (L), intima (I) and media (M).
5.2.7 - *In vitro* aortic ring angiogenesis assay

Segments from 129J mouse aortas were collected and cultured in Matrigel (BD Biosciences, Mississauga, ON). Whole bone marrow was harvested from C57 mice by flushing femurs and tibias with cold PBS/2% fetal bovine serum (FBS; Hyclone, Logan, UT) solution. To mimic the allogeneic mismatch in our transplant model, co-incubation of the aortic segments with bone marrow was performed using the transwell inserts. Co-cultures were maintained in endothelial basal medium (EBM-2; Lonza, Guelph, ON) supplemented with EGM-MV SingleQuots®.

To analyze microvascular outgrowth from the aortic segment, *NIH ImageJ* imaging software was used to measure outgrowth length and area. Briefly, measurements of outgrowth length were obtained at 0°, 90° and 180° from the aortic ring segment. Measurements of outgrowth area were obtained by tracing the circumferential area of outgrowth to determine the total area and subtracting that from the area of the aortic ring segment alone.

5.2.8 – Statistical analysis

Analysis of variance (ANOVA) was first performed between all the groups for each dataset of interest. When a significant difference was found within the groups, Student’s *t*-test (*p* < 0.05) was performed to determine the significance between specific data points.
5.3 – Results

5.3.1 – Heterotopic cardiac transplantation and response to soluble VEGFR1

Through the course of this study, 6/6 vehicle-treated transplants and 4/6 sVEGFR1-treated transplants reached the predetermined experimental endpoint of 21 days. Of the two mice in the sVEGFR1 group which did not reach endpoint, one was sacrificed the morning after transplant due to post-surgical complications, while the other was sacrificed on day three post-transplant due to no detectable heartbeat. Alongside daily observations of animal health and well-being, transplant recipient body weight changes were used as a primary measure of recovery from the heterotopic transplantation surgical procedure and toxicity to administration of sVEGFR1. Over the time course of the experiment, there was no apparent significant difference between vehicle- and sVEGFR1-treated animals (Figure 31).

Figure 31 – Treatment with soluble VEGFR1 does not result in a change in mouse body weight. Intraperitoneal injections of soluble VEGFR1 (sVEGFR1) does not significantly affect mouse body weight after heterotopic implantation of 129J mouse donor hearts as compared to vehicle control injections (PBS).
5.3.2 – Treatment with soluble VEGFR1 does not affect lipid levels

Triglyceride levels trended to a decrease in sVEGFR1-treated animals when compared to vehicle-treated animals (11.74±1.81mg/dL vs 36.97±11.37mg/dL), although this difference was not statistically significant (p = 0.15) (Figure 32).

There were no significant differences in HDL serum concentration (201.34±3.02mg/dL vs 197.41±3.07mg/dL; p = 0.4122) or LDL serum concentration (114.33±12.77mg/dL vs 102.66±12.58mg/dL; p = 0.5541) when comparing vehicle-treated animals and those treated with sVEGFR1 (Figure 32).

![Figure 32 – Treatment with soluble VEGFR1 does not change plasma lipid levels. No significant difference in plasma levels of triglycerides (TG), high-density lipoprotein (HDL) or low-density lipoprotein (LDL) was observed when comparing vehicle-treated (PBS) and soluble VEGFR1 (sVEGFR1)-treated animals. Data are represented as mean±SE.](image-url)
5.3.3 – Treatment with soluble VEGFR1 significantly increases plasma levels of VEGF

Interestingly, animals treated with sVEGFR1 had significantly greater serum concentrations of VEGF as compared to vehicle-treated controls (56.61±4.45pg/mL vs 36.16±4.93pg/mL; p = 0.0316) (Figure 33).

Figure 33 – Treatment with soluble VEGFR1 significantly increases plasma VEGF concentrations. Plasma levels of VEGF protein were measured using ELISA. Soluble VEGFR1 (sVEGFR1)-treated animals had significantly increased plasma VEGF concentrations as compared to vehicle-treated (PBS) animals. Data are represented as mean±SE; *, p < 0.05.
5.3.4 – Treatment with soluble VEGFR1 significantly reduces luminal narrowing

Micrographs were digitally captured of all visible intramyocardial arteries within native and transplanted hearts in vehicle- and sVEGFR1-treated animals (Figure 34a). When comparing the percentage luminal narrowing between transplant and native hearts in both treatment groups, transplanted hearts had significantly increased percentage luminal narrowing in both groups, despite being on 6mg/kg FK506 daily immunosuppression. In vehicle-treated animals, transplanted hearts had significantly greater luminal narrowing as compared to native hearts (51.15±3.92% vs 19.16±1.47%; p = 1.75 x 10^-5) (Figure 34b). As well, in sVEGFR1-treated animals, transplanted hearts had significantly greater percentage luminal narrowing as compared to native hearts (36.56±1.70% vs 14.11±1.95%; p = 0.0010) (Figure 34b). This serves to validate this mouse model, which is able to present with significant development of luminal narrowing in arteries of the transplanted heart, which is a hallmark of CAV, in a minor MHC-mismatched allogeneic transplant model in the presence of the immunosuppressant FK506.

Animals treated with sVEGFR1 had significantly reduced degrees of luminal narrowing as compared to vehicle-treated animals (36.56±1.70% vs 51.15±3.92%; p = 0.0413) (Figure 34b). Although percentage luminal narrowing trended to be decreased in native hearts of sVEGFR1-treated animals as compared to vehicle-treated animals (14.11±1.95% vs 19.16±1.47%), this difference was not statistically significant (p = 0.828) (Figure 34b).
Figure 34 – Quantitation of luminal narrowing in intramyocardial arteries. (a) Representative micrographs of Movat’s pentachrome-stained intramyocardial arteries from native and transplant hearts in vehicle- and sVEGFR1-treated animals. (b) Treatment with sVEGFR1 resulted in a significant decrease in luminal narrowing in transplanted hearts as compared with vehicle-treated controls (36.56±1.70% vs 51.15±3.92%, respectively; *, p = 1.75x10^{-5}). Data are represented as mean±SE. Scale bar = 50µm.
5.3.5 – Treatment with soluble VEGFR1 reduces edema in transplanted hearts

Micrographs were digitally captured of ventricular cross-sections of native and transplanted hearts in vehicle- and sVEGFR1-treated animals (Figure 35a). The average wet heart weight in sVEGFR1-treated transplanted hearts was significantly less than vehicle-treated transplanted hearts (0.15±0.01g vs 0.25±0.05g; p = 0.0423). There was no significant difference between the weights of vehicle- and sVEGFR1-treated native hearts (0.13±0.01g vs 0.14±0.01g, respectively), nor between sVEGFR1-treated transplanted hearts and vehicle- or sVEGFR1-treated native hearts (Figure 35b).

Digital micrographs of mid-ventricular cross-sections of native and transplant hearts from vehicle- and sVEGFR1-treated mice were captured and ImagePro Plus® software was used to quantitate cross-sectional area of histological sections. When comparing the mean cross-sectional area of vehicle- and sVEGFR1-treated transplant hearts, no significant difference was measured (11.96±1.50µm² vs 10.43±0.67µm²; p = 0.4843) (Figure 36).
Figure 35 – Quantitation of ventricular cross-sectional area.
(a) Representative Movat’s pentachrome-stained micrographs of ventricular cross-section of native and transplant hearts from vehicle- and sVEGFR1-treated mice. (b) There was no significant difference between native heart or transplant heart ventricular cross-sectional area in vehicle- or sVEGFR1-treated animals. Data are represented as mean±SE. Scale bar = 500µm.
Figure 36 – Wet heart weight is significantly reduced in soluble VEGF1-treated transplanted hearts. Wet heart weight (g) was significantly reduced in transplanted hearts from sVEGFR1-treated animals as compared to vehicle-treated animals (0.15±0.01g vs 0.25±0.05g; p = 0.0423). Data represent mean±SE.

Consolidating the results from Figures 35 and 36, transplant hearts from vehicle-treated animals had significantly greater wet heart weights compared with transplanted hearts from sVEGFR1-treated animals. This result, coupled with the measurement of no significant difference in cross-sectional area of either transplant or native hearts from vehicle- or sVEGFR1-treated animals suggests that treatment with sVEGF1 reduces edema in transplanted hearts.
5.3.6 – Treatment with soluble VEGFR1 reduces capillary growth induced by bone marrow

Aortic segments were cultured for 5 days alone, or co-incubated with BM and either vehicle or sVEGFR1 (Figure 37a). When aortic ring segments were incubated with whole bone marrow from C57 male mice, the average length of outgrowth increased 123% as compared to control aortic rings which were co-cultured with an empty transwell insert (p = 0.000463). For aortic ring segments cultured with only bone marrow, the average outgrowth area was not significantly different as compared to control (104%; p = 0.4037) (Figure 37b).

When aortic ring segments were co-incubated with bone marrow and sVEGFR1, there was a significant decrease in the average outgrowth length as compared to control (51%, p = 5.35 x 10⁻⁵), and this difference was also significantly decreased as compared to bone marrow co-culture alone (51% vs 123%; p = 5.92 x 10⁻⁸). As well, samples treated with sVEGFR1 had significantly decreased outgrowth area (relative to aortic ring area) compared with control (43%; p = 4.69 x 10⁻⁶) and compared with bone marrow co-culture alone (43% vs 104%; p = 2.56 x 10⁻⁶) (Figure 37b).
**Figure 37 – In vitro aortic ring angiogenesis assay co-culture with bone marrow (BM)-derived cells.**

(a) Representative phase contrast micrographs of aortic ring segments without co-culture or cultured with BM-derived cells and either vehicle or sVEGFR1. (b) Co-culture with BM significantly increased average length of outgrowth (120% as compared with control; *, p = 0.000463), while addition of sVEGFR1 with BM significantly reduced average length of outgrowth (51% as compared with control; **, p = 5.36x10^{-5}). sVEGFR1-treated groups also had significantly shorter outgrowth length than vehicle-treated groups co-cultured with BM (+, p = 5.92x10^{-5}). Co-culture with BM did not significantly increase average outgrowth area; however, co-culture with BM and sVEGFR1 significantly reduced average outgrowth area (***, p = 4.69x10^{-6}). sVEGFR1-treated groups also had significantly smaller outgrowth area as compared with vehicle-treated groups co-cultured with BM (++, p = 2.56x10^{-6}). Data are represented as mean±SE; n = 3 per group.
5.4 – Discussion

During the conceptualization of this proof-of-principle in vivo verification of my doctoral dissertation thesis, the model system and potential therapeutic avenues explored have evolved to change from a rat to a mouse model of heterotopic cardiac transplantation, has included much troubleshooting and optimization of appropriate immunosuppressive regimens to balance inhibition of acute rejection while producing significant CAV, and a fundamental shift in my approach to better understand the role of VEGF in the pathogenesis of CAV. I moved from administration of VEGF protein in vivo, to the use of antisense deoxynucleotide molecules directed against the VEGF internal ribosomal entry sequence, to the use of signal transduction pathway inhibitors, to more specific VEGF receptor tyrosine phosphorylation inhibitors and anti-VEGF neutralizing antibodies. In this chapter, I focus on the utilization of soluble VEGFR1, which not only had a significant effect in reducing luminal narrowing in CAV, but also was well-tolerated by the transplant recipients and resulted in no unexpected post-surgical complications or any evident drug toxicity.

One of the primary reasons for my shift from a rat model to a mouse model of heterotopic cardiac transplantation was the desire for a model system which was capable of sustaining a pro-atherogenic hyperlipidemic environment. This particular point was tantamount as our laboratory was the first to demonstrate the presence and involvement of lipids in the pathogenesis of CAV. In my experiments, animals were placed on a Western diet which resulted in significantly increased LDL cholesterol levels in transplanted mice as compared to untransplanted C57 and 129J mice on normal rodent chow diet (data not shown). In the examination of the effect of administration of soluble VEGFR1, I observed no statistically
significant difference in triglyceride, LDL cholesterol or HDL cholesterol levels (Figure 32). This suggests that the role VEGF plays in CAV may not directly relate to lipids, at least in this model at the time point investigated; however, this hypothesis needs to be further explored. To clearly rule out the contribution of lipids to the soluble VEGFR1-mediated reduction in CAV, a series of further experiments is required to specifically examine the changes in lipid profile during the pathogenesis of CAV using greater animal numbers, more numerous time points (especially early post-transplantation) and tail vein collections of blood through the time course of pathogenesis; however, these investigations are beyond the scope of my thesis.

One interesting observation I made was the significant increase in plasma VEGF concentrations in animals treated with soluble VEGFR1 (Figure 33). Initially, this result seemed counter-intuitive, as my entire VEGF-centered focus in my doctoral dissertation project began with the observation of increased VEGF expression in human heart allografts with CAV, whereas in this circumstance, we have increased plasma VEGF concentrations coupled with a reduction in CAV. It may be possible that the addition of soluble VEGFR1, an agent which can bind circulating VEGF, may result in a compensatory increase in the expression or secretion in a feedback-like manner. It should be noted that the particular antibody used in the ELISA to profile VEGF in mouse plasma samples can also function in vivo as an anti-VEGF neutralizing antibody, and thus, the observed increased in VEGF plasma levels is not likely as a result of measurement of VEGF-sVEGFR1 complexes.

My immunohistochemical profiling of the transplant heart tissues from this chapter for VEGF expression revealed no significant difference in VEGF immunoreactivity between vehicle- and soluble VEGFR1-treated animals (data not shown). Overall, the time point chosen for this
experiment produces a significant degree of luminal narrowing and CAV; however, in comparison to the cases from human heart allografts used in Chapter 3, the human CAV samples were from archival tissue from explanted heart transplants or those obtained at autopsy, suggesting severe CAV and/or end-stage heart failure. To mirror the results obtained in my immunohistochemical investigations in human heart allografts, it would be necessary to significantly extend the experimental time point. However, I believe that as this research direction continues in the laboratory, it is more vital to examine the changes in VEGF secretion, expression and localization during the early transplant time course before significant luminal narrowing or CAV may be detected. To truly elucidate the role of VEGF in CAV, it is necessary to separate the potentially beneficial effects which may be involved in healing ischemia/reperfusion and/or alloimmune injury initially post-transplantation from the potentially deleterious effects of persistent pathological expression of VEGF may have in the long term. Irregardless, my experiments clearly demonstrate that abrogation of the VEGF axis with soluble VEGFR1 has an overall positive effect in reducing luminal narrowing and CAV at 21 days post-transplantation (Figure 34). Further optimization of dosing regimen to enhance beneficial VEGF effects while minimizing deleterious ones may, in the future, provide an even greater degree of prevention or reduction of CAV.

In exploring the potential mechanisms of the soluble VEGFR1-mediated reduction in CAV, there are two emerging directions from my work. One, related to the overarching hypothesis of Chapter 4 and this thesis, relates to VEGF-induced permeability – *in vivo*, this permeability can be directly observed as edema. I have demonstrated that in comparison to vehicle-treated transplants, transplants which received soluble VEGFR1 have a significant
reduction in wet heart weight (Figure 35). Coupled with my morphometric assessment of no change in heart cross-sectional area (i.e. change in size or hypertrophy) (Figure 36), this suggests that abrogation of the VEGF axis in CAV reduces edema, and this may be a mechanism related to the significant reduction of CAV in animals treated with soluble VEGFR1.

The other emerging direction is the relationship of the VEGF axis to the bone marrow response post-transplantation. Research performed in our laboratory has demonstrated a significant increase in bone marrow mobilization to the transplanted heart. The results from my in vitro aortic ring angiogenesis assays, which were co-cultured with bone marrow, suggest that the bone marrow plays a role in at least directing microvascular growth. Usage of soluble VEGFR1 in aortic rings co-cultured with bone marrow significantly reduced both outgrowth length and overall outgrowth area (Figure 37). This result suggests that this may, in part, be another potential mechanism of action when attempting to decipher the mechanism of soluble VEGFR1-mediated reduction of CAV. This in vitro model also serves to verify that the dose and nature of the soluble VEGFR1 used in my in vivo investigations does, indeed, inhibit classic VEGF functions of induced endothelial migration, proliferation and angiogenesis.

Ultimately, further investigation into the evolving role of VEGF in CAV will allow for better manipulation of the VEGF axis to promote beneficial physiological functions while reducing deleterious pathological ones. The key findings from this chapter are reviewed in Figure 38.
Figure 38 – Diagrammatic representation of the key findings from Chapter 5.

The proof-of-principle series of experiments detailed in this chapter provide the first characterization of the ability of administration of soluble VEGFR1 (sVEGFR1) to significantly reduce percentage luminal narrowing (intimal area / luminal area + intimal area) in a hypercholesterolemic mouse model of heterotopic cardiac transplantation. Percentage luminal narrowing is the primary measure of cardiac allograft vasculopathy (CAV) in humans and animal models. As well, administration of sVEGFR1 also reduced edema in transplanted hearts, suggesting that abrogation of the VEGF axis can reduce permeability in the allogeneic transplant setting. A complementary investigation using an *in vitro* aortic ring angiogenesis assay co-cultured with bone marrow cells demonstrated that sVEGFR1 can inhibit bone marrow-mediated microvascular growth. Taken together, these provide two possible mechanisms for the reduction in CAV observed after application of sVEGFR1.

Moving forward, the results from this thesis provide significant new knowledge with respect to the role of VEGF in the pathogenesis of CAV. These prospects, a summary of my work, and a revised concept diagram which includes the role of VEGF in the pathogenesis of CAV are provided in the final chapter of this thesis.
Chapter 6 – Closing remarks

The clear conclusions from the summation of my doctoral studies are: i) VEGF is aberrantly expressed in the coronary arteries of human heart allografts with CAV, and other atheromatous diseases; ii) VEGF is able to induce significant increases in endothelial permeability to LDL in human coronary or cardiac-specific macro- and microvascular endothelial cells, likely through the disruption of tight junctions via an ERK1/2-dependent pathway; and iii) Alteration of the balance of the VEGF axis in a hyperlipidemic model of heterotopic cardiac transplantation in mice significantly reduces the severity of CAV.

These main points solidify a role for VEGF in the pathogenesis of CAV. Continued diligence and investigation along these lines will undoubtedly further our understanding of the pathogenesis of CAV such that therapeutic avenues such as the use of soluble VEGFR1 may one day be utilized to significantly reduce or even prevent the pathogenesis of CAV and AV in all solid organ transplants.

Specifically related to the concepts covered in my doctoral dissertation thesis, the highest priority hypothesis to test would be to delineate the expression and localization of VEGF family members and receptors during the time course of progression of CAV. Optimally, this investigation could be performed in an expanded version of the studies performed in Chapter 3, where a significantly larger sample size for CAV cases may allow the delineation of VEGF expression and localization during different time periods of the pathogenesis of CAV, possibly to delineate “early” and “late” disease. Complementary to this approach, larger sample sizes will allow for the correlation of VEGF expression and CAV pathogenesis with lipid and lipoprotein insudation and retention. The main challenge to this approach, however, remains
the acquisition of sufficient numbers of CAV case materials to adequately power this investigation. As well, implant duration does not directly correlate with the time course of progression of CAV, as many adjacent factors such as donor- and host-specific risk factors and variable levels of immunosuppression post-transplantation may serve as determinants for the onset and progression of CAV.

As such, this hypothesis could be more appropriately addressed in the murine model of heterotopic cardiac transplantation used in Chapter 5, as it provides a controlled model of CAV where significant CAV develops within 3-4 weeks post-transplantation with a minor MHC-mismatch in the presence of a common immunosuppressive agent to inhibit acute rejection. With this time course of VEGF expression and localization in relation to progression of CAV, one could appropriately inhibit VEGF signaling and action using soluble VEGFR1, or other inhibition strategies such as anti-VEGF neutralizing antibodies or chemical inhibitors of VEGFR2 tyrosine kinase phosphorylation within specific time periods post-transplantation (i.e., within 1 week; between 1-2 weeks; after 2 weeks; etc.) to determine whether VEGF may play beneficial or deleterious roles within different time periods during the pathogenesis of CAV. As well, one of the primary reasons for transitioning from a rat model of CAV to a mouse model of CAV was to be able to take advantage of hyperlipidemic models in the mouse, be it through diet or transgene modification, or both. It would be interesting to examine the direct relationship between VEGF expression and localization with lipid and lipoprotein insudation in the vessel wall in the pathogenesis of CAV, and whether augmentation of VEGF through the aforementioned inhibition strategies may also modify lipid and lipoprotein permeability.
Overall, continued investigation is warranted to determine the predominant mechanism for the reduction of luminal narrowing by administration of sVEGFR1.

During my doctoral thesis studies, I have been fortunate to be directly or peripherally involved with a variety of transplant-related programs. This began with my involvement in Program Project Grant awarded to multiple investigators from the Heart and Stroke Foundation of BC and Yukon. In this project, the primary model of cardiac allograft vasculopathy was performed in the Fisher-to-Lewis rat model of heterotopic cardiac transplantation. One of the primary observations that arose from this series of experiments was the disruption of the endothelium, both functionally and structurally. A key observation was, at 21 and 42 days post-transplantation, the presence of intercellular gaps in cyclosporine-treated animals. This observation, along with others, solidified the hypothesis that a hyperpermeability-inducing agent may play a role in the pathogenesis of CAV. Coupled with my observations in human heart allografts of the aberrant expression of VEGF, this line of thinking lead to the exploration of the role of VEGF in the permeability or hyperpermeability of human cardiac-specific primary endothelial cells in culture, explored in Chapter 4.

In addition, as our animal transplant program and model evolved from the rat to the mouse, I was fortunate to have the opportunity to be involved with the development of the model and the optimization of immunosuppressive and transplant regimens to produce a murine model of heterotopic cardiac transplantation rooted in a C57/Bl6 recipient which would facilitate vertical expansion in the future for application of transgenic mouse models. As well, the transition to a mouse model of transplantation allowed for the augmentation of lipid profiles, either by diet, transgene, or both, to more closely resemble that of humans.
Expositing, several studies have observed the clinical benefit of statin therapy post-transplantation.\textsuperscript{427-430} It has also been demonstrated by other investigators that greater change in serum LDL cholesterol levels during the first year post-transplantation is associated with more severe vasculopathy.\textsuperscript{431} Most recently, there has been an intriguing case report describing the use of dextran sulphate cellulose LDL adsorption apheresis in a 50-year-old male orthotopic heart transplant recipient with familial hyperlipidemia resulting in stabilization and reversal of cardiac allograft vasculopathy.\textsuperscript{432} I believe that in the context of CAV, and other atheromatous diseases, VEGF may contribute to their pathogenesis, in part, through increased LDL permeability. Taken together with my previous report of a similar role for VEGF-A in CAV,\textsuperscript{412} it is likely that there may be disruption of several VEGF family members and receptors which augment the balance of physiological healing and pathological atherogenic outcomes.

In one of our laboratory’s transplant-related focuses, examining the role of granzyme B and perforin in CAV, I was able to contribute technically and conceptually, and we observed a reduction of CAV and endothelial damage in perforin knockout mice.\textsuperscript{426} This line of experimentation served to further highlight the immune contribution to CAV, and specifically, its relationship to endothelial damage. Our laboratory has demonstrated a role for apoptosis,\textsuperscript{111} and both the Fas-mediated\textsuperscript{407} and granzyme B-mediated\textsuperscript{402,425,426,433} pathways in the pathogenesis of CAV. This immune-mediated damage to the graft may be another initiating stimulus to early endothelial damage and dysfunction resulting in the overexpression of VEGF in CAV characterized in Chapter 3.

Another transplant-related focus I was peripherally involved in was the investigation of the role of bone marrow-derived cells in transplantation and CAV.\textsuperscript{359,360} This work highlighted
an increased mobilization of the bone marrow to the transplanted heart, and revealed an interesting possibility of relevance to my doctoral thesis focus. As the field of stem cells emerged, the role of VEGFR1 and VEGF in the mobilization of bone marrow-derived cells became more apparent. The revelations from our work, and that in the literature highlighted an additional possibility for the role of VEGF in the pathogenesis of CAV.

Finally, a disparate line of investigation which occurred in our laboratory was the investigation of a differentially regulated proteoglycan, versican, which had previously been shown by our laboratory to be increased in human heart allografts. Work performed by Dr. Maziar Rahmani delineated the role of the Wnt-signaling pathway in the transcriptional regulation of versican. An interesting complement I was fortunate to be involved in was the investigation of Wnt-related transcriptional regulation of the VEGF gene as well. We determined the ability of T cell factors (TCF) to increase the transcriptional activity of the VEGF gene. This result, coupled with my observations of both increased beta-catenin in arteries from a rat model of aortic stenosis, introduced another possible role for VEGF in the pathogenesis of atheromatous disease. Specifically, it is hypothesized that a Wnt-related gene program may regulate a host of downstream genes including VEGF, versican and matrix metalloproteinase-9, among others, to be a relevant axis determining neovascularization in the plaque.

Taken together, the convergence of what began as several disparate lines of investigation serves to highlight the interplay between all networks, be it within a cell, tissue, organ or organ system, or throughout the body. What began in my thesis with the simple observation of aberrant VEGF expression in CAV and atheromatous disease has expanded into a complex multifaceted research program, which reflects the complex multifactorial etiology of
CAV. Moving forward, research efforts should focus not simply on VEGF alone and its role in CAV; but to begin to elucidate its role, one must consider the balance of numerous factors which may alter the VEGF axis in either a beneficial reparative or deleterious pathological manner, including the role and relativity of splice variants, the variety of primary receptors (and soluble forms), their primary tyrosine phosphorylation-dependent actions and control mechanisms which include homo- and heterodimer configurations of VEGF ligands and also VEGF receptors. Coupled with the function of “decoy” soluble receptors and competitive binding of VEGF family members such as PlGF, there are numerous positive and negative feedback mechanisms, whose ultimate balance may dictate phenotype.

The changes in endothelial permeability are intricate and the studies to understand them must be multifaceted. Much remains to be learned about these processes. Foremost are the components of the tight and adherens junctions, especially the sealing elements in tight junctions. Complementing these elements is the function of the different individual junctional components found, and how these differ in cells from different vascular beds. At the other side of the cell lies the extracellular cellular matrix and the role it plays in permeability in vivo. Inside the cell, the role of the cytoskeleton remains to be elucidated. The second messengers are yet another mystery. It is unclear which messenger, subtype or isoenzyme is utilized by each permeability-altering agent. Determining what the targets of the messengers are and how the different pathways interact with each other, as well as how these pathways may be differentially utilized in different vascular beds may offer targets for therapeutic intervention. Certainly, opportunities are wide-open for important discoveries in this area.
A revised current concept for the pathogenesis of CAV highlighting the roles of VEGF in CAV is detailed in Figures 39 and 40.

![Diagram](image)

**Figure 39 – Revised concept of the early pathogenic mechanisms in cardiac allograft vasculopathy.** There are several injurious stimuli, including ischemia/reperfusion injury, hypoxia, reactive oxygen species, hypercholesterolemia and the alloimmune response against the graft, which contribute to the initiation and progression of cardiac allograft vasculopathy (CAV). Early endothelial damage and dysfunction result in the release of not only pro-inflammatory cytokines and chemokines, but also growth factors and other molecules, which enhance the immune response against the graft and induce reparative responses which attempt to maintain endothelial barrier integrity and vascular function. Smooth muscle cells (SMC) in the media produce vascular endothelial growth factor (VEGF) in a physiological attempt to repair the endothelium either through endothelial migration and proliferation along the endothelium or by inducing the homing of endothelia progenitor cells. This expression of VEGF may result in deleterious effects on the vessel wall, resulting in increased endothelial permeability to low-density lipoproteins (LDL) and enhanced chemotaxis of monocytes to the vessel wall. Subsequent to LDL insudation and retention on proteoglycans within the extracellular matrix (ECM), native LDL itself can further induce SMC to produce and secrete VEGF.
During the progression and propagation of cardiac allograft vasculopathy (CAV), chronic, aberrant expression of vascular endothelial growth factor (VEGF) can continue to alter the vessel microenvironment. It has been well documented that oxidized low-density lipoproteins (oxLDL) can not only induce macrophages to produce and secrete VEGF, but also do so independent of oxLDL uptake in macrophages. As well, VEGF has been shown to have pro-survival effects on macrophages, providing a possible mechanism whereby macrophages which uptake excess low-density lipoproteins (LDL) or oxLDL may be kept alive long enough to form lipid-laden foam cells, which subsequently may contribute to the concentric, lipid-rich lesions characteristic of CAV. Conversely, VEGF may also maintain some beneficial roles within the vessel wall. It has been demonstrated that VEGF can inhibit oxLDL-induced endothelial cell apoptosis. As well, a central role for VEGF in the chemotaxis, signaling, survival and differentiation of stem cells is emerging. It may be possible in the future to augment the VEGF signaling axis to promote the physiological, beneficial effects of VEGF, while minimizing or inhibiting potentially deleterious, pathological effects.

Figure 40 – Revised concept of the later events in the pathogenesis of cardiac allograft vasculopathy.
References


## Appendix – Supplemental information for case materials from Chapter 3

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Condition</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>28</td>
<td>M</td>
<td>Self-inflicted gunshot wound</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>18</td>
<td>F</td>
<td>Blunt trauma to the head and chest</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>17</td>
<td>M</td>
<td>Blunt trauma to the head and chest</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>34</td>
<td>F</td>
<td>Drug toxicity</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>20</td>
<td>M</td>
<td>Carbon monoxide poisoning</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>17</td>
<td>F</td>
<td>Suffocation</td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>19</td>
<td>M</td>
<td>Shotgun wound to the head</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>29</td>
<td>F</td>
<td>Gunshot to the abdomen</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>18</td>
<td>M</td>
<td>Shotgun wound to the head</td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>17</td>
<td>M</td>
<td>Multiple stab wounds to the chest and abdomen</td>
</tr>
<tr>
<td>11</td>
<td>Normal</td>
<td>19</td>
<td>F</td>
<td>Blunt trauma to the head, chest and abdomen</td>
</tr>
<tr>
<td>12</td>
<td>Normal</td>
<td>22</td>
<td>M</td>
<td>Blunt trauma to the head, chest and abdomen</td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>26</td>
<td>F</td>
<td>Blunt trauma to the head, chest and abdomen</td>
</tr>
<tr>
<td>14</td>
<td>Normal</td>
<td>15</td>
<td>F</td>
<td>Blunt trauma to the head and chest</td>
</tr>
<tr>
<td>15</td>
<td>Normal</td>
<td>21</td>
<td>F</td>
<td>Blunt trauma to the head, chest and abdomen</td>
</tr>
<tr>
<td>16</td>
<td>Normal</td>
<td>32</td>
<td>M</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3 – Case listing for Normal group *(Pathobiological Determinants of Atherosclerosis in Youth* study).

M – male; F – female; N/A – not available.
**Table 4 – Case listing for Native Atherosclerosis (NA) group.**
M – male; F – female; N/A – not available; CAD – coronary artery disease; ARDS – acute respiratory distress syndrome; CLL – chronic lymphocytic leukemia; AIDS – acquired immune deficiency syndrome; HepC – hepatitis C; HepB – hepatitis B.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Condition</th>
<th>% occlusion</th>
<th>Primary diagnosis</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Atherosclerosis</td>
<td>&gt;50%</td>
<td>N/A</td>
<td>78</td>
<td>M</td>
<td>Respiratory, cardiac, renal failure</td>
</tr>
<tr>
<td>2</td>
<td>Atherosclerosis</td>
<td>100%</td>
<td>CAD</td>
<td>76</td>
<td>M</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>3</td>
<td>Atherosclerosis</td>
<td>&gt;25%</td>
<td>ARDS</td>
<td>51</td>
<td>M</td>
<td>Ruptured aortic aneurysm</td>
</tr>
<tr>
<td>4</td>
<td>Atherosclerosis</td>
<td>60-70%</td>
<td>CAD</td>
<td>69</td>
<td>F</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>5</td>
<td>Atherosclerosis</td>
<td>75-90%</td>
<td>CLL</td>
<td>53</td>
<td>F</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>Atherosclerosis</td>
<td>50%</td>
<td>CAD</td>
<td>41</td>
<td>F</td>
<td>AIDS, mycobacterium, HepC, HepB</td>
</tr>
<tr>
<td>7</td>
<td>Atherosclerosis</td>
<td>65%</td>
<td>CAD</td>
<td>52</td>
<td>M</td>
<td>Massive pulmonary embolism</td>
</tr>
<tr>
<td>8</td>
<td>Atherosclerosis</td>
<td>50-60%</td>
<td>CAD</td>
<td>77</td>
<td>F</td>
<td>Hemopericardium, aortic dissection</td>
</tr>
<tr>
<td>9</td>
<td>Atherosclerosis</td>
<td>80%</td>
<td>N/A</td>
<td>84</td>
<td>M</td>
<td>Subendocardial necrosis</td>
</tr>
<tr>
<td>10</td>
<td>Atherosclerosis &lt;25%</td>
<td>N/A</td>
<td>CAD</td>
<td>24</td>
<td>M</td>
<td>Arrhythogenic right ventricular dysplasia</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Condition</th>
<th>% occlusion</th>
<th>Primary diagnosis</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Atherosclerosis</td>
<td>40-50%</td>
<td>CAD</td>
<td>74</td>
<td>M</td>
<td>Focal myocyte necrosis, ischemia</td>
</tr>
<tr>
<td>12</td>
<td>Atherosclerosis</td>
<td>30-40%</td>
<td>CAD</td>
<td>69</td>
<td>M</td>
<td>Cerebral infarction, thrombus from heart</td>
</tr>
<tr>
<td>13</td>
<td>Atherosclerosis</td>
<td>65-70%</td>
<td>CAD</td>
<td>76</td>
<td>M</td>
<td>Aortic dissection, ischemia</td>
</tr>
<tr>
<td>14</td>
<td>Atherosclerosis</td>
<td>75-100%</td>
<td>CAD</td>
<td>52</td>
<td>M</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>15</td>
<td>Atherosclerosis</td>
<td>35%</td>
<td>CAD</td>
<td>78</td>
<td>M</td>
<td>Chronic obstructive, sudden death</td>
</tr>
<tr>
<td>16</td>
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<td>65%</td>
<td>CAD</td>
<td>87</td>
<td>F</td>
<td>Cardiac arrest</td>
</tr>
<tr>
<td>17</td>
<td>Atherosclerosis</td>
<td>25-30%</td>
<td>CAD</td>
<td>68</td>
<td>F</td>
<td>Acute myocardial infarction</td>
</tr>
</tbody>
</table>

**Table 5 – Case listing for Diabetes Mellitus (DM) group.**
M – male; F – female; N/A – not available.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Condition</th>
<th>% occlusion</th>
<th>Primary diagnosis</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Cause of Death</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Diabetic</td>
<td>20-40%</td>
<td>CAD</td>
<td>63</td>
<td>M</td>
<td>Heart transplant</td>
</tr>
<tr>
<td>2</td>
<td>Diabetic</td>
<td>&gt;75%</td>
<td>CAD</td>
<td>49</td>
<td>M</td>
<td>Heart transplant</td>
</tr>
<tr>
<td>3</td>
<td>Diabetic</td>
<td>50-75%</td>
<td>CAD</td>
<td>55</td>
<td>M</td>
<td>Heart transplant</td>
</tr>
<tr>
<td>4</td>
<td>Diabetic</td>
<td>50-75%</td>
<td>CAD</td>
<td>54</td>
<td>M</td>
<td>Heart transplant</td>
</tr>
<tr>
<td>5</td>
<td>Diabetic</td>
<td>&lt;25%</td>
<td>CAD</td>
<td>57</td>
<td>M</td>
<td>Heart transplant</td>
</tr>
<tr>
<td>6</td>
<td>Diabetic</td>
<td>25-50%</td>
<td>CAD</td>
<td>67</td>
<td>F</td>
<td>Congestive heart failure</td>
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<tr>
<td>7</td>
<td>Diabetic</td>
<td>100%</td>
<td>CAD</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>Diabetic</td>
<td>N/A</td>
<td>CAD</td>
<td>69</td>
<td>F</td>
<td>Arrhythmia, severe atherosclerotic heart disease</td>
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<tr>
<td>9</td>
<td>Diabetic</td>
<td>40%</td>
<td>CAD</td>
<td>77</td>
<td>F</td>
<td>Rhythm disturbance, myocardial necrosis</td>
</tr>
<tr>
<td>10</td>
<td>Diabetic</td>
<td>50%</td>
<td>CAD</td>
<td>65</td>
<td>M</td>
<td>Myocardial ischemia</td>
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<tr>
<td>11</td>
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<td>75%</td>
<td>CAD</td>
<td>69</td>
<td>M</td>
<td>Respiratory failure, severe acute lung disease</td>
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<tr>
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<td>CAD</td>
<td>85</td>
<td>F</td>
<td>Myocardial infarction</td>
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<tr>
<td>13</td>
<td>Diabetic</td>
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<td>CAD</td>
<td>59</td>
<td>F</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>14</td>
<td>Diabetic</td>
<td>70%</td>
<td>CAD</td>
<td>64</td>
<td>M</td>
<td>Acute myocardial infarction, non-insulin dependent diabetes mellitus</td>
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<td>Diabetic</td>
<td>75%</td>
<td>CAD</td>
<td>71</td>
<td>F</td>
<td>Cardiac arrest, non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>Case no.</td>
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<td>% occlusion</td>
<td>Primary diagnosis</td>
<td>Implant Duration (days)</td>
<td>Age (yr)</td>
<td>Sex</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-------------</td>
<td>-------------------</td>
<td>------------------------</td>
<td>----------</td>
<td>-----</td>
</tr>
<tr>
<td>1</td>
<td>heart transplant</td>
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<td>Heart failure</td>
<td>540</td>
<td>48</td>
<td>F</td>
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<tr>
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<td>heart transplant</td>
<td>100%</td>
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<td>611</td>
<td>16</td>
<td>M</td>
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<tr>
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<td>60</td>
<td>M</td>
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<td>360</td>
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<td>F</td>
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<tr>
<td>5</td>
<td>heart transplant</td>
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Table 6 – Case listing for Cardiac Allograft Vasculopathy (CAV) group.