

**Glycogen Synthase Kinase 3 β Inhibition for Improved Endothelial Progenitor Cell
Mediated Arterial Repair**

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Abstract:

Increasingly, cell-based therapy with autologous progenitor populations, such as endothelial progenitor cells (EPC), are being utilized for treatment of vascular diseases. However, both the number and functional capacity are diminished when cells are derived from patients with established risk factors for coronary artery disease (CAD). Herein, we report that inhibition of glycogen synthase kinase 3 β (GSK) can improve both the number and function of endothelial progenitor cells in patients with CAD or diabetes mellitus (DM) leading to greater therapeutic benefit. Specifically, use of various small molecule inhibitors of GSK (GSKi) results in a 4-fold increased number of EPCs. Moreover, GSKi treatment improves the functional profile of EPCs through reductions in apoptosis, improvements in cell adhesion through up-regulation of very-late antigen-4 (VLA-4), and by increasing paracrine efficacy by increasing vascular endothelial growth factor (VEGF) secretion. Therapeutic improvement was confirmed *in vivo* by increased re-endothelialization (RE) and reductions of neointima (NI) formation achieved when GSKi-treated cells were administered following vascular injury to CD-1 nude mice. Because cell-based therapy is technically challenging, we also tested a strategy of local delivery of GSKi at the site of arterial injury through GSKi-eluting stents. *In vitro*, GSKi elution increased EPC attachment to stent struts. *In vivo*, GSKi-eluting stents deployed in rabbit carotid arteries resulted in systemic mobilization of EPCs, improved local RE, and important reductions in in-stent NI formation. Finally, we tested the ability of GSKi to improve EPC-mediated arterial repair in patients with DM. As in patients with CAD, GSKi treatment improved EPC yield and diminished *in vitro* apoptosis. Utilizing a proteomics approach, we identified Cathepsin B (catB) as a differentially regulated

protein necessary for reductions in apoptosis. Indeed, antagonism of catB prevented GSKi improvements in GSKi treated EPC mediated arterial repair in a xenotransplant wire injury model. Thus, our data demonstrates that GSKi treatment results in improvements in EPC number and function *in vitro* and *in vivo* resulting in enhanced arterial repair following mechanical injury. Accordingly, GSK antagonism is an effective cell enhancement strategy for autologous cell-based therapy with EPCs from high risk patients such as CAD or DM.

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I would especially like to thank my wife Rebecca for her unwavering patience, understanding and support during the many years of training. As I am writing this – I am already late for dinner.

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List of Abbreviations (in order of appearance):

CAD – coronary artery disease
PTCA – percutaneous transluminal coronary angioplasty
ISR – in-stent restenosis
NI – neointima
PCI – percutaneous coronary intervention
SMC – smooth muscle cell
DES – drug eluting stent
SES – sirolimus eluting stent
PES – paclitaxel eluting stent
BMS – bare metal stent
RE – re-endothelialization
ST – stent thrombosis
ACS – acute coronary syndrome
STEMI – ST elevation myocardial infarction
ECM – extracellular matrix
IEL – internal elastic lamina
EEL – external elastic lamina
EPC – endothelial progenitor cells
EGM – endothelial growth media
PBMC – peripheral blood mononuclear cell
ECFC – endothelial colony forming cell
CFU – colony forming unit
cEPC – circulating endothelial progenitor cell
KDR – kinase domain receptor
EC – endothelial cell
RBC – red blood cell
WBC – white blood cell
GSK3 β – glycogen synthase kinase 3 β
 β cat – β -catenin
DM – diabetes mellitus
HSC – hematopoietic stem cells
GFP – green fluorescent protein
FRZ – frizzled
DVL – disheveled
APC – adenomatous polyposis coli
GSKi – glycogen synthase kinase inhibitor
VEGF – vascular endothelial growth factor
VLA4 – very late antigen 4
CatB – cathepsin B
EDTA – ethylenediaminetetraacetic acid
LDL – low density lipoprotein
acLDL – acetylated low density lipoprotein
LiCl – lithium chloride

UEAI – *ulex europaeus* agglutinin I
 FITC – fluorescein isothiocyanate
 DAPI – 4',6-Diamidino-2-Phenylindole
 PVDF – polyvinylidene fluoride
 TBS – tris buffered saline
 ECL – enhance chemiluminescence
 PCR – polymerase chain reaction
 RT – reverse transcription
 C-EPC – control treated EPCs
 G –EPC – glycogen synthase kinase inhibitor treated EPCs
 H&E – hematoxylin and eosin
 ANOVA – analysis of variance
 I:M – intima to media ratio
 G-CSF – granulocyte colony stimulating factor
 CIHR – canadian institutes of health research
 NA – neointimal area
 GS – glycogen synthase kinase inhibitor coated stent
 VS – vehicle coated stent
 RS – rapamycin coated stent
 RGS – rapamycin and GSKi coated stent
 NIH – national institutes of health
 HPF – high power field
 DMSO – dimethyl sulfoxide
 IC50 – half maximal inhibitory concentration
 BrdU – bromodeoxyuridine
 NBF – neutral buffered formalin
 LA – lumen area
 SEM – scanning electron microscopy
 GSL I-B4 – Griffonia (bandeiraea) simplicifolia lectin I – isolectin B4
 LSD – least significant difference
 PBS – phosphate buffered saline
 2D PAGE – two dimensional polyacrylamide gel electrophoresis
 IPG – immobilized pH gradient
 IEF – isoelectric focusing
 LC-MS – liquid chromatography mass spectrometry
 HPLC – high performance liquid chromatography
 OHRI – ottawa hospital research institute
 RIPA – radioimmunoprecipitation assay buffer
 HUVEC – human vascular endothelial cells
 EBM – endothelial basal media
 DMII – type 2 diabetes
 DM I – type 1 diabetes
 PVD – peripheral vascular disease
 SCaMC1- calcium-binding mitochondrial carrier protein
 GDI-2 – GDP dissociation inhibitor 2
 DM-EPCs- EPCs derived from patients with diabetes

PAI-2 – plasminogen activator inhibitor 2
uPAR – urokinase-type plasminogen activator receptor

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Chapter 1: Introduction

1.1 Clinical Problem: In-stent restenosis

Coronary artery disease (CAD), the most common cause of death and cardiovascular morbidity in industrialized nations such as Canada,(102) results from blockages in arteries that form due to atherosclerosis. The most common method of revascularizing patients with CAD is to open blockages with balloon devices. Gruentzig performed the first percutaneous transluminal coronary angioplasty (PTCA) in 1977, and since then percutaneous interventions have rapidly become the preferred method of revascularization.(59) Early results with PTCA were encouraging, but long-term vessel patency was hampered by restenosis at the site of intervention – a complication occurring in approximately 40% of patients.(122) Major limitations of PTCA were arterial dissections and elastic recoil of the vessel – both of which played a significant role in abrupt closure of the dilated vessel and in many instances subsequent restenosis. Thus, to address this limitation, endoluminal metal scaffolds – called coronary stents (**Figure 1.1**) – were developed as a means of preventing early arterial recoil. Coronary stents act as luminal scaffolds and resultantly decrease the rate of in-stent restenosis (ISR) – renarrowing of the artery in the stent – by half. (46,140,141) Nonetheless, despite the marked reductions in need for revascularization procedures, neointima formation (NI) causes flow limiting disease requiring a second PCI in about 10-30% of patients receiving bare metal stents.(141) With over 1.2 million PCIs being performed annually in the United States alone,(93) the clinical importance of preventing ISR is evident.

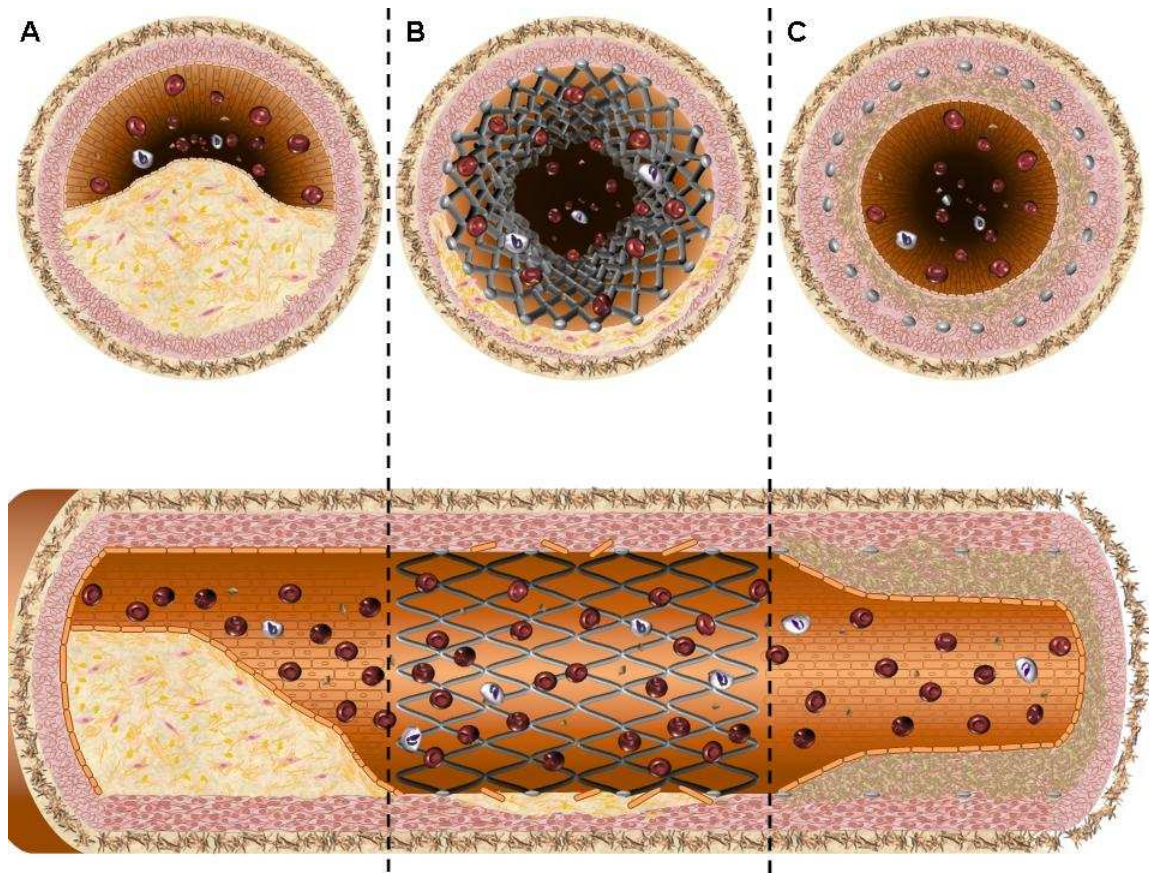


Figure 1.1 – Progression of in-stent restenosis. Cross-sectional and longitudinal views of artery depicting chronological progression of in-stent restenosis. **(A)** Obstructive atheromatous plaque causing flow-limiting stenosis of arterial lumen with reduced luminal diameter. **(B)** Following percutaneous endoluminal stenting which restores the native vessel diameter by compressing the atheromatous plaque into the vessel wall with resultant denudation of the endothelial layer. **(C)** In-stent restenosis following inappropriate neointimal hyperplasia in response to percutaneous stent insertion resulting in recurrence of flow-limiting stenosis.

The mechanisms underlying ISR following PCI have recently been reviewed and to this day remain incompletely understood.(122) Bare-metal stents (BMS) were the first devices employed for coronary stenting. Interestingly, while these devices reduced the rates of restenosis compared to balloon angioplasty, ISR continued to occur in 20-30% of cases.(46,110) While stenting was able to address arterial recoil and abrupt vessel closure, the residual cases of ISR were thought to relate primarily to NI formation through medial smooth muscle cell (SMC) migration, proliferation and synthesis of proteoglycan matrix.(55,122) In turn, compounds targeting cellular proliferation were coated onto stents giving rise to modern day drug-eluting stents (DES).

DESs are composed of a metallic stent, a polymer-based drug delivery platform, and a pharmacologic agent – typically an immunosuppressant and/or antiproliferative compound. The goal of DES technology is to minimize PCI-related vascular inflammation and cellular proliferation which are thought to lead to NI formation, and ultimately, ISR. Indeed, early trials of DESs assessing sirolimus (SES) and paclitaxel eluting stents (PES) reduced the rate of ISR to 5-8%.(49,107,148) These randomized trials were followed by a large meta-analysis reporting outcomes on over 18,000 patients and demonstrated substantial reductions in target lesion revascularization with DESs compared to BMSs.(147)

The advent of DESs improved outcomes in PCI; however, these incremental advancements unfortunately came at the cost of an increased risk of stent thrombosis (ST). While both ISR and ST are known complications of stent implantation, their proposed pathophysiology remains diametrically opposed, with ST thought to result from the pro-thrombotic state arising at the site of an exposed stent strut. DESs were designed to mitigate ISR by inhibiting the normal healing response at the site of stent insertion by blocking cell infiltration, proliferation and matrix formation. Nonetheless, one unintended consequence is that normal re-endothelialization (RE) is delayed resulting in a prolonged period in which the stent struts are exposed to the circulation – acting as a nidus for platelet adherence, activation, and ultimately thrombus propagation. In simplified terms, ISR is related to an overzealous response of the vessel to injury whereas ST arises as a result of impaired or delayed healing resulting in acute thrombotic events. Consequently, while DESs have improved outcomes from an ISR perspective, they may also increase the risk of abrupt vessel closure due to thrombosis leading to myocardial infarction.(3,78,96)

While initial studies comparing BMSs to DESs reported similar rates of sub-acute ST (24 hours to 30 days post-PCI) and late ST (30 days to 1 year post-PCI), long-term follow-up showed an increase in very late ST (>1yr post-PCI).(149) Indeed, autopsy studies confirmed that DESs delayed arterial healing, with BMSs demonstrating complete RE at 6-7 months while first generation DESs failed to fully RE even beyond 40 months.(78) In contrast, while the risk of ST was higher, rates of TLR were reduced from 23.6% with BMSs to 7.8% with first generation DESs.(149) Thus, clinicians continue to be

challenged with balancing the benefit of reductions in ISR with the risks associated with delayed healing and potential ST.

Historically, ISR was believed to be a benign clinical entity, with patients experiencing recurrence of their angina 3-12 months following revascularization. However, recent studies have reported ISR presenting as an acute coronary syndrome (ACS) in 9.5-18% of cases, of which 2-2.2% were ST-elevation MIs (STEMIs).(18,124) In contrast, ST is much rarer, occurring in 0.2-0.5% of cases per year, but typically presents as either a STEMI or sudden cardiac death.(49,50) Hence, the ongoing challenge remains to develop a device that minimizes ISR while not adversely affecting arterial healing and the risk of ST. These concerns are of particular importance as indications for PCI continue to expand such that even low rates of ISR and ST will impact large numbers of patients.

1.2 Cellular mechanisms and pathophysiology of ISR

The accepted pathogenesis of ISR remains in flux with numerous models proposed in an attempt to explain the observed epiphenomenon described during development of a NI. We recently reviewed these models and their implication for therapeutic intervention (**Appendix VII**).(122) The most widely accepted model is an adaptation of the “response-to-injury” model originally proposed by Ross and Glomset in 1976,(129) whereby the mechanical disruption of the endothelial lining by PCI serves as an initiating factor.(128) Subsequent endothelial dysfunction and the ensuing inflammatory response is thought to stimulate the remodeling process, initiating migration and proliferation of SMCs from the media (**Figure 1.2B**). NI growth is then further exacerbated as SMCs

adopt a synthetic phenotype depositing excess extracellular matrix proteins which compromise lumen patency. Yet disparity in findings from animal models and human pathologic specimens has made reconciliation with this model difficult.

Initial studies performed in murine and rat models focused predominately on the origin of cells involved in NI progression, suggesting that medial SMCs migrate and proliferate to generate the NI.(13,23,24,115,116) For example, in rats, SMC proliferation was abundant after balloon angioplasty, peaking at 96 hours then returning to basal rates following RE of the artery.(23) Additionally, evidence from human studies have suggested a role for medial SMCs in generating NI. In sex-mismatched cardiac transplant recipients examination of vessels using fluorescent *in situ* hybridization techniques demonstrated >90% of cells appeared to be derived from the donor vessel.(54) However, these analyses are limited to transplanted specimens and the confounder of transplant vasculopathy makes drawing definitive conclusions difficult.

Interestingly, studies of human pathologic samples have repeatedly failed to demonstrate high levels of proliferation – a finding inconsistent with the media as a major contributor to NI formation.(57,103,109) Furthermore, in models of wire or balloon vascular injury, NI formation is typically preceded by apoptosis within the medial layer.(119,131) Accordingly, researchers have focused on other potential sources of intimal cells and accumulated evidence that circulating progenitor cells may play a role in generating NI lesions.

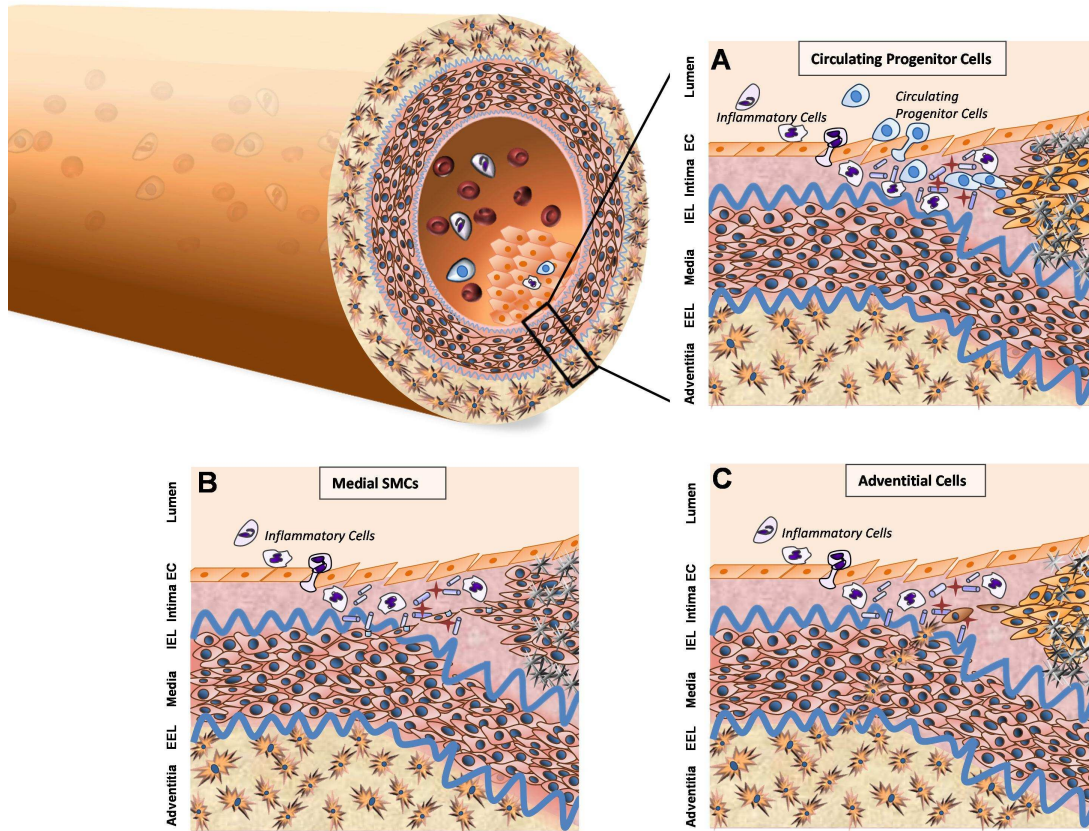


Figure 1.2. Models of neointima formation in coronary arteries. Damage to vascular endothelial cells (EC) leads to infiltration of inflammatory cells and subsequent pro-inflammatory cytokine release (blue rods), resulting in neointima formation via three postulated mechanisms. **(A)** Circulating Progenitor Cells –Cytokine release stimulates influx of circulating progenitor cells then differentiate under the influence of cytokines and growth factors (red stars) into a smooth muscle cell/myofibroblast phenotype with a secretory phenotype. Production of extracellular cellular matrix (ECM, grey stars) contributes to NI volume. **(B).** Medial Smooth Muscle Cells Inflammatory cell influx and cytokine release stimulates proliferation and migration of medial SMCs the internal elastic lamina (IEL) towards the intima. Once in the subendothelial space SMCs acquire a synthetic phenotype producing an abundant ECM. **(C)** Adventitial Cells – Cytokine release stimulates migration of adventitial fibroblast cells across the external elastic lamina (EEL), through the tunica media, and across the IEL to the intima. These migratory fibroblasts then differentiate into SMC-like cells known as myofibroblasts with subsequent ECM secretion under the influence of pro-inflammatory cytokines and growth factors. Reproduced with permission.(122)

Early experimental studies using dacron grafts implanted in pigs, dogs and sheep revealed the potential to develop endothelial and vascular SMCs independent of input from the native vessel.(43,47,79,113,151-153) These studies were the first to suggest that cellular components of the vessel could organize independently of paracrine factors or cells from mature vessels *in vivo*. More recently, studies have confirmed that circulating populations of progenitor cells can populate the subintimal space and transdifferentiate into a mature SMC phenotype.(62,98,145) These studies have also found support from transplant studies in animals using the *LacZ* reporter system,(130) fluorescence *in situ* hybridization(69,70) and more recently by our group (**Appendix VII**) using quantitative polymerase chain reaction for donor/recipient specific markers to track cells from sources other than the artery itself.(98) Moreover, both qualitative and quantitative evidence now suggests that cells comprising NI following vascular injury are at least in part derived from circulatory sources (**Figure 1.2A**).

Lastly, rather than the circulation, some authors have contended that the vessel adventitia may be a major contributor of NI cells (**Figure 1.2C**). Fibroblasts comprise the majority of cells within the adventitia and these cells have been shown to react to the inflammatory response that occurs following vascular injury.(142,143) Specifically, these cells respond to changes in local paracrine factors, adopting a synthetic phenotype as well as expressing SMC α -actin – a feature typical of NI cells.(32,142) Furthermore, unlike the media which shows increased levels of apoptosis and low levels of proliferation, the adventitia enters a state of hyper-proliferation in response to injury with numerous reports

documenting transmigration of adventitial cells into the NI. Thus, while Ross' "response to injury" hypothesis continues to dominate scientific studies of NI formation, considerable evidence supports a potential role for circulating progenitor cells and adventitial cells in the generation of a NI following vascular injury.

1.3. Role of Endothelial Progenitor Cells in Arterial Repair Following Vascular Injury.

We have previously reviewed the role of EPCs in both vascular lesion formation (122) and vascular repair (**Appendix XI**). (65,66) Indeed, while results from early studies suggested the potential to derive endothelial cells from blood alone (79,151-153), it wasn't until 1997 when Asahara and colleagues isolated putative EPCs. (8) Since this initial report the study of EPCs, their role in both disease pathogenesis and potential use as therapeutics have been studied extensively.

Interestingly, while researchers continue to focus on the therapeutic potential of EPCs, the very definition of what constitutes a "true endothelial progenitor cell" continues to evolve. (40,71,164,165) Indeed, lack of a uniform definition has led to conflicting results in both pre-clinical and clinical studies hampering our progress and understanding of EPC biology. For the purpose of this thesis, the term EPC will be reserved for blood-derived cells isolated through adherence to fibronectin and cultured for four to seven days in endothelial growth media (EGM) as recently reviewed (**Figure 1.3**). (30,40) Alternatively, EPCs have been defined and enumerated by flow cytometry based on the

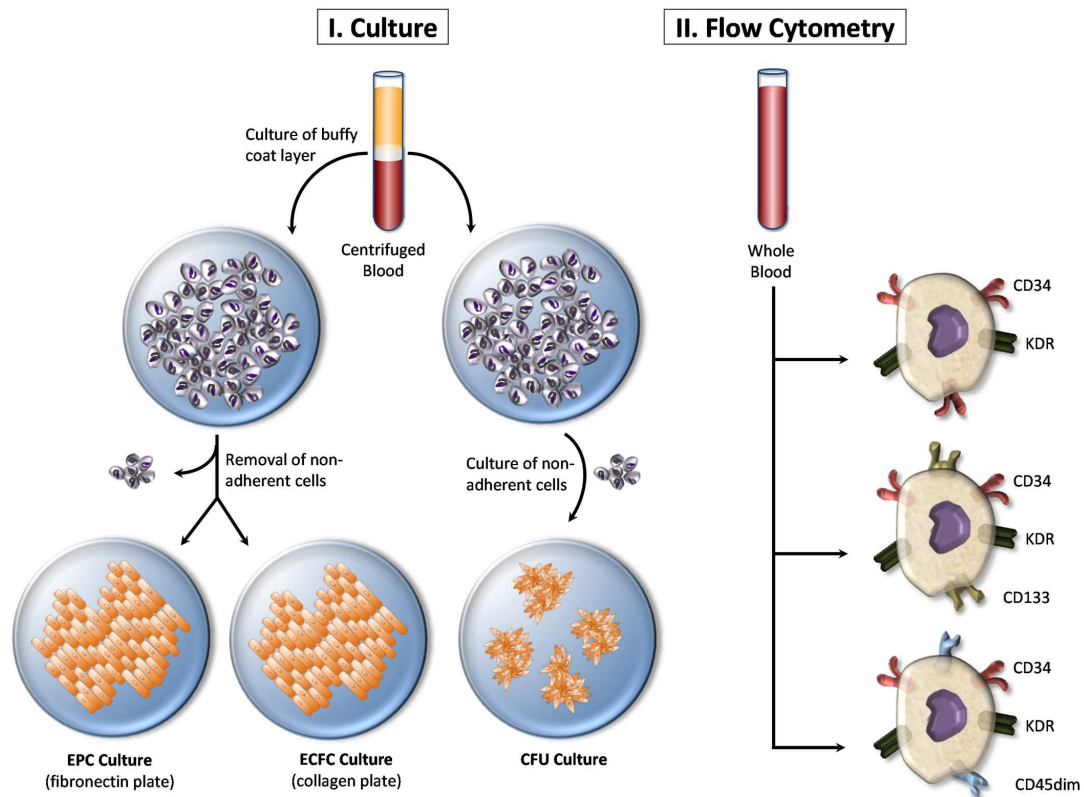


Figure 1.3: Phenotypic characterization of endothelial progenitor cells. (I) Culture. Culture based methodology utilizing Ficoll-based differential centrifugation of whole blood, extraction of buffy coat layer enriched with peripheral blood mononuclear cells (PBMCs) and subsequent culture. When cultured on fibronectin, with non-adherent cells removed, adherent cells develop into endothelial progenitor cells (EPCs). When initially cultured on collagen-coated plates, adherent cells differentiate into endothelial colony forming cells (ECFC). Alternatively, the colony-forming unit (CFU) assay entails replating of non-adherent cells with subsequent development of CFU's. (II) Flow Cytometry. Analysis of whole blood samples following either red blood cell lysis or isolation by differential centrifugation of PBMCs. Several gating strategies have been employed using various combinations of surface markers including: CD34⁺/KDR⁺, CD34⁺/KDR⁺/CD133⁺, CD34⁺/KDR⁺/CD45dim. Used with permission.(30)

presence or absence of various surface markers. While culture-derived EPCs are derived primarily from early monocyte populations,(126) the circulating EPCs (cEPC) defined by flow cytometry represent a distinct population. Exact gating strategies and the panel of markers needed to ensure reproducibility and specificity continues to be debated (**Figure 1.3**), (12,28,29,64,134,135) but cEPCs typically are defined as CD45 dim or negative, CD34 positive, and either CD133 or kinase domain receptor (KDR) positive.(15,117,175)

A number of studies have clearly established an epidemiological link between EPC and cEPC number in peripheral blood and future adverse cardiovascular events.(68,168,172,174) In patients with fewer EPCs *or* with EPCs with lower functional capacity, events such as myocardial infarction, ISR and death are known to occur at a higher frequency.(52) Indeed, this has led to the hypothesis that specific subsets of progenitors can contribute to arterial homeostasis through both direct incorporation into the endothelium and through secretion of pro-healing paracrine factors (**Figure 1.4**). This has led our laboratory to study EPCs as both a marker and as a potential therapeutic agent for the prevention of ISR following percutaneous revascularization. Indeed, Padfield *et al.*(111) recently reviewed the data linking EPC mediated arterial repair and improved clinical outcomes, such as reductions in ISR, but noted that no targeted therapies currently exploit this well-documented endogenous repair mechanism.

Pre-clinical animal studies provided the first evidence that circulating EPCs play an integral role in arterial homeostasis. The earliest studies examined the role of statin

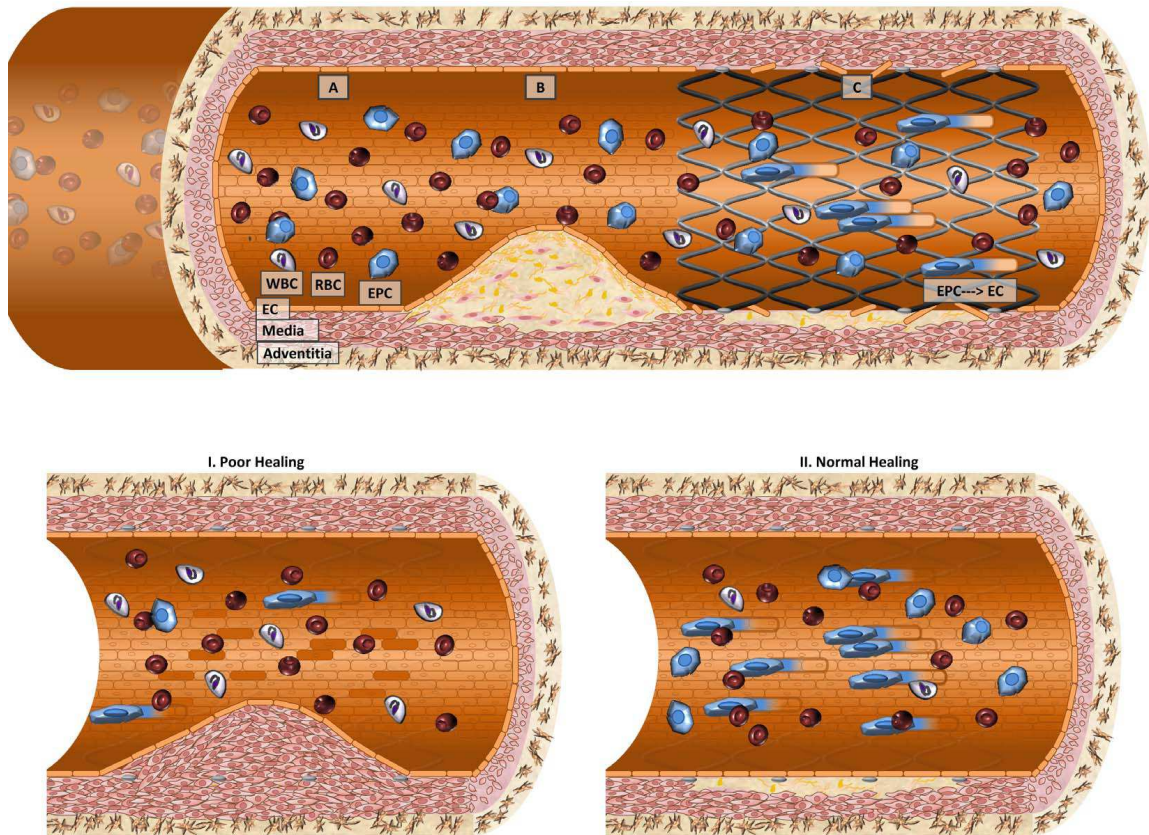


Figure 1.4. Role of EPCs in Arterial Homeostasis following PCI. Arterial cross section (upper panel) displaying vascular anatomy composed of outer adventitial layer with fibroblasts and extracellular matrix (ECM), medial layer with smooth muscle cells, and inner endothelial cell (EC) layer. Erythrocytes (RBCs), leukocytes (WBCs), and endothelial progenitor cells (EPCs) circulate within the vessel's lumen. **Region (A)** Normal arterial vessel with intact endothelium. **Region (B)** Atherosclerotic lesion containing a fibrous cap and cholesterol crystals lined by endothelium. **Region (C)** Atherosclerotic lesion treated via insertion of metal stent, compressing the plaque while denuding the endothelial layer in this region. Re-endothelialization then occurs via EPCs adhering to the vessel wall where they differentiate into endothelial cells (EPC → EC) or secrete paracrine factors which promote re-endothelialization by local mature ECs. Lower panels illustrate time lapse healing of stented region (C). **I. Poor Healing** due to diminished EPC numbers and/or function results in incomplete re-endothelialization with exposed stent struts and neointima formation. This delayed arterial homeostasis results in in-stent restenosis. **II. Normal Healing** displays completely re-endothelialized stented segment with no exposed struts or neointima formation owing to EPC numbers and/or function stimulating normal arterial homeostasis. Reproduced with permission.(66)

medications (HMG Co-A reductase inhibitors) on mobilization of EPCs and RE.

Dimmeler *et al.* first demonstrated that statin therapy resulted in mobilization of EPCs in mice through the PI3-kinase (PI3K) pathway.(34) Subsequent studies in both mice(173) and rats(170) confirmed that statin therapy could mobilize bone-marrow-derived EPCs which homed to sites of vascular injury, incorporated into the vessel wall and accelerated RE. Subsequently, Werner and colleagues confirmed that EPC administration alone could yield therapeutic effects by injecting labeled spleen-derived EPCs into mice who had undergone femoral artery injury.(171) In this model, cells homed to the arterial site of injury rapidly leading to RE and ultimately a reduction in NI formation – providing the first evidence that EPCs could serve as a cell-based therapeutic for reduction in NI formation. Subsequently, numerous therapies which mobilize EPCs, such as estrogens,(150) granulocyte colony stimulating factor,(87) and leptin(137) have all been demonstrated to increase EPC number and enhance arterial repair in models of vascular injury. Thus, these studies have provided strong mechanistic evidence that EPCs, either through increased number or improved function, may enhance arterial homeostasis following injury.

In addition to animal studies, there are a number of clinical studies that have supported the link between EPCs and ISR. First, vascular injury by PCI is known to mobilize EPCs into the circulation (**Appendix VI**)(11,36,48,64) with numerous studies linking the degree of mobilization to clinical ISR.(74,118,136) Furthermore, in addition to absolute EPC number, mobilization of EPCs with reduced adhesive capacities has been shown to be predictive of stent renarrowing.(52,62) Indeed, in a study by our group, cultured EPCs

were reduced by 50% in patients who developed ISR compared to patients in whom stents remained patent.(62) While there remain no studies investigating the systemic administration of EPCs on the incidence of ISR, there nonetheless remains both strong pre-clinical and clinical evidence suggesting improved EPC-mediated arterial repair may lead to less ISR. Hence, identifying therapies which enhance either EPC mobilization or EPC function may lead to clinically relevant treatments for the prevention of ISR.

1.4. Glycogen Synthase Kinase 3 β (GSK3 β) Inhibition and Progenitor Cells

GSK3 β is a ubiquitously expressed serine/threonine protein kinase which is negatively regulated by Wnt signaling. Under basal conditions, GSK3 β phosphorylates the nuclear transcription factor β -catenin (β cat) resulting in its proteosomal degradation (**Figure 1.5**). While originally described and named for its role in inactivating glycogen synthase(26), subsequent studies have identified GSK3 β as an important regulator in a number of pathological processes including cancer(95), Alzheimer's disease(72), and diabetes mellitus (DM).(38) More recently, GSK3 β has been garnering increasing attention as an important regulator of stem and progenitor cell self-renewal and mobilization – a finding with broad implications for progenitor cell-based therapies.

Trowbridge and colleagues first demonstrated the ability of GSK3 β inhibition to regulate both *in vivo* and *in vitro* hematopoietic stem cell (HSC) repopulation.(162) Following transplantation of either human or mouse HSC expressing green-fluorescent protein (GFP+) into nonobese diabetic-severe combined immunodeficient mice, GSK3 β

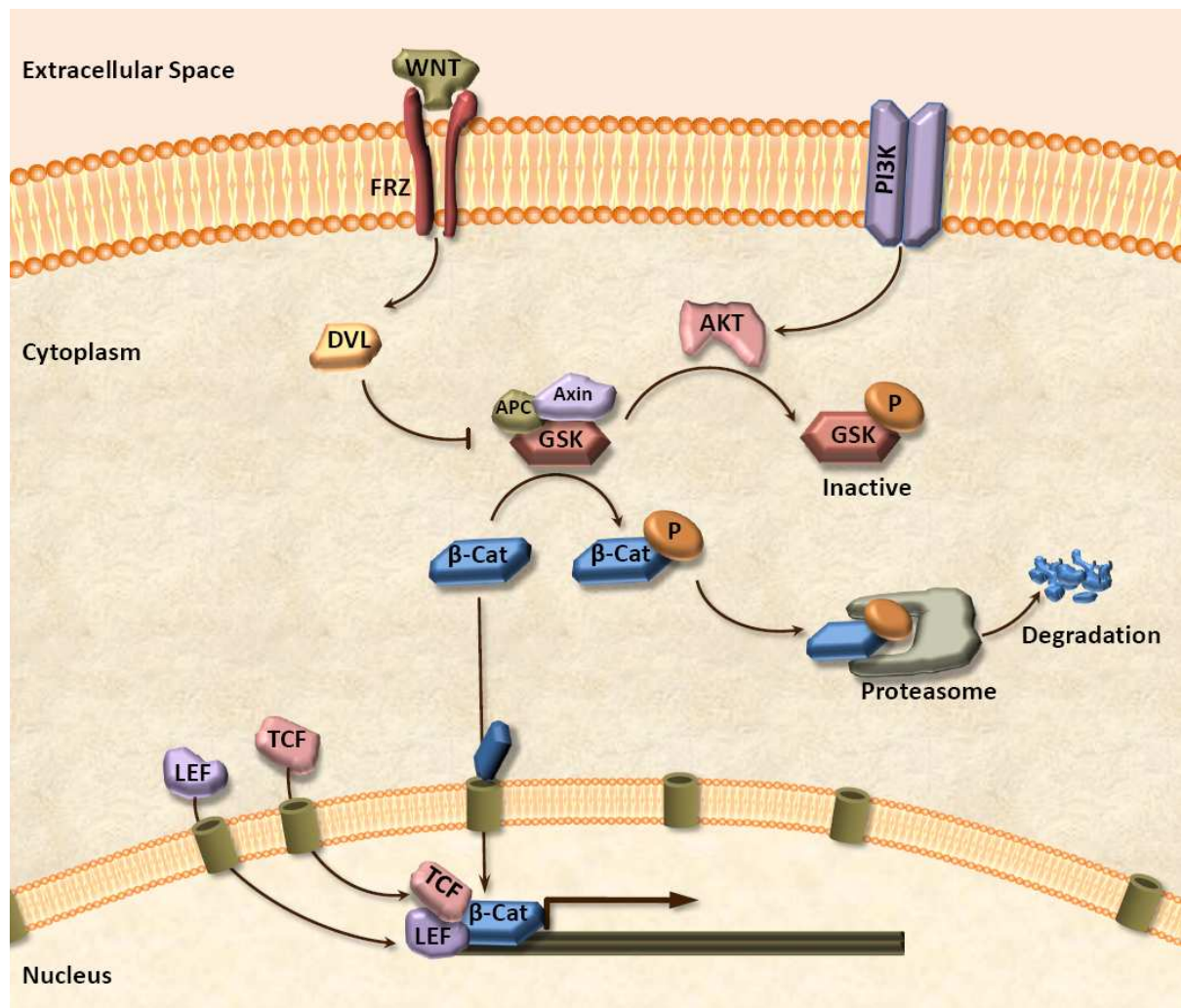


Figure 1.5. Glycogen synthase kinase 3 Signaling Pathway. Binding of WNT to frizzled (FRZ) receptor activates disheveled (DVL) which inactivates a complex consisting of adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase 3-beta (GSK). This complex results in activation of GSK-3 which can be inactivated when phosphorylated by AKT. The activated GSK complex is responsible for phosphorylating beta-catenin (β cat) resulting in its ubiquitination and ultimate degradation by proteasomes. Conversely, un-phosphorylated β -catenin translocates to the nucleus where, in combination with transcription factors (LEF and TCF), it leads to the transcription of a number of gene products.

inhibition resulted in more rapid expansion and recovery of hematopoietic cell lines. Moreover, inhibitor treatment resulted in regulation of multiple gene targets known to be regulated by Wnt, Hedgehog, and Notch pathways. These findings have been corroborated in numerous types of stem and progenitor populations with GSK3 β inhibition promoting stem cell pluripotency(85,176), proliferation and differentiation.(106,182) Moreover, although a number of downstream targets are regulated by GSK3 β , a number of recent studies have demonstrated the necessity of β cat in causing downstream effects.(88,176) Thus, evidence linking the importance of GSK3 β as a conserved regulator of progenitor cell function in numerous cell populations led to the hypothesis that inhibition of this enzyme may lead to improved function in EPC based therapies.

Choi and colleagues performed the first studies of GSK3 β inhibition and it's effects on therapeutic angiogenesis achieved with EPC-based therapy.(22) In this study, EPCs were transduced with a dominant negative GSK3 β mutant which led to an expected increase in nuclear β cat levels. Interestingly, transfected EPCs generated greater vascular networks *in vitro* and when transplanted into ischemic hind limbs of CD1 nude mice led to greater perfusion, capillary density, and ultimately greater limb salvage. While compelling, this study had several limitations for cell-based therapy, namely the use of cells from healthy volunteers *and* the use of viral transfection of cells to achieve GSK3 β inhibition which may have negative safety implications for therapeutic administration of cells.(35) Nonetheless, this study provided the first evidence that GSK3 β may be a target for improving therapeutic effect of cell based therapy with EPCs.

1.5 Statement of Objective and Hypotheses

Although PCI with stent implantation represents a significant advancement in revascularizing patients with obstructive CAD, ISR and impaired RE at the site of stent implantation remains a major limitation in treating patients. Thus, the current thesis starts at the bedside with an important clinical problem for which our current understanding and treatment options remain limited. Accordingly, the overall **objective** of this thesis was to determine mechanisms by which EPC-mediated repair could be enhanced in order to improve arterial homeostasis after stent implantation. To achieve this objective we tested the following hypotheses:

- 1) Inhibition of GSK3 β by small molecules can enhance EPC yield *in vitro* and improve arterial repair *in vivo* following mechanical injury.
- 2) Local delivery of small molecule GSKi using drug-eluting stents can improve arterial healing via augmented EPC-mediated RE.
- 3) GSK3 β dysregulation contributes to decreased EPC number and function in patients with DM.

Herein data from both *in vitro* studies using primary human cells and *in vivo* experiments using validated pre-clinical models will demonstrate that GSK3 β antagonism is an effective cell enhancement strategy for autologous cell-based therapy with EPCs from high risk patients such as CAD or DM. Moreover, data presented confirms that mechanistically, increased paracrine activity through vascular endothelial growth factor (VEGF) secretion, improved cell adhesion through up-regulation of very late antigen 4 (VLA4) and reductions in apoptosis achieved by increased cathepsin B (CatB) activity contribute to improved EPC arterial repair achieved with GSKi.

Chapter 2: Inhibition of Endothelial Progenitor Cell GSK 3 β Results in Attenuated Neointima Formation and Enhanced Re-endothelialization after Arterial Injury

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2.1 Abstract

Aims: Endothelial progenitor cells (EPCs) are circulating pluripotent vascular cells capable of enhancing re-endothelialization and diminishing neointima formation following arterial injury. Glycogen synthase kinase 3 β (GSK) is a protein kinase that has been implicated in regulation of progenitor cell biology. We hypothesized that EPC abundance and function could be enhanced with the use of an inhibitor of GSK 3 β (GSKi) thereby resulting in improved arterial repair.

Methods: EPCs were expanded *ex vivo* and treated with a specific GSKi then assessed for both yield and functional characteristics by *in vitro* assays for adherence, apoptosis, and survival. *In vivo* functionality of treated human EPCs was assessed in immune tolerant mice subjected to femoral artery wire injury. Re-endothelialization was assessed at 72 hours and neointima formation at 7 and 14 days following injury.

Results: GSKi treatment resulted in an improvement in the yield of EPCs and a reduction in apoptosis in cells derived from both healthy controls and patients with coronary artery disease. Treatment also increased vascular endothelial growth-factor secretion, up-regulated expression of mRNA for the α -4 integrin subunit, and improved adhesion, an effect which could be abrogated with a α -4 integrin blocking antibody. EPCs without or with *ex vivo* GSKi treatment enhanced re-endothelialization 72 hours following injury as well as reduced neointima formation at 7 days (e.g., endothelial coverage: $7.2 \pm 1.7\%$ vs. $70.7 \pm 5.8\%$ vs. $87.2 \pm 4.1\%$; intima to media ratios: 1.05 ± 0.19 vs. 0.39 ± 0.08 vs. 0.14 ± 0.02 ; $p < 0.05$ for all comparisons) an effect persistent at 14 days.

Conclusions: GSKi improves the functional profile of EPCs and is associated with improved re-endothelialization and reduced neointima formation following injury.

2.2 Introduction

Injury to the vascular endothelium is thought to be the initiating process in development of atherosclerotic disease(127) and has also been implicated in the pathogenesis of other vascular disorders such as transplant vasculopathy and neointimal hyperplasia following balloon angioplasty.(114) Data from observational clinical studies have noted an association between circulating numbers of endothelial progenitor cells (EPCs) and both the presence of coronary artery disease(68) as well as the likelihood of future cardiovascular events.(172) These findings, as well as evidence in both animal(171) and clinical studies(133) of improved outcomes through delivery of isolated populations of progenitor cells have reinforced the importance of re-establishing endothelial integrity following injury as a key step in prevention of vascular disease.

Circulating progenitor cell populations - specifically EPCs - play a key role in arterial repair following injury.(181) For example, statins,(170) granulocyte colony stimulating factor,(87) and direct transplantation of *ex vivo* cultured EPCs(171) improve cell-mediated repair and ultimately reduce neointima formation in animal models of arterial injury. Moreover, stents that effectively “capture” EPCs by means of CD34 antibodies(4) loaded on stent struts demonstrate enhanced re-endothelialization and safety in preliminary human trials.(25) While transplantation of a patient’s progenitor cells is a conceptually attractive strategy for enhancing vessel re-endothelialization it is important to note that EPCs from patients with coronary artery disease (CAD) are typically low in abundance and show attenuated functional properties compared to cells from healthy controls.(61) Ultimately, pharmacologic regulation of these endogenous cell populations

represents the most likely manner in which EPC biology will be exploited for clinical benefit.

Glycogen synthase kinase 3 β (GSK) is a serine/threonine protein kinase known to negatively regulate Wnt signaling through phosphorylation of the nuclear transcription factor β -catenin and hence, direct its degradation.(180) Recently, GSK inhibition has been shown to stimulate progenitor and hematopoietic stem cell capacity *in vivo* through modulation of Wnt, Hedgehog and Notch signaling.(162) Wnt signaling is also known to play a key role in the mobilization of vascular progenitor cells and enhancement of neovascularization, as Aicher and colleagues demonstrated using the Wnt signaling antagonist Dickkopf-1.(2) However, to date little is known about the effects of GSK signaling on EPC function in CAD patients or intima development following arterial injury. The purpose of the current study was to determine if a specific inhibitor of GSK (GSKi) could augment the *in vitro* yield and functions of human EPCs as well as improve arterial repair *in vivo*.

2.3 Materials and Methods

2.3.1 EPC Culture and Cell Labeling

Human EPCs derived from normal controls and patients with established CAD were cultured in the usual manner.(37,62,71) Patients with CAD had to have established disease as defined by previous MI, flow-limiting stenoses requiring stent insertion or surgical revascularization. All patients were on medical therapy at time of EPC collection including maximally titrated statin, aspirin, beta-blocker and ace inhibitor or angiotensin

receptor blocker therapy. Blood was collected by venipuncture and anticoagulated with EDTA. Peripheral blood mononuclear cells (5×10^6) isolated by ficoll centrifugation were cultured in EGM-2 media (Cambrex) and plated on fibronectin-coated plates. Inhibition of GSK was achieved using a specific GSK inhibitor N-(4-Methoxybenzyl)-N'-(5-nitro-1,3-thiazol-2-yl) urea (referred to as GSKi, Calbiochem) or LiCl (Sigma) at the indicated doses. Confirmation of effective GSK inhibition was achieved by western blot.

Adherent cells were maintained for 7 days prior to enumeration. For the task of enumeration, EPCs were defined as cells dually positive for AcLDL uptake and *ulex europaeus* agglutinin I (UEAI) binding. DiI-AcLDL (2.5 $\mu\text{g/ml}$, Molecular Probes) was incubated with cultured EPCs for 1 hour in a cell incubator. Subsequently, cells were washed and fixed with Cytofix Buffer (BD) and incubated with FITC-UEAI (5 $\mu\text{g/ml}$, Sigma) for 30 minutes. Plates of cells were again washed, and incubated with a DAPI nuclear counterstain before a coverslip was applied to the well and double positive cells were counted in 6 random high power fields ($\times 200$ magnification).

2.3.2 Western Blots

β -catenin nuclear levels were assayed by western blot of nuclear extracts from both control and treated EPCs. Briefly, nuclei were separated by differential centrifugation and total nuclear protein was extracted. Protein was then run on an acrylamide gel and transferred onto a PVDF membrane overnight. After transfer, the membrane was blocked for one hour in 5% skim milk powder in 0.1% Tween-20 in Tris buffered saline, and then incubated for 48 hours with primary antibody of a monoclonal anti- β -catenin (Cell

Signaling Technologies) diluted 1:1000 in 5% skim milk powder in TBS-T. After incubation the membrane was washed 3 times for 5 minutes each with TBS-T. The membrane was then incubated in secondary antibody of goat anti-rabbit IgG (H+L) conjugated with horseradish peroxidase (1:5000 in TBS-T) for 24 hours before being washed and quantitated using ECL Plus (Amersham Biosciences).

2.3.3 EPC survival, apoptosis, VEGF secretion, and adherence.

Seven day old EPCs were used for all experiments unless otherwise indicated. For the cell survival assay, six high power fields were enumerated for each individual.

Subsequently, cells were washed and the media changed every four days after which cells were again enumerated. Survival was expressed as a percentage of cells present on day 7.

For apoptosis studies, cells from patients and healthy controls were cultured then lifted and recoated at a density of 2×10^6 mature EPCs per well. The cells were then allowed to incubate for an additional four days prior to being lifted with EDTA supplementation of the media and gentle agitation. Cells were pelleted and resuspended in hank's balanced salt solution (HBSS). Prior to analysis by flow cytometry, 10 μ L of propidium iodide was added to the cell suspension. EPCs were identified by uptake of acLDL-alexa488

(Invitrogen) and UEA-1 FITC labeling in separate experiments. Inability to exclude propidium iodide identified apoptotic cells. A total of ten thousand events were analyzed and the data expressed as a percentage of total EPCs being apoptotic. All experiments were conducted on a Beckman Coulter Cytomics FC 500 cytometer.

The secretion of vascular endothelial growth factor (VEGF) by EPCs was measured using a VEGF ELISA kit (R&D Systems) using the manufacturer's provided protocol. Briefly, EPCs were plated in equal numbers and incubated in VEGF-free EGM-2 for 24 hrs. Subsequently, 200 μ L of the culture supernatant was added to a 96 well plate coated with anti-human VEGF antibody. After 2 hours of incubation, the conjugated secondary antibody was added and allowed to incubate for another 2 hours. Substrate solution was added and the wells interrogated for absorption at 450 nm using a Bio-Rad micro-plate reader.

For adherence studies, EPCs were detached from their fibronectin-coated plates by incubation with 0.5 mmol EDTA. Cells were pelleted, resuspended in EGM-2 and enumerated. Subsequently, 100,000 EPCs were plated in 24-well fibronectin-coated plates and incubated for 30 minutes before being washed three times with HBSS and adherent cells enumerated in six random high power fields. Blocking experiments were performed using a specific antibody to the α -4 integrin subunit (MAB16983, Millipore) at a concentration of 10 μ g/mL and allowing cells to incubate for 2 hours with the antibody.

Quantitative PCR was performed on the Light Cycler Q-PCR System (Roche) and the data were analyzed using the accompanying software package. Total RNA was isolated from EPCs using Trizol (Invitrogen) and RT performed using standard techniques. Amplicons were cloned into the pGEM-T vector, sequenced, and isolated using the PhasePrep BAC DNA kit (Sigma). The plasmids were then linearized, purified, and

diluted to generate standard curves for quantitative PCR analysis. Primers and probes were designed using the PrimerQuest software and are as follows:

GAPDHfwd: CGCCTGGAGAAAGCTGCTAAGTAT,

GAPDH rev: GCTTCACAAAGTGGTCATTGAGGG,

α -1fwd: ACAAGTGACAGCGAAGAACCTCCT

α -1rev: TGGGTACAGCACAGGGTAACCATT,

α -2fwd: ACTTTATCTCCAGCGGTACAAAGT,

α -2rev: TGGGCCTTATCCCAATCTGACCAA,

α -3fwd: CAAAGACAGGCAAACGGCAACGTA,

α -3rev: TTATTGGTCGCGGTGAGAAGCCTA,

α -4fwd: AGGGCAAGGAAGTTCCAGGTTACA,

α -4rev: ACATGAGGACCAAGGTGGTAAGCA,

α -4probe: AGCATTTATGCGGAAAGATGTGCGGG

α -5fwd: TGCCTGAGTCCTCCCAATTTTCAGA,

α -5rev: ACATGAGGACCAAGGTGGTAAGCA.

PCR was performed with an annealing temperature of 56 degrees for all primer combinations. The QPCR reagents utilized were the QuantiTect SYBR PCR system and the QuantiTect Probe PCR system (Qiagen). All primer combinations were confirmed to have a single amplicon on agarose gel and SYBR green PCR was utilized for quantification of alpha integrins 1-5 and GAPDH. Confirmation of α -4 integrin mRNA upregulation was done using a probe-specific quantitative PCR technique. The α -4fwd and α -4rev primers were used in conjunction with a 5' 6-FAM labeled and 3' TAMRA

modified α -4probe. For these experiments, Qiagen QuantiTect Probe PCR system was utilized.

2.3.4 CD-1 Nude mouse femoral artery wire injury model

CD-1 nude mice were acquired from Charles River Laboratories and acclimatized in our facilities for 2-6 weeks prior to surgeries. To assess the ability of *ex vivo*-treated EPCs to enhance arterial repair, the femoral artery of the mice were injured by insertion of a 32 gauge blunt needle (Strategic Applications Inc) to induce neointima formation as previously described.⁽¹³¹⁾ Following arterial incision the needle was introduced into the lumen, advanced proximally and passed five times in order to denude endothelium and mechanically stretch the vessel. Subsequently, 5×10^5 of control-EPCs (C-EPCs), GSKi-treated EPCs (G-EPCs) or vehicle (n=6 per group) were injected and the artery ligated proximal to the incision site. Arterial injuries were performed proximal to major branches and the femoral artery was ligated distal to these branches. Hence, blood flow was maintained in the main arterial segment due to run-off via the intact branch vessels. None of the animals exhibited signs of ischemia to their hind limbs and all animals had full use of the limb immediately post surgery. General anesthesia was achieved during surgery using isoflurane.

For assessment of re-endothelialization, mice were sacrificed at 24 (n=2), 48 (n=2) and 72 hours (n=6) post injury. Mice sacrificed at 24 and 48 hours had enface preparation and staining with DAPI and were examined by fluorescent microscopy. To permit quantification of re-endothelialization, mice were perfused with 0.5% Evans blue, then

perfusion-fixed with formaldehyde until clear of dye. For assessment of neointimal formation, mice were sacrificed at 7 and 14 days (n=6 per time point). Femoral arteries were fixed in buffered formalin then dehydrated with ethanol. Arteries were mounted in paraffin blocks and sectioned in 5 μ m sections. Sections were hematoxylin and eosin (H&E)-stained and analysis performed using a computer-assisted digital imaging system (Image-Pro Plus, Media Cybernetics).

2.3.5 Ethics and Statistics

Animal procedures followed the University of Ottawa Animal Care Committee and the Canadian Council on Animal Care guidelines. All protocols involving human donors were approved by the Ottawa Heart Institute Research Ethics Committee. These studies conform with the Declaration of Helsinki for the use of human tissue and animal experiments conformed with the Guide for the Care and Use of Laboratory Animals. For statistical procedures a p-value less than 0.05 was considered significant. Analyses were performed using the Sigmastat 3.5 package. Two-way comparisons were performed with a student's t-test and multiple comparisons using one-way ANOVAs with Holm-Sidak *post-hoc* test. Data are expressed as means $\pm$ standard error of the mean.

2.4 Results

2.4.1 GSK-3 β Inhibition Improves EPC Yield

Given the paucity of EPCs found in patients with CAD, we first tested the effects of GSKi treatment on EPC yield *in vitro* after seven days. Outgrowth of EPCs was reproducibly achieved in all subjects and the phenotype confirmed using the commonly

accepted parameters of acLDL uptake and *UEA-1* binding (**Figure 2.1A&B**).⁽⁷¹⁾ Media was supplemented with either 104 nM (IC₅₀, 1×) or 208 nM (2×) GSKi. In order to confirm the specificity of the GSKi, nuclear β -catenin levels were assessed by western blotting. Under control (untreated) conditions GSK-3 β phosphorylates β -catenin thereby targeting it for degradation. However with GSKi treatment the degradation of β -catenin is interrupted thereby resulting in increased nuclear levels of the protein (**Figure 2.1C**). Compared to control treatment media supplemented with GSKi at both 1× and 2× concentrations led to a progressive increase in the yield of EPCs derived from healthy controls (48.5±4.6 vs. 68.6±6.6 vs. 75.7±6.8; respectively) and more than quadrupled the yield of CAD EPCs (14.0±8.0 vs. 41.7±17.0 vs. 58.5±15.8;**Figure 2.1D**). To confirm that the observed effects on EPC yield were specific to GSK-3 β inhibition, EPCs were cultured with varying doses of a second GSK-3 β inhibitor, Lithium Chloride (LiCl; **Figure 2.2**). Similar to GSKi, LiCl augmented the yield of EPCs derived from both healthy controls and CAD patients in a dose-dependent manner. Combination treatment with GSKi and LiCl did not result in further improvement in EPC yield (**Figure 2.1E**), thereby suggesting that the increase in EPC number was achieved through a specific inhibitory effect on the GSK-3 β isoform that was maximal with one or the other treatment and not altered in a synergistic manner due to an off-target effect of one of the drugs.

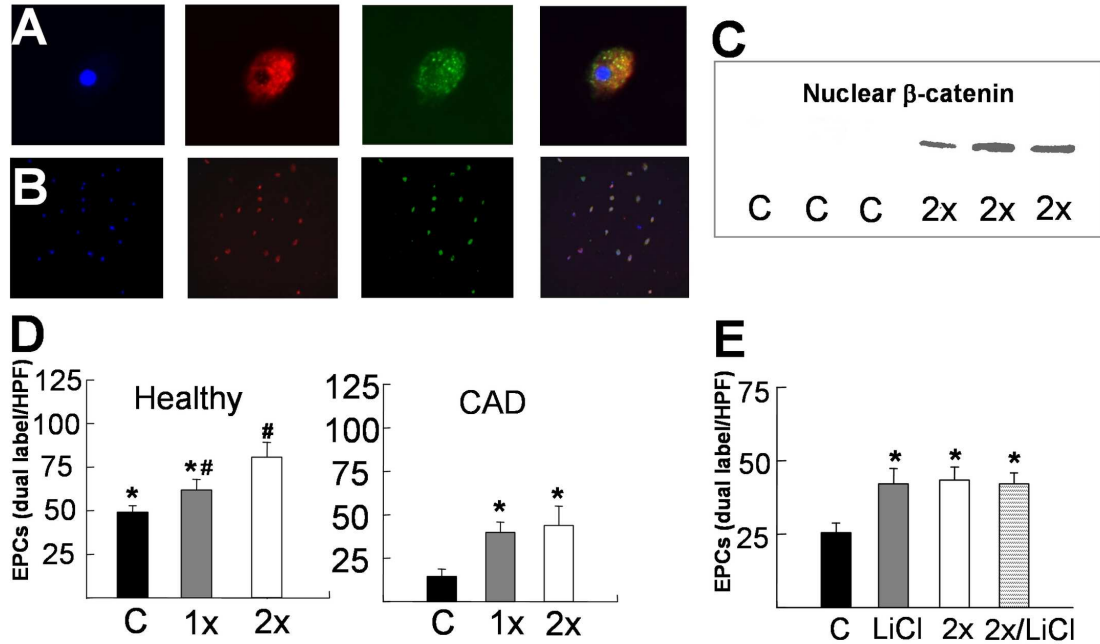


Figure 2.1. GSK 3 β inhibitor improves attenuated levels of EPCs from patients with CAD. **A,B** - High and low power magnification of EPCs at 7 days labeled with DAPI (blue), AcLDL-DiI (Red), UEA-1-FITC (green), and merged image used for enumeration. **C** - Western blot of nuclear fraction from EPCs cultured in control (C) media and media supplemented with GSKi (2x) showing increased levels of β -catenin. **D** - Comparison of EPC yields in both healthy controls and patients with CAD when treated with control (C) media, the 104 nM (1x) and 208 nM GSKi (2x), n=6. **E** - Dual treatment of EPCs with both GSKi and LiCl does not synergistically improve EPC yields, n=6. * or # denote statistical significance, p<0.05.

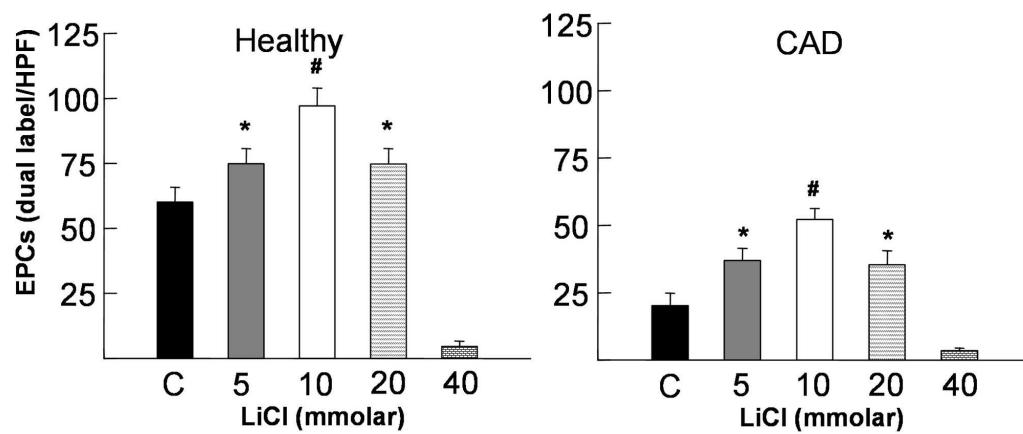


Figure 2.2. GSK-3 β inhibition with LiCl increases the yield of EPCs derived from both healthy controls and CAD patients. n=6. * or # denote statistical significance, p<0.05 or less.

Given the known role of GSK in the regulation of apoptosis, we hypothesized that, in part, the improved EPC yields with GSKi may be linked to lower levels of apoptosis.(68) To test this hypothesis we cultured EPCs for 7 days, replated them at equal densities then treated them with either control EGM-2 media or media supplemented with 2× GSKi. EPCs from CAD patients had a faster rate of attrition than EPCs from healthy control subjects (**Figure 2.3A**). GSKi treatment markedly improved EPC survival for both cell populations. In fact, EPCs from CAD patients survived to the same extent as EPCs from control subjects when treated with GSKi. At baseline, EPCs from CAD patients demonstrated a higher rate of apoptosis compared to EPCs from controls (**Figure 2.3B**). In both groups, 2× GSKi caused a reduction in the percentage of apoptotic cells - a result which may explain the improved survival observed in GSKi-treated cells.

2.4.2 GSK-3 β Inhibition Improves the Functional Profile of EPCs

Clinical studies suggest that not only the abundance of EPCs but also the functional capacity determines the *in vivo* biological effects transplanted cells.(139) Hence, we tested the ability of GSKi to affect the secretion of a relevant endothelial growth factor, VEGF. Equal numbers of EPCs were incubated for 24 hours in VEGF-free media before VEGF levels were measured in the media by ELISA. There was a dose-dependent effect of GSKi on VEGF secretion by EPCs, with the 2× dose of GSKi resulting in a more than 6× increase over control conditions (**Figure 2.3C**).

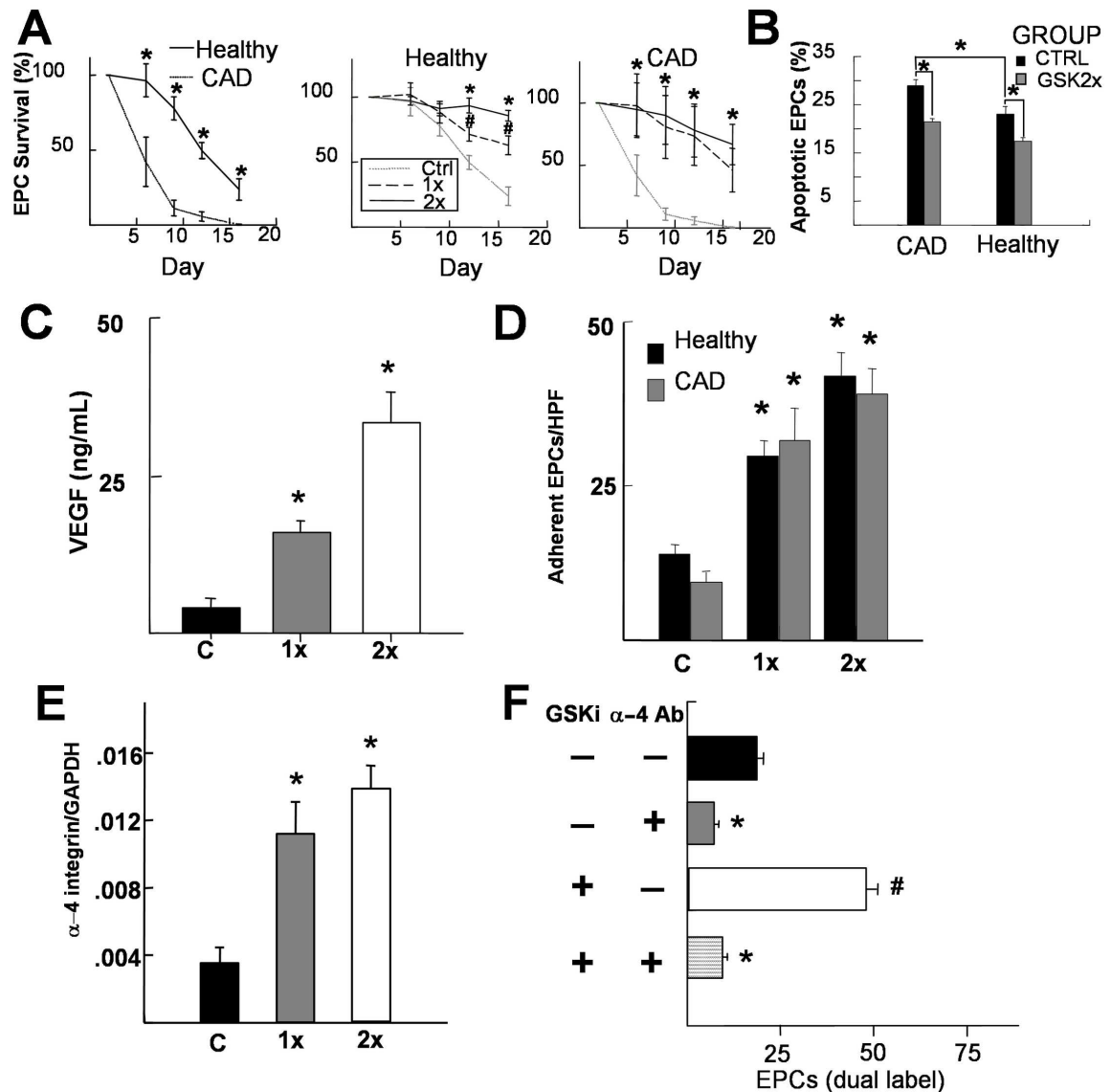


Figure 2.3. GSKi Enhances EPC Survival and Adherence. **A** - EPC survival is impaired in patients with CAD. Treatment with GSKi in both healthy controls and patients with CAD significantly improves long term viability, n=6. **B** - GSKi decreases apoptosis in both EPCs derived from healthy controls and patients with CAD. **C** - VEGF secretion by EPCs is enhanced by GSKi in a dose dependant manner. **D** - GSKi improves adhesive properties of EPCs derived from both healthy controls and CAD patients, n=12. **E** - GSKi treatment upregulates mRNA of the α -4 integrin isoform as measured by Q-PCR, n=6. **F** - Introduction of a specific α -4 integrin subunit blocking antibody (α -4 Ab) demonstrates the reversibility of improved EPC adhesion and implicates α -4 in EPC adhesion, n=6. * or # represent significant differences p<0.05.

Previously, our group and others reported that EPCs are incorporated into healing arteries post-injury (e.g., after stent implantation).(52,62,65) As the steps that lead to progenitor cell homing and adhesion into the vessel wall are incompletely understood we began by characterizing the adhesive characteristics of EPCs to fibronectin. Adherence to fibronectin was similar for control and CAD EPCs. However, treatment with GSKi resulted in an approximate 4-fold increase cell adherence for both populations of cells (**Figure 2.3D**). Next we determined if GSKi treatment upregulated the expression of one or more of α -integrin subunits as this integrin family of cell surface receptors plays a key role in vascular cell adhesion. We cloned α -integrin subunits 1 through 5 as well as the GAPDH gene from human PBMCs and performed SYBR green quantitative PCR on mRNA from control, GSKi 1 $\times$, and GSKi 2 $\times$ treated EPC samples. Levels of mRNA for the α -1, α -2, α -3 and α -5 integrin subunits did not change with GSKi treatment (data not shown). In contrast, the α -4 integrin subunit was upregulated with both GSKi concentrations (**Figure 2.3E**). To confirm that the observed increase in adhesion was mediated via α -4 regulation, we tested the ability of an α -4 integrin subunit blocking antibody to impair EPC adhesion in a fibronectin assay (**Figure 2.3F**). As observed in the previous experiments, addition of GSKi resulted in an increased number of adherent cells. However, this effect was completely abrogated by pretreatment of the cultured cells with the α -4 integrin subunit blocking antibody – thereby suggesting that the observed increase in EPC adhesion with GSKi treatment is mediated via upregulated expression of the α -4 integrin subunit.

2.4.3 GSK-3 β Inhibition Enhances EPC Mediated Arterial Repair

Werner *et al.*(171) first described the ability of *ex vivo* cultured EPCs to mediate arterial repair *in vivo*. We hypothesized that the enhanced *ex vivo* function achieved by GSK inhibition would translate into improved *in vivo* function. To test this hypothesis vehicle, C-EPCs or G-EPCs were systemically injected into immune compromised mice subjected to femoral artery injury. Notably, cells from CAD patients were used for these experiments despite the fact that at baseline these cells are less abundant and functionally deficient compared to cells from healthy control subjects. Re-endothelialization was assessed by enface examination at 24, 48 and 72 hours post blunt needle injury. At 24 hours post injury there was near complete disruption of the endothelium with only few cells overlying the media (**Figure 2.4A**). By 48 hours post-injury re-endothelialization was evident, particularly in the cell treatment groups. Thus, a 72 hour time point was selected for quantitative assessment of the re-endothelialization area of arteries perfused with Evan's blue solution (**Figure 2.4B**). Treatment with C-EPCs alone resulted in a ten-fold increase of re-endothelialization area, an effect that was further enhanced by EPCs pretreated with GSKi (e.g., vehicle: $7.2 \pm 1.7\%$, C-EPCs: $70.7 \pm 5.8\%$ and G-EPCs: $87.2 \pm 4.1\%$; $p < 0.05$, **Figure 2.4C**). These findings suggest a marked enhancement of endothelial regeneration by EPC transplantation - a benefit which was further augmented through abrogation of GSK 3 β / β -catenin signaling.

To assess neointima formation in these mice euthanasia was carried out seven and fourteen days after blunt injury (**Figure 2.5A**). Compared to sham (uninjured) arteries, all of the injured arteries in each groups was nearly 3 times larger due to the uniform

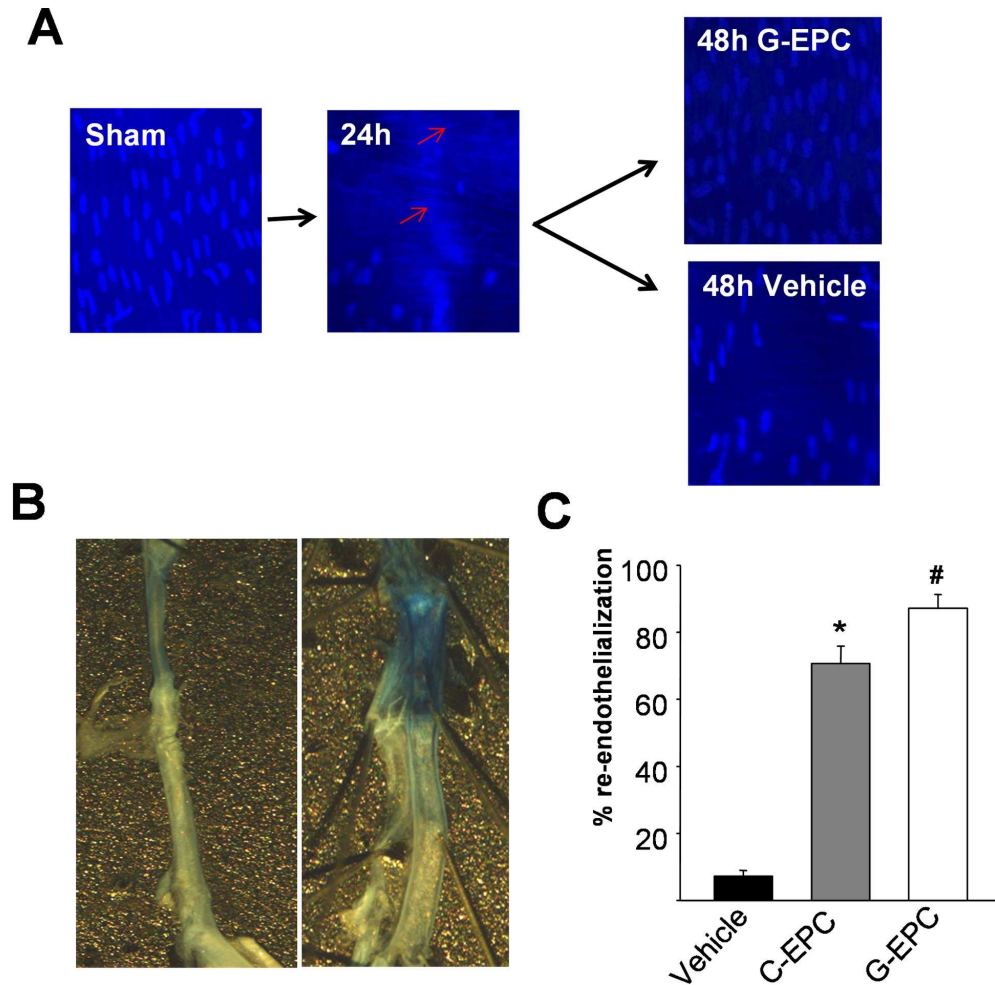


Figure 2.4. Rapid re-endothelialization of injured arteries is promoted by EPC transfusion and further enhanced by GSKi pretreatment. **A** - Wire injury results in significant denudation of endothelium by 24 hours exposing underlying smooth muscle cells (red arrows). EPC infusion results in nearly complete re-endothelialization by 48 hours when compared to vehicle treated arteries. **B** - Intact and enface isolated femoral arteries. Mice were perfused with Evans blue prior to dissection. Intact endothelium excludes the blue dye. **C** - Percentage of arterial re-endothelialization as assessed by Evans blue perfused arteries, n=5 for vehicle and n=6 for C-EPC and G-EPC groups. * or # denote statistical significance, $p < 0.05$ or less.

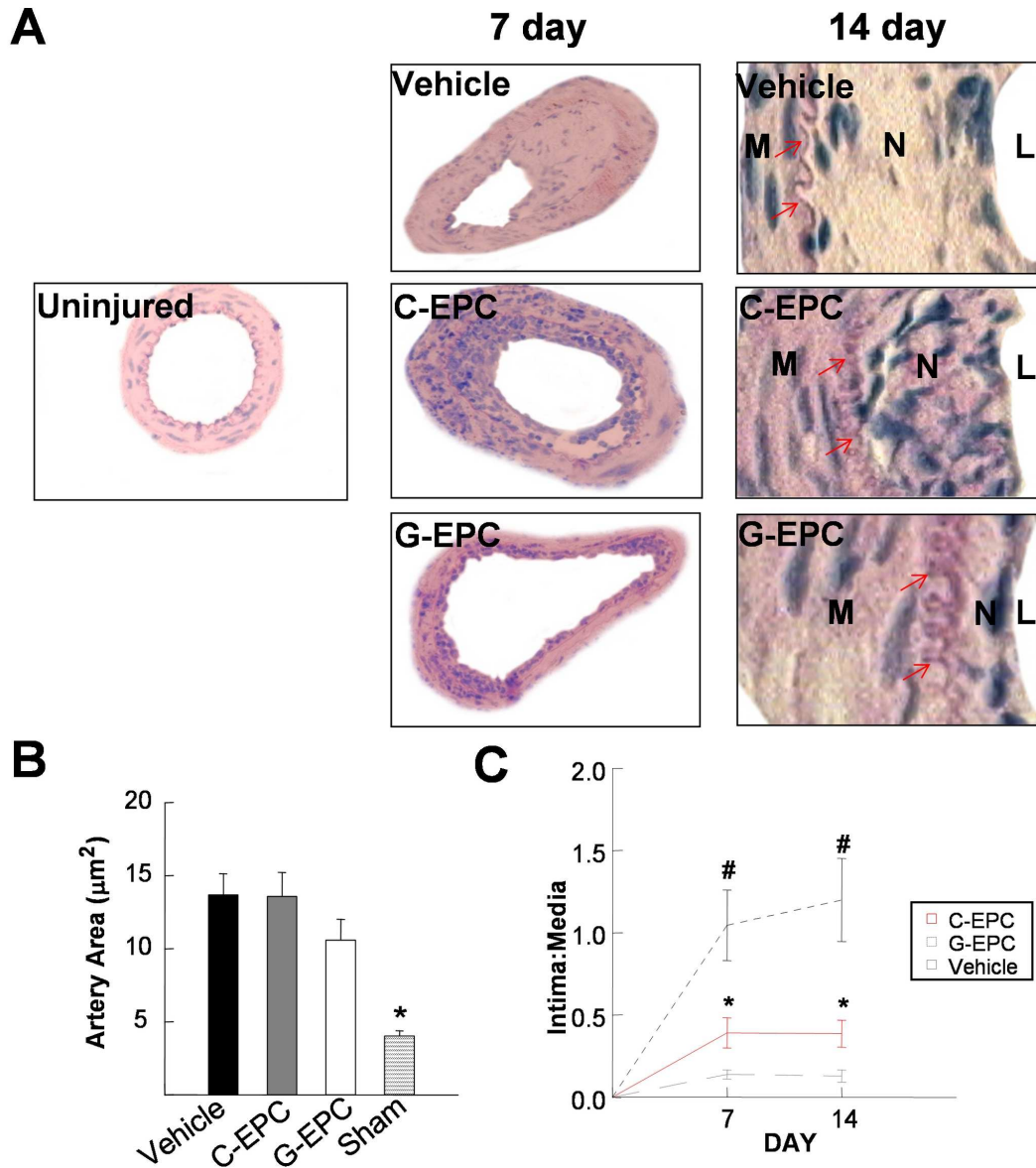


Figure 2.5. Mouse femoral artery wire injury model of neointima formation. **A** - Wire injury results in significant intima formation at both 7 and 14 days. Representative 7 day cross-sections at low magnification and 14 day high power cross-sections are shown with hematoxylin and eosin staining. Media (m), neointima (n), and lumen (l) are labeled for reference. Arrows indicate the internal elastic lamina. **B** - Wire injury results in significant increase in artery volume compared to sham. No significant differences exist between the vehicle group, the control EPCs (C-EPC) and the GSKi treated EPC (G-EPC) groups, n=6. **C** - Transplantation of C-EPCs reduces both 7 and 14 day intima to media ratios (I:M) an effect further enhanced by pretreatment of EPCs with GSKi. No significant differences in intima/media ratios were seen between 7 and 14 days. * or # denote statistical significance, $p < 0.05$ or less.

(mechanical) dilatation that resulted from the intra-luminal passage of the blunt needle (**Figure 2.5B**). Assessment of intimal area was adjusted for media area, and expressed as the intima to media ratio (I:M). At seven days the I:M was attenuated by C-EPCs, and reduced to less than one sixth of controls by infusion with G-EPCs (e.g., vehicle: 1.05 ± 0.19 , C-EPCs: 0.39 ± 0.08 , G-EPCs: 0.14 ± 0.02 ; $p < 0.05$ for all comparisons; **Figure 2.5C**). Intimal lesions showed a marked increase in cellularity in both groups of mice that received EPC infusions, but the intima of the vehicle-treated mice consisted primarily of extracellular matrix. The comparison of the I:Ms 14 days post-injury was similar to that performed on the data recorded at 7 days post-injury (e.g., vehicle: 1.20 ± 0.23 , C-EPCs: 0.39 ± 0.07 and G-EPCs: 0.13 ± 0.03 ; $p < 0.05$ for all comparisons).

2.5 Discussion

Relative to healthy controls, patients with CAD have a paucity of circulating EPCs – cells that are now thought to be of central importance for the maintenance of vessel wall homeostasis.(68,139) Hence, it is postulated that increasing EPC number and function may facilitate arterial repair – particularly the restoration of the endothelium. However, it is now evident that the functional properties of EPCs are of at least equal importance to their abundance. In this study we describe a pharmacological strategy of inhibiting GSK that results in an expansion and functional enhancement of EPCs *in vitro* as well as amelioration of vascular healing *in vivo*. Originally, GSK was identified as a kinase that phosphorylates glycogen synthase, however subsequent studies identified a broader range of substrates. Indeed, when GSK 3β is inactivated β -catenin levels rise. β -catenin is the

principal mediator of Wnts, a family of secreted glycoproteins that regulate a multitude of cell processes, including haematopoiesis and stem cell function.

In the current study we begin by showing *in vitro* that GSKi enhances survival and reduces apoptosis of EPCs. As well, GSKi upregulates expression of the α -4 integrin subunit mRNA and enhances EPC adhesion in a manner that can be inhibited by means of an α -4 blocking antibody. *In vivo*, we note that GSKi-treated human EPCs attenuates neointima formation and enhances re-endothelialization of injured femoral arteries in immune tolerant mice. Although the reduction in the I:M may be due to a direct intimal effect, it is more likely that enhanced re-endothelialization was instrumental in producing this result. Nonetheless, this study is the first to demonstrate the importance of GSK signaling in EPC mediated arterial repair while also highlighting functional capacity of the cells as an important mediator of effect. Perhaps of greater importance, is the fact that GSKi-mediated augmentation of EPC number and function is possible in feeble cells harvested from patients with CAD – a hurdle that previously had not been adequately addressed or overcome.

Rapid restoration of the endothelium and its role as a protective barrier and/or regulator of the local milieu (e.g., fibrinolysis) is of critical importance in vascular repair. While the re-endothelialized area increased dramatically with infusion of just the C-EPCs (e.g., approximately 10-fold compared to vehicle treatment) there was an important additional enhancement in re-endothelialization area with G-EPC infusion (e.g., from 70.7% to 87.2%). Indeed, in a remarkably short period (72 hours) the arteries were almost

completely re-endothelialized with GSKi-treated EPCs. Perhaps if we had lowered the number of cells infused to less than 5×10^5 or if the caliber of the arteries were larger (e.g., larger animal or human coronary artery) the degree of re-endothelialization with the control-EPCs would have been smaller - and there might have been a higher incremental difference in the re-endothelialized area with the G-EPCs. Clinically, those patients in acute need of repaving their endothelium (e.g., acute coronary syndrome patients) are also the same individuals with remarkably low EPC counts; hence, their need for even a modest augmentation in potent EPC number and/or function will be dear – particularly in the first 24-72 hours after an acute ischemic event. Finally, it is attractive to speculate as to how GSKi may be modulating re-endothelialization. Certainly, GSKi-mediated prevention of EPC apoptosis and/or increased VEGF secretion may play key roles in the regrowth of the endothelium in these arteries. Previously, Kim *et al.* demonstrated that transduction of a dominant negative GSK3 β is protective against EC apoptosis and enhances endothelial cell migration towards VEGF.(83) As well, Choi and colleagues demonstrated an increase in VEGF secretion in EPCs transfected with a GSK 3 β dominant negative mutant.(22) Our data also suggests that GSKi facilitates EPC adhesion via an up-regulation of the α -4 integrin subunit. It is important to point out that the potentially important role of the α -4 integrin in enhancing arterial repair seen in the current study is somewhat at odds with our previous publication that demonstrated a reduction in stent neointimal formation when the α -4 integrin subunit was blocked using a systemically administered antibody.(99) However, the apparent difference in outcome for these two studies may be the result of the cells that were targeted. In the current study the effect is limited to EPCs treated *ex vivo* before being infused *in vivo*, whereas in our

previous study all cells, including monocytes and neutrophils were affected by the systemically delivered anti- α -4 blocking antibody.

Our study is not without potential limitations. First, while *ex vivo* GSKi treatment results in robust and durable reductions in lesion formation following arterial injury, the clinical feasibility of this strategy may be limited as transplantation of *ex vivo* manipulated cells can be labor intensive and impractical. Second, in the current study it is impossible to definitively ascribe all of the beneficial vascular healing effects to the infusion of G-EPCs as it is conceivable that the observed increase in re-endothelialization might be due to paracrine effects of GSKi-treated EPCs. For example, could these infused cells adhere at or near the site of re-endothelialization where they then secrete pro-endothelial cytokines, such as VEGF, and thereby attract adjacent (endogenous) endothelial cells that then repopulate the endothelium? In fact, while robust re-endothelialization is observed with G-CSF treatments that mobilize EPCs, there is little evidence to support the direct contribution of (tagged) bone marrow-derived EPCs to endothelial reconstitution.(179) Finally, inhibition of GSK is becoming an attractive target for vascular healing with evidence suggesting benefits in myocardial neovascularization following infarction in rats, in addition to our data implicating GSK signaling in arterial repair following injury.(177) However, given the known roles of GSK signaling in both energy metabolism and oncogenic transformation, careful animal studies of any systemically based therapies must be undertaken before clinical studies can be embarked upon.

In summary, our studies demonstrate that GSK 3 β signaling is a key modulator of progenitor cell mediated vessel wall homeostasis following mechanical injury – a novel observation with significant potential for therapeutic development. Specifically, inhibition of this enzyme enhances the yield of EPCs *in vitro* and promotes EPC-mediated arterial healing *in vivo*. Further studies with systemic and device-based delivery systems targeting the GSK-3 β signaling pathway to reduce intima formation are ongoing.

2.6 Funding

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2.7 Conflicts of Interest

None declared.

**Chapter 3: Delayed Re-Endothelialization with Rapamycin-coated Stents is Rescued
by the Addition of a Glycogen Synthase Kinase-3 β Inhibitor**

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3.1 Abstract

3.1.1 Aims

Drug eluting stents (DESs) reduce neointima area (NA) and in-stent restenosis but delay re-endothelialization (RE). Recently, we demonstrated that pharmacological expansion and functional enhancement of endothelial progenitor cells (EPCs) can be achieved by treatment with a glycogen synthase kinase 3-beta inhibitor (GSKi) - even for feeble cells derived from coronary artery disease (CAD) patients. GSKi treatment enhanced EPC adhesion via upregulated expression of the alpha-4 integrin, ameliorated RE and reduced neointima formation in denuded murine arteries. Hence, we hypothesized that GSKi-coated stents will enhance EPC adhesion and attenuate delayed vascular healing associated with rapamycin, a key DES agent.

3.1.2 Methods and Results

In vitro human EPCs adhered to stents coated with GSKi (GS) with affinities that were 2×, 14× and 13× greater than vehicle (VS), rapamycin (RS) and rapamycin plus GSKi (RGS) coated stents, respectively. Stents were inserted in rabbit carotid arteries and at 14 days NA was 45% and 49% lower in GS stents compared to bare metal (BMS) and VS stents. Moreover, RS stents had a 47% larger NA than GS stents, but RGS stents reduced NA to a level comparable to GS stents. Seven days after stenting GS stents displayed RE that was 40%, 33% and 42% greater than BMS, VS and RS respectively. Moreover, RGS stents had 41% more RE than RS stents. At 14 days the 7-day RE patterns persisted.

3.1.3 Conclusions

GSKi efficiently ameliorates the vascular response to stent implantation and has an important redeeming effect on the deleterious endothelial effects of rapamycin coated stents.

3.2 Introduction

Coronary artery stent implantation is annually used to treat >1 million patients with obstructive coronary artery disease (CAD), however, the infrequent yet important problems of in-stent restenosis (ISR) and stent thrombosis limit clinical efficacy. ISR is believed to be due to dysfunctional arterial healing involving primarily hyperplastic inflammatory pathways, and recently it has been shown that circulating progenitor cells may play a major role in stent neointima formation.(165,171,181) With the advent of drug-eluting stents (DESs) neointima growth and revascularization rates are lower compared to bare metal stents (BMSs).(141) However, recent clinical studies suggest that DESs delay re-endothelialization (RE) and, in some studies, appear to be accompanied by a higher prevalence of stent thrombosis.(78) Conversely, increased RE after stent implantation correlates with reduced neointima formation.(167,169)

Glycogen synthase kinase-3 β (GSK-3 β) is a ubiquitously expressed constitutively active serine/threonine kinase. While inhibition of GSK maintains self-renewal and enhances differentiating capacity of embryonic stem cells, GSK-3 inhibition also increases hematopoietic repopulation in recipients transplanted with mouse or human hematopoietic stem cells and enhances survival of endogenous stem cells after irradiation.(132,162) Indeed, inhibition of GSK-3 β by transduction of a catalytically

inactive GSK-3 gene into endothelial progenitor cells (EPCs) enhances their survival, proliferation, differentiation and angiogenic properties *in vitro*.⁽²²⁾ In the context of arterial repair, we recently demonstrated pharmacological expansion and functional enhancement of feeble EPCs derived from patients with CAD by *ex vivo* treatment with a pharmacological inhibitor of GSK-3 β (GSKi).⁽⁶³⁾ GSKi enhanced EPC adhesion via the upregulation of the α -4 integrin subunit as well as ameliorated RE and neointima formation in denuded murine arteries. In the current study we hypothesize that local delivery of GSKi using coated stents may improve arterial healing via augmented EPC-mediated RE. Furthermore, we tested if the addition of GSKi rescues the delayed arterial healing observed with rapamycin-coated stents.

3.3 Materials and Methods

3.3.1 Ethics

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were performed with the approval of the University of Ottawa Animal Care Committee (HI-283, Ontario, Canada) and followed the guidelines of the Canadian Council on Animal Care. All protocols involving human donors were approved by the Ottawa Heart Institute Research Ethics Committee. The investigation conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects.

3.3.2 *Ex vivo* EPC Attachment Assay

Blood was collected from healthy young volunteers by venipuncture and anticoagulated with EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated and plated on fibronectin-coated plates and cultured in EGM-2 media (Lonza) for 4 days. Adherent cells were harvested and then cultured in wells with BMSs and various drug-coated stents (described below) for 48 hours prior to examination. Cells attached to the surface of stent struts were stained with DAPI and observed using fluorescent microscopy. The number of attached cells was counted in 6 random high power fields (HPFs; magnification $\times 200$) per strut. The collection of blood samples and the consent protocol for the volunteers were approved according to institutional guidelines. EPCs were defined as cells dually positive for DiI-acLDL uptake and *ulex europaeus* agglutinin I (UEAI) binding. While we acknowledge that circulating EPCs are better defined by CD34/KDR co-expression for functional assays(51) we utilized cultured cells as previously described(20,65) which meet the currently accepted definition of a cultured vascular progenitor cell.

3.3.3 Stent coating and stent implantation

Forty eight bare metal stents (BMSs; Driver 3.0 $\times$ 24 mm, Medtronic AVE, Inc., Santa Rosa, CA) were manually coated with 45 μ l of one of the following coating materials to make either vehicle-coated stents (VSs), GSKi-coated stents (GSs), rapamycin-coated stents (RSs), or a combination of rapamycin plus GSKi –coated stents (RGSs). Twelve BMSs were used as a control.

Drug-coated stents were made in our laboratory using a method previously described by our laboratory.(20) Each balloon-mounted stent was individually coated with 45 μ L of

lubricating jelly (Medline Industries, Inc., Mundelein, IL) with or without the active treatment compound(s). The GSK-3 β inhibitor used in our study is GSK-3 β inhibitor VIII (Calbiochem) also called AR-A014418. It is a highly specific inhibitor of GSK-3 β and has been shown to inhibit GSK-3 β with high potency (IC₅₀ = 104 nM). Moreover, GSK-3 β Inhibitor VIII does not inhibit CDC2 and CDK5 - two GSK3-related kinases that are inhibited by other published GSK3 inhibitors.(14) For GSs, GSK-3 β Inhibitor VIII was dissolved in DMSO and reconstituted in water (25 mM) before being mixed with vehicle jelly at a ratio of 1:9 in order to achieve a final concentration that was 20 $\times$ the IC₅₀ (2.08 μ M).The dose of GSK inhibitor was determined using an *in vitro* EPC attachment assay. EPCs were cultured with GSKi (incremental dose escalation at 2 $\times$, 4 $\times$, 8 $\times$, 16 $\times$, 20 $\times$, 40 $\times$, 80 $\times$, 160 $\times$ IC₅₀) and cell viability was examined. For the *in vivo* studies we selected the maximum tolerated GSKi dose *in vitro* (20 $\times$ IC₅₀). For RSs, rapamycin was reconstituted at a concentration of 4 μ g/ μ l – a dose previously tested and found to be efficacious in pre-clinical studies.(86) For RGS stents, GSKi and rapamycin were formulated in the same manner as for stents containing each drug alone, but blended together to achieve the same concentrations on a combination drug stent. The control vehicle-coated gel was made by mixing 0.01% DMSO in water with Lubricating Jelly at ratio of 1:9. All stents underwent air drying for 18 hours before being implanted *in vivo*.

Thirty male New Zealand white rabbits (3.0 –3.5 kg, Charles River Laboratories, Quebec, Canada) were studied. Under general anesthesia with ketamine [25 mg/kg, intramuscularly (i.m.)], midazolam (2–4 mg/kg, i.m.), and isoflurane (via an endotracheal tube), one BMS, VS, GS, RS, or RGS stent was deployed at six atmospheres in each

carotid artery via the iliac artery. All rabbits received one stent in each carotid artery, and the two stents inserted in each rabbit were identical. All animals received Heparin (125 U/kg, Leo Pharma Inc., Ajax, Ontario, Canada) as an i.v. bolus at the outset of the procedure. To limit stent thrombosis all rabbits were given acetylsalicylic acid (rectal gel, 10 mg/kg) every day, starting 3 days before stenting and continuing until euthanasia. As per usual clinical practice, the complementary antiplatelet agent clopidogrel bisulfate (Sanofi-Synthelabo Canada, Montreal, Quebec) was administered transdermally, beginning with a loading dose of 4 mg/kg on the day before stenting and continued as 1 mg/kg/day thereafter until euthanasia.

3.3.4 Tissue Harvest and Quantitative Histomorphologic Analyses

Seven or fourteen days after stent implantation, animals were euthanized using intravenous Euthanyl (Schering-Plough, Quebec), and the stented carotid arteries were harvested. All animals received BrdU (50 mg/kg, i.v. Sigma, St. Louis, MO) one hour before euthanasia to allow immunolabeling of proliferating cells. The stented arteries were evenly divided into 4 segments. The first segment was embedded in methylmethacrylate after overnight fixation with 10% neutral-buffered formalin (NBF). Cross-sections (5 μ m-thick) were cut with a D-Profile tungsten carbide knife (Delaware Diamond Knives Inc., Wilmington), and stained with hematoxylin and eosin in order to obtain the following quantitative histomorphological parameters. The lumen area (LA) as well as the area circumscribed by the internal elastic lamina was measured using a computer-assisted digital system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Neointima area (NA) was defined as the internal elastic lamina area minus the LA.

The second segment was opened longitudinally before manually removing the stent and immersion fixed in 10% NBF before embedding in paraffin. For both methylethacrylate and paraffin blocks, serial 5 μm cross-sections were cut at subsegment intervals of 350 μm . For morphometric analyses, nine cross-sections from three subsegments (three cross-sections per subsegment) were examined. The third segment was opened longitudinally, the stent was manually removed and the tissue was snap frozen in liquid nitrogen. The fourth segment devoted to scanning electron microscopy (SEM; XL 30 ESEM, Philips Electronics Ltd., Markham, Ontario, Canada) was opened longitudinally, flattened and fixed in 1.6% glutaraldehyde before being dehydrated, dried with liquid CO_2 and coated with gold. SEM photomicrographs of each specimen were examined using a computer-assisted digital assessment system to determine the percentage area re-endothelialized compared to the total luminal surface area. For each specimen, 5 separate SEM photomicrographs were taken at $\times 400$ magnification and the RE area for each artery was represented by the sum of the data for the 5 photographed sub-areas.

For each specimen, the extent of arterial wall injury was graded using the standardized protocol developed by Schwartz *et al.*, which tabulates in a semi-quantitative manner the degree of injury imparted to the vessel wall at each stent strut.(138) For example, a score of zero corresponds with an intact internal elastic lamina and the maximum score (three) is defined by medial laceration that extends through the external elastic lamina. Similarly, the extent and density of inflammatory cells surrounding each stent strut were scored using a system devised by Kornowski *et al.*(89) For example, a score of zero is assigned

if no inflammatory cells are present, and the maximum score of three is used if there is a dense, circumferential collection of lymphohistiocytic cells around a stent strut.

3.3.5 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded tissue. The following primary antibodies were used: i) anti-smooth muscle actin (α -SMA; dilution 1:400; Sigma-Aldrich) ii) RAM-11 for macrophages (dilution 1:50; Dako) iii) an anti-BrdU antibody for immunolabeling actively proliferating cells (dilution 1:50; Dako) and iv) biotinylated Griffonia (bandeiraea) simplicifolia Lectin I - isolectin B4 (GSL I-B4, dilution 1:100; Vector) was used for labeling endothelial cells.(5,98) After incubating with the primary antibody, a species-specific biotinylated secondary antibody was applied (except for GSL I-B4) followed by incubation with an avidin-biotin peroxidase complex (Elite ABC kit, Vector) and visualization with 3,3-diaminobenzidine (Sigma). Slides were counterstained with hematoxylin. Immunolabeled SMCs, macrophages, proliferating cells or GSL I-B4 positive cells in the neointima (NI) were counted and expressed as a percentage of the total number of NI cells per HPF (magnification: $\times 400$). NI cell density was expressed as the ratio of the total NI cell number to the NA per HPF. For the quantitative analysis 3 HPFs were counted per arterial cross-section and 3 cross-sections were examined per arterial sample.

3.3.6 In vivo EPC recruitment assay

On the same day of stent implantation blood was withdrawn from rabbits and PBMCs were isolated. These PBMCs were *ex vivo* labeled with PKH26, a red fluorescent dye

previously used for long term *in vivo* cell tracking,(31,75) and then injected back to the same rabbit immediately after VS or GS stent implantation. One week after stent plantation, the stented arteries were harvested, opened flat and the luminal surface was visualized by *en face* confocal microscopy.

3.3.7 Circulating EPC Culture Assay

Peripheral blood was withdrawn from rabbits before and 3 days after either GS or VS stent implantation. PBMCs were isolated and cultured with Clonetics EGM-2-MV-SingleQuots (Lonza) supplemented with 5% FBS on fibronectin-coated chamber slides (Becton Dickinson).(73) At day seven after culture, adherent cells were identified as EPCs by DiI-acLDL / lectin double staining. Endothelial colony forming cells were identified as well-circumscribed monolayers of cobblestone-appearing cells(178) and the colonies were counted in 8 randomly selected fields of each cultured slide.

3.3.8 Statistics

Comparison of two groups was performed by using a Student *t*-test and multiple comparisons were performed by using one-way ANOVA with a Fisher LSD comparison. Values are expressed as means $\pm$ standard error of the mean. As the re-endothelialization data was not normally distributed (e.g., skewed towards 50% and higher), the percentage re-endothelialization was arcsin transformed before being subjected to ANOVA. Statistical significance was defined as $p < 0.05$.

3.4 Results

3.4.1 GSKi enhanced EPC Attachment to Coated Stents

First, we assessed the *in vitro* effects of different stent drug coatings on the adherence of human EPCs. GSs showed levels of EPC attachment that were 2×, 14× and 13× higher than VSs, RSs and RGSs respectively (e.g., 21.5 ± 2.7 vs. 10.2 ± 1.6 , 1.5 ± 0.8 and 1.7 ± 0.4 , respectively, $p < 0.001$; **Figure 3.1**).

3.4.2 Arterial Response to the Implantation of GSKi-Coated Stents

The arterial response to stent-based delivery of GSKi was compared to that observed with vehicle control stents. There were no differences in the arterial injury and inflammatory scores observed with either stent at 7 and 14 days post stent implantation (**Table 3.1**). NI cell density at 14 days was similar in arteries containing GS and VS stents. The abundance of NI SMCs and macrophages was similar between arteries with GS and VS stents (e.g., SMCs: $44.1 \pm 1.5\%$ vs. $46.7 \pm 2.6\%$; macrophages: $2.5 \pm 0.3\%$ vs. $2.2 \pm 0.3\%$). Macrophages were primarily located adjacent to stent struts. There were more proliferating cells in the NI of arteries with GS compared to VS stents ($3.9 \pm 0.2\%$ vs. $2.4 \pm 0.2\%$; $p < 0.05$) and although the identity of these cells is unclear, their location was scattered throughout the NI and not confined to the luminal surface where endothelial cells were located. The percentage of NI cells that were positive for GSL I-B4, the rabbit marker for cells of endothelial lineage, was less with GS compared to VS stents ($7.9 \pm 1.1\%$ vs. $11.8 \pm 1.1\%$, $p < 0.05$).

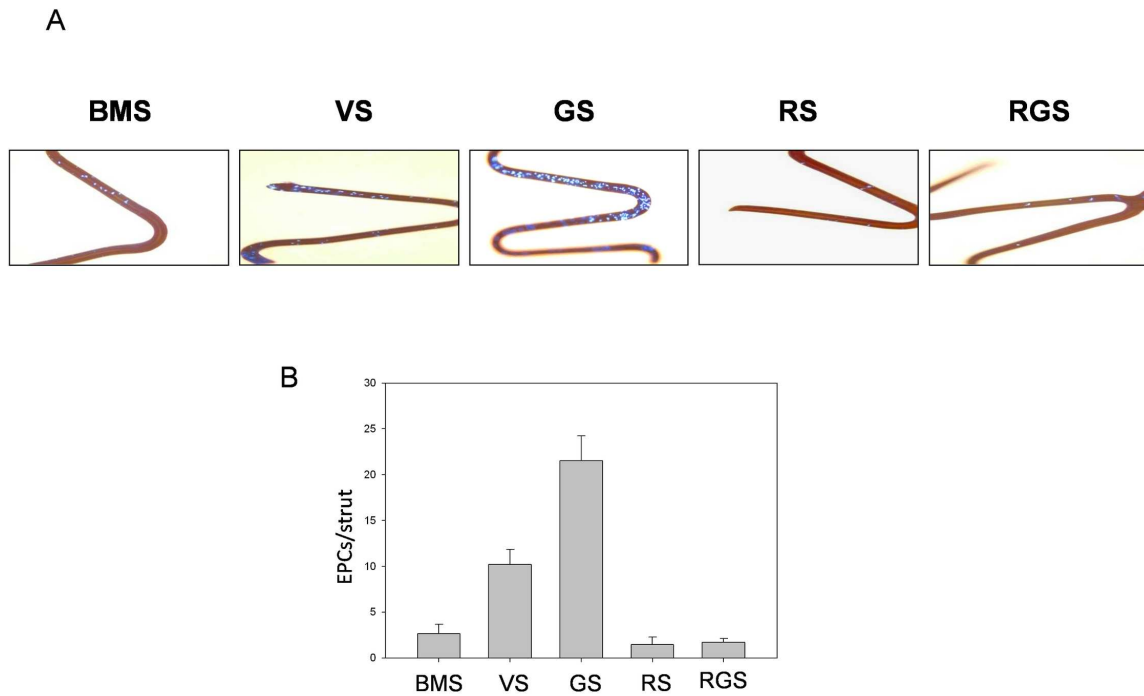


Figure 3.1. *In vitro* EPC attachment to coated stents. (A) Representative photos show cells attached to VS, GS, RS, RGS stent strut. Cell nuclei were stained with DAPI as blue color (magnification, 100×). (B) Bar graph demonstrating number of EPCs attached to the stent struts. GSs showed levels of EPC attachment that were 2×, 14× and 13× higher than VSs, RSs and RGSs.

Table 3.1 Injury and Inflammatory Scores for Stented Vessels

	BMS	VS	GS	RS	RGS	Time point
Injury score	1.02±0.04	1.01±0.03	1.04±0.03	1.04±0.05	1.02±0.03	1wk
Injury score	1.00±0.04	0.98±0.03	1.01±0.05	1.03±0.05	1.00±0.04	2wk
Inflammatory score	1.78±0.05	1.83±0.07	1.80±0.05	1.84±0.05	1.84±0.06	1wk
Inflammatory score	1.84±0.05	1.87±0.06	1.85±0.05	1.87±0.03	1.84±0.04	2wk

3.4.3 Neointima Response to GSKi vs. Rapamycin Coated Stents

At 7 days post stent implantation mean stent NA varied (e.g., BMS $0.47 \pm 0.08 \text{ mm}^2$, VS $0.39 \pm 0.05 \text{ mm}^2$; GS $0.34 \pm 0.06 \text{ mm}^2$, RS $0.28 \pm 0.05 \text{ mm}^2$, and RGS $0.17 \pm 0.04 \text{ mm}^2$; ANOVA: $p=0.02$, **Figure 3.2**). Specifically, RS stents had lower NA than BMS stents ($p=0.03$); yet RGS stents had a NA that was lower than BMS, VS and GS stents (p values: 0.002, 0.02 and 0.04; respectively). However, at 14 days the mean NAs were slightly higher in all groups (e.g., BMS $0.55 \pm 0.04 \text{ mm}^2$, VS $0.59 \pm 0.06 \text{ mm}^2$; GS $0.30 \pm 0.02 \text{ mm}^2$; RS $0.44 \pm 0.07 \text{ mm}^2$, and RGS $0.28 \pm 0.02 \text{ mm}^2$, ANOVA: $p<0.001$; **Figure 3.2**). Indeed, GS stents had 45% and 49% less NA than BMS and VS stents ($p<0.001$ for both). Moreover, RS stents had a 47% larger NA than GS stents, but RGS stents reduced NA to a level comparable to GS stents.

3.4.4 GSKi Rescues Rapamycin-Associated Attenuation of Stent Re-Endothelialization

At 7 days post stent implantation the RE area for the various stents were as follows: BMS $46.7 \pm 3.8\%$, VS $49.4 \pm 3.2\%$, GS $65.6 \pm 6.4\%$, RS $46.0 \pm 4.7\%$, and RGS $65.0 \pm 3.3\%$ (ANOVA: $p=0.005$; **Figure 3**). GS and RGS stents were equally efficient at increasing RE compared to BMS, VS, and RS stents. At 14 days the RE areas for all stents were higher, but with important differences: BMS $62.3 \pm 2.6\%$, VS $64.8 \pm 6.9\%$, GS $92.5 \pm 2.6\%$, RS $81.0 \pm 3.4\%$, and RGS $90.9 \pm 2.8\%$ (ANOVA: $p<0.001$; **Figure 3.3**). Again, at this later interval GS stents showed increased RE compared to BMS and VS and the combination of GSKi and rapamycin treatment rescued RE compared to stents coated with rapamycin alone ($p=0.03$). Notably, one animal was followed out to 3 months with GS implantation

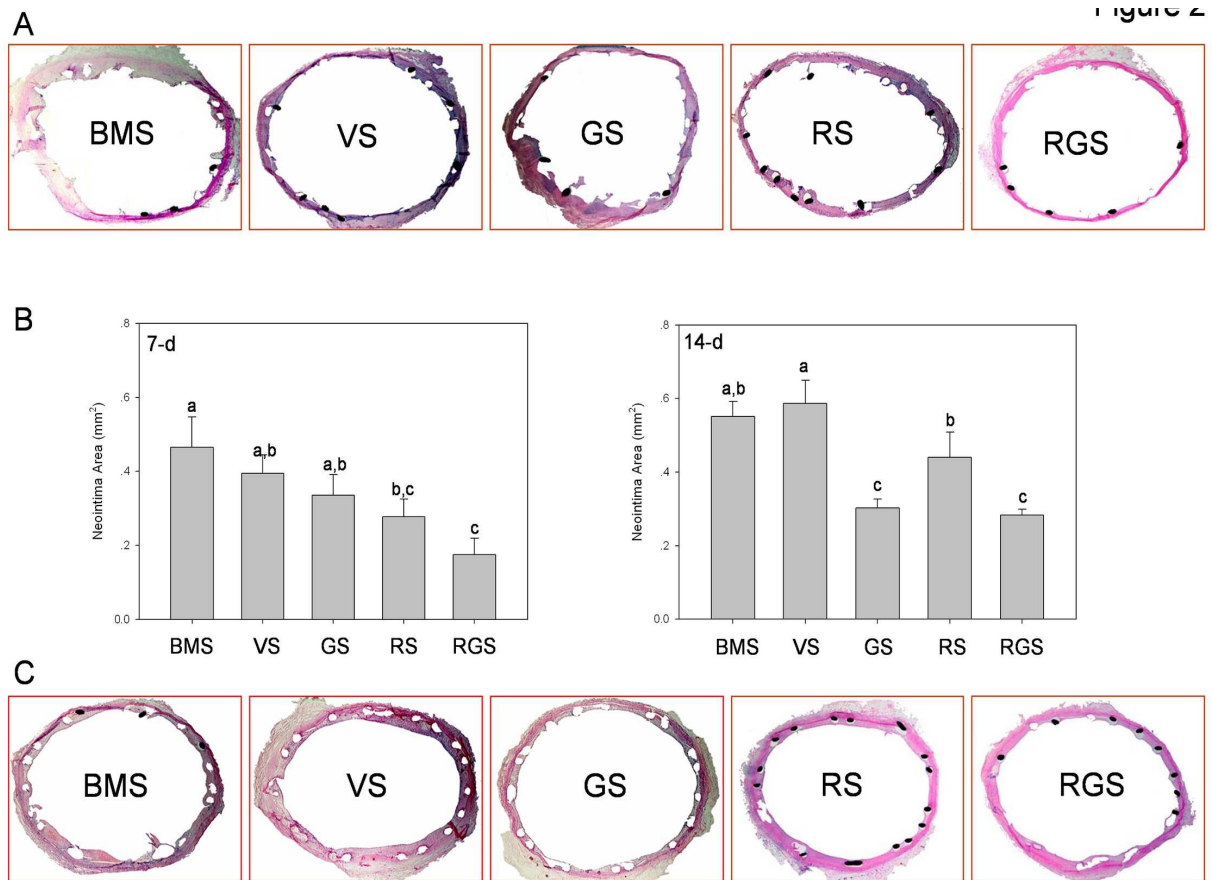


Figure 3.2. Neointima formation of stented arteries. Representative photos of H&E stained cross-sections of arteries at 7 days (**A**) and 14 days (**C**) post stent implantation (magnification, 40×). (**B**) Bar graphs demonstrating NA of stented arteries. At 7 days RS stents had lower NA than BMS stents ($p=0.03$); RGS stents had a NA that was lower than BMS, VS and GS stents (p values: 0.002, 0.02 and 0.04; respectively). At 14 days, GS stents had 45% and 49% less NA than BMS and VS stents ($p<0.001$ for both). Moreover, RS stents had a 47% larger NA than GS stents, but RGS stents reduced NA to a level comparable to GS stents. Letters indicate statistical differences, $p<0.05$.

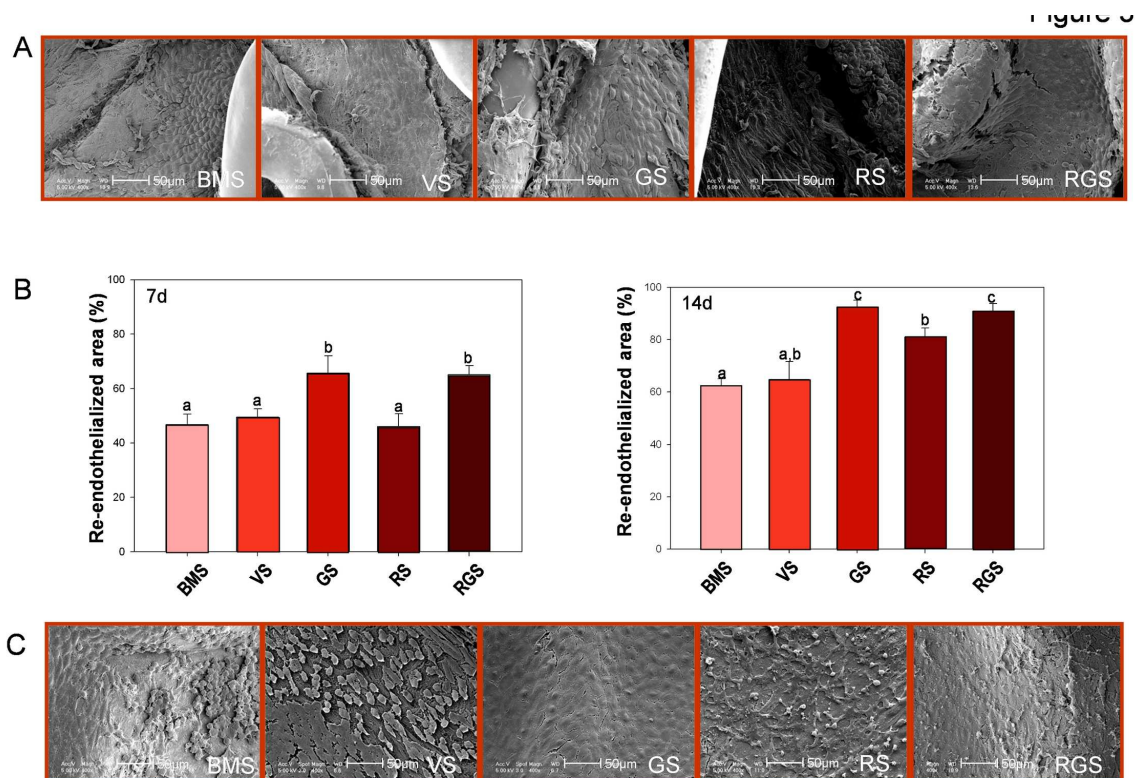


Figure 3.3. Re-endothelialization assessed by SEM. En-face scanning electron micrographs of arteries 7 days (A) and 14 days (C) post stent implantation. (B) Bar graphs demonstrating percentage of re-endothelialized area of total stented area. At both 7 days and 14 days, GS stents showed increased RE compared to BMS and VS, and the combination of GSKi and rapamycin treatment rescued RE compared to stents coated with rapamycin alone. Letters indicate statistical differences, $p < 0.05$.

and, as expected, was found to have complete re-endothelialization (**Figure 3.4**)

3.4.5 Re-Endothelialization through Recruitment of Circulating EPCs

To determine the effects of GSKi-eluting stents on mobilization and differentiation of circulating EPCs in the re-endothelialization of stented arterial segments two experiments were performed. First, approximately 1% of rabbit circulating PBMCs were tagged with a fluorescent dye and re-injected immediately post stent implantation and 7 days later confocal microscopy was used to visualize tagged cells that incorporated into re-endothelialized GS-stented rabbit carotid artery segments. **Figure 3.5** shows an example of a fluorescently tagged cell that was incorporated into the newly formed endothelial layer covering a stent. Second, PBMCs were isolated before (baseline) and 3 days after stent implantation and cultured for 7 days. For baseline samples the total WBC count and the number of adherent EPCs after 7 days in culture were similar for rabbits that received VS and GS stents. However, there was a 4-fold increase in EPC colony forming capacity for blood samples harvested from rabbits receiving GS compared VS stents ($p < 0.05$, **Figure 3.6**).

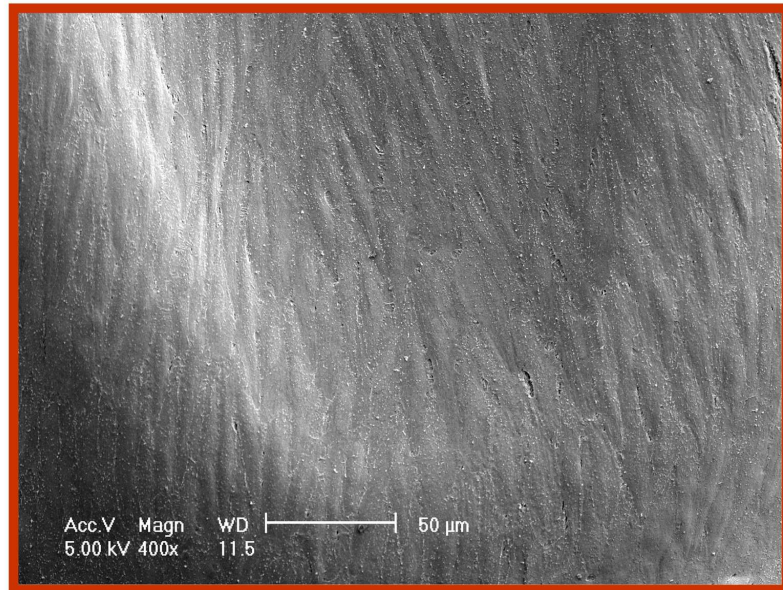


Figure 3.4. Photomicrograph demonstrating intact endothelium overlying a GSKi coated stent at 3 months.

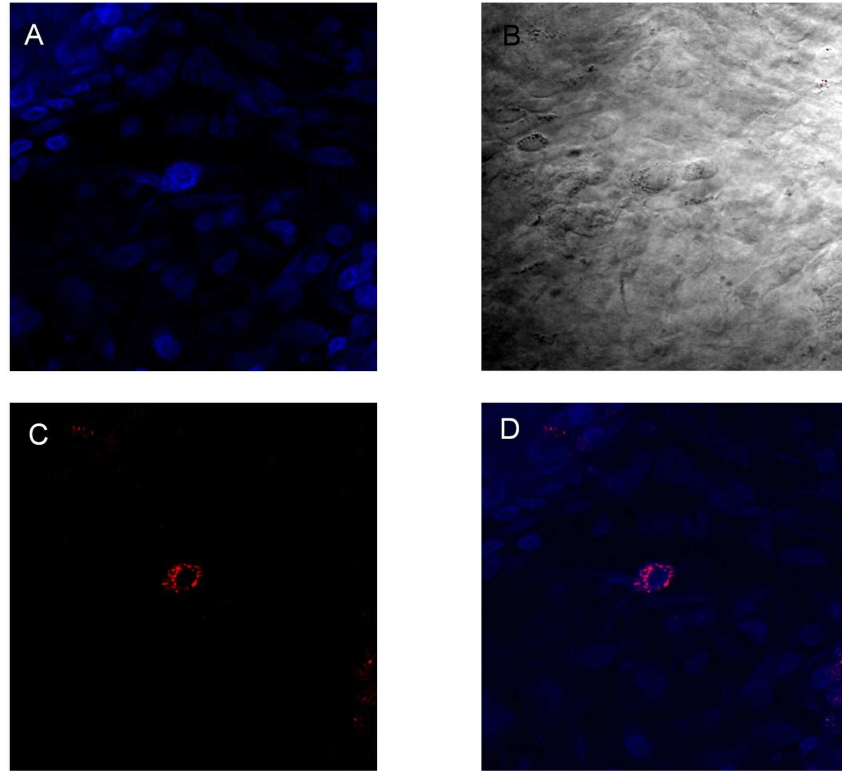


Figure 3.5 Circulating EPCs participate in re-endothelialization visualized by en face confocal microscopy. (A) Cells of the endothelial layer of stented artery were visualized with DAPI nuclei staining as blue color. (B) Phase contrast image of A. (C) PKH26 labeled cell from circulation was visualized as red color. (D) Merged image of A and C showing incorporation of the PKH26 labeled cell into the newly formed endothelial layer of stented artery.

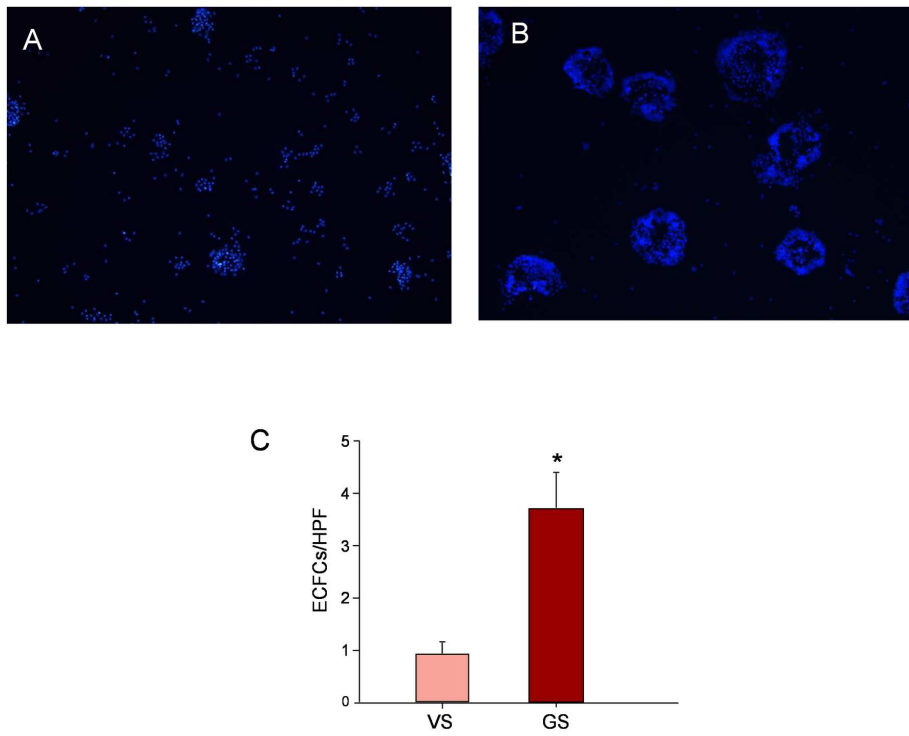


Figure 3.6. Effects of GS stents on circulating progenitor cells. Microscopic photos showing cultured EPCs from rabbits with either VS (A) or GS (B) stents (magnification, 100 $\times$). (C) there was a 4-fold increase in EPC colony forming capacity for blood samples harvested from rabbits receiving GS compared VS stents ($p<0.05$).

3.5 Discussion

In this study we sought to determine if a pharmacological inhibitor of GSK-3 β is beneficial for arterial healing after stent implantation. The principal findings are that stent NA was reduced and RE was augmented by local, stent-based delivery of GSKi. However, perhaps of greater significance is that the salutary endothelial effects of GSKi both *in vitro* and *in vivo* were superior to rapamycin, a common agent in clinically utilized DESs. Moreover, GSKi combined with rapamycin in effect rescued the deficiencies of rapamycin alone on arterial healing.

Stents are implanted into arterial segments with severe atherosclerotic lesions that are either deficient in a luminal surface endothelium or contain endothelial cells that are essentially dysfunctional. After stent implantation the involved arterial segment may be highly prothrombogenic – particularly if stent struts are incompletely apposed to the luminal surface. Recent reviews of the efficacy of coronary stenting have focused on the rare but potentially catastrophic occurrence of acute stent thrombosis and highlight the need for dual anti-platelet aggregation therapy in order to minimize thrombus formation within stents.(27) Traditionally, it is believed that endothelial cells originating from intact adjacent arterial segments populate the stent luminal surface as a result of local proliferation and migration. However, this concept is now under review as recent studies demonstrate that circulating progenitor cells are also involved in both NI formation and RE.(98) Indeed, there is an expanding experimental literature that points to the potential restorative potential of EPCs – yet often with disregard of a clinically important caveat. Relative to healthy controls, patients with CAD have a paucity of circulating and/or

functional EPCs.(68,139) Hence, for CAD patients who have the most to gain there is a dire need to increase both the abundance and functional capacity of EPCs in order to ameliorate arterial repair. One interesting strategy aimed at capturing EPCs in the artery wall involves the use of stents that are coated with an antibody to CD34; however from the results of preliminary human studies it is unclear if this novel stent is superior to conventional DESs.(4) Recently we illustrated that GSKi treatment of EPCs not only enhances their abundance and adhesion *in vitro* but *ex vivo* GSKi-treatment of EPCs improves the ability of these cells to ameliorate arterial healing *in vivo* – even when feeble cells are harvested from patients with CAD.(63)

Current commercially available DESs are popular for their tendency to reduce ISR - primarily by preventing vascular SMC proliferation and migration, as well as local inflammatory pathways. Unfortunately, DESs may also compound the problem of stent thrombosis by impairing RE.(96) The delay of RE with DESs has been observed in both animal models and human clinical studies.(44,78) Restoration of an intact endothelium therefore represents a crucial process for the prevention of both ISR (presumably related to the restoration of the local vessel wall homeostatic milieu) as well as stent thrombosis (particularly in the first days post-implantation). Rapamycin-coated stents are popular clinically and known to inhibit proliferation, migration, and differentiation of human EPCs.(16,19) Hence, there is at least a theoretical if not real need to correct the delayed RE observed in human arteries treated with rapamycin-coated stents. In the current study we have attempted to address this by quantifying the kinetics of re-endothelialization in the context of rapamycin alone and with GSKi. While in humans, the time to re-

endothelialization is incompletely understood, studies suggest that it is likely complete by 3 to 4 months with bare metal stents(42,58) and with DESs much later.(45) While we observed nearly complete re-endothelialization by 14 days, this is in keeping with the kinetics observed by our group(99) and others in similar animal models. As well, at both 7 and 14 days no differences existed between our BMS and VS groups in NI formation, RE, or inflammation scores suggesting no differences existed with regards to the biological reaction to stent implantation with our coating method. In the current study we show for the first time that combining GSKi with rapamycin results in an additional benefit – not only inhibiting NI growth but also minimizing the deleterious effects of rapamycin on circulating EPCs and enhancing RE in the critical early period.

While it is attractive to speculate that GSKi-induced augmentation in RE is instrumental in reducing NI formation, such a hypothesis is difficult to prove *in vivo* and we are only able to speculate that GSKi promotes the restoration of an endothelial barrier that blocks the accumulation of blood-borne cells that might contribute to NI formation. Nonetheless, conceptually we do establish using a combination of dye-labeling and *en-face* confocal microscopy that circulating EPCs are incorporated into the reconstituted endothelial layer of the stented arterial segment. However, as only 1% of the total circulating PBMC population was labeled quantification of the contribution of circulating progenitor cells to RE could only be addressed in principle and not quantitatively. One unexpected finding is the apparent systemic effects of GS stents. As shown *in vitro*, there was a 4-fold increase in PBMC-derived EPC colony forming units in GS compared to VS stented rabbits – an effect that may be important not only for the stented arterial segment but

throughout the vasculature of subjects prone to the complications of atherosclerosis. Indeed, this observation may also help explain why *in vitro* there was no difference in EPC attachment to RGS and RS stents, yet *in vivo* RGS stents are clearly superior. In fact, coating stents with the vehicle alone increased EPC adhesion compared to BMS - an effect that is presumably related to the composition of the vehicle. Interestingly, with the addition of rapamycin to the vehicle, EPC adhesion diminished, thereby highlighting the toxicity of rapamycin to EPCs. Moreover, adding GSKi to rapamycin could not attenuate rapamycin's toxicity *in vitro*. In contrast, when added to rapamycin *in vivo*, GSKi results in a 41% increase in RE at 7 days post-stent implantation compared to RS stents, likely due to the recruitment of additional EPCs. Interestingly, the percentage of NI cells that labeled with the rabbit marker of endothelial lineage, GSL I-B4, was less in the GS compared to the VS stents. At this juncture we are unsure of the significance of this finding, as there are limitations with this cell marker including a lack of information about the state of differentiation of labeled cells (e.g., perhaps it is a late marker of endothelial differentiation and does not recognize EPCs).

As GSK-3 β is universally expressed, one would expect inhibition of GSK-3 β may also have effects on SMCs and potentially their involvement in ISR. Indeed, Park and colleagues observed in a rat balloon injury model that delivery of the active GSK-3 β gene inhibited proliferation yet produced sustained apoptosis of SMCs.(112) The same group also showed that decreased SMC proliferation by rosiglitazone was reversed by inactivation of GSK-3 β .(91) In the current study NI proliferation was greater in the GS compared to VS arteries and while this may be a GSKi-related effect we do not know the

precise identity of the proliferating cells (e.g., SMCs or endothelial cells). Regardless, the frequency of proliferating cells was rather low in both the GSKi-treated and control arteries and consistent with previous observations.(55,109)

In conclusion, coating stents with substances that potentially facilitate RE may represent a novel therapeutic approach for improving the efficacy and long-term safety of patients undergoing vascular stenting procedures. Inhibition of GSK-3 β by means of a GSKi coated stent provides a novel strategy for the augmentation of EPC recruitment and RE post stent implantation. These data provide a foundation for the development of improvements in the safety and efficacy of DESs.

3.6 Funding

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3.7 Acknowledgements

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3.8Conflict of Interest

None declared

**Chapter 4: Glycogen Synthase Kinase-3 β Inhibition Augments Diabetic Endothelial
Progenitor Cell Abundance and Functionality via Cathepsin B: Novel Therapeutic
Opportunity for Arterial Repair**

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as submitted for publication

4.1 Abstract

4.1.1 Rationale:

Progenitor cell therapy is hindered in patients with diabetes mellitus (DM) because of cell senescence. Glycogen synthase kinase 3 β (GSK3 β) activity is increased in DM potentially exacerbating impaired cell based therapies. Thus, we hypothesized that GSK3 β antagonism may improve cell-based therapy in patients with DM.

4.1.2 Objectives:

This study aimed to determine if and how a small molecule GSK3 β inhibitor (GSKi) can improve therapeutic efficacy of endothelial progenitor cells (EPC) from patients with DM.

4.1.3 Methods and Results:

Patients with DM had 50% fewer EPCs and increased rates of apoptosis. DM-EPCs also exhibited higher levels of GSK3 β activity resulting in increased levels of phosphorylated β -catenin. Proteomic profiling of DM EPCs treated with GSKi identified 37 non-redundant, differentially regulated proteins compared to untreated or non-DM EPCs. Target proteins were validated by Q-PCR and Western blot. Cathepsin B (CatB) was differentially regulated by GSKi and showed 40% less baseline activity in DM-EPCs but responded by 400% with GSKi treatment resulting in attenuation of apoptosis. Finally, *in vivo* efficacy of cell based therapy was assessed in a xenotransplant mouse model 14 days post-wire injury. DM EPCs produced a 41% reduction in the intima:media – an effect

that was further augmented by 40% when DM EPCs were pre-treated with GSKi, yet absent when CatB was antagonized.

4.1.4 Conclusions:

Increased basal GSK3 β activity contributes to accelerated EPC apoptosis and cellular senescence in patients with DM. Up regulation of CathB by small molecule antagonism of GSK3 β reduces apoptosis and enhances cell-based therapy following vascular injury.

4.2 Introduction

Use of endothelial progenitor cell (EPC) populations for cell-based therapies is beneficial in a host of cardiovascular conditions including peripheral vascular disease, pulmonary arterial hypertension, and myocardial infarction.(65) However, the administration of autologous EPCs in patients with established disease is often hindered due to attenuated cellular yield and biologic activity. Accordingly, a myriad of strategies have been employed with the goal of improving EPC cellular yield, survival, and function, including our own efforts to over-expressing endothelial nitric oxide synthase(90) or administer small molecule inhibitors of glycogen synthase kinase 3-Beta (GSK3 β). (63)

GSK3 β is a ubiquitously expressed serine/threonine protein kinase which is negatively regulated by Wnt signaling. Under basal conditions GSK3 β phosphorylates β -catenin (β cat) resulting in proteasomal degradation of this important nuclear transcription factor. Pharmacologic GSK3 β inhibition or Wnt3a stimulation promotes hematopoietic stem cell self-renewal and repopulation of cell lineages *in vitro* and *in vivo*.(161,162) In EPCs, GSK3 β inhibition by either transfection of a dominant negative mutant or small molecule inhibition improves the therapeutic capacity of cells, thereby augmenting angiogenesis in ischemia models and improving arterial repair following vascular injury.(22,97) This is of particular interest given that GSK3 β expression and activity are dysregulated in patients with diabetes mellitus (DM)(38) a population in whom EPC function is severely attenuated.(33,76)

Hence, we sought to explore the potential for pharmacologic inhibition of GSK3 β to improve vascular homeostasis and repair in patients with DM. Herein we demonstrate that inhibition of GSK3 β in DM EPCs abrogates apoptosis and improves EPC yields *in vitro*. Moreover, using a proteomic approach we identify and confirm the differential regulation of candidate proteins for the observed benefits of GSK3 β inhibition. Among the identified proteins, increased cathepsin-B (catB) activity is demonstrated to be essential for reductions in EPC apoptosis and necessary for increased efficacy with cell based therapeutic arterial homeostasis. These findings suggest that inhibition of GSK3 β is an important strategy for improving autologous cell-based therapy in patients with DM and acts via a novel mechanism involving increased activity of catB.

4.3 Materials and Methods

4.3.1 Cell Isolation

Endothelial progenitor cells were isolated as previously described.(63,64,98) Briefly, blood was collected by venipuncture and anti-coagulated with EDTA. Subsequently, density gradient centrifugation was performed with Ficoll Histopaque 1077 (Sigma) to isolate PBMCs. Cells were washed with PBS and plated on human fibronectin (Sigma) coated 6-well plates at a density of 5.0×10^6 PBMCs/well in endothelial growth media -2 (EGM2, Lonza). Following 4 days in culture, non-adherent cells were removed and plates washed with PBS. All experiments performed with day 4-7 cells with samples from individual donors representing a single replicate. For enumeration, EPCs were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethyliodocarbocyanine-acetylated LDL (acLDL, 2.5 μ g/mL; Invitrogen) followed by FITC-conjugated *Ulex europaeus* agglutinin I (5

μg/mL, Sigma) then counterstained with DAPI. Six optical fields were blindly evaluated per patient with the mean results reported.

4.3.2 GSK3β Inhibitors

GSK-3β small molecule and peptide inhibitors (GSKi) were purchased and assayed for efficacy in increasing cell yield and blocking phosphorylation of β-catenin (βcat). All inhibitors were diluted in DMSO to a final concentration of 0.1% *or* sterile PBS if water soluble. Vehicle controls were utilized in all experiments. Specifically, AR-A014418 (Sigma), CHIR98014 (Cedarlane), (22,3E)-6-Bromoindirubin-3-oxime (Calbiochem), GSK peptide inhibitor (Calbiochem), and LiCl (20nmol/L, Sigma) were tested for *in vitro* efficacy.

4.3.4 Apoptosis Assay

EPCs were maintained under basal culture conditions *or* serum-starved for 24 hours as indicated. Non-adherent cells were removed by washing with PBS. Subsequently, adherent cells were lifted by gentle agitation with 1 mmol/L of EDTA. Cells were enumerated and 1×10^5 EPCs were stained with Annexin V-FITC and propidium iodide (PI) as per manufacturers instructions (BD). All flow studies were performed on a Beckman Coulter Cytomics FC 500 cytometer. Early apoptotic cells were defined as AnnexinV+/PI-.

4.3.5 Protein lysate preparation

Media was aspirated and cells were washed twice with PBS. After all PBS was removed, TrypLE was added for 5 minutes at 37°C to lift off the cells. The reaction was neutralized by adding complete growth culture media (EGM-2MV 5% FBS) and cells were collected. The cells were then centrifuged at 220 g for 5 minutes at RT. After the centrifuge, supernatant was discarded and cells were resuspended in DPBS (Lonza) to wash them. An aliquot of cells was then taken before the next centrifugation at 220 g for 5 minutes at RT in order to count the cells. After the centrifugation, the supernatant was removed and cell pellets in the 50-ml tubes were frozen down in liquid nitrogen before being put at -80°C. Once needed, cell pellets were put on ice and cell lysis buffer (7M Urea (w/v), 2M Thiourea (w/v), 4% CHAPS (w/v), 1% DTT (w/v)) was added immediately according to the recorded cell number. Cell lysates were vortexed and kept at room temperature for 30 minutes to enable protein solubilisation. Cell lysates were then sonicated in a 4°C ultrasonic bath sonicator with the following program: 5 second pulse followed by 10 seconds off and for 10 cycles. Cell lysates were vortexed and then centrifuged at 14,000 g for 15 minutes at room temperature. The supernatant was transferred and protein quantification was realized with 2D Quant kit (GE Healthcare). Before being used for the 2D gel experiments, 1% 3–10 ampholytes (Bio-Rad) was added to the sample.

4.3.6 Two-dimensional polyacrylamide gel electrophoresis – 2D PAGE

The total proteins (30 µg) were passively rehydrated overnight in 220 µL rehydration/sample buffer (same buffer as described in 2-D sample preparation) and applied to immobilized pH gradient (IPG) strips (11 cm, pH 4–7; Bio-Rad). Isoelectric focusing (IEF) was carried out using the Agilent fractionator (Agilent) in the in-gel mode

and programmed as follows: the voltage was initially held at 300 V for 1 min, then linearly increased to 3500 V over 90 min, and focused at 3500 V for 5 hours until it reached 18,000 Vh. The current did not exceed 50 μ A per strip. Each focused strip was subsequently equilibrated in 4.0 ml of equilibration buffer I [(6 M urea (w/v); 50 mM Tris-Cl, pH 8.8; 2% SDS (w/v); 30% glycerol (v/v); bromophenol blue (trace); 1% DTT (w/v)] for 15 min with gentle agitation followed by the equilibration buffer II [equilibration solution I with DTT replaced by 2.5% iodoacetamide (w/v)] for 15 min with gentle agitation. The second-dimensional separation was performed on a 10% SDS-PAGE gel in Ettan DALT six electrophoresis system (GE Healthcare) at 10 mA per gel at 25°C for approximately 18h until the bromophenol blue reached the bottom of the gel. Two technical replicates were done independently for each biological sample, for a total of 18 gels.

4.3.7 Sypro Ruby gel stain

The gels were stained with Sypro Ruby gel stain (Sigma) for total protein staining. Briefly, the gels were fixed in 10% methanol and 7% acetic acid for 30 minutes before being stained with Sypro Ruby gel stain overnight. The next day, gels were washed in 10% methanol and 7% acetic acid for 30 minutes before being imaged on a Gel Doc imaging system (Bio-Rad).

4.3.8 Image acquisition and 2-D gel analyses

The same scanning conditions were used for each Sypro Ruby-stained gel. The scanned gels were analyzed using PDQuest 2-D analysis software (advanced version 8.0; BioRad)

according to the protocol provided by the developer. The 2-D gel analysis software PDQuest was used for gel-to-gel matching and identifying differences between the different groups. Each spot was visually inspected for proper matching and localization on the other gels of the same group using the group consensus tool. The gel images were normalized in the PDQuest software with the local regression model to even out differences in staining intensities between gels. Each matched protein spot was assigned a unique SSP (sample spot protein) number. For gel comparison, a statistical approach was applied for determining statistically differentially regulated proteins using the PDQuest software. Student's t-test was performed with 95% significance level to determine which proteins were statistically differentially regulated between the healthy cells and the patient cells non-treated and the patient cells non-treated and treated with GSKi. A minimum of 1.5-fold change was considered for the upregulated proteins and 0.67-fold for downregulated proteins. Each analysis set was visually inspected to match PDQuest software spot detection and 5 spots were removed from the final analysis due to poor quality. Protein spots with differential expression patterns on 2-DE maps were excised with the automated spot excision robot, the EXQuest spot cutter (Bio-Rad).

4.3.9 Protein identification

LC-MS analysis was performed at the OHRI Proteomics Core Facility (Ottawa, Ontario, Canada). Gel bands were in-gel digested according to the method of Shevchenko (Nat Protocols 2006;1(6):2856-60). Peptide extracts were concentrated by vacufuge (Eppendorf) and diluted in 0.1% trifluoroacetic acid. Peptides were loaded onto a peptide trap (Agilent) for 5 minutes at 15 microlitres per minute using a Dionex UltiMate 3000

RSLC nano HPLC. Peptides were eluted over a 20 minute gradient of 3% - 45% acetonitrile with 0.1% formic acid at 0.3 microlitres per minute onto a 10-cm analytical column (New Objective Picofrit self-packed with Zorbax C18) and sprayed directly into a LTQ Orbitrap XL hybrid mass spectrometer using a nanospray source (Thermo Scientific, USA). Mass spectra were acquired in a data-dependent fashion, with MS scans acquired in the FT cell while MS² scans were acquired in the ion trap module.

MS/MS spectra were matched against a custom database (2011_07_human_con) comprised of human sequences from SwissProt (2011_07 version of uniprot_sprot.fasta.gz from ftp.uniprot.org) concatenated with a database of common contaminants (Contaminant db downloaded from maxquant.org, downloaded june 9th 2011) using MASCOT 2.3.01 software (Matrix Science, UK) with MS tolerance of ± 5 ppm and MS/MS tolerance of 0.6 Da. Oxidation of methionine, carbamidomethylation of cysteine, deamidation, protein N-terminal acetylation, conversion of peptide N-terminal Glu or Gln to Pyro-Glu and phosphorylation of serine or threonine were allowed as potential modifications.

4.3.10 Quantitative PCR

Total RNA was isolated using Trizol (Invitrogen) and purified utilizing RNeasy mini kits. Subsequently, RNA was quantified utilizing a NanoDrop 1000 (Thermo Scientific) and reverse transcription performed using Omniscript kit as directed (Qiagen). All real-time PCR experiments were performed using the SYBR Green Jumpstart Taq Ready Mix

(Sigma) on a Lightcycler 480 (Roche) and analyzed with accompanying software as per the Pfaffl method.(120,121)

4.3.11 Western Blots

Western blots were performed using standard techniques. Briefly, protein was isolated in RIPA buffer using a ratio of 50 μ L per 1 million cells. The sample was then allowed to incubate on ice for 30 minutes followed by centrifugation. The supernatant was assayed using a standard BCA assay (Thermoscientific). Protein was then separated on 10% acyladmide gels and transferred to PVDF membranes using iBlot as directed (Invitrogen). After transfer, the membrane was blocked for one hour with 5% skim milk in TBS-T at room temperature. Primary antibodies were incubated overnight at 4 degrees. Primary antibodies included: PAI-2 (Abgent, AP6562c, 8:1000), β -actin (Sigma, 1:100000), Gelsolin (Abcam, ab11081, 1:1000), GDI2 (Abcam, ab49193, 1:2000), SCaMC-1 (Santa Cruz, sc-133987, 1:500), CatB (Abcam, ab58802, 1:10000). Membranes were then washed and incubated with biotinylated secondary antibodies (Santacruz) for a period of 1 hour then visualized using ECL Plus (Amersham Biosciences).

4.3.12 Cathepsin B activity assay

Cathepsin B activity was assayed in day 5 EPCs using a standardized CatB fluorometric assay kit as directed (Abcam). Briefly, EPCs were washed with PBS, lifted with EDTA and 5×10^6 cells collected by centrifugation. Cells were lysed by incubation with cell lysis buffer, pelleted, and 50 μ L transferred to a 96 well plate. Subsequently, 2

μ L 10 mmol/L AC-RR-AFC was added and samples incubated for 2 hours. Plates were read on the SynergyMx microplate reader (Bio Tek).

4.3.13 VEGF Secretion Assay

EPCs were cultured using standard techniques to day 4. Cells were subsequently lifted, counted, and replated in 96 well plates at equivalent densities in VEGF free media with treatment as indicated. Following 24 hours, media was removed and assayed for VEGF levels using a standard VEGF ELISA kit (R&D Systems) using the manufacturers protocol.

4.3.14 HUVEC Adhesion Assay

HUVEC were cultured to confluence in 96 well plates then treated with 10ng/mL of TNF- α for 6 hours to activate cells. 10^6 EPCs were cultured in with 5 μ mol/L calcein for 30 minutes, lifted with EDTA, pelleted and resuspended in EGM-2. Subsequently, 4×10^4 cells were plated on the activated HUVECs and allowed to adhere for 1 hour. Plates were read on the SynergyMx microplate reader, washed 3 times with PBS, and re-read. Adherence is expressed as % fluorescence retained after washing.

4.3.15 Cell Invasion Assay

Day 5 EPCs were treated as indicated. Using a modified Boyden chamber and a nucleopore filter (12 μ m BD) with a matrigel matrix (BD), EPC invasiveness was assayed. Briefly, 5×10^6 EPCs were placed in the upper chamber with EBM with EGM-2

in the lower chamber. Cells were permitted to migrate for 24 hours at 37 degrees. Cells were counterstained with DAPI and enumerated in 6 random high power fields.

4.3.16 CD-1 Nude Femoral Artery Wire Injury Model

Thirty-two CD-1 nude athymic male mice were purchased from Charles River Laboratories and permitted to acclimatize for 2-6 weeks prior to surgery. Under isoflurane anesthesia, mice underwent blunt dissection of the femoral neurovascular bundle with isolation of the femoral artery.(131) A branch of the femoral artery was isolated, incised and a 0.014-inch intravascular guidewire was introduced and passed in the main lumen denuding the artery and initiating neointima formation as previously described. Flow was restored to the main femoral artery and the incised branch was ligated. Subsequently, 2×10^5 EPCs from patients with DM were infused into the adjacent vein utilizing a blunt needle. None of the animals exhibited ischemia in the hind limb. Mice were recovered and were sacrificed at 14 days for tissue analysis.

4.3.17 Tissue Processing

Mice underwent perfusion fixation with buffered formalin at time of sacrifice. Arteries were then fixed for 24 hours in formalin and dehydrated in ethanol. Arteries were mounted in paraffin blocks and sectioned in 5 μ m sections till 100 μ m from the branch vessel site. These sections were then haematoxylin and eosin stained and analysis performed using a computer-assisted digital imaging system (Image-Pro Plus, Media Cybernetics).

4.3.18 Ethics and Statistics

All protocols involving human donors were approved by the Ottawa Heart Institute Research Ethics Committee with participants providing written informed consent. These studies conform with the Declaration of Helsinki for the use of human tissue. Animal experimental protocols were approved by the University of Ottawa Animal Care committee and adhere to the Canadian Council on animal Care guidelines. Data are expressed as mean plus or minus standard error of the mean. Statistical significance was determined for $p < 0.05$. Pairwise comparisons were performed using a paired student t-test with multiple comparisons performed with a one-way ANOVA with Holm-Sidak *post hoc* testing.

4.4 Results

4.4.1 Diabetes Accelerates Apoptosis in EPCs Through Increased GSK3 β Activity

EPCs isolated from human subjects were cultured for seven days and then characterized using immunolabeling for *ulex europeus agglutinin-1* and acetylated-LDL uptake (**Figure 4.1A**). The baseline characteristics of the human subjects are presented in **Table 4.1**. Samples derived from patients with DM yielded fewer EPCs than those derived from healthy controls ($n=12$, 14.9 ± 4.6 vs 38.5 ± 6.7 cells per high power field, $p < 0.01$, **Figure 4.1B**). Several GSK3 β inhibitors were supplemented in increasing concentrations to identify the optimal inhibitor and concentration. CHIR98014 at a concentration of

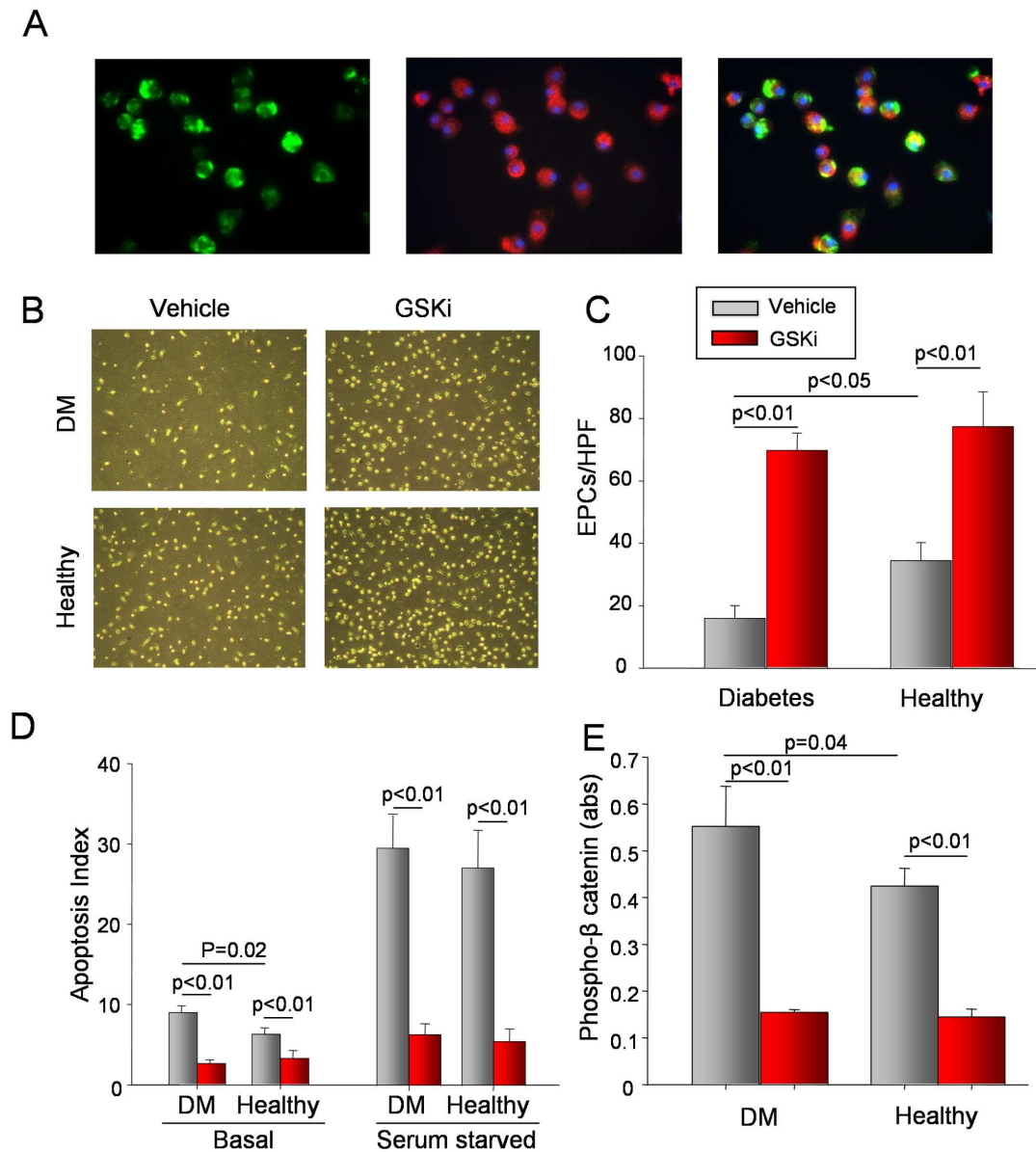


Figure 4.1. Increased GSK3 β Signaling in Diabetic EPC reduces *in vitro* cell yield. **A** - EPCs at 7 days labeled with DAPI (blue), AcLDL-DiI (Red), UEA-1-FITC (green), and merged image. **B** - Light microscopy images of cells from patients with DM and healthy controls under basal conditions or treated with GSKi. **C** - GSKi treatment increases EPC yield in both healthy controls and patients with DM. **D** - GSKi treatment attenuates apoptosis under both basal and serum-starved conditions. **E** - EPCs from patients with DM have higher levels of phospho- β catenin. GSKi treatment in both groups markedly reduced these levels.

Table 4.1 – Baseline Characteristics

	Diabetes (n=12)	Healthy (n=12)	p-value
Age – years (SD)	45.5 (13.8)	52.3 (17.4)	0.30
Males – no. (%)	6 (50.0)	8 (66.7)	0.68
Hypertension – no. (%)	1 (8.3)	0 (0.0)	1.00
Diabetes – no. (%)	12 (100.0)	0 (0.0)	<0.001
Dyslipidemia – no. (%)	3 (25.0)	1 (8.3)	0.59
Smoking – no. (%)	1 (8.3)	2 (16.6)	1.00
Family History – no (%)	2 (16.6)	2 (16.6)	1.00
CAD – no. (%)	0 (0.0)	0 (0.0)	1.00
Fasting Glucose – mmol/L (IQR)	8.4 (7.3-9.0)	5.3 (5.0-5.8)	<0.001
HbA1C – mean (SD)	8.5 (1.3)	5.3 (0.5)	<0.001
LDL cholesterol – mmol/L (SD)	2.5 (1.1)	3.0 (0.8)	0.18

CAD: coronary artery disease; HbA1C: hemoglobin A1C; LDL: low density lipoprotein

1 μ M yielded optimal EPC yields and significantly greater inhibition of GSK3 β (**Figure 4.2B**). Subsequently, CHIR98014 was used as the preferential GSK3 β inhibitor (GSKi) in all experiments.

Notably, supplementation of the culture media with GSKi resulted in an approximately 300% increases in the yields of EPCs in both DM and healthy controls ($p < 0.01$, **Figure 4.1C**). Under basal conditions, the apoptosis index at 96 hours was higher in patients with diabetes as measured by Annexin V and propidium iodide double labelling (9.2 ± 0.9 vs 7.3 ± 0.9 , $p = 0.02$, **Figure 4.1D**) an effect attenuated through GSK3 β inhibition. As expected, serum starvation, used to reproduce cell stress following therapeutic transplantation, resulted in marked increases in apoptosis index in both DM and healthy cells – an effect abrogated with GSKi treatment to near basal levels (**Figure 4.1D**). Importantly, higher levels of phosphorylated β -catenin (p β cat), the product of active GSK3 β activity, in EPCs derived from diabetic patients (0.55 ± 0.08 vs 0.42 ± 0.04 , $p = 0.04$) were markedly reduced in both cohorts of cells following GSKi treatment (**Figure 4.1E**, $p < 0.01$). These findings demonstrate that increased basal activity of GSK3 β in EPCs from patients with DM results in accelerated apoptosis *in vitro* – an effect abrogated by use of isoform specific small molecule inhibitors.

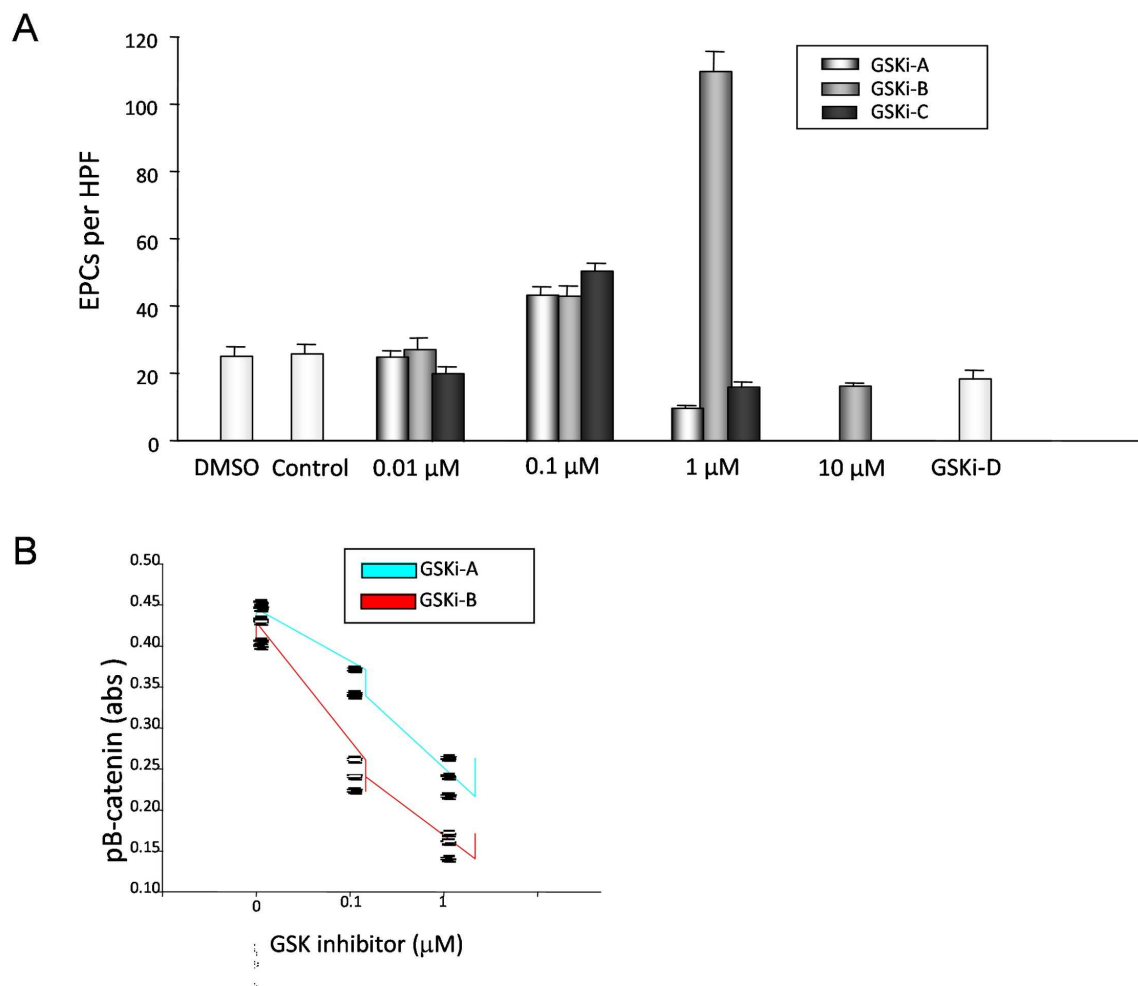


Figure 4.2 Identification of optimal GSK inhibitor. **A** – Effect of increasing doses of GSKi-A (AR-A014418), GSKi-B (CHIR98014), and GSKi-C (Indirubin-3'-monoxime) on EPC yield. GSKi-B at 1μM doses yielded optimal EPC yields with no toxicity. **B** – Comparison of efficacy of GSK3β inhibition between GSKi-A and GSKi-B. At equimolar dosing GSKi-B demonstrated more efficient inhibition.

4.4.2 Proteomic Profiling of EPCs in DM

To ascertain mechanistic insight into the beneficial effects of GSKi on EPCs, analyses of the proteome of EPCs from patients with DM, DM treated with GSKi, and healthy controls were performed (n=3 for each). Isolation protocols were scaled up to yield sufficient cellular yields for the analyses. Differential yields between healthy controls and diabetic patients were maintained in scaled up protocols as were the effects of GSKi (**Table 4.2**). Following 2D gel electrophoresis and digital image analysis, 242 unique protein spots were identified. Differentially regulated candidate targets were identified if there was either a 2.0-fold up regulation or 0.5 down regulation of spot intensity identified between the groups (**Figure 4.3**). In total, 37 non-redundant proteins met these criteria for significant differential expression ($p < 0.05$). These spots were excised from the Sypro-Ruby stained gels and submitted to in-gel trypsinization and the peptide mixtures were analyzed by LC-MS/MS analysis and the results of mass spectroscopy identification are presented in (**Table 4.3**).

Western blot analysis of three target proteins of interest was performed. Specifically catB up regulation (1.9 ± 0.06 vs. 5.8 ± 2.0), Gelsolin down regulation (5.6 ± 1.5 vs. 1.9 ± 0.9), and Plasminogen activator inhibitor-2 (PAI-2) up regulation (2.1 ± 0.7 vs. 5.1 ± 0.5) was confirmed in EPCs from patients with DM ($p < 0.05$ for all comparisons, **Figure 4.4B&C**). As β cat acts as a transcription factor with Transcription factor 7 and Lymphoid enhancer-binding factor 1, we hypothesized that regulation of protein levels seen by GSKi were most likely transcriptional in nature. Indeed, quantitative PCR of mRNA isolated from DM EPCs under basal and GSKi-treated conditions revealed a 4.6 fold increase in catB (± 1.2 , $p < 0.01$, **Figure 4.4D**), 0.5 fold reduction in Gelsolin

Table 4.2 – Proteomics baseline characteristics and cellular yields

	Diagnoses	Cell number	Total μg protein
Healthy 1	None	5.0×10^6	217
Healthy 2	None	7.5×10^6	252
Healthy 3	None	5.0×10^6	275
DM 1	DMII, CAD	3.5×10^6	178
DM 1 + GSKi	DM II, CAD	4.5×10^6	374
DM 2	DMI, PVD	2.5×10^6	90
DM2 + GSKi	DMI, PVD	6.0×10^6	429
DM 3	DMII	3.0×10^6	192
DM 3 + GSKi	DMII	8.0×10^6	726

DM: diabetes mellitus; DMII: type II diabetes mellitus; CAD: coronary artery disease; GSKi: glycogen synthase kinase 3 β inhibitor; DMI: type I diabetes mellitus; PVD: peripheral vascular disease

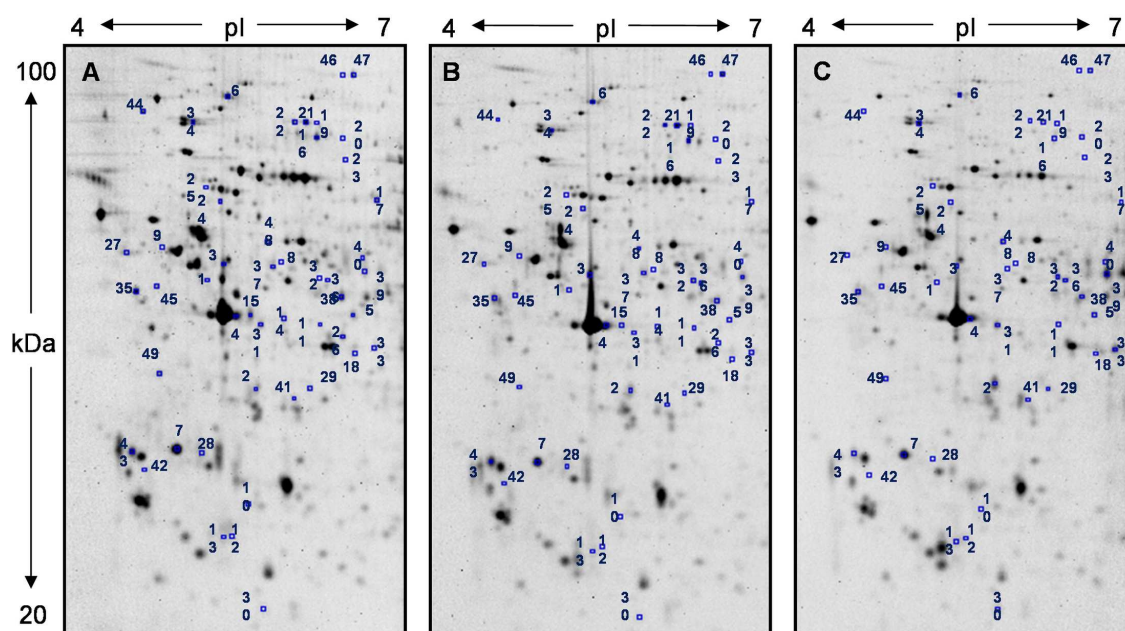


Figure 4.3 Sample 2D gel electrophoresis. Sample protein gels from EPCs derived from a patient with DM, DM EPCs treated with GSKi, and a healthy control. Numerical designation of spots correspond to proteins in **Table 4.3**.

Table 4.3 – Proteomics Analysis

Dot No.	Protein Identity	Swissprot No.	MS Score	Theoretical MW	# of statistically significant matches	Coverage (%)	Ratio (DM/Healthy)	Ratio (DM+GSKI vs. DM)
1	26S protease regulatory subunit 6A	P17980	401	49172	26	46	0.94	1.73
2	Actin, cytoplasmic 1	P60709	1796	41710	82	55	1.53	2.99
3	Actin, cytoplasmic 1	P60709	2638	41710	115	50	2.9	0.42
4	Actin, cytoplasmic 1	P60709	635	41710	27	33	2.09	1.79
5	Adenosylhomocysteinase	P23526	1246	47685	71	31	0.64	1.84
6	Alpha-actinin-4	O43707	364	104788	18	35	1.46	0.51
7	Annexin A5	P08758	2639	35914	159	52	0.65	1.42
8	ATP-dependent RNA helicase DDX39A	O00148	292	49098	13	33	0.86	1.54
9	Beta-hexosaminidase subunit alpha	P06865	786	60664	38	29	0.98	2.12
10	Beta-hexosaminidase subunit beta	P07686	83	63071	8	14	0.55	0.97
11	Calcium-binding mitochondrial carrier protein SCaMC-1	Q6NUK1	325	53320	16	34	1.12	1.76
12	Cathepsin B	P07858	559	37797	21	33	0.45	4.06
13	Cathepsin B	P07858	522	37797	15	30	0.56	3.08
14	Cathepsin D	P07339	788	44524	33	32	0.42	nd
15	Cathepsin D	P07339	1212	44524	55	31	0.49	nd
16	Coagulation factor XIII A chain	P00488	594	83215	28	25	1.62	0.51
17	Dihydropyrimidinase-related protein 2	Q16555	1478	62255	59	62	0.6	0.47
18	Galactokinase	P51570	269	42246	13	26	0.57	1.77
19	Gelsolin	P06396	447	85644	20	30	1.57	0.8
20	Gelsolin	P06396	144	85644	8	10	1.41	1.82
21	Gelsolin	P06396	3025	85644	108	38	1.3	0.38
22	Gelsolin	P06396	2006	85644	72	45	1.97	0.34
23	Glycyl-tRNA synthetase	P41250	560	83113	40	32	0.79	1.51
24	Heterogeneous nuclear ribonucleoprotein K	P61978	603	50944	44	53	0.77	0.62
25	Lamin-B1	P20700	1251	66368	51	49	0.91	2.45
26	Leukocyte elastase inhibitor	P30740	1214	42715	48	49	0.47	nd
27	Lymphocyte-specific protein 1	P33241	600	37169	35	44	0.94	0.34
28	Microtubule-associated protein RP/EB family member 1	Q15691	602	29980	33	59	0.56	0.33
29	N-acetyl-D-glucosamine kinase	Q9UJ70	313	37352	12	24	0.95	1.56
30	Peroxisiredoxin-2	P32119	578	21878	32	38	1.65	0.88
31	Plasminogen activator inhibitor 2	P05120	2097	46566	80	56	0.47	4.43
32	Pyruvate kinase isozymes M1/M2	P14618	3019	57900	96	59	2.89	1.9
33	Pyruvate kinase isozymes M1/M2	P14618	1053	57900	34	35	0.94	2.62
34	Ras GTPase-activating-like protein IQGAP1	P46940	1155	189134	58	13	1.5	0.86
35	Ribonuclease inhibitor	P13489	769	49941	28	37	1.65	0.94
36	Sorting nexin-6	Q9UNH7	259	46620	16	27	1.76	1.44
37	Spliceosome RNA helicase DDX39B	Q13838	887	48960	42	37	1.8	0.38
38	Synaptic vesicle membrane protein VAT-1 homolog	Q99536	1046	41893	55	50	0.68	1.73
39	Tissue alpha-L-fucosidase	P04066	2138	53655	71	43	0.96	5.41
40	Tissue alpha-L-fucosidase	P04066	995	53655	35	21	0.18	2.59
41	Transaldolase	P37837	142	37516	9	19	1.08	1.63
42	Tropomyosin alpha-1 chain	P09493	271	32689	12	20	2.56	0.47
43	Tropomyosin alpha-4 chain	P67936	2259	28504	107	39	1.82	0.43
44	Vimentin	P08670	51	53619	1	6	0.68	1.51
45	Vimentin	P08670	735	53619	34	35	2.03	1.26
46	Vinculin	P18206	4302	123722	142	58	2.1	0.77
47	Vinculin	P18206	2976	123722	119	41	2.05	0.4
48	V-type proton ATPase subunit B, brain isoform	P21281	1523	56465	64	48	0.99	1.95
49	V-type proton ATPase subunit d 1	P61421	271	40303	12	25	0.63	1.55

The protein identities for each spot are listed. The other columns depict the SWISS-PROT No: SWISS-PROT accession number; MS Score: Mass spectrometry score indicating the significance of protein identification from peptide mass finger print according to MASCOT software application 2.3.01 (Matrix Sciences, London, UK) (score value > 50 for $p < 0.05$); Theor. Mr: Theoretical molecular weight of the matching protein; No of significant peptide matches: Number of spectrum-to-peptide matches that were evaluated to be statistically significant (Expect <0.05); Density values were normalized by the local regression model. Student's t-test (two-tail, 95% level of confidence) was calculated for pair-wise comparisons to identify proteins that were expressed at significantly different levels Mean fold change values are indicated in the final two columns and significant changes are indicated in bold. By computational 2D gel image comparison, a total of 49 protein spots were found to be differentially expressed, each exhibiting ≥ 1.5 fold-change (either increase or decrease) of mean value spot intensity among the three different samples. ND: non-detectable.

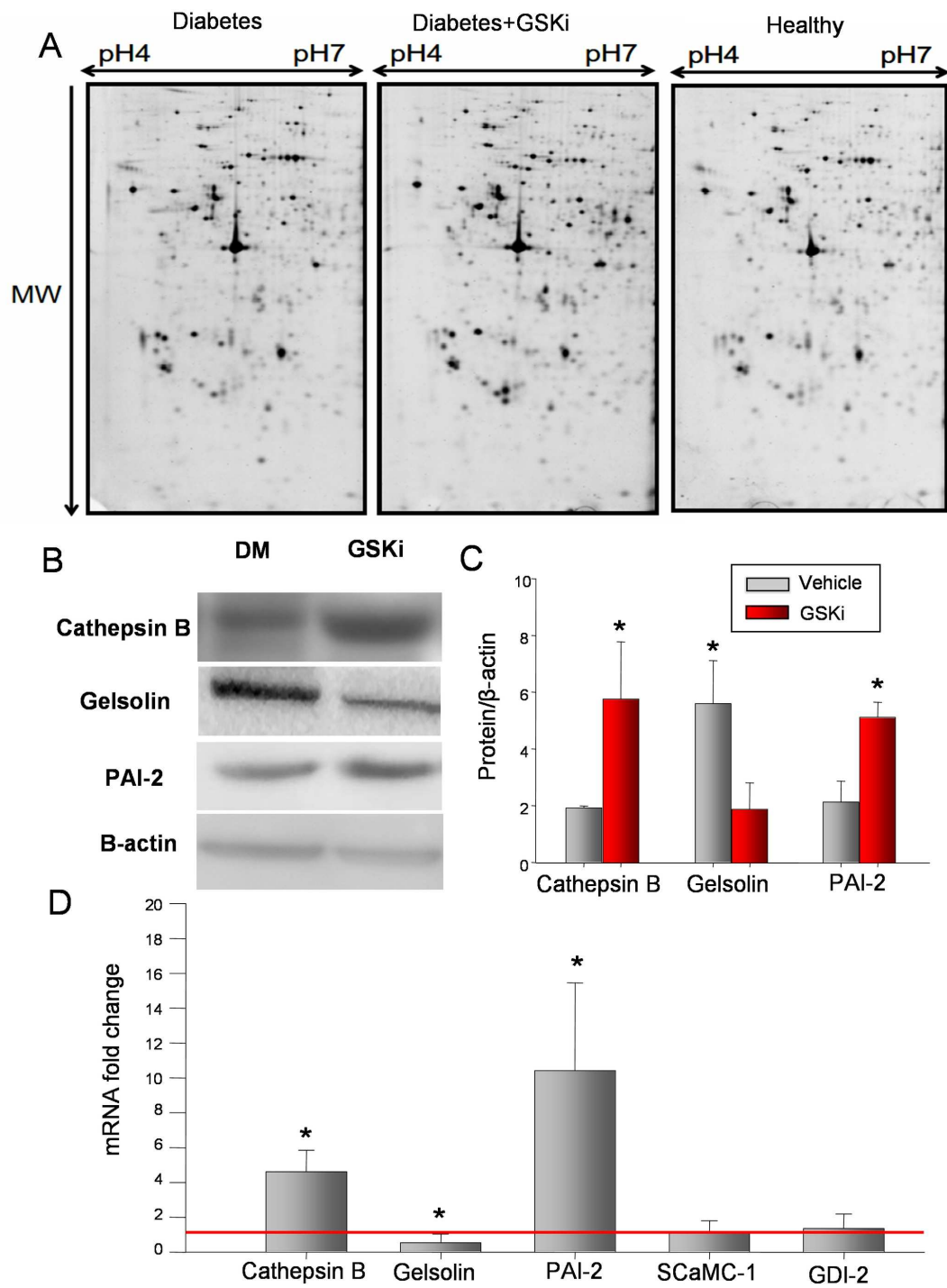


Figure 4.4. Proteomic Analyses of EPCs from patients with DM with and without GSK3 β Inhibition. **A** - Sample 2D gels utilized in proteomics analysis. **B,C** – Western blot analysis of catB, Gelsolin, and PAI-2 (n=6). **D** – Quantitative PCR analysis of candidate genes identified by proteomics analysis. Fold change represents GSKi sample compared to control sample in EPCs derived from patients with DM (n=6). * represents $p < 0.05$.

(0.4, $p < 0.05$), and 10.4 fold increase in PAI-2 (± 5.0 , $p < 0.01$). Neither Calcium-binding mitochondrial carrier protein SCaMC-1 nor GDP dissociation inhibitor-2 (GDI-2) appeared differentially regulated at the mRNA or protein level. These findings confirmed the validity of the proteomics results and identified several targets known to regulate apoptosis *and* to be expressed in EPCs. In this manuscript, we summarize the relevant findings and experiments that evolved from our study of catB.

4.4.3 Cathepsin-B is required for GSKi mediated reductions in apoptosis

To ascertain the role of catB activity in EPC dysfunction in patients with DM, we assayed enzyme activity levels. As predicted by mRNA and protein levels, EPCs from patients with diabetes had 40% less measurable activity compared to healthy control cells (5587.3 ± 1455.1 vs. 8251.2 ± 771.9 RFU, $p = 0.03$, **Figure 4.5A**). Treatment with GSKi raised measurable activity 4-fold in both groups ($p < 0.01$). To ascertain if catB activity was required for changes in phenotype associated with GSKi, we used a specific inhibitor of catB, CA074.(155) Supplementation of the culture media with CA074 abrogated a GSKi-induced dose-dependent increase in catB activity at all levels assayed (**Figure 4.5B**). In turn, functional assays were performed to ascertain the necessity of intact catB activity for phenotypic changes in apoptosis rates, VEGF secretion, and endothelial adhesion by EPCs cultured from patients with DM. Notably, the effect of GSKi on apoptosis could be attenuated when cells were cultured in the presence of the catB inhibitor CA074 (**Figure 4.5C**). Blockade of catB activity demonstrated no effect on VEGF secretion *or* binding of EPCs to activated HUVECs (**Figure 4.5D,E**). Instead, only EPC invasive capacity as assessed by matrigel invasion was also catB dependent, as this parameter increased 3-fold

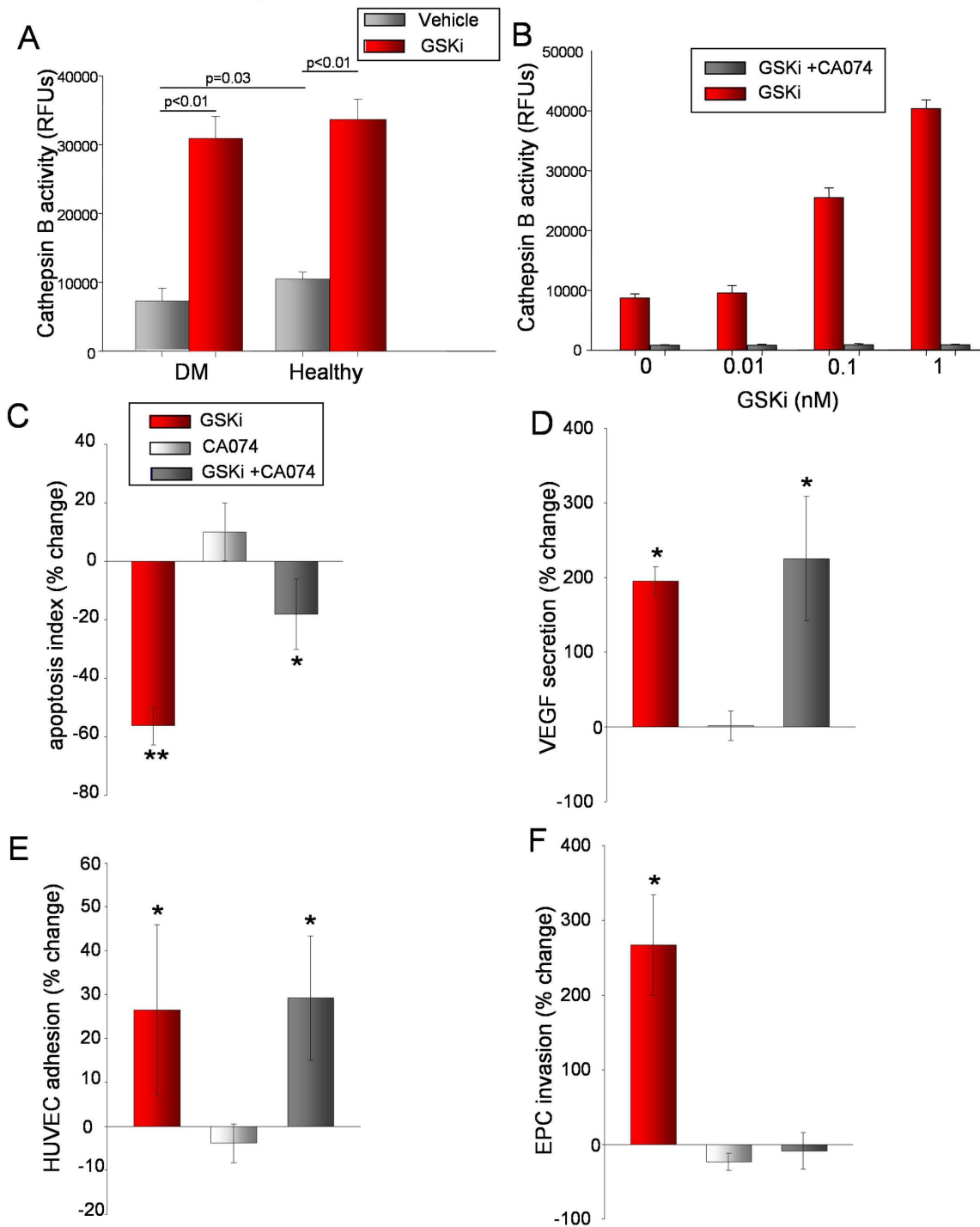


Figure 4.5 Cathepsin B is Required for GSKi Mediated Reduction in the Rate of Apoptosis in Diabetic EPCs. **A** – Cathepsin B activity is reduced at baseline in DM-EPCs and increased by treatment with GSKi (n=6). **B** – Dose dependent increase in cathepsin B activity is achieved with GSKi and effect abrogated by treatment with the cathepsin B inhibitor, CA074 (n=12). **C** – The basal rate of apoptosis is reduced by 60% in EPCs treated with GSKi. This effect is lost with cathepsin B inhibition (n=12). **D** – Improvements in VEGF secretion achieved with GSKi occur independently of cathepsin B activity (n=12). **E** – Increased EPC adhesion achieved with GSKi occur independently of cathepsin B activity (n=12). **F** – Improvement in EPC invasion achieved with GSKi is dependent of cathepsin B activity (n=6).

with GSKi treatment (**Figure 4.5F**, $p < 0.01$). These findings demonstrate that EPCs derived from patients with DM have intrinsically lower catB activity and that induction of expression by GSKi attenuates higher levels of apoptosis and improves invasiveness *in vitro*.

4.4.4 GSK3 β inhibition mediated improvements in EPC based cell therapy require Cathepsin B activity

Efficacy of cell based therapies depends on the dose, functional capacity, and survival of cells following administration. To investigate the therapeutic necessity of catB up regulation to EPC-mediated arterial healing, we administered cells from 6 diabetic patients in a xenotransplant femoral artery wire injury model in CD-1 nude mice (**Figure 4.6A**). Following wire injury, 2.5×10^5 cells were administered intravenously. Administration of cells alone reduced the intima:media (IM) ratio 41% (e.g., 1.37 ± 0.15 vs. 0.81 ± 0.12 , $p < 0.01$, **Figure 4.6B**) at 14 days. Pre-treatment of cells with GSKi resulted in a further 40% reduction in IM ratio compared to untreated EPCs (0.50 ± 0.07 , $p = 0.02$) – an effect lost when cells were co-cultured with GSKi and CA074 (0.77 ± 0.11 , $p = 0.03$ vs. EPC+GSKi). Of note, effects were observed in each individual subject (**Figure 4.6C**). These findings confirm that reductions in apoptosis achieved through up regulation of catB by GSK3 β inhibition in part explain the therapeutic enhancement of EPC based therapy in patients with DM.

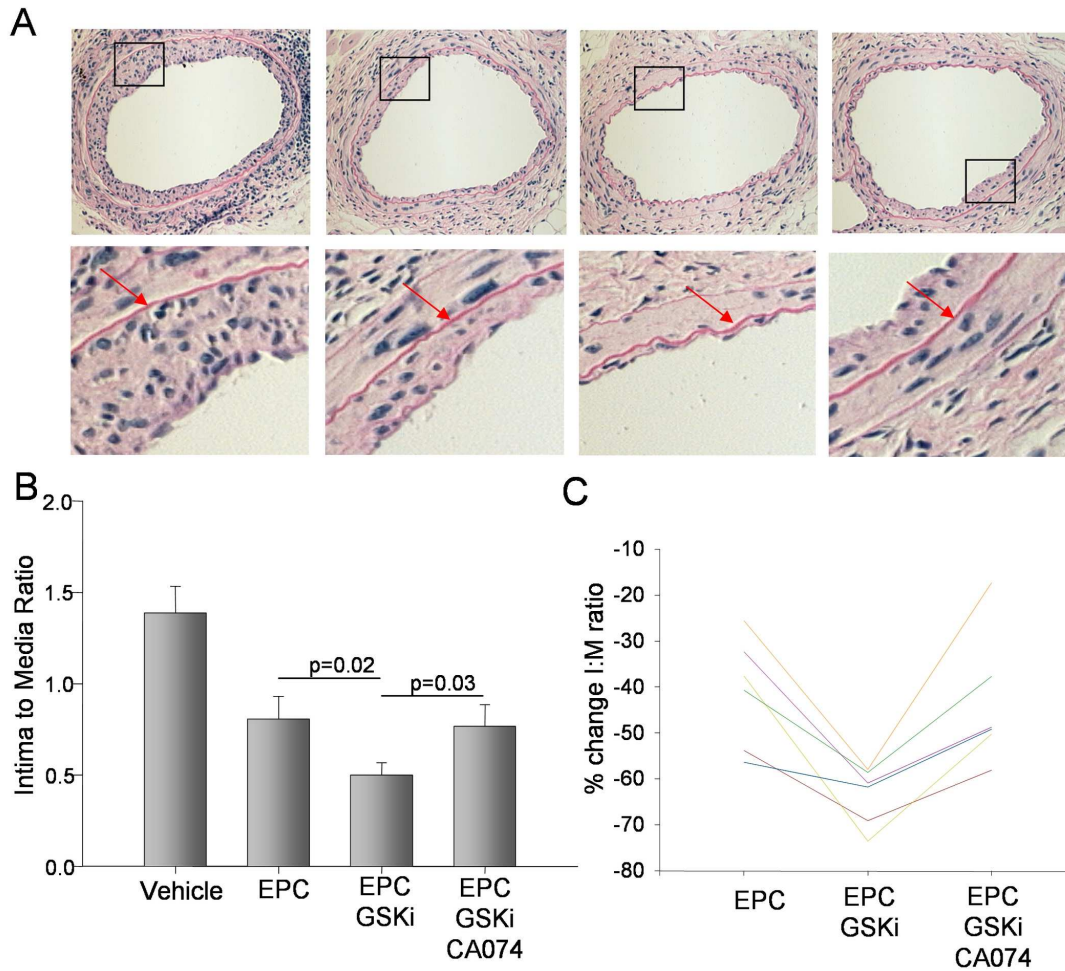


Figure 4.6 Cathepsin B is Required for Improvement in DM-EPC mediated Arterial Repair Achieved with GSKi. **A** - Representative 14 day cross-sections at low magnification with magnified regions of NI highlighted. Arrows indicate the internal elastic lamina. **B** – GSKi treatment of DM-EPCs results in important reductions in NI formation, an effect lost with cathepsin B inhibition (n=6). **C** – Changes in NI formation of individual patients as per treatment group. All patients demonstrated improvement in arterial homeostasis with GSKi treatment (n=6).

4.5 Discussion

Identifying cell enhancement strategies, be it genetic modification,(90) small molecule antagonism of signaling cascades,(63) or use of adjuvant biomaterials is essential to improve the therapeutic efficacy of cell based therapies. Indeed, transplanted cells in a wide variety of models demonstrate poor engraftment with high rates of cell attrition limiting therapeutic benefit. Herein, we highlight important differences in GSK3 β signaling as a factor for enhanced EPC senescence in DM resulting in accelerated rates of apoptosis, decreased VEGF secretion and attenuated adhesion. Moreover, using a proteomics approach we identified up regulation of catB as essential for GSKi-induced reductions in basal and stress induced apoptosis. Finally, in a xenotransplant model, we confirm that catB activity is required for GSKi-induced improvements in EPC mediated arterial repair.

Patients with DM have increased rates of cardiovascular disease and markedly higher rates of in-stent restenosis following revascularization due to impaired arterial healing.(60) This, in part, is owing to attenuated EPC function in patients with DM who have not only fewer circulating cells(39) but higher rates of EPC apoptosis and increased senescence.(41,183) Multiple mechanisms of EPC dysfunction have been identified including eNOS uncoupling,(160) increased reactive oxygen species and the effects of advanced glycation end products.(76) GSK3 β is known to be highly upregulated in a number of tissues in DM,(38) with our data now confirming increased p β -cat, the end product of GSK3 β , in EPCs derived from diabetics. Thus, dysregulation of β -cat

signaling represents a new target for cell enhancement of EPCs in patients with DM - the current study noting improvements in both yield of available cells as well as EPC function.

To date, three studies suggest a beneficial effect of GSK3 β antagonism in EPC-based therapy.(22,63,97) The current report is the first to use an unbiased proteomic approach to identifying differentially regulated proteins in EPCs derived from patients with DM and with GSKi treatment. Using this technique, we identified catB, a protein with known roles as both a pro- and anti-apoptotic factor. Similar to observations in several cell lines,(100,108,154) we noted catB downregulation in DM-derived EPCs resulted in enhanced apoptosis – an effect rescued with GSKi therapy. Interestingly, catB has been linked to cell invasiveness in several cancer cell lines that in turn reduces apoptosis and increases metastatic potential. Our data replicates these findings from the cancer literature, with striking parallels in which transplanted cells with more “malignant” phenotypes are able to degrade extra-cellular matrix and integrate into surrounding tissues more efficiently – in the case of cell based therapy improving therapeutic effect.

This study is not without limitations. Indeed, the definition of EPCs continues to be in flux(40) and we cannot be certain that the mechanisms described in the current study apply to circulating EPC populations. However, the current experiments were performed in primary cells commonly used for therapeutic administration from patients with DM. Second, while there are clear improvements in apoptosis and invasiveness, we do not demonstrate increased cell retention in our *in vivo* model. However, it is well documented

that in numerous animal models of cell based therapy that cellular retention is a rare event that is noted at later time points, while the paracrine effect of cell therapy may be an important early beneficial mechanisms.(56) Despite these limitations, our study is the first to highlight catB regulation by GSK3 β as a potential cell enhancement strategy for patients with DM and our unbiased proteomic approach highlights potential future targets, such as PAI-2, for future investigation.

4.6 Conclusions

Inhibition of GSK3 β activity in EPCs from patients with DM results in up regulation of catB expression and activity. Increased catB activity improves EPC invasiveness, reduces apoptosis, and ameliorates therapeutic effect of cell based therapy. Small molecule antagonism of GSK3 β is a cell enhancement strategy for patients with DM.

4.7 Sources of Funding

The Canadian Institute for Health Research and Medtronic collectively provide EOB with a peer-reviewed Research Chair (URC #57093; IGO 94418) and operating grant.

4.8 Disclosures

None

Chapter 5 General Discussion

5.1 Summary of Findings

Previous studies have clearly established an epidemiological link between EPC number and cardiovascular risk factors, outcomes, and arterial homeostasis post injury.(68,135,168) However, strategies that seek to harness the ability of EPCs to reduce arterial lesion formation following injury have been hampered by poor yields of EPCs from high risk patients *or* poor qualitative properties such as survival, cytokine secretion and adhesion.(61-63,139)

In the current thesis we report three completed studies which demonstrate improved EPC function and improved progenitor cell-mediated arterial repair using primary cells from patients with established CAD or DM. Specifically, in our first report(63) we have shown the ability of inhibiting GSK3 β to increase the yield and functionality of EPCs derived from CAD patients when cultured *in vitro*. We further complemented these studies by demonstrating that the ameliorated function of EPCs translated into improved RE and resultanted in decreased NI formation in a CD-1 nude mouse xenotransplant model of arterial injury.

Subsequently, we have demonstrated in a rabbit model of carotid artery stenting that local delivery of GSKi by drug eluting stent (DES) improves arterial healing.(97) Specifically, GSKi-coated stents showed improved RE as demonstrated by SEM of stented arteries as

well as reductions in NI formation to levels consistent with an established clinically proven drug, sirolimus. Furthermore, the mechanism of this benefit appeared to be limited to effects on EPCs as levels of circulating EPCs were augmented with no significant effect observed on medial SMCs.

Finally, in our last study we tested the strategy of GSK3 β inhibition on EPCs derived from a high risk patient population, those with DM. Having shown that higher activity of GSK3 β in derived EPCs resulted in accelerated EPC apoptosis, we utilized an unbiased proteomics approach to identify increased catB activity as required for improved EPC survival. Moreover, we were able to demonstrate that catB activity was essential for GSKi-mediated improvements in arterial repair.

Overall, these studies provide convincing evidence that cell based therapy with GSKi-treated EPCs and/or local delivery by DES is a feasible therapeutic approach to improve arterial homeostasis following mechanical injury.

5.2 Importance of cell function

The concept of cells as a therapeutic agent, while new to the field of vascular medicine, has existed since the 1950's and is derived from initial studies of bone marrow transplantation.⁽¹⁵⁹⁾ Cell therapy can be subdivided into allogeneic, the transplantation cells from one or many donors, or autologous, whereby cells are harvested, purified, treated or expand and then reintroduced to the patient for therapeutic effect. To date, while there remains no clinically approved cell based therapies for cardiovascular

diseases, a number of early clinical studies have shown promise using autologous sources. However, one of the main hurdles remains the relatively poor function of cells derived from patients with already established disease.

Hill and colleagues(68) first noted an inverse relationship between cultured EPC CFUs and the number of cardiovascular risk factors. Subsequently, these findings have been confirmed by numerous other groups(61,168) who also noted that in addition to fewer cells, the EPCs isolated exhibited attenuated functional activity.(139) Indeed, conditions like DM, hypertension, and smoking have all been shown to adversely affect EPC senescence, apoptosis, migration and paracrine function. While these observations may in part explain the pathophysiology of endothelial dysfunction in these conditions, the attenuated functional capacity is of particular importance in the context of cell-based therapy. Indeed, the lack of therapeutic efficacy derived in some cell-therapy studies has been suggested to be the result of important differences in cell isolation procedures and the resultant blunting of EPC migratory capacity.(94,133,139) Thus, understanding the mechanisms which impair EPC function and deriving therapies to correct innate functional limitations will be paramount in advancing autologous EPC-based therapies clinically.

One of the first papers that linked EPC function to ISR was by George and colleagues.(52) In this seminal study, EPCs from 16 patients with ISR were compared to 11 patients with patent stents. In this study, patients with ISR, EPC adhesion to fibronectin was reduced by 50%, providing the first evidence that impaired EPC function

may play an important role in impaired homeostasis following arterial injury. Our studies(67,98) complement these findings and extend the importance of cell function to use of cells for therapeutic purposes. Specifically, in both of our studies in which EPCs were administered to CD-1 nude mice, the same number of cells (5×10^5) was administered meaning differences in treatment arms were owing only to important differences in therapeutic efficacy. Indeed, in our study of patients with CAD these differences accounted for a further 50% reduction in NI formation. Thus, as suggested by epidemiologic data from clinical cohorts, changes in EPC adherence, survival, and paracrine activity play an important role in EPC mediated arterial repair.

Gnecchi and colleagues recently reviewed the importance of paracrine effects in stem and progenitor based therapies.(56) Indeed, the authors highlight that a number of animal studies have demonstrated significant benefit without evidence of transdifferentiation of progenitor cells into an end-organ phenotype – suggesting that the benefits observed are likely derived through paracrine effects. To this end, one of the major angiogenic factors secreted by EPCs is VEGF, a factor previously shown to be essential in EPC-mediated postnatal neovascularization and endothelial proliferation.(6,7,144) In this thesis, one of the major effects observed with GSKi therapy was increased VEGF secretion. Indeed, in patients with CAD GSKi therapy augmented EPC secretion 600% with a 200% increase achieved in patients with DM. Moreover, co-culture of EPCs treated with GSKi stimulated proliferation of mature human endothelial cells leading to more robust RE *in vitro*. Thus, given the *in vitro* findings and the robust *in vivo* efficacy of GSKi pre-

treatment, important differences in paracrine efficacy likely in part contributed to the observed differences.

A second important limitation of cell therapy is the high rates of cell death of progenitor cells – both *in vitro* during expansion and furthermore once cells are transplanted.(158) Indeed, cell enhancement strategies which improve cell retention following therapeutic administration is critical for therapeutic efficacy.(17,157,158) The current thesis highlights an important improvement in cell survival using GSKi treatment in EPCs. Moreover, these reductions in apoptosis were observed under basal and stress conditions (serum-starved) and were shown to be dependent on catB. Our findings are similar to those by other groups who have identified strategies which reduce cell apoptosis can enhance therapeutic efficacy.(21,105) Indeed, our studies confirmed these findings demonstrating that antagonism of catB both abrogated reductions in apoptosis *in vivo* and blocked therapeutic efficacy *in vivo*. Thus, these results suggest that reductions in apoptosis are critical for therapeutic efficacy and in our studies accounted for the majority of GSKi-mediated benefit.

5.3 Progenitor Cell Preconditioning, Cathepsins and EPCs

A growing body of literature now supports progenitor survival and retention following transplantation as the greatest hurdle in cell-based therapies.(1) Indeed, while paracrine efficacy is important, cell retention at the site of transplantation may play a rate-limiting role in therapeutic efficacy with some authors estimating close to 90% cell loss due to apoptosis in as little as 24 hours.(10)Accordingly, cell preconditioning has become a

preferred strategy for enhancing therapeutic efficacy and therapies such as insulin-like growth factor -1 have been shown to reduce rates of apoptosis in transplanted cells and resultantly improve efficacy.(80) Currently our understanding of the mechanisms which limit cell engraftment are incomplete and the results presented in this thesis provide insight not only into a novel mechanism by which to improve therapeutic efficacy but also highlights limitations in using autologous cells derived from patients with CAD or DM.

Cathepsins are a family of proteases classified based on their structure and catalytic targets. CatB belongs to the family of cysteine cathepsins and it has been linked to regulation of a numerous cell functions through both intracellular and extracellular activity.(125) Cathepsin L (catL) has also been previously implicated in EPC activity. Urbich and colleagues first demonstrated that catL activity is essential for EPC-mediated neovascularization, primarily by mediating cell invasion.(166) Subsequently the same group noted that EPCs cultured in high glucose media *or* derived from patients with DM exhibited profoundly diminished catL activity – and concluded that this would likely limit *in vivo* activity.(163) Results in the current thesis now complement these findings and highlights catB, also a cysteine protease, as dysregulated in DM and as essential for *in vivo* therapeutic effect.

Data regarding the role of catB in the vascular system are somewhat limited; however, an abundance of data exists linking increased catB activity with both GSK3 β and enhanced metastatic capacity and cell survival. Specifically, increased catB activity has been

demonstrated to be essential for matrix degradation with antagonism hindering a number of primary cancer cell lines' invasive capacity.(81,104,154,156) Furthermore, inhibition of catB and urokinase-type plasminogen activator receptor (uPAR) have been shown to increase rates radiation-induced apoptosis.(100,101) Finally, catB activity has also been demonstrated to be essential for malignant stem-cell self-renewal.(123) Thus, our finding that GSK3 β inhibition restores catB activity reducing apoptosis in EPCs is not surprising given the previously reported data in cancer cells. Rather, it highlights the parallels in signaling cascades which in one instance enables a cancer cell to invade and survive causing disease while in another instance enabling an EPC to survive transplantation and exert therapeutic benefit. It remains to be seen if similar parallels between metastatic potential in cancer cell lines can be exploited for therapeutic effect in cell-based therapies.

5.4 Limitations of Experimental Studies and Barriers to Clinical Translation

Certainly, the data presented in the current thesis are not without limitations. First and foremost, use of cells from GSK3 β knockout mice would offer an ideal experimental model to confirm our findings. However, knockout of GSK3 β results in embryonic lethality(82) and an EPC specific knockout of the enzyme is not yet available.(84) Nonetheless, to ensure specificity we utilized numerous small molecule inhibitors confirming increased nuclear localization of β cat. Alternatively, transformation of EPCs with a dominant negative GSK3 β mutant(22) could have been performed, however, genetic manipulation of progenitor cells introduces greater complexity, enhances cell death *in vitro*, and introduces barriers to potential use as a clinical therapeutic.

A second major limitation to the described strategies is the potential off target effects of GSK3 β inhibition. For example, GSK3 β inactivation in SMCs has been shown to increase migration, proliferation and in some models increase NI formation.(53,112,146) However, our strategy of preconditioning EPCs with GSKi enables EPC specific inhibition without causing any off-target effects. Additionally, in our studies using a strategy of local delivery at the site of injury(97), GSKi ultimately reduced the arterial response to injury. Thus, based on the results presented in the current thesis off target effects do not seem to complicate either elution of GSKi from a stent or preconditioning of cells. Nonetheless, future studies testing these strategies should be cognizant of this potential limitation and further animal studies are needed prior to clinical studies.

Lastly, perhaps the greatest limitation of the current studies are the barriers inherent in any cell-based therapy.(65,77,92) Namely, current technology for cell isolation and preparation is labor intensive and requires specialized techniques to use EPCs for therapeutic purpose. Moreover, isolating autologous cells from patients often requires mobilization with agents such as G-CSF or obtaining samples from enriched sources such as bone marrow. Nonetheless, early clinical studies demonstrate promise(9,94,133) and by better understanding the mechanisms that impair their function and identifying treatments which enhance their therapeutic effect we move closer to realizing the promise of cell-based therapies.

5.5 Future Directions

Our studies highlight a novel strategy for enhancing cell-based therapy and EPC-mediated arterial repair through isoform-specific inhibition of GSK3 β . However, these studies are limited to a single progenitor population and experiments performed using a model of arterial repair following vascular injury. Ideally, future studies will determine if similar preconditioning regimens yield improved cell function and therapeutic benefit using both diverse cell populations, such as mesenchymal stem cells or skeletal muscle progenitor cells, as well as in other cardiovascular conditions, such as cardiac repair following myocardial infarction. Indeed, given the parallels in cell repair mechanisms and the fidelity of the biological effect of GSK3 β inhibition in various cancer types it is attractive to hypothesize that the effect reported may be generalized to other types of cell-based therapy. Data from diverse experimental models will provide not only additional evidence of efficacy and safety but also afford a greater impetus for future clinical studies.

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7.1 Contributions & Collaborators

Chapter 2

XM – taught me the femoral artery technique and histology

AP – assisted in *in vitro* assays

EH – measured in a blinded fashion I:M volumes

KR – assisted in *in vitro* assays

YXC – taught me histology and *in vitro* assays

JS – assisted in *in vitro* assays

LF – taught me flow cytometry and assisted in data collection

EO – designed and supervised the experiments

Chapter 3

XM – performed the rabbit surgeries and histology

BD & TS – assisted in histology

XZ & YXC – assisted in *in vitro* assays

EO – designed and supervised the experiments

Chapter 4

JL – performed the proteomics and analyzed the proteomics data

XM & YXC – taught surgical techniques and histology

TS, JR, TS – assisted in *in vitro* assays and western blot optimization

DS & EO – designed and supervised the experiments

8.1 Appendices

Appendix I

Citation Information:

Costiniuk, C.T., **Hibbert, B.**, Simard, T., Ghazawi, F.M., Angel, J., O'Brien, E.R. (2013)
Circulating Endothelial Progenitor Cells in HIV Infection: A Systematic Review. *Trends
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Author Contributions:

Conceived and designed the study: CC, BH, TS

Wrote the manuscript: CC BH TS FG

Supervised: ERO, JA

Circulating Endothelial Progenitor Cells in HIV Infection: A Systematic Review

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Running Title: EPCs in HIV Infection

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Abstract

Human Immunodeficiency Virus (HIV)-infected individuals have a cardiovascular disease risk that is almost tripled that of their HIV-uninfected counterparts. Given the critical role of endothelial progenitor cells (EPCs) in vascular homeostasis and arterial repair post-injury, coupled with their strength as biomarkers predictive of cardiovascular events, interest has arisen in characterizing EPCs in the context of HIV infection. We performed a systematic review of the literature to determine the current state of knowledge on EPCs in the context of HIV infection. Herein we summarize the pertinent findings of these studies and discuss important differences in the subpopulations of EPCs examined and the methodologies used for their enumeration which likely contributed to the heterogeneity observed across studies.

Key Words: Endothelial Progenitor Cells, EPCs, Cardiovascular Disease, HIV

Introduction

Despite the use of highly active antiretroviral therapy (HAART), human immunodeficiency virus (HIV)-infected individuals are at higher risk of myocardial infarction (MI) (SMART Study Group 2006), in-stent restenosis (Ren et al. 2009) and stroke (Sen et al. 2012). In fact, HIV-infected individuals with sustained undetectable blood plasma viral loads (VL) are at a risk of cardiovascular disease that is almost tripled that observed in individuals with similar cardiovascular risk factors but without HIV infection (Vittecoq et al. 2003; Currier et al. 2008).

Circulating endothelial progenitor cells (EPCs) were first described by Asahara et al. (1997) in a landmark study whereby they demonstrated that human CD34⁺ hematopoietic progenitor cells have the capacity to differentiate *in vitro* to an endothelial phenotype. Although a number of definitions of what constitutes a “true” EPC have emerged (see review by Fadini (2012)), EPCs are typically characterized by the presence of CD34⁺ and vascular endothelial growth factor (VEGF) receptor kinase insert domain (KDR). When derived from mononuclear cells in culture, they are referred to as early or late EPCs or cultured angiogenic cells (CACs) (Hibbert 2009; Hirshi 2008). EPCs are well-described mediators of arterial homeostasis and vascular repair (Fadini 2012; Hibbert et al. 2009; O’Brien et al. 2011). From a clinical perspective, interest in EPCs has rapidly escalated due to their role both as a biomarker for the prediction of cardiovascular events (Hill et al. 2003; Schmidt-Lucke et al. 2005; Werner et al. 2005) and for promising results as a therapy following myocardial infarction (Assmus et al. 2006; Schachinger et al. 2006).

Given the rapidly growing interest in EPCs with respect to HIV, we performed a systematic review of the literature to describe the current state of knowledge regarding EPC levels and functional capacity within the HIV-infected population. We also reviewed the literature to determine whether these cells have been found to be correlated with markers of HIV disease status, such as CD4⁺ count, VL and duration of HIV infection.

Methods:

We performed a computerized literature search of MEDLINE and SCOPUS up until September 17th 2012 for English language studies. Searches using permutations of the search terms human immunodeficiency virus, HIV, acquired immune deficiency syndrome, AIDS, endothelial progenitor cells, EPC, cultured angiogenic cells, CAC, endothelial colony-forming cells, and ECFC were performed. Secondarily, additional references were identified via searching all references and citing articles of the selected studies utilizing the SCOPUS database. This practice was repeated for each article selected to ensure inclusion of all relevant studies. In this manner, a total of 3,146 titles and abstracts were reviewed for potential study eligibility. From this initial collection, abstracts were reviewed by a single individual who removed articles not relevant to the study. From this, 36 articles were collected for further review. Two reviewers independently evaluated the identified studies for eligibility, with any discrepancies being resolved by consensus, to yield a final list of 6 articles for further analysis.

Patient Characteristics

Characteristics of 6 articles retrieved (Costiniuk et al. 2012; Da Silva et al. 2011; Gomez-Garre et al. 2012; Lopez et al. 2011; Papasavvas et al. 2012; Toefili et al. 2010) and the study participants are summarized in Table 1. Numbers of HIV-infected participants ranged from 14 (Teofili et al. 2010) to 200 (Gomez-Garre et al. 2012) and the age range was from 29 (Lopez et al. 2011) 55 to years (Papasavvas et al. 2012). One

study excluded women (Costiniuk et al. 2012) in order to avoid the confounding effects of estrogen (Fadini et al. 2008) whereas the percentages of women ranged from 7% (Lopez et al. 2011) to 21% (Da Silva et al. 2011) in the other 5 studies. Similarly, only 1 study specifically excluded smokers (Costiniuk et al. 2012) whereas the percentages of HIV-infected participants who were smokers ranged from 16% (Lopez et al. 2011) to 61% (Papasavvas et al. 2012) in the remaining studies. Notably, the only study which included diabetics was that performed by Gomez-Garre whereby almost 10% of HIV-infected individuals were diabetic (Gomez-Garre et al. 2012). Frequently, dyslipidemia was the only cardiovascular risk factor identified in the HIV-infected individuals in these studies (Costiniuk et al. 2012; Da Silva et al. 2011; Gomez-Garre et al. 2012; Teofili et al. 2010). Other classical cardiovascular risk factors in HIV-infected participants included hypertension and obesity. However, these latter two risk factors were uncommon overall. With regards to HAART use, 4 of the 6 studies included exclusively HAART-naive individuals (Costiniuk et al. 2012; Da Silva et al. 2011; Lopez et al. 2011; Teofili et al. 2010), whereby 2 studies included individuals who were HAART-naive in addition to individuals already on HAART (Gomez-Garre et al. 2012; Papasavvas et al. 2012).

Techniques for the Quantification of EPCs

The two methods currently utilized for EPC enumeration include flow cytometry and cell culture. Five groups performed flow cytometry to enumerate EPCs (Costiniuk et al. 2012; Da Silva et al. 2011; Gomez-Garre et al. 2012; Lopez et al. 2011) whereas Teofili et al. (2010)

employed cell culture. Furthermore, 5 studies quantified EPCs once per study participant and 1 study, performed by Papassavas et al. (2012), quantified EPCs twice per individual. In this latter study, EPCs were enumerated at baseline and one year later as the aim was to determine whether rate of carotid intima media thickness (cIMT) progression was related to EPC frequency (Papassavas et al. 2012).

Given previous observations that cells obtained using different protocols vary in their propensity to differentiate into endothelial cells and undergo angiogenesis (Fadini et al. 2012), the International Society of Haematotherapy and Graft Engineering (ISHAGE) protocol was developed in an attempt to facilitate standardization of methods (Sutherland et al. 1996). This protocol consists of a gating strategy for the analysis of rare cells expressing CD34⁺ (Sutherland et al. 1996). Recently, the ISHAGE protocol was modified by Schmidt-Lucke (2010) by adding a fluorochrome-labelled surface marker for kinase insert domain receptor (KDR) to the original protocol which has, in turn, increased accuracy in EPC enumeration. One of the studies included in this review utilized the modified ISHAGE protocol for cell enumeration (Costiniuk et al. 2012) whereas the other studies used non-standardized protocols for cell enumeration. Overall, there was significant heterogeneity in the methods used to enumerate EPCs (Table 2).

Populations of EPCs Examined and Study Outcomes

The term “EPC” was never established using a unique or specific cell marker set and there is considerable debate within the scientific community with regards to the optimal way to define an EPC. However, a common belief is that a cell should bear at least one marker indicating that it is a stem cell and at least 1 marker demonstrating its endothelial

commitment in order to be designated as an EPC. The former typically includes CD34 and/or CD133 while the latter commonly consists of KDR [also known as vascular endothelial growth factor receptor-2 (VEGFR-2) and fetal liver kinase-1 (Flk-1)]. Frequently EPCs are defined as CD34+KDR+ as this population can stimulate angiogenesis *in vivo* (Asahara et al. 1997). Isolation methods and commonly used terminology are highlighted in Figure 2.

Using flow cytometry, both Gomez-Garre et al. and Da Silva et al. found lower frequencies of CD34+KDR+ cells in HIV-infected, compared to non-infected, individuals (Gomez-Garre et al. 2012; Lopez et al. 2011). In contrast, Papasavvas et al. found that HIV-infected individuals had higher percentages of CD34+/KDR+ cells in two measures over a 1 year follow-up period compared with HIV-uninfected controls. Higher numbers of EPCs in HIV-infected individuals, compared to controls, were observed for both HIV-infected individuals receiving HAART and those who were HAART-naïve (Papasavvas et al. 2012).

In earlier years, an issue of debate within the scientific community was whether to include CD45+ or CD45- cells in studies involving EPCs. However, now it is well-recognized that most EPCs are hematopoietic cells. In fact, greater than 90% of progenitor cells express CD45 at low intensity (CD45^{dim}) whereas less than 10% are CD45- (Fadini et al. 2012). The cell populations which are CD45^{dim} form endothelial colonies *in vitro* and those which are CD45- are referred to as late outgrowth endothelial cells. Furthermore, CD45^{dim}/CD34+/KDR+ cells have been shown to correlate best with future cardiovascular events and progression of atherosclerotic disease (Schmidt-Lucke et al. 2005). Our group examined CD45^{dim}/CD34+/KDR+ cells between HIV-infected

versus non-infected men and did not find any significant differences in levels between these two groups (Costiniuk et al. 2012). We also examined CD45^{dim}/CD34+/CD184+ cells and CD45^{dim}/CD34+/CD117+ cells as secondary endpoints given that CD184 corresponds to the receptor for stromal-derived factor-1 and CD117 is the cognate receptor for stem cell factor. As with CD45^{dim}/CD34+/ KDR+ cells, levels of CD117+ and CD184+ progenitors did not differ between HIV-infected men and controls (Costiniuk et al. 2012).

Although not expressed on endothelial cells, CD133 is another marker which has garnered interest. It is expressed on many hematopoietic stem, progenitor and epithelial cells and it is thought to be a more immature marker than CD34. Although Papasavvas et al. (2012) examined primarily CD34+/KDR+ cells, they also confirmed that HIV-infected individuals had higher levels of CD133+/KDR+ and CD34+/KDR+ cells. In contrast, Da Silva et al. (2011) also examined CD34+/CD133+ and KDR+/CD133+ cells and did not detect a difference between HIV-infected individuals compared with controls. Along a similar vein, Lopez et al. (2011) differentiated between EPCs based on their level of maturity and examined immature EPCs (CD45-/CD34+/CD31+CD133+), EPCs (CD45^{dim}/CD34+/CD31+/CD133+/CD309+), late EPCs (CD45^{dim}/CD34+/CD133-) and circulating endothelial cells (CECs) (CD45-/CD34+/CD31+/CD133-). They found that numbers of late EPCs were similar between HIV-infected and HIV-uninfected individuals whereas EPCs were lower in HIV-infected compared to HIV-uninfected individuals (Lopez et al. 2011).

Gomez-Garre et al. (2012) examined yet another subpopulation of EPCs. In addition to CD34+/KDR+ cells, they also examined levels of progenitor cells bearing CD34+/VE-

cadherin⁺ and CD14⁺Endoglin. VE-cadherin mediates important interactions between endothelial cells (Dejana et al. 2004) and helps maintain their integrity and ability to function as barriers (Corada et al. 1999). Meanwhile, endoglin is a Transforming Growth Factor- β co-receptor expressed in endothelial cells which plays a key role in vascular homeostasis, angiogenesis and vascular remodeling (Lopez-Novoa and Bernabeu 2010). In comparison to HIV-negative individuals, HAART-naïve HIV-infected individuals displayed lower levels of CD34⁺/KDR⁺ and CD34⁺/KDR/VE-cadherin⁺ cells. HIV-infected individuals on HAART had even lower levels of CD34⁺/KDR⁺ and CD34⁺/VE-cadherin⁺ cells compared to HIV-infected individuals not on HAART. However, levels of CD14⁺Endoglin⁺ cells did not differ statistically between HIV-infected and non-infected individuals. Furthermore, within the HIV-infected cohort, HAART use was associated with higher CD14⁺Endoglin⁺ levels than being HAART-naïve (Gomez-Garre 2012).

In the only study to enumerate EPCs by culture assays, Teofili et al. (2010) examined colony-forming unit endothelial cells (CFU-EC) and endothelial colony-forming cell (ECFC) progenitors. “Early EPCs,” or CFU-EC, facilitate the formation of new blood vessels by the secretion of growth factors. In contrast, “Late EPCs,” or ECFC, are able to produce actual blood vessels. Teofili et al. (2010) found that CFU-EC, but not ECFC, were significantly reduced among HIV-infected individuals compared to healthy controls. Furthermore, Teofili et al. (2010) were the only group to examine a marker of EPC function by conducting assays for the expression of VEGF in CFU-EC from both HIV-infected and HIV-uninfected individuals. CFU-EC colonies from HIV-infected individuals produced similar amounts of VEGF RNA as CFU-EC obtained from healthy

controls despite the fact that CFU-EC levels were reduced among HIV-infected individuals (Teofili et al. 2010).

EPC Levels and Parameters of HIV Infection

A noteworthy feature of all studies is that the time from diagnosis of HIV was relatively short, with a mean of 3-4 years in 2 studies (Costiniuk et al. 2012; Da Silva et al. 2011) and less than 1 year in one study (Lopez et al. 2011). Furthermore, although cIMT was not associated with EPC frequencies in the study by Papassavas et al (2012), a positive association was detected between CD34+/CD133+/KDR+ cell frequency and peripheral CD4+ counts. In contrast, 4 studies did not find a correlation between CD4+ count and EPC levels (Costiniuk et al. 2012; Gomez-Garre et al. 2012; Lopez et al. 2011; Teofili et al. 2010). Similarly, 3 studies (Costiniuk et al. 2012; Lopez et al. 2011; Teofili et al. 2010) did not find a correlation between EPC levels and VLs. Interestingly, Gomez-Garre et al. (2012) found that VL positively correlated with CD34+/KDR+ cell levels but was negatively correlated with CD14+/Endoglin+ cell levels.

In this latter study by Gomez-Garre et al. (2012), HAART use was the main predictor for low CD34+KDR+ cell levels and high CD14+/Endoglin+ cell levels after adjustment for cardiovascular risk factors and duration of infection. These investigators found that accumulated exposure time of greater than 5 years to a non-nucleoside reverse transcriptase inhibitor (NRTI) and a protease inhibitor (PI) was associated with lower levels of CD34+/KDR+ cells in addition to greater cIMT (Gomez-Garre et al. 2012). In contrast, while Papasavvas et al. (2012) found that HIV-infected individuals on HAART had higher EPC frequencies compared to HIV-uninfected individuals regardless of CD4+

count, there were no differences in EPC levels between HIV-infected individuals on HAART or off of HAART. Interestingly, a relatively large proportion of individuals on HAART in this latter study still had CD4⁺ counts <350 cells/ μ L at time point one (15%) and one year later at the second time point (23%) (Papasavvas et al. 2012).

Discussion

To date, only 6 studies have examined EPCs in the context of HIV infection and these studies have yielded divergent results. While 2 studies demonstrated that CD34⁺KDR⁺ cells were reduced in HIV-infected individuals compared to uninfected individuals (Gomez-Garre et al. 2012; Da Silva et al. 2011), one study revealed no difference between these populations (Costiniuk et al. 2012). The one study which examined EPCs bearing CD45^{dim}/CD34⁺/CD31⁺/CD133⁺/CD309⁺ surface markers also found that these cells were reduced in HIV-infected individuals compared to controls (Lopez et al. 2011). In contrast, one study found that CD34⁺KDR⁺ cells were elevated at two separate time points (Papasavvas et al. 2012). In the one study which used culture-based assays to enumerate EPCs, CFU-EC but not ECFC were significantly reduced in HIV-infected, compared to HIV-uninfected individuals (Teofili et al. 2010). In this latter study, which was the only study to examine VEGF expression, a marker of EPC function, CFU-EC colonies from HIV-infected individuals were observed to produce similar amounts of VEGF RNA as CFU-EC from healthy controls (Teofili et al. 2010).

There were several confounders present in these studies which could have influenced EPC levels. Notably, all studies except for one (Costiniuk et al. 2012) included smokers. Nicotine can induce anomalies in progenitor cell proliferation and can

induce their apoptosis. It can also influence their migratory and angiogenic potential (Cardinale et al. 2012). In the general population, smoking has been associated with reduced EPC levels as well as diminished adhesion and proliferative capacity (Puls et al. 2011). Likewise, diabetes is also an important factor to consider when examining studies involving EPCs. EPCs in diabetics possess reduced adhesive and proliferative properties, hampering their ability to incorporate into vascular structures (George et al. 2003). Meanwhile, dyslipidemia results in the accumulation of remnant-like lipoproteins which have been implicated in premature EPC senescence through the inhibition of telomerase (Thorin et al. 2011). Furthermore, as EPCs have been shown to increase in a dose-dependent manner in response to estrogen and may vary throughout the menstrual cycle (Ruifrok et al. 2009), variability in the proportions of women across these studies is an important consideration.

Whether participants were receiving HAART is another important factor to take into account when examining outcomes from these studies. Results from The Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) Study Group (2007) demonstrated an adjusted relative risk of acute MI of 1.16 per year of ART exposure. The use of a PI was also found to be a risk factor for increased risk of MI (Sabin et al. 2008). Although numerous other observational studies have since been published with overall conflicting results (Cruciani et al. 2011), studies at the basic science level have demonstrated possible mechanisms by which ARVs may promote endothelial damage (Zhong et al. 2002) thus supporting the notion that HAART may, indeed, contribute to atherosclerosis independently of other factors. Furthermore, given the link between ongoing HIV replication, inflammation and endothelial dysfunction (Hsue et al. 2012) it

is very clear that individuals on HAART differ immunologically from those who are not on HAART which may, in turn, also influence EPC levels and functional capacity. As previously alluded to, in the study by Papasavvas et al. (2012), several individuals on HAART had CD4⁺ counts <350 cells/ μ L at both time points separated by a year. As the authors indicate, this may be reflective of suboptimal immune recovery in these individuals or very low CD4⁺ T cell levels at first presentation.

The different enumeration techniques used across studies is a pivotal factor contributing to the divergent outcomes. Both flow cytometry and cell culture methods are well-accepted for the enumeration of EPCs and each method has revealed important findings. For example, EPCs analyzed by each method have been shown to differ significantly between individuals with and without cardiovascular disease (Urbich and Dimmeler 2004; Werner et al. 2005). Circulating EPCs, as determined by flow cytometry, have been shown to predict the occurrence of cardiovascular events and death from cardiovascular causes (Werner et al. 2005). When assessed by culture, a strong correlation has been observed between the number of EPCs and cardiac risk scores and brachial-artery reactivity (Hill et al. 2003). An advantage of flow cytometry is its ability to identify cells based on surface antigens without manipulation (Fadini et al. 2012). In contrast, when cells are obtained by culture methods, there is concern that culture methods alter antigen expression such that they no longer represent antigen expression or differentiation capacity within the body (Fadini et al. 2012).

In summary, relatively few studies have been conducted on EPC levels in the HIV-infected population and only one of these studies examined functional capacity of EPCs (Teofili et al. 2010). Significant heterogeneity in enumeration techniques and reporting

among studies precluded meta-analysis and thus the ability to quantitatively estimate the effect of HIV on EPC numbers. Furthermore, as varying flow cytometry protocols were used, the biological significance for many “EPC subpopulations” examined remains unclear. When studying cell populations with increasing antigenic complexity, these cells are often present in very low numbers and these events frequently do not follow a Gaussian distribution limiting the reproducibility of cell enumeration (Fadini 2012).

Although the studies are few in number, this review summarizes the entirety of the current state of knowledge regarding EPCs in the context of HIV. Future studies should strive to utilize standardized techniques for EPC enumeration and reporting. EPC functional capacity and the effects of HIV are likely to be as important as absolute levels of these cells and should also be evaluated in future reports. The role of EPCs in the vascular health of HIV patients remains to be defined.

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Conflicts of Interest

None of the authors have any conflicts of interest to declare.

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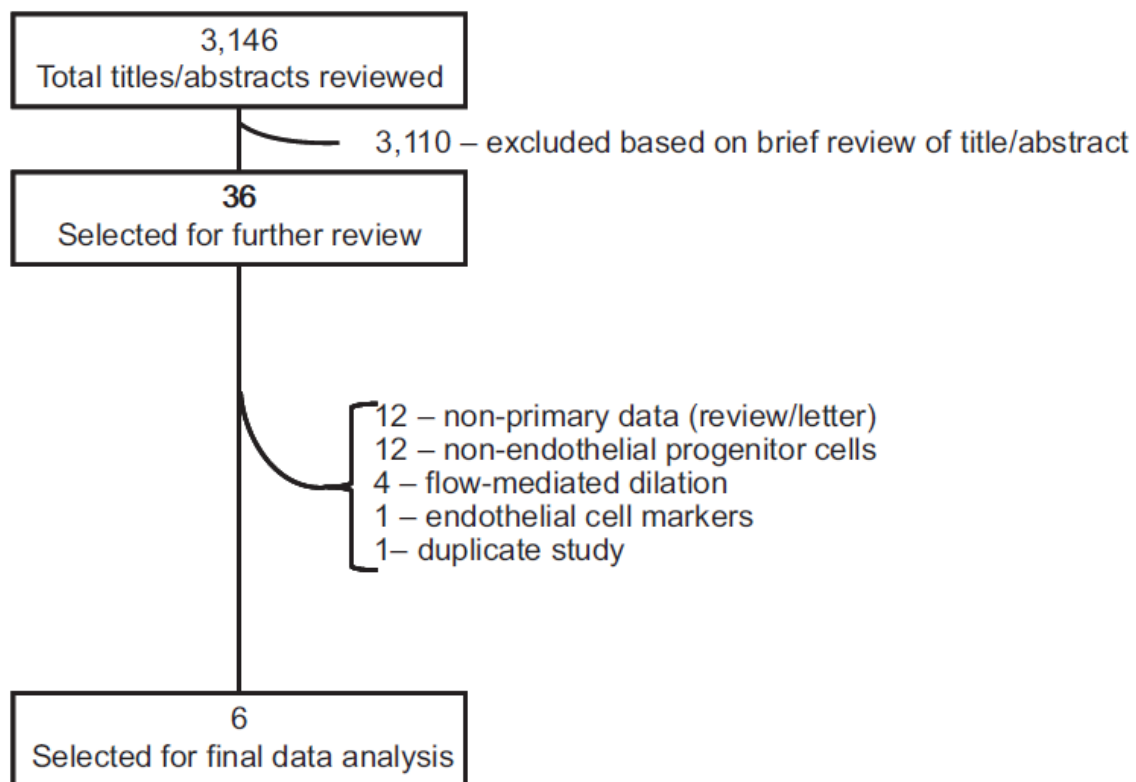


Figure 1: Results of literature search of MEDLINE and SCOPUS for articles examining endothelial progenitor cells (EPCs) in human immunodeficiency virus (HIV)-infected individuals

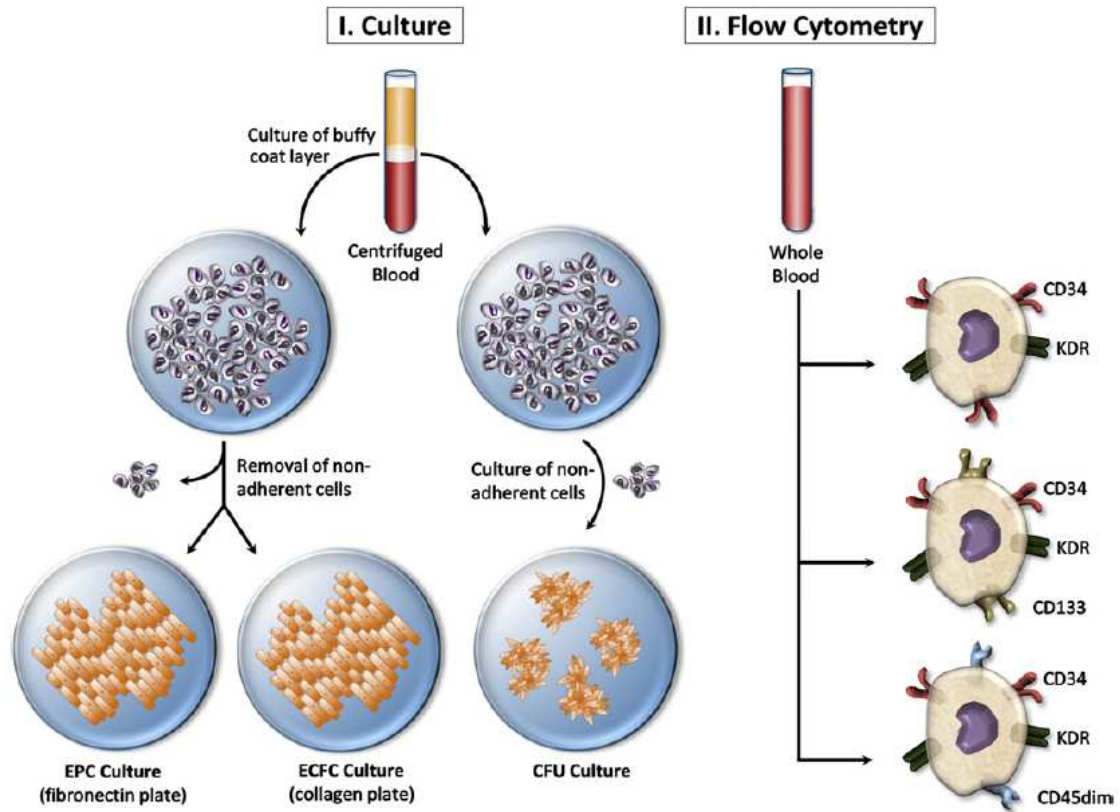


Figure 2: Phenotypic characterization of endothelial progenitor cells (EPCs). (I) Culture: Culture-based methodology utilizing Ficoll-based differential centrifugation of whole blood, extraction of buffy-coat layer enriched with peripheral blood mononuclear cells (PBMCs) and subsequent culture. When cultured on fibronectin after removal of non-adherent cells, adherent cells develop into EPCs. When initially cultured on collagen-coated plates, adherent cells differentiation into endothelial colony forming cells (ECFC). Alternatively, the colony-forming unit (CFU) assay entails replating of non-adherent cells with subsequent development of CFUs. (II) Flow cytometry: Analysis of whole-blood samples following either red blood cell lysis or isolation by differential centrifugation of PBMCs. Several gating strategies have been employed using various combinations of surface markers including: CD34⁺/KDR⁺, CD34⁺/KDR⁺/CD133⁺, and CD34⁺/KDR⁺/CD45dim.

Author	Number of participants	Differences between HIV+ and HIV– groups	HAART status of HIV-infected participants	Age of HIV+/HIV– participants (years)	Number of HIV+ males/females	History of cardiovascular disease	Smokers excluded	CD4+ count at study enrollment (cells/ μ L)	Viral load at study enrollment (log10copies/mL)	Time from diagnosis of HIV infection to study enrollment (years)
Gomez-Garre et al. (2012)	200 HIV+ 27 HIV–	Not specifically indicated; HIV+ patients on HAART were older and had higher SBP, cholesterol and TGs than HIV+ patients not on HAART	20 HAART-naïve 180 on HAART	See Table 2* All > 18 years	172/28	No	No	See Table 2* Range 314–747	See Table 2*	See Table 2* Range 3–19
Costiniuk et al. (2012)	30 HIV+ 36 HIV–	HIV+ higher TGs, SBP and ESR and lower HDL	All HAART-naïve	33 $\pm$ 6/30 $\pm$ 4 (Mean $\pm$ SD)	30/0	No	Yes	478 $\pm$ 303 (Mean $\pm$ SD)	3.86 $\pm$ 1.01 (Mean $\pm$ SD)	3.0 $\pm$ 2.3 (Mean $\pm$ SD)
Da Silva et al. (2011)	35 HIV+ 33 HIV–	HIV+ lower total cholesterol and HDL	All HAART-naïve	34 (21–48)/31 (22–45) [Median (range)]	29/6	No	No	590 (Mean)	3.76 (Mean)	3.8 (Mean)
Lopez et al. (2011)	15 HIV+ 15 HIV–	HIV+ lower total cholesterol, HDL and platelet levels than controls	All HAART-naïve	29 (13/27 (10) [Median (IQR)])	14/1	Low CVDR scores [†] in both groups	HIV+ 16% smokers (between groups)	359 (145) [Median (IQR)]	4.38 (1.14) [Median (IQR)]	10 months (27) [Median (IQR)]
Papasavvas et al. (2012)	66 HIV+ 50 HIV–	No differences indicated	59 on HAART at baseline (time point 1); 60 on HAART 1 year later (time point 2)	48 (45–55)/48 (43–54) [Median (range)]	59/7	Not indicated	HIV+ 61% smokers (smoking status not indicated for HIV–)	354 (221–651) [Median (range)]	75 (50–1592) copies/mL (range)	Not indicated
Teofili et al. (2010)	14 HIV+ 15 HIV–	No differences indicated	All HAART-naïve	40 $\pm$ 12 (Mean $\pm$ SD)	11/3	No	Not indicated	420 $\pm$ 141 (Mean $\pm$ SD)	132,568 $\pm$ 146,917 (Mean $\pm$ SD)	Not indicated

HAART = highly active antiretroviral therapy; SBP = systolic blood pressure; TG = triglycerides; HDL = high density lipoprotein; SD = standard deviation; SEM = standard error of the mean; IQR = interquartile range; CVDR = Cardiovascular Disease Risk.

* In order to determine whether there were any relationships between levels of EPC subpopulations and various risk factors, patients were grouped based on whether they had high vs low EPC levels (i.e., mean ages, median CD4 counts, number of individuals with undetectable VLs and median time from HIV diagnosis were expressed for individuals with high vs low levels of EPC subpopulations).

[†] Risk <5% with Framingham and REGICOR or <3% with SCORE.

Author	Primary outcomes	Definition of EPC/cell populations examined	Methods used to study EPCs	EPC levels in HIV+/HIV–	Other findings
Gomez-Garre et al. (2012)	EPC levels in HIV+ and HIV– controls and their relationship to cIMT	CD34+/KDR+	Flow cytometry	Reduced number of CD34+/KDR+ in HIV+ [0.02 (0.01–0.03)] vs HIV– [0.08 (0.04–0.14)%], $p < 0.01$	HAART was the main predictor for low CD34+/KDR+ cells and high CD14+/Endoglin+ cells after adjustment by cardiovascular risk factors and HIV duration and HAART treatment; levels were only partially associated with high c-IMT in HIV+ patients
		CD34+/VE-cadherin+		Reduced CD34+/VE-cadherin+ cells in HIV+ [0.07 (0.04–0.11)] vs HIV– [0.03 (0.02–0.05)%], $p < 0.01$	
		CD14+/Endoglin+		CD14+/Endoglin+ cells tended to be lower in HIV+ [3.2 (1.7–5.1)] than HIV– [2.1 (1.2–3.7)%], $p < 0.067$ although difference not statistically significant Compared to HIV–, HIV+ who were HAART-naïve had lower CD34+/KDR+, CD34+/VE-cadherin+ and CD14+/Endoglin+ cells; HIV+ on HAART had further reduced levels of CD34+/KDR+ and CD34+/VE-cadherin+ cells CD14+/Endoglin+ levels higher in HAART-treated than HAART-naïve patients HIV–, CD34+/KDR+ 15 (8–12) cells CD34+/VE-cadherin+ cells 22 (14–33) cells	
Costiniuk et al. (2012)	EPC levels between HIV+ and HIV– controls	CD45dim/CD34+/KDR+ CD45dim/CD34+/CD117+	Flow cytometry	EPC levels similar between HIV+ and HIV– CD45dim/CD34+/KDR+ 1.4 ± 0.5 vs 3.7 ± 2.2 cells/ μ L, $p = 0.92$ CD45dim/CD34+/CD117+ 30 ± 7.3 vs 35.1 ± 5.4 cells/ μ L, $p > 0.05$ CD45dim/CD34+/CD184+ 5.5 ± 2.2 vs 6.6 ± 1.7 , $p > 0.05$	EPC levels were not associated with disease parameters such as CD4 count or VL
		CD45dim/CD34+/CD184+			
Da Silva et al. (2011)	EPCs %	CD34+/KDR+	Flow cytometry	% of CD34+/KDR+ cells lower in HIV+ than in HIV– [0.02 $\pm$ 0.01% vs 0.09 $\pm$ 0.03%, $p = 0.045$]	EMP concentration higher in HIV+ than HIV– (1963 $\pm$ 62 vs 436 $\pm$ 30 microparticles/ μ L PPP, $p = 0.003$)
	EMP and FMP levels between HIV+ and HIV– controls;	CD34+/CD133+		No difference between CD34+/CD133+ cells (0.01 $\pm$ 0.02% vs 0.01 $\pm$ 0.02, $p = 0.25$) or KDR+/CD133+ (0.01 $\pm$ 0.02% vs 0.01 $\pm$ 0.03%, $p = 0.21$) (Mean $\pm$ SD)	Similar number of FMP in both groups (24,992 vs 26,642 microparticles/ μ L PPP $p = 0.83$)
	Endothelial function as measured by FMD	KDR+/CD133+ EMP (CD31+)			(Mean $\pm$ SD) Negative correlation between hsCRP and EPCs (KDR+/CD133, $p = 0.029$) but for other EPC subpopulations or microparticles Flow-mediated dilation lower in HIV+ than HIV– controls (10 $\pm$ 1% vs 16 $\pm$ 2%, $p = 0.03$) (Mean $\pm$ SEM) No correlation between microparticles and FMD
		FMP (CD31+/CD42+)			

Author	Primary outcomes	Definition of EPC/cell populations examined	Methods used to study EPCs	EPC levels in HIV+/HIV-	Other findings
Lopez et al. (2011)	CEC levels, EPC levels and vascular index (CEC/EPC ratio) in HIV+ vs HIV- controls	CD45dim/CD34+/CD133- (late EPC) CD45dim/CD34+/CD31+/CD133+/CD309+ (EPC) CD45-/CD34+/CD31+/CD133- (CEC) CD45-/CD34+/CD31+/CD133+ (immature CEC)	Flow cytometry	Numbers of late EPCs similar between groups CEC level higher in HIV+ than HIV- [0.042 (0.027)% vs 0.016 (0.011), $p = 0.007$] EPC levels lower in HIV+ than HIV- [0.002 (0.003)% vs 0.009 (0.009), $p = 0.002$]; vascular index CEC/EPC ratio increased in HIV+ vs HIV- [12(15) vs 2 (3), $p < 0.0001$] [Median (IQR)]	No association between EPC and/or CEC and CD4 counts, blood plasma HIV-RNA level
Papavasvas et al. (2012)	EPC frequency and c-IMT changes over 1 year in HIV+ vs HIV- controls	CD34+/KDR+ CD133+/KDR+ CD34+/CD133+/KDR+	Flow cytometry	Two distinct groups of HIV+ subjects identified a priori: rapid IMT progressors ($n = 13$, Ac-IMT > 0.2 mm) and slow c-IMT progressors (slow or no c-IMT progression, $n = 53$, Ac-IMT < 0.2 mm); Median progression of c-IMT 0.30 mm (0.22-0.41) for rapid progressors and 0.01 (-0.03 to 0.12) for slow progressors Baseline EPC frequency similar between rapid and slow c-IMT progressors EPC frequency in HIV+ significantly higher than in HIV- ($p = 0.01$) No association between AEPC frequencies over time and Ac-IMT	HIV+ on HAART maintained higher EPC frequency compared to HIV- No differences between EPC levels in HIV+ on HAART vs HIV+ off HAART EPC frequency not associated with c-IMT, TG, total cholesterol, HDL, LDL or CRP EPC frequency correlated positively with CD4+ T count
Teofilii et al. (2010)	CFU-EC and ECFC levels in HIV+ vs HIV- controls Expression VEGF and APOBEC subunits in CFU-EC compared to circulating monocytes Proviral DNA content of CFU-EC	N/A	CFU-EC and ECFC assays	CFU-EC but not ECFC significantly lower in HIV+ persons [number of circulating CFU-ECs in HIV+ $0.6/2.5 \times 10^5$ plated cells (0-10) vs HIV- $13/2.5 \times 10^5$ plated cells (1.9-42.5); 0.4×10^7 cell (0-3); ECFC in HIV+ $0.4/10^7$ cells (0-3) vs $0.1/10^7$ plated cells (0-0.8)] [Median (range)] Patients with HIV had significantly lower CFU-EC than HIV- controls ($p < 0.0001$) but ECFC frequency higher in HIV+ than in HIV-, although differences not significant ($P = 0.297$)	Proviral DNA was detectable only in CFU-EC but not in ECFC The expression of APOBEC 3 A subunits was significantly lower in CFU-EC than in circulating monocytes of HIV+; VEGF levels similar between HIV+ and HIV- CFU-EC contained high levels of HIV proviral DNA copies No relationship between CD4+ T-cell count for CFU-EC and ECFC or plasma VL

cIMT = carotid intima media thickness; SD = standard deviation; SEM = standard error of the mean; KDR = kinase insert domain receptor; VL = viral load; CEC = circulating endothelial cells; CVDR = cardiovascular disease risk; CFU-EC = colony-forming unit endothelial cells; ECFC = endothelial colony-forming cells; c-IMT = carotid artery intima media thickness; ECFC = endothelial colony-forming cell; hsCRP = high sensitivity C-reactive protein; TG = triglycerides; LDL = low density lipoprotein; PPP = platelet poor plasma; PMP = platelet-derived microparticle; EMP = endothelial-derived microparticles; VEGF = vascular endothelial growth factor; APOBEC = apolipoprotein B mRNA-editing enzyme catalytic polypeptide.

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Appendix II

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Performed the experiments: FDR, BH, TS, AP, RH, MK

Performed statistical analysis: KW, SH

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Natural history and management of aortocoronary saphenous vein graft aneurysms: a systematic review of published cases

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Despite ongoing advances in percutaneous revascularization, coronary artery bypass grafting (CABG) continues to be performed in a large number of patients with over 400,000 operations reported in 2007 in the United States alone.¹ Though arterial conduits are generally preferred, saphenous vein grafts (SVGs) continue to be regularly employed. First described by Riahi and colleagues in 1975,² aneurysmal dilatation of aortocoronary SVGs remains a rare yet widely reported phenomenon. Indeed, subsequent literature on the topic consists almost exclusively of case reports and small case series. As such, the precise incidence of aortocoronary SVG aneurysms (SVGAs) remains difficult to ascertain, though in one case series an incidence of 0.07% has been estimated based on a review of over 5500 grafts at a single institution.³ However, this likely underestimates the true figure as SVGAs often remain clinically silent and no guidelines exist to screen for their development. Given the infrequent identification of SVGAs, our current understanding of the epidemiology and pathogenesis of these aneurysms remains limited.

Aneurysms are generally defined as a focal dilatation of vessels greater than 1.5 times the proximal reference diameter; however, aneurysmal dilatation of aortocoronary SVGs have led to ‘giant’ aneurysm formation with reports of cases exceeding 10 cm.⁴⁻⁶ SVGAs are often incidentally identified on imaging, but cases of rupture,⁷ fistula formation with neighbouring anatomy,⁸ and of hemodynamic compromise due to compression of adjacent cardiac and vascular structures have been reported.⁹ To date, two reviews have been published briefly summarizing 108 cases of SVGAs.^{10, 11} Traditionally their management has been surgical – generally resection of the aneurysm with or without bypass of the affected territory. However, with refinement of percutaneous techniques, including the use of Amplatzer devices, covered stents, and arterial coiling, the

management options for affected patients are becoming increasingly diverse and consensus on the optimal approach to managing SVGAs remains unclear.

Therefore we performed a systematic review of published cases as has been reported for other very rare clinical conditions to determine the natural history and management of SVGAs.^{12, 13} Details regarding the methodology, search strategy, and results can be accessed online (see online Expanded Methods and Results). Briefly, 168 articles reporting 209 patients with 229 SVGAs were identified (Figure 1) and analyzed (online Supplemental Table).

Interestingly, though the first SVGa case was reported in 1975,² of the 168 articles (209 cases) published as of 2010, only 19 (26 cases) were reported prior to 1990. In contrast, more than one third of available reports (73 cases) were published after December 2005. This increased reporting in recent years remains to be fully explained and, though many reasons have been postulated, it is likely that none alone fully accounts for the trend.

However, given the improving long-term outcomes of patients following CABG¹⁴ as well as the increasing sophistication and use of diagnostic investigations,¹⁵⁻¹⁷ it is likely that a growing number of patients with SVGAs and their associated complications will continue to be identified.

Etiology

The mechanism responsible for aneurysmal dilatation of aortocoronary SVGs is poorly understood though several pathophysiologies have been proposed. Late aneurysm formation (i.e. greater than five years after CABG) is felt to occur secondary to SVG

atherosclerotic degeneration. This process contributes to weakening of the vessel wall leading to graft dilatation.^{11, 18, 19} However, the transposition of a vein into arterial circulation represents a unique setting in which additional factors may exacerbate and accelerate these processes. One such potential factor is vessel wall ischemia that can occur following disruption of vasa vasorum during the harvesting and grafting process. Some authors have also suggested that the abrupt change in wall stress when the graft is subjected to the high pressure and pulsatile flow of an arterial system may play a role in aneurysm formation.²⁰ However, the delayed presentation and low incidence of these lesions would suggest that arterial pressures alone unlikely contribute significantly to aneurysm formation. Rather, atherosclerotic changes, graft endothelial dysfunction and changes in medial smooth muscle cell orientation in the vicinity of valve sites likely play a more pivotal role in late aneurysm development.^{21, 22}

Conversely, early SVGAs have been reported within a matter of months following surgery^{2, 23-25} with one case detected 16 days post-operatively.²⁶ These early aneurysms (i.e. identified less than 12 months following surgery) undoubtedly occur secondary to a different pathophysiology. Infection of the implanted graft;²⁶ intrinsic weakness of the venous wall (i.e. undetected varicosities)²³; technical factors relating to conduit harvesting, preparation, and grafting, including conduit injury with or without dissection,^{27, 28} anastomotic suture disruption,²⁹ and failure to reverse the SVG at the time of grafting²¹ have been implicated in the formation of early SVGAs. Indeed, early and late SVGAs seem to be the products of unique pathophysiologies that give rise to similar clinical entities.

Patient Demographics and Clinical Presentation

SVGAs typically arise remotely from the initial bypass surgery, being identified an average of 13 years post-CABG. In fact, in only 4.2% of patients were SVGAs diagnosed within the first year following CABG with 6.1% subsequently identified between one and five years, 21.2% between five and ten years, and 68.5% at greater than ten years after surgery. Though SVGAs were detected in both men and women on average within the sixth decade of life, SVGAs are predominantly reported in men who account for 87% of cases – likely relating to the higher incidence of atherosclerotic coronary disease in that population (Table 1).³⁰

Patients with SVGAs have varied clinical presentations, but have most commonly presented with chest pain/angina (46.4%), followed by shortness of breath (12.9%), and myocardial infarction (7.7%). However, nearly a third of reported cases of SVGAs were discovered incidentally, often first noted as abnormalities on routine chest x-rays or posthumously identified on autopsy. In contrast, few patients have been reported to present with shock or hemoptysis (4.3% and 3.8% of patients, respectively). All other presentations have been noted in fewer than 3% of identified patients (Figure 2). In keeping with this observation, the majority of patients with SVGAs were hemodynamically stable at presentation (55%) though nearly 10% were unstable with the remainder of reports providing insufficient details to determine hemodynamic stability (Table 1).

Aneurysm Characteristics and Natural History

In reported cases of SVGAs, the mean aneurysm size at diagnosis was large at 60.4 mm with the subset of cases specifically reporting pseudoaneurysms documenting even larger dimensions, averaging 68.9 mm (Table 1). However, though larger aneurysms may elicit greater concern and serve as impetus for intervention, whether SVGAs continue to increase in size once identified as well as the nature of the relationship between aneurysm dimensions and adverse events have thus far remained unexamined. We therefore identified all publications that serially reported SVGA size during follow-up. Nearly all such cases share the common finding of aneurysmal growth.^{6, 15, 23, 31-59} In fact, SVGA growth has resulted in appreciable increases in size in as little as six months.³⁷ In our institution, for instance, one graft aneurysm can be seen growing in size from 2 cm to over 8 cm in four years (Figure 3D-F). However, though SVGA growth is commonly reported, the rates of growth appear to vary greatly. Indeed, in the nine cases reporting detailed size assessments over time, considerable variation in the rates of growth can be seen (Figure 4A).^{23, 37, 43, 44, 49, 55, 56, 58}

Aneurysmal conduits have been most commonly grafted to the right coronary artery (RCA) (38%), followed by the left anterior descending (LAD) (25.3%), obtuse marginal (10.9%), and left circumflex arteries (10.5%). This predominance of aneurysms in SVGs to the right coronary distribution is intriguing. One potential explanation for this finding relates simply to an increased frequency of use of SVGs to this coronary. Indeed, left internal mammary arterial conduits are preferentially grafted to the LAD when used, which may lead to a relative over-representation of grafts to the RCA. Furthermore, bilateral internal thoracic arteries tend to more often be used on the left coronary system.⁶⁰ However, given the limited information provided in the case reports regarding

patient anatomy it is impossible to quantify the frequency with which venous versus arterial conduits were utilized to graft each coronary territory. Another hypothesis for this imbalance relates to intraoperative technical considerations. The RCA is often deliberately grafted first and, due to size-matching considerations, with the most proximal piece of vein and therefore the largest segment of the harvested conduit. Thus, at baseline, the segment of vein grafted to the RCA will usually have the largest luminal diameter. On being exposed to arterial pressures, a larger luminal diameter will lead to greater wall tension as per LaPlace's law. Thus, it stands to reason that if larger vein conduits are utilized preferentially as grafts to the RCA, this may in part explain the increased frequency of aneurysms in this distribution. Overall, however, though an interesting observation, the explanation for this finding is likely multi-factorial and the discussion of these potential mechanisms remains speculative.

Because of their size and proximity to intrathoracic structures, SVGAs causing mechanical complications are well described in the literature, being reported in 35.9% of cases. Furthermore, likely due to the predominance of SVGAs to the right coronary distribution, right-sided cardiac structures have been most commonly affected. For example, right atrial compression has been documented in 11.5% of cases, right ventricular compression in 7.2%, and fistula formation in 7.7% (over half of which formed between the SVG and the right atrium). Aneurysm rupture, the most feared complication of SVGAs, has been reported as a presenting feature in only a minority of cases; however, it remains an important complication given its poor prognosis (Table 1). As with aneurysms in other vascular beds there appears to be a positive relationship between SVGA size and adverse events. There was no 'safe' size for SVGAs below

which surveillance alone can be proposed with aneurysms measuring as little as 20 mm being associated with a 33.3% event rate of a composite endpoint of mechanical complications, myocardial infarction, aneurysm rupture, an/or death in cases of conservative management, which increased to as high as 69.2% for diameters exceeding 100 mm. Interestingly, this increased risk was predominantly driven by increased risk of mechanical complications (not myocardial infarction, rupture, or death) (Figure 4B). Thus, as with other vascular aneurysms, SVGAs appear to continue to increase in size and at larger sizes portend an increased risk of complications. Given that event rates even with smaller aneurysm sizes were high, these findings have clear implications for surveillance and management.

Diagnostic Assessment

The goals of imaging suspected SVGAs should be to confirm the diagnosis, establish the size of the aneurysm, and rule out common complications such as fistulisation, compression of adjacent structures, and/or rupture. Furthermore, clinicians should evaluate the patency of the affected conduit and/or other grafts, the status of the native coronaries, and assess for other indications for cardiac surgery (for instance significant valvular disease). This is of particular importance given the emerging role of percutaneous interventions for SVGAs as clinicians may be able to identify candidates suited for less invasive management.

In our review of the literature a number of diagnostic imaging modalities were employed to evaluate SVGAs. Two thirds (66.5%) of cases incorporated cardiac catheterization for diagnosis and/or management planning. Similarly, computed tomography (CT) was

widely employed with 60.3% of patients undergoing CT studies with or without contrast while only 12.9% utilized magnetic resonance imaging (MRI). Chest x-rays were reported in 54.1% of cases though in many instances they were performed for unrelated investigations and were not diagnostic. Echocardiograms (both transthoracic and transesophageal) were documented in only 28.2%.

Though there is no consensus regarding the optimal approach to the assessment of SVGAs, as reported above, the use of coronary angiography is highly prevalent in the literature. However, despite few reports explicitly addressing the limitations of specific imaging modalities, a small number have described difficulties in diagnosing and/or delineating SVGAs via this modality due to the presence of intraluminal thrombi.^{52, 61-63} For instance, in one case, the aneurysm was shown to compress both the right atrium and ventricle using echocardiography though coronary angiography showed no evidence of an SVGA. It was subsequently confirmed on CT, however, and at surgery was found to measure 12 x 10 x 6.5 cm.⁶⁴ Similarly, echocardiography has limitations in that not all SVGAs can be properly imaged depending on their location with several reports describing their misdiagnoses as intracardiac masses using the technique.⁶⁵⁻⁶⁷ Therefore, given the potential limitations of coronary angiography and echocardiography, a cross-sectional modality such as CT or MRI should be employed in the initial assessment to definitively establish the presence of an SVGA and to assess for mechanical complications. Furthermore, with the increasing proficiency of coronary CT angiography, information regarding native and graft vessel anatomy may be derived in some instances without catheterization (Figure 2A-B).⁶⁸ Echocardiography, in most instances, would be

indicated for an assessment of left ventricular function and to rule out concomitant valvular heart disease.

Based on our review, it therefore seems prudent to employ a multi-modality approach when investigating SVGAs in order to fully assess their true dimensions, rule out complications, and establish the underlying cardiac status of the patients affected.

Management Approaches and Outcomes

Due to the infrequent detection of SVGAs, approaches to the management of these lesions have thus far been primarily derived from case reports or small case series. Dieter et al retrospectively reviewed 13 patients with SVGAs, two of which were treated surgically and 11 of which were managed conservatively. The authors concluded that early surgical treatment of SVGAs did not result in improved short-term survival compared with conservative management.³ Subsequently, Sareyyupoglu et al retrospectively reviewed 16 patients who underwent surgical SVGA repair and proposed that surgical revascularization should be recommended for symptomatic patients, for patients with SVGAs of diameters exceeding 1 cm, or if graft flow is diminished.⁶⁹ Finally, Memon et al recommended surgical resection and revascularization though stated that coil embolization was a viable option in cases of high surgical risk.¹¹

On review of the literature, the most commonly reported management of SVGAs is surgical (58.4% of all cases) with either aneurysmal resection or ligation performed followed by bypass grafting in cases with significant myocardium in jeopardy. Percutaneous intervention (with coil embolization, Amplatzer vascular occlusion, or

covered stent placement) was reported in only 15.8% of cases, most of which were published within the last ten years. Interestingly, conservative management was employed in 20.1% of reports.

Despite a large proportion of SVGAs being identified incidentally, the in-hospital and/or 30-day mortality rate in the overall cohort was high at 13.9% (Table 1). Individually, mortality rates for reported cases of surgical, percutaneous, and conservative management were 13.9%, 6.1%, and 23.8%, respectively (Table 2). Of note, as described above, it is important to note that adverse outcomes occurred in one third of cases of SVGAs measuring ≤ 20 mm, rendering it impossible to propose a ‘safe’ size for SVGa surveillance.

Our review suggests that both percutaneous and surgical interventions are viable management options for SVGAs. Additionally, with the advent and development of percutaneous methods, minimally-invasive interventions are gaining popularity. Our findings suggest that, once identified, clinicians must balance the risks of observation with those of intervention and be cognizant that SVGAs can be expected to continue to increase in size. However, if conservative management is preferred, our results do support the notion that smaller SVGAs can be more safely monitored than their larger counterparts.

Management Algorithm

While comparative studies are not possible, the observed low mortality rate for percutaneous options is encouraging in light of the high risk of repeat sternotomy⁷⁰

(Table 3). Given the emerging role of percutaneous management of SVGAs, both anatomical features and patient factors should be considered in selecting the optimal intervention. If possible, we propose that patients should be assessed by a heart team comprised of a cardiac surgeon and an interventionalist with experience in structural heart disease. In patients with alternative indications for cardiac surgery (i.e. multiple territories requiring revascularization or concomitant valve surgery) surgical intervention should be pursued. However, in the absence of these alternative indications we have proposed a management algorithm (Figure 5).

The first step in deciding on management options for SVGAs is to assess for the presence of mechanical complications. The presence of fistula, rupture, or compression of adjacent cardiac or vascular structures renders surgery the only option and aneurysm resection with subsequent bypass should be performed when technically feasible. However, the majority of patients present without mechanical complications and in these individuals the patency of the affected vein graft should be established. In cases in which the affected graft remains patent despite aneurysmal dilatation percutaneous management with a covered stent in patients with suitable anatomy offers a less invasive approach while maintaining myocardial perfusion. In this select group distal embolization protection is indicated to prevent embolization of thrombus⁷²⁻⁷⁴ with successful stenting permitting exclusion of the aneurysm.⁷⁵ In patients with patent affected grafts in whom the graft anatomy is not favorable to covered stenting, surgical resection with repeat bypass grafting should be pursued.

In a large number of patients the affected graft is occluded with proximal patency and persistent flow into the aneurysmal segment (Figure 3H, Supplemental Video 2).⁷⁶ In this subset preservation of myocardial blood supply is not a concern. Similarly, in patients in whom the affected graft supplies a small territory, the aneurysm neck can be occluded by Amplatzer vascular plugs⁷⁷ (Figure 3I, Supplemental Video 3) or the aneurysm thrombosed by endovascular coiling.^{9, 44} In patients with moderate or large territories supplied by the affected graft, there may be a role for assessment of myocardial viability. Patients with scar in the territory supplied by the culprit SVG will not benefit from revascularization⁷⁸ and percutaneous closure of the aneurysm could be considered. Conversely, those with viable myocardium may benefit from revascularization, particularly in cases of large viable territories.⁷⁹ Numerous studies have demonstrated that the use of either fluorodeoxyglucose positron emission tomography (FDG-PET), cardiac MRI, dobutamine stress echocardiography, or single photon emission CT (SPECT) imaging to detect hibernating myocardium can help predict recovery of regional function with revascularization.⁸⁰ Thus, viability testing may enable the selection of patients in whom the affected SVG supplies predominantly viable myocardium and thus may benefit from repeat revascularization. Of course, in patients in whom repeat surgical intervention is not an option due to co-morbidities or patient wishes, percutaneous closure of the SVGA remains the preferred choice.

Limitations

Ideally evidence for optimal investigation and management strategies for patients with SVGAs would be derived from randomized controlled trials or large cohort studies, yet

given the rarity of the condition it is unlikely that such a study would be feasible. Thus, we performed the current systematic review in order to summarize the available knowledge on this rare clinical entity. We acknowledge that such an approach has potential limitations in that it is retrospective and comprised of individual case reports and small case series. Thus, reporting bias may influence the available cases for analysis – particularly as adverse outcomes following novel interventions or any type of outcome after commonly used therapies, such as surgical treatment, may be less likely to be reported. As well, given the lack of routine screening for SVGAs, symptomatic patients may be relatively over-represented in the literature as they are arguably more likely to be investigated. Furthermore, the lack of both patient randomization to their interventions and of standardized reporting of baseline characteristics precludes a comparative analysis of outcomes based on management strategies. Despite these limitations, a systematic review of cases represents the only available method to evaluate SVGAs and provides clinicians with the best available evidence on which to base their clinical decisions.

Conclusions

SVGAs represent a rare and typically late complication of CABG with the potential for significant morbidity and mortality. Our analysis suggests that the risk of complication increases with size and that, once identified, these aneurysms continue to grow at variable rates. Multi-modality imaging is often required to establish the size of the SVGA, the myocardial territory supplied by the affected graft, and to assess for complications. The proposed management algorithm takes into account patient and clinical characteristics, identifying individuals best suited for percutaneous or surgical intervention based upon

the published experience. This systematic review should help clinicians in the evaluation and management of patients presenting with SVGAs.

Disclosures

None.

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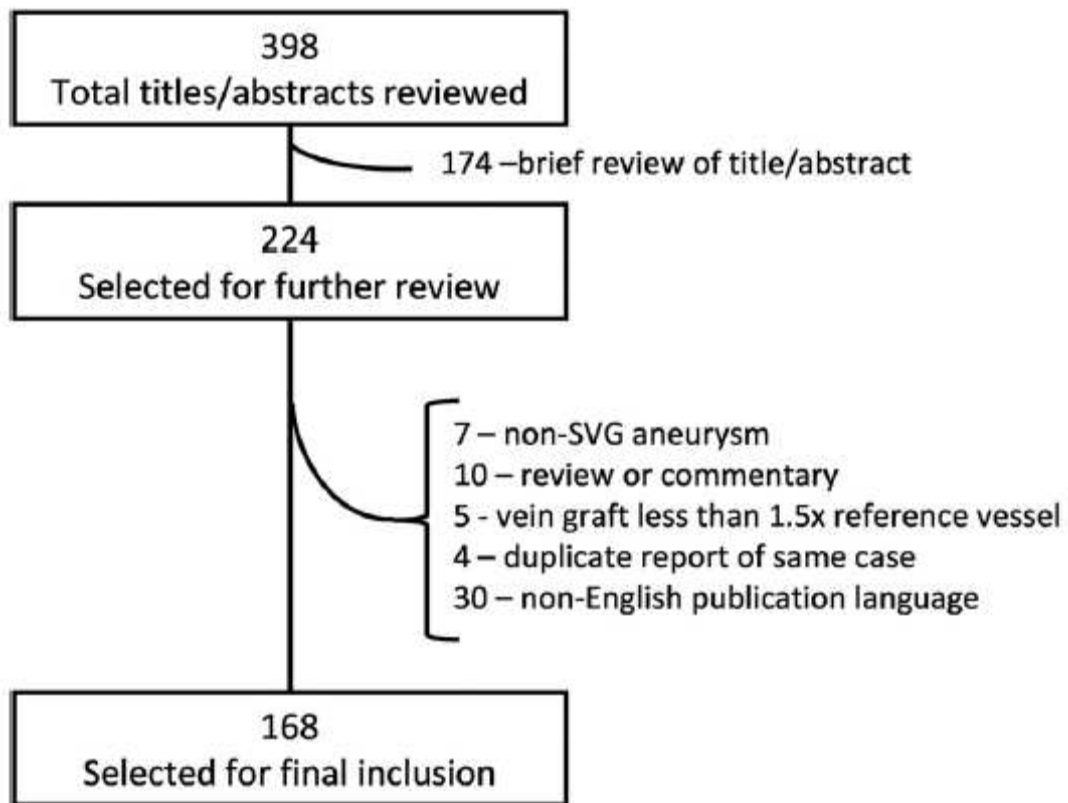


Figure 1. Outcome of literature search for identification of reported cases of aortocoronary saphenous vein graft aneurysms. SVG: saphenous vein graft.

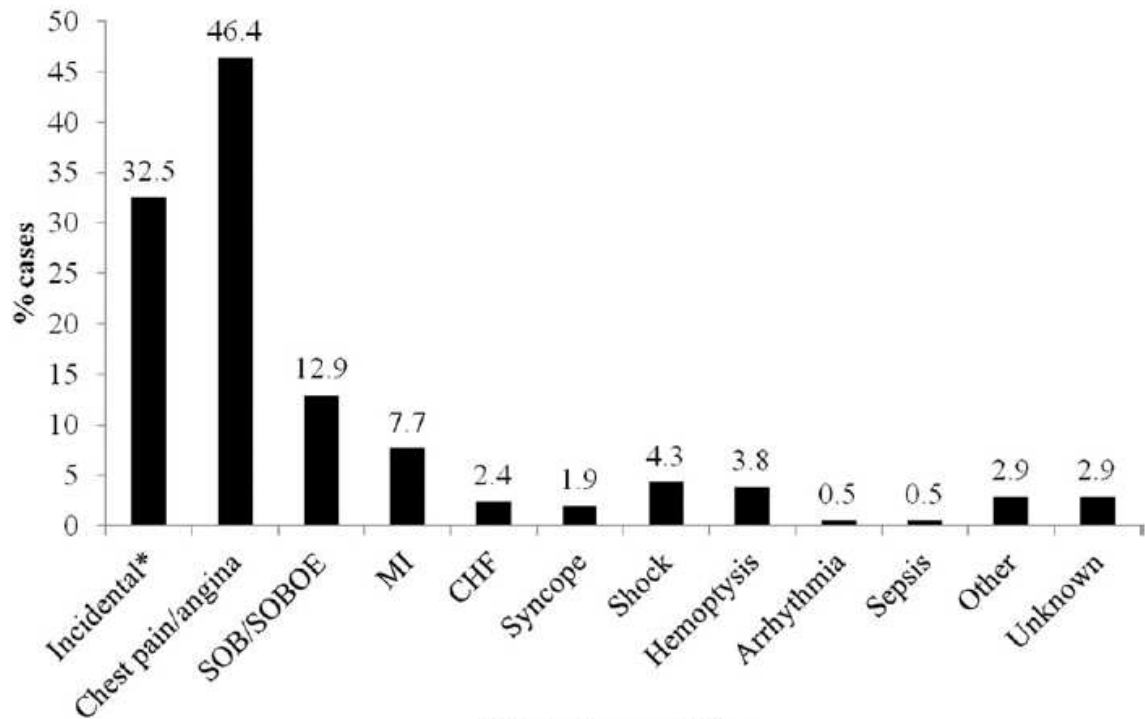


Figure 2. Clinical presentation of patients with saphenous vein graft aneurysms. Chest pain/angina was reported in 46.4% of patients, shortness of breath in 12.9%, myocardial infarction in 7.7%, and in 32.5% the pathology was discovered incidentally (*including two cases discovered at autopsy). CHF: congestive heart failure, MI: myocardial infarction, SOB: shortness of breath, SOBOE: shortness of breath on exertion. Note that values do not add to 100% as many patients presented with more than one complaint/finding.

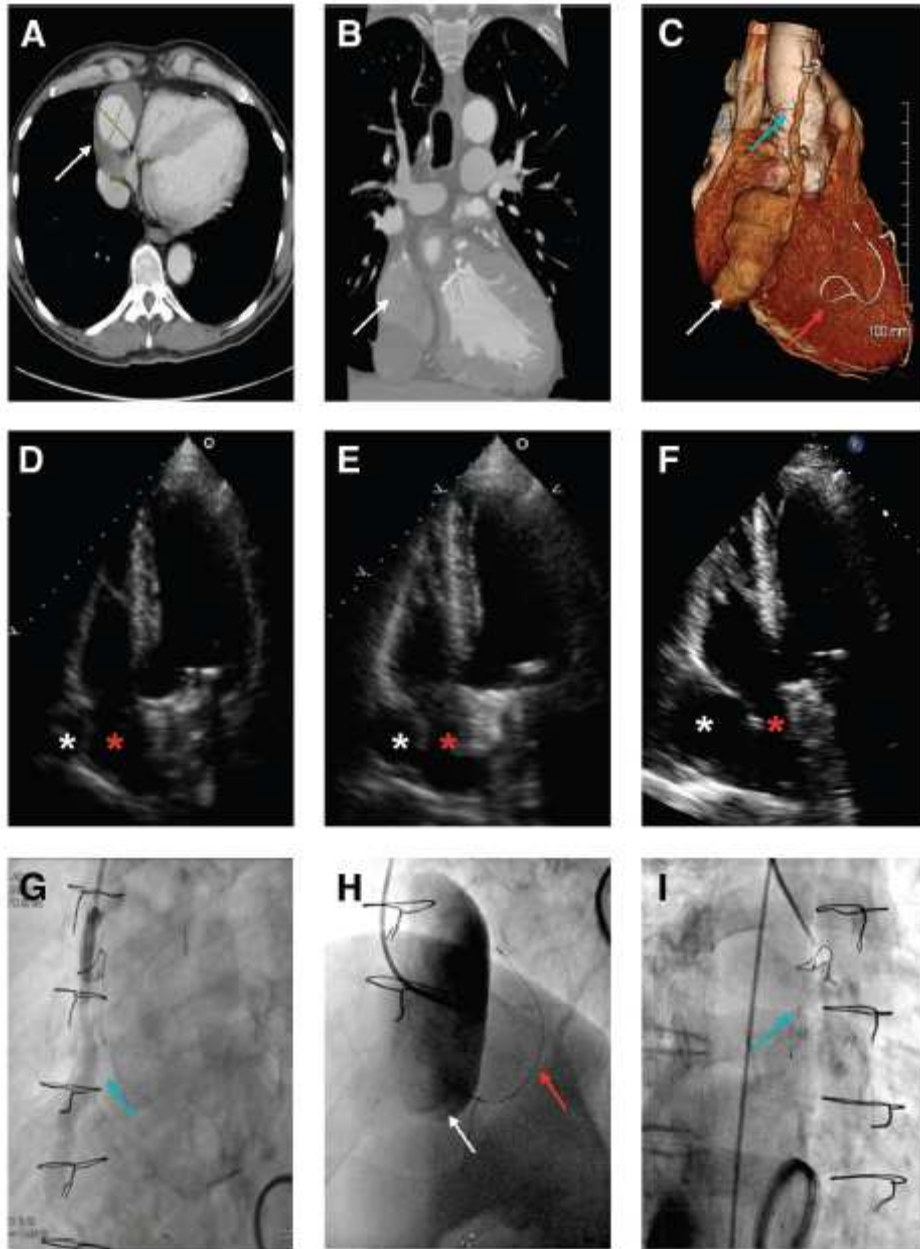


Figure 3. Multi-modality imaging of a 66 year old male with a history of mechanical aortic and mitral valve replacements as well as coronary artery bypass grafting with a saphenous vein graft (SVG) to the RCA 12 years prior. (A) CT scan of the abdomen incidentally identified a mass adjacent to the right ventricle suggestive of an aneurysm of either a coronary artery or a bypass graft (white arrow). (B) Coronal section from a dedicated CT angiogram demonstrating a saphenous vein graft aneurysm (SVGA, white arrow). (C) 3D reconstruction showing the relationship of the SVGA (white arrow) to the right atrium and ventricle. A residual epicardial pacing wire is present (red arrow). (D-F) Serial transthoracic echocardiograms from 2006, 2009, 2010 showing growth of the SVGA (white asterisk) with further compression of the right atrium (red asterisk). Measurements of the aneurysm were 2.3 x 2.1 cm, 7.8 x 3.1 cm, and 8.9 x 4.2 cm in 2006, 2009, and 2010, respectively. (G) Injection of the SVG (blue arrow) for

measurement of graft diameter (maximum diameter of 9 mm). (H) Injection of the SVGA (white arrow) demonstrated no distal flow to the bypassed RCA. Again the epicardial pacing wire is seen (red arrow). (I) Angiography after deployment of the Amplatzer vascular plug in the neck of the SVG (blue arrow) demonstrates markedly reduced flow, which is expected to resolve. Online videos available for G-I (Supplemental Videos 1-3).

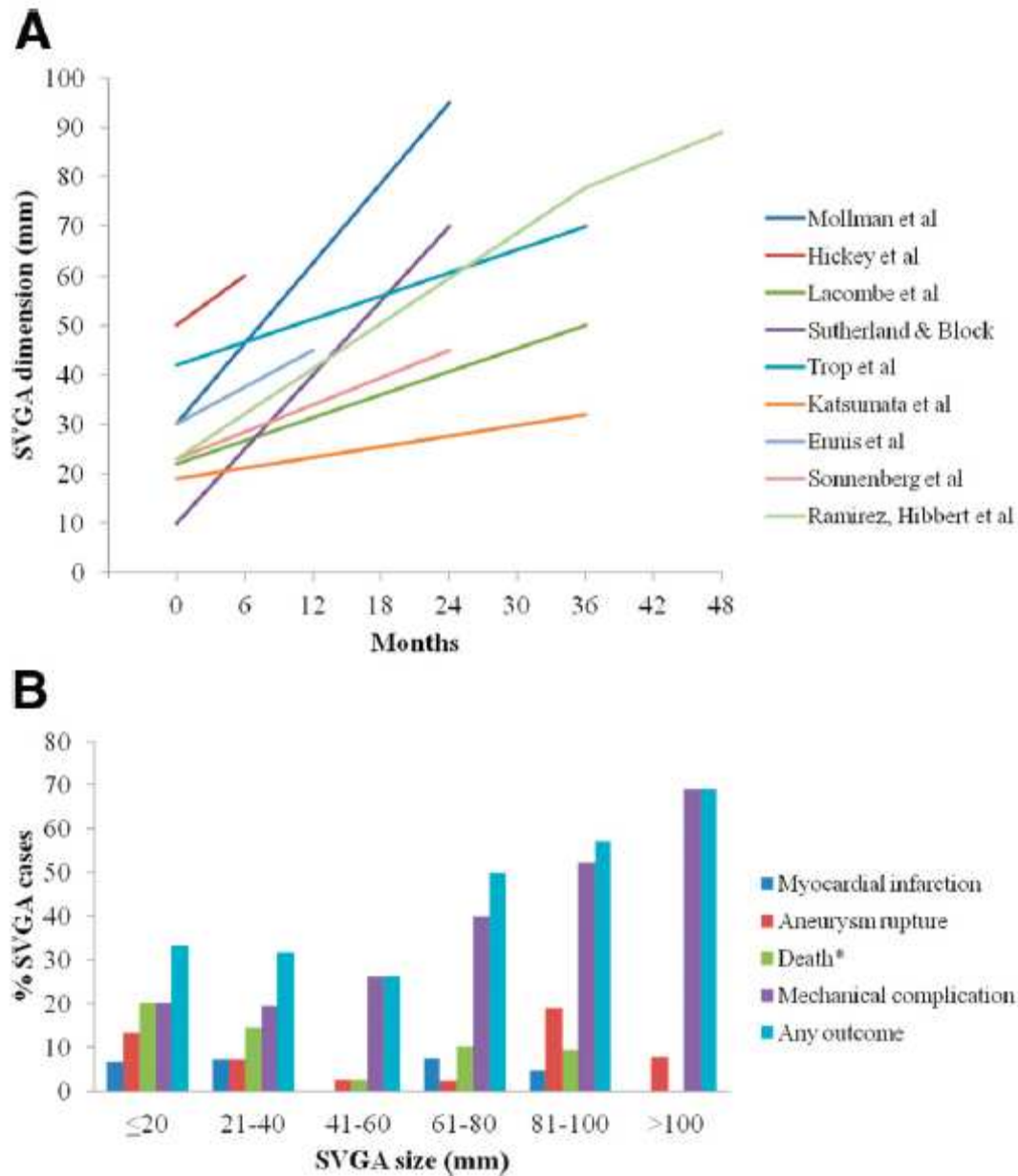


Figure 4. Natural history of aortocoronary saphenous vein graft aneurysms (SVGAs). (A) Reported SVGA dimensions over time. Time zero refers to time of diagnosis.^{23, 37, 43, 44, 49, 55, 56, 58} (B) Relationship between rates of reported adverse events (mechanical complications, myocardial infarction, aneurysm rupture, and/or death) and SVGA size. Mechanical complications encompass compression of adjacent structures and fistula formation. Any outcome: any of the above complications. *Death from any cause while in hospital and/or within 30 days of diagnosis; includes only cases of conservative management.

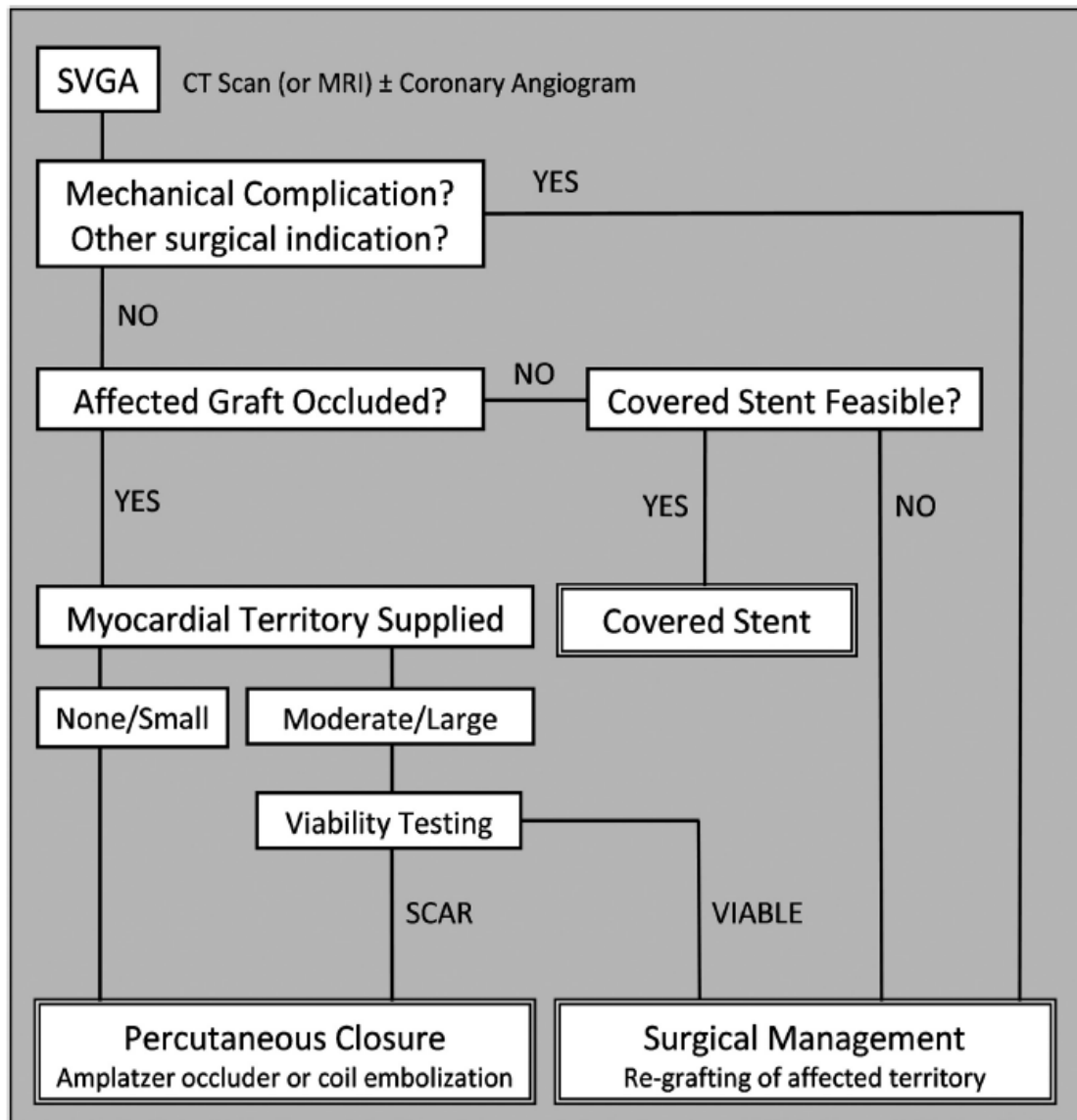


Figure 5. Algorithm for the investigation and management of aorticoronary saphenous vein graft aneurysms (SVGAs).

Table 1. Summary of case data

Characteristic	n (%) or mean $\pm$ SD
Sex	
Male	181 (86.6)
Female	25 (12.0)
Unknown	3 (1.4)
Age (years)	65.2 $\pm$ 10.5
Men	64.7 $\pm$ 9.9
Women	69.2 $\pm$ 13.8
Years post-CABG†	13.2 $\pm$ 6.0
0 to 1	7 (4.2)
>1 to 5	10 (6.1)
>5 to 10	35 (21.2)
>10	113 (68.5)
Unknown	44 (21.1)
Graft target vessel (artery)	
Left anterior descending	58 (25.3)
Diagonal	7 (3.1)
Left circumflex	24 (10.5)
Posterior left ventricular	3 (1.3)
Retroventricular artery	1 (0.4)
Right coronary	87 (38.0)
Obtuse marginal	25 (10.9)
Posterior descending	8 (3.5)
Unknown	16 (7.0)
Average aneurysm size (mm)	60.4 $\pm$ 31.7
Pseudoaneurysm*	68.9 $\pm$ 34.4
Suspected or confirmed pseudoaneurysm	62 (29.7)
Presence of intraluminal thrombi	127 (60.8)
Mechanical complications	75 (35.9)
Fistula	16 (7.7)
Right atrium	9 (4.3)
Right atrial compression	24 (11.5)
Right ventricular compression	15 (7.2)
Pulmonary artery compression	8 (3.8)
Superior/inferior vena cava obstruction	8 (3.8)
Left atrial compression	7 (3.3)
Aneurysm rupture	17 (8.1)
Hemothorax	4 (1.9)
Other	15 (7.2)
Presented hemodynamically stable	
Yes	115 (55.0)
No	20 (9.6)
Unknown	74 (35.4)
Management	
Percutaneous coronary intervention	33 (15.8)
Stent placement	13 (6.2)
Coil embolization	13 (6.2)
Vascular plug/septal occluder	4 (1.9)
Other	3 (1.4)
Surgery	122 (58.4)
Conservative	42 (20.1)
Unknown	12 (5.7)
Management-independent outcomes‡	

Survival	151 (72.3)
Death	29 (13.9)
Unknown	29 (13.9)

*Data based on 39 cases of diagnosed pseudoaneurysms with provided dimensions.

†Percentage values based on the total number of cases in which the data were provided.

‡30-day/in-hospital mortality

Values may not add to 100% due to rounding. CABG: coronary artery bypass grafting;
SD: standard deviation.

Table 2. Patient characteristics, aneurysm sizes, and outcomes by management employed*

	Surgery (n=122)	Percutaneous (n=33)	Conservative (n=42)
Outcome, n (%)†			
Death	17 (13.9)	2 (6.1)	10 (23.8)
Unknown	15 (12.3)	1 (3.0)	1 (2.4)
Patient age (years)	63.1 ± 10.3	71.7 ± 7.7	66.1 ± 10.7
Aneurysm size (mm)‡	65.1 ± 31.4	64.7 ± 36.6	51.2 ± 30.7
Hemodynamic stability at presentation, n (%)			
Stable	67 (54.9)	22 (66.7)	22 (52.4)
Unstable	12 (9.8)	5 (15.2)	3 (7.1)
Unknown	43 (35.2)	6 (18.2)	17 (40.5)

* Excludes 12 cases in which management strategies were not specified

†30-day/in-hospital mortality

‡Refers to largest aneurysm in cases of patients with ≥ 1 saphenous vein graft aneurysm

Table 3. Type of percutaneous intervention, patient characteristics, and outcomes

	Covered stent (n=15)*	Coil embolization (n=11)	Amplatzer device (n=4)	Other/ combined (n=3) [†]
Outcome, n (%)				
Alive	13 (86.7)	11 (100.0)	4 (100.0)	3 (100.0)
Death	2 (13.3)	0 (0.0)	0 (0.0)	0 (0.0)
Patient age (years)	71.7 ± 6.3	69.0 ± 8.1	79.8 ± 4.3	71.3 ± 11.9
Aneurysm size (mm)	47.6 ± 28.6 [‡]	64.2 ± 31.4 [§]	110.5 ± 36.4	60.0 ± 21.2
Hemodynamic stability at presentation, n (%)				
Stable	9 (60.0)	6 (54.5)	4 (100.0)	3 (100.0)
Unstable	3 (20.0)	2 (18.2)	0 (0.0)	0 (0.0)
Unknown	3 (20.0)	3 (27.3)	0 (0.0)	0 (0.0)

* Includes one case employing a “homemade” covered aortic endograft³⁴

† Comprises one case of ethylene-vinyl alcohol copolymer injection,⁵⁵ one case employing both a covered stent and coil embolization,⁷¹ and one case using coil embolization and an Amplatzer vascular plug³⁶

‡ Based on ten cases in which data were available

§ Based on six cases in which data were available

|| Based on two cases in which data were available

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Appendix III

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Author Contributions:

Conceived and designed the study: BH &ML

Collected Data: MB, EO, DS, ML, AD, CG, MF, JM

Performed statistical analysis: BH, GW, ML

Wrote the manuscript: BH, AD, ML

Supervised: ML

**Bivalirudin for Primary Percutaneous Coronary Interventions: Outcome
Assessment in the Ottawa STEMI Registry**

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Abstract:**Background:**

Data from randomized trials has demonstrated the superiority of bivalirudin to GPI *plus* heparin in patients undergoing primary percutaneous coronary intervention (PPCI). Real-world performance of bivalirudin in PPCI and the benefit of bivalirudin over heparin remains unknown in an era of routine dual antiplatelet therapy.

Methods and Results:

From July 2004 to December 2010, 2317 consecutive patients were indexed in the University of Ottawa Heart Institute STEMI registry. During this period 748 patients received bivalirudin, 699 patients received GPI, and 676 patients received unfractionated heparin alone. The primary outcome was the rate of non-coronary artery bypass graft (CABG) related Thrombolysis In Myocardial Infarction (TIMI) major bleeding. Bivalirudin significantly reduced the primary outcome compared to heparin plus GPI (2.7% vs. 7.3%, adjusted OR 2.96, 95% CI 1.61-5.45, $p<0.001$) and the composite endpoint of death, stroke, re-infarction and major bleed (OR 1.66, 95% CI 1.12-2.45, $p=0.01$). Compared to heparin alone, a reduction in major bleeds (OR 1.21, 95% CI 0.60-2.44, $p=0.59$) or the composite endpoint (1.05, 95% CI 0.68-1.63, $p=0.83$) with bivalirudin could not be demonstrated. Notably, major bleeding was associated with a five-fold increase in the risk of mortality both in-hospital (3.5% vs 20.6%) and out to 180 days (5.6% vs 25.8%).

Conclusions:

Bivalirudin use compared to GPI plus heparin as an antithrombotic strategy in PPCI results in less major bleeding in contemporary practice. A benefit of bivalirudin over

heparin could not be established with this registry and requires additional investigations to either confirm or refute.

Key words:

Primary PCI, Bivalirudin, Complications, Bleeding

Introduction:

Primary percutaneous coronary intervention (PPCI) for treatment of ST-elevation myocardial infarction (STEMI) is the preferred treatment strategy in patients in whom revascularization can be achieved within 90 to 120 minutes. Indeed, in the United States nearly 85% of patients with STEMI undergo PPCI as a revascularization strategy.¹

Adjuvant pharmacotherapy including oral antiplatelet regimens², heparins, and glycoprotein IIb/IIIa inhibitors (GPI)^{3, 4} have demonstrated variable efficacy and safety profiles in these patients. However, bleeding remains a potent predictor of prognosis in both STEMI and PCI⁵ where implementation of bleeding avoidance strategies are becoming increasingly important.⁶ In the Harmonizing Outcomes with RevascularIZatiON and Stents in Acute Myocardial Infarction (HORIZONS-AMI) trial bivalirudin use resulted in an absolute 2.9% reduction in 30 day net adverse clinical events and 1.0% reduction in 30 day mortality when compared to a GPI *plus* heparin in STEMI patients undergoing PPCI.⁷ More recently, in a comparison of bivalirudin versus abciximab plus heparin in NSTEMI patients, no differences existed in a combined endpoint of death, recurrent MI, urgent revascularization or major bleeding; however, bivalirudin significantly reduced the risk of major bleeding from 4.6% to 2.6%.⁸

Clinical trials designed to demonstrate efficacy and safety often exclude high-risk patients or those patients at greatest risk of complications from the studied therapy, thus limiting their generalizability. Data from registries allow ‘real world’ evaluation of adjuvant pharmacotherapy permitting a comparison of strategies in a broader population. However, real-world data comparing outcomes with the use of bivalirudin to other anti-thrombotic strategies in PPCI have yet to be reported.

Thus, as a high volume regional STEMI program, we chose to evaluate various anti-thrombotic strategies in patients undergoing PPCI. Specifically, we set out to evaluate clinical outcomes between bivalirudin, GPI, and heparin alone.

Methods:

Study Design, Data Source and Patients:

The University of Ottawa Heart Institute (UOHI) regional STEMI program services a population of approximately 1.3 million residents in eastern Ontario.^{9, 10} The PPCI program receives patients from 9 referral hospitals as well as a direct transport of STEMIIs identified by paramedics evaluating patients in the field. Patients are prospectively indexed in the UOHI-STEMI registry and, demographic data, therapies and clinical outcomes are recorded.

Inclusion in the study required a confirmed diagnosis of STEMI with the revascularization strategy being PPCI. For the purpose of this study STEMI was defined as ST elevation of at least 1 mm in two contiguous leads on a 12 lead electrocardiogram and presentation within 12 hours of onset of symptoms. Patients were excluded if they received thrombolytics, underwent coronary artery bypass graft (CABG) surgery immediately as a revascularization strategy, declined angiography, or received medical therapy alone. Patients were classified based on adjuvant anti-thrombotic therapy into three groups: bivalirudin with provisional GPI use, GPI, or heparin alone. All patients received aspirin 160 mg to chew, clopidogrel 600 mg load followed by 75 mg daily, and an unfractionated heparin bolus of 60 units/kg to a maximum of 4000 units at time of first medical evaluation by a physician. Notably, this protocol is similar to that in HORIZONS AMI in which 65% of patients received an upfront bolus of heparin followed by bivalirudin in the catheterization laboratory. These therapies are as per our regionalized STEMI protocol and low molecular weight heparins are not utilized. Subsequent use of bivalirudin, GPIs, or continuing with heparin alone was at the discretion of the

interventionalist. This study was reviewed and approved by the UOHI institutional human research ethics board and was deemed not to require informed consent.

Outcome Measures & Definitions:

The primary outcome of this study was the rate of non-CABG related Thrombolysis In Myocardial Infarction (TIMI) major bleeding (defined as a fall in hemoglobin of >5g/dL once adjusted for transfusion or intracranial bleeding).¹¹ Secondary outcomes included a composite endpoint of in-hospital death, stroke, re-infarction, or non-CABG related TIMI major bleeding. Stroke was defined as either ischemic or hemorrhagic stroke resulting in a new neurologic deficit of more than 24 hours' duration as diagnosed by a treating neurologist. Re-infarction was defined as recurrent chest pain associated with re-elevation of the ST segments in association with either re-elevation of the cardiac enzymes (twice the upper limit of the normal range) or angiographic documentation of re-occlusion of the infarct-related artery. Additional secondary outcomes of interest included death at 30-day and 180-day follow-up, bleeding outcomes using the TIMI definitions, need for transfusion, and probable and definite stent thrombosis as defined by the ARC criteria.¹² Mortality is reported as both percent of patients with available follow-up *and* as an adjusted mortality rate calculated by censoring patients lost to follow-up at 180 days.

Statistical Analysis:

All continuous variables were described as mean ($\pm$ standard deviation) or median (and interquartile range) and categorical variables as number (%), as appropriate. For composite endpoints, all components are reported individually.¹³ For patient and procedural characteristics, categorical variables were compared by Chi-square and continuous variables by analysis of variance or Kruskal-Wallis test as appropriate. For adjusted analysis of the primary and composite outcome, multiple logistic regression (MLR) was performed and odds ratio (OR) with 95% confidence intervals (CI) and adjusted p-values are reported. Variables in the MLR model were identified by univariate analysis (**Supplemental Tables 1 & 2**) using bivalirudin as the reference group, with indicator variables for heparin and GPI. Independent variables demonstrating a $p < 0.15$ were retained in the final MLR models. Lastly, to account for baseline differences in the cohorts, propensity scores were calculated using baseline clinical variables in **Table 1** and baseline angiographic variables in **Table 2**. A propensity score adjusted logistic regression analysis was then performed and adjusted ORs and 95% confidence intervals were reported. As there were 3 treatment arms, the outcome in the propensity score model was the treatment group. A logistic regression model with a generalized logit link function was utilized to model 3 treatment arms as the outcome. All analyses were performed using SAS software version 9.2.

Results:

Population and Baseline Characteristics

From July 2004 to December 2010, a total of 2317 consecutive patients were indexed in the registry from which a cohort of 2123 patients who underwent PPCI was included in the analysis (**Figure 1**). Of the patients initially transferred for PPCI, 0.7% received thrombolytics, 2.4% underwent emergent CABG as a revascularization strategy, 0.6% declined angiography, and 4.7% had medical therapy alone. Of patients undergoing PPCI, 748 patients received bivalirudin, 699 patients received GPI, and 676 patients received heparin alone.

Despite the non-randomized nature of the cohort study, groups were similar in a number of baseline characteristics including age, sex and cardiac risk factors (**Table 1**). However, important differences were present between cohorts – namely fewer patients receiving bivalirudin (1.5%) presented in Killip class IV compared to GPI (4.9%) or heparin (3.7%, $p<0.001$). Procedure related factors including infarct related artery, percent of patients receiving stents, and post PCI TIMI flow did not differ significantly amongst the groups (**Table 2**). More patients in the heparin group had a radial approach employed (26.2%) compared to GPI (5.7%) or bivalirudin (5.6%, $p<0.001$). Anti-platelet regimen, including admission and discharge aspirin and clopidogrel use was similar amongst the groups.

Clinical Outcomes

The incidence of the primary and secondary outcomes are reported in **Table 3**. The primary outcome, major bleeds, occurred in 2.7% of bivalirudin patients, 7.3% of

GPI patients, and in 3.3% of patients treated with heparin alone. The composite endpoint of in-hospital death, major bleeds, stroke, and re-infarction, occurred in 7.6% of patients receiving bivalirudin compared to 11.4% of patients receiving GPI and in 9.5% of patients receiving heparin. Other than marked differences in the rates of major bleeding, other components of the composite endpoint (in-hospital mortality, stroke or re-infarction) were similar amongst the strategies.

Bleeding complications in this cohort of patients was common, with 11.8%, 20.5%, and 12.9% of patients experiencing either a major or minor bleed in the bivalirudin, GPI, and heparin group respectively. Similarly, patients receiving either bivalirudin (3.1%) or heparin (3.6%) experienced approximately half the number of any major bleeding complication than patients receiving GPI (7.4%). Lastly, the risk of TIMI minor bleeds was also markedly lower in patients who did not receive GPI (bivalirudin 4.1% vs. GPI 13.2% vs. heparin 9.5%). Interestingly, the risk of transfusion did not differ between the cohorts.

At 180 days, mortality in the total study population was low at 6.2%. However, in patients in whom a major bleeding complication was identified, mortality increased significantly. Comparatively, major bleeding events increased the incidence of unadjusted mortality approximately 5 fold occurring in 3.5% vs. 20.6%, 3.8% vs. 21.1%, and 5.6% vs. 25.8% during the hospitalization, at 30 days, and at 180 days, respectively ($p < 0.001$ for all comparisons, **Figure 2**). Notably, a numerically higher frequency of stent thrombosis was seen with bivalirudin use (1.9% bivalirudin vs. 1.0% GPI vs. 0.6% heparin).

Adjusted analyses

Because of the non-randomized design of this study, univariate logistic regression was first performed to identify predictors of each endpoint and the results are presented in **Supplementary Table 1** and **Supplementary Table 2** (available online). Variables with significant association with the primary and composite outcome ($p < 0.15$) were retained in the MLR models. In-hospital major bleeds occurred less frequently with bivalirudin compared to GPIs (OR 2.78, 95% CI 1.53-5.06, $p < 0.001$). In contrast, bivalirudin did not demonstrate significant benefit when compared to heparin alone (OR 1.15, 95% CI 0.59-2.24, p 0.68). An elevated initial heart rate, anterior MI, Killip class, and renal failure were also independent predictors of the primary outcome. A propensity score adjusted logistic regression analysis was also performed which yielded results similar to the MLR analysis. In-hospital major bleeds occurred less frequently with bivalirudin compared to GPIs (OR 2.96, 95% CI 1.60-5.45, $p < 0.001$, **Figure 3A**). Again, bivalirudin did not reduce the risk of major bleeds compared heparin alone (OR 1.21, 95% CI 0.60-2.43, p 0.58).

The composite endpoint of death, stroke, re-infarction, and major bleeds, did not meet criteria for significance when comparing bivalirudin to either GPI *and* heparin (OR 1.49, 95% CI 0.98-2.28, $p = 0.06$) or heparin alone (OR 0.97, 95% CI 0.62-1.51, $p = 0.88$), although there was a strong trend favoring bivalirudin over GPI. Notably, propensity score adjustment demonstrated that the composite outcome occurred less frequently with bivalirudin compared to GPI *and* heparin (OR 1.62, 95% CI 1.07-2.44, $p = 0.02$). However, as with the primary endpoint, no difference observed when bivalirudin was compared to heparin alone (OR 1.05, 95% CI 0.68-1.63, $p = 0.83$).

Discussion:

The current study is the first to compare bleeding and clinical outcomes between anti-thrombotic strategies in real-world patients in the context of PPCI. Compared to GPIs, we found that bivalirudin use resulted in a 4.6% absolute reduction in the number of major bleeds resulting in a reduction in the composite endpoint of in-hospital death, major bleeding, stroke, and re-infarction. In our study bivalirudin did not result in significant benefit in bleeding or the composite endpoint when compared to heparin alone. Finally, though not powered to assess differences in mortality, we noted a 5-fold increase in the risk of death out to 180 days in patients in whom major bleeding occurred confirming the prognostic implication of bleeding seen in data from randomized control trials.^{14, 15}

Monitoring real-world performance of antithrombotic pharmacotherapy provides complimentary information to results derived from clinical trials. Specifically, our cohort included all patients presenting with a diagnosis of STEMI in whom PPCI is the revascularization strategy of choice. This allows for evaluation of outcomes in a broader population and validates findings from randomized trials. Despite these important differences, we observed a similar pattern of outcomes compared to the HORIZONS AMI study.⁷ For example, we found a 3.8% decrease in our composite endpoint (7.6% vs 11.4%, OR 1.66, 95% CI 1.12-2.45 p=0.01) when comparing bivalirudin to GPI – compared to a 2.9% reduction in net adverse clinical events in HORIZONS (12.1% vs 9.2%). Thus, our findings compliment data from HORIZONS and confirm the efficacy and safety of bivalirudin in a cohort of unselected patients managed by PPCI.

However, our data also permits a comparison of outcomes between patients in whom heparin alone was utilized as an antithrombotic strategy – a comparison not previously performed in PPCI. Most recently, it has been reported that heparin plus bivalirudin compared to bivalirudin alone reduced the rates of death and definite target vessel thrombosis during PPCI in the Swedish Coronary Angiography and Angioplasty Register.¹⁶ Our study has two important differences. First, all patients (including patients receiving bivalirudin) received unfractionated heparin upfront as part of our standardized protocol. Secondly, our data provides a cohort of patients in whom heparin alone was utilized for comparison. Interestingly, in the current study we could not detect a reduction in major bleeding (2.7% vs 3.3%, $p=0.59$) or the combined endpoint (7.6% vs 9.5%, $p=0.83$) with bivalirudin when compared to heparin alone. Notably, while this comparison did not meet significance, our bleeding rates and reductions in bleeding are similar to those seen in a randomized comparison of bivalirudin and heparin in patients undergoing elective PCI (3.1% vs 4.6%) and the lack of difference may simply reflect the small sample size and resultant lack of statistical power.¹⁷ Nonetheless, while it is evident from the randomized data and now the current study that GPIs increase bleeding events in patients undergoing PPCI, the use of bivalirudin preferentially over heparin in PPCI remains to be confirmed in randomized studies.

Certainly, our data add to the growing evidence that bivalirudin confers equivalent reductions in ischemic endpoints with an improved safety profile. For example, Marso and colleagues demonstrated marked reductions in bleeding events when bivalirudin was used in patients undergoing PCI in their analysis of the National Cardiovascular Data Registry (NCDR) – an effect magnified when used in conjunction

with vascular closure devices.¹⁸ Moreover, bivalirudin use has recently been shown in two studies to be strongly associated with reductions in bleeding events and length of stay in STEMI patients - resulting in the additional benefit of a significant reduction in overall cost of care.^{19, 20} However, despite the abundance of evidence, a recent analysis of the NCDR database suggests only 14% of STEMI patients receive bivalirudin as an anti-thrombotic therapy, while 65% of patients receive unfractionated heparin of which 80% receive a concomitant GPI.²¹ This suggests that the majority of STEMI patients continue to receive GPI plus heparin despite the overwhelming evidence of increased bleeding.

Stent thrombosis in patients undergoing PPCI is an exceedingly rare event²² although mortality has been reported to be as high as 30% for patients with definite or probable stent thrombosis.^{23, 24} In HORIZONS AMI, acute stent thrombosis occurred more frequently with bivalirudin than GPI (1.3% vs 0.3%, $p<0.001$). Subsequent analyses of these patients have suggested that an upfront heparin bolus and 600mg clopidogrel loading could in part mitigate the increased risk seen with bivalirudin.^{2, 25} Interestingly, in our study all patients received upfront heparin and clopidogrel 600mg loading yet we still observed a numerically higher incidence of acute stent thrombosis with bivalirudin compared to GPI or heparin alone (1.6% vs. 0.6% vs. 0.3%, respectively). Though the low event rates preclude adjustment of our observational data for known risk factors – our study suggests that in a real world population acute stent thrombosis remains a risk with bivalirudin that remains despite upfront heparin and high dose clopidogrel.

Our study has limitations, both inherent in the observational nature of the cohort study and due to the limited sample sizes achieved. First, our study is not adequately powered to assess differences in hard clinical outcomes such as mortality, stroke or re-

infarction. Accordingly, we did not observe differences amongst the groups on these individual endpoints. However, bleeding complications have been strongly associated with higher mortality in patients with MI^{15, 26} and thus we elected to use non-CABG related TIMI major bleeding as our primary endpoint. Indeed, in our analysis patients in whom bleeding complications occurred had a 5 fold increase in risk of mortality during their index hospitalization. Secondly, the patients were not randomized to antithrombotic strategies. Accordingly, we used accepted statistical modeling to control for differences in risk factors which varied between groups when analyzing our outcomes. However, important differences in the use of radial access existed amongst the groups (26.2% in heparin vs 5.6% in the bivalirudin group) – a magnitude of difference difficult to fully adjust. Indeed in the STEMI cohort of the The RadIal Versus femorAL (RIVAL) access for coronary intervention trial, radial access reduced a composite outcome of death, MI, stroke, and non-CABG related major bleeding from 5.2 to 3.1% (p=0.026). Thus, this large difference in the use of radial access in the heparin cohort may minimize the potential benefit of bivalirudin seen in this non-randomized study. Nonetheless, despite these limitations, the data represent the largest real world cohort of patients undergoing PPCI in which bivalirudin has been compared to GPI plus heparin and to heparin alone. Finally, our results very closely replicate both the trends and absolute event rates seen from randomized control trials – suggesting the applicability of their findings to clinical practice.

Conclusion:

Our analysis confirms that bivalirudin with provisional GPI use compared to upfront GPI plus heparin as an antithrombotic strategy in patients undergoing PPCI results in significantly less bleeding complications. A benefit of bivalirudin over heparin alone could not be demonstrated in our analysis and remains to be established in this population.

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Disclosures:

None.

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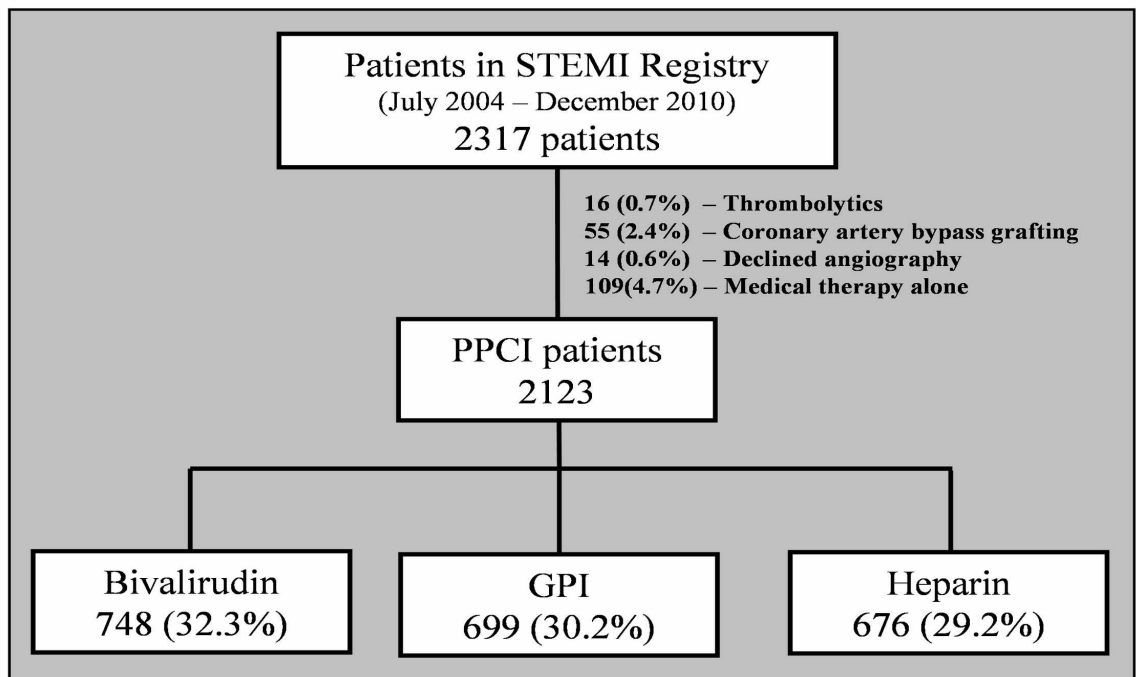


Figure 1: Selection of study population. Patients managed by a regionalized STEMI program where prospectively enrolled in the University of Ottawa Heart Institute STEMI registry. Patients who underwent primary PCI were identified and grouped based anti-thrombotic management used. Patients who received thrombolytics as a primary reperfusion strategy, late presentation infarctions, false positive STEMIs, patients managed medically, or patients revascularized by CABG were excluded. STEMI – ST elevation myocardial infarction; GPI – glycoprotein IIb/IIIa inhibitor; PPCI – primary percutaneous coronary intervention.

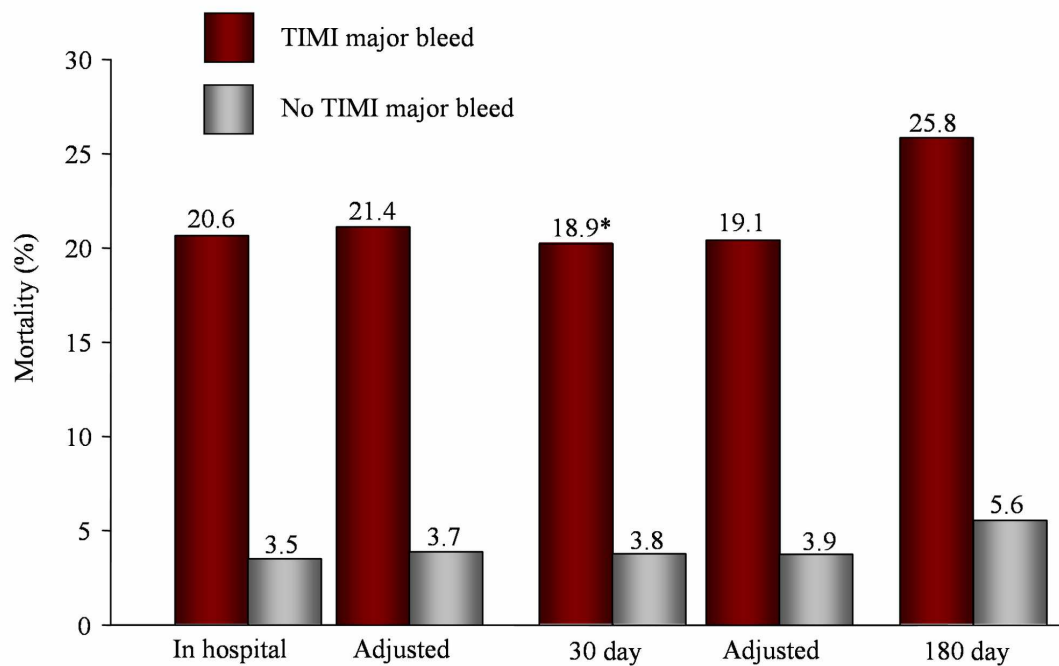
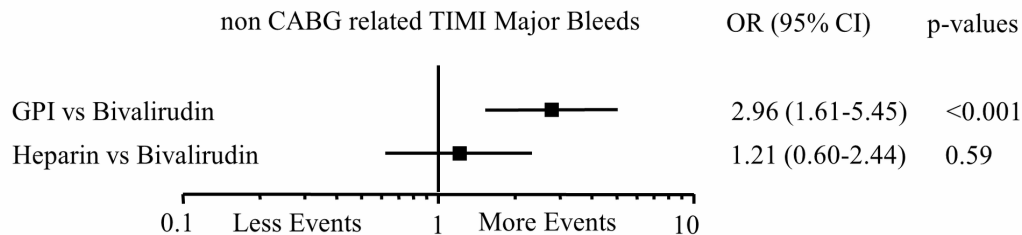


Figure 2. Effect of bleeding on in hospital, 30 day, and 180 day mortality. Red bars indicate patients with TIMI major non-CABG bleeds in-hospital. Grey bars indicate patients without a TIMI major non-CABG bleeding complication. Mortality rates are reported for the total cohort in whom follow-up is available. Adjusted mortality rates are reported by censoring patients lost to follow-up at 180 days. * - mortality is lower at 30 days due to patients who died in-hospital but after 30 days.

A



B

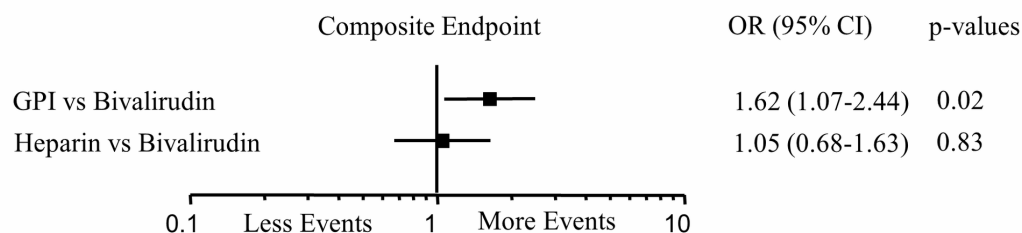


Figure 3. Outcome of propensity score adjusted logistic regression analysis for primary and secondary outcomes. (A) Effect of anti-thrombotic regimen on rates of non CABG related TIMI major bleeds. (B) Effect of anti-thrombotic regimen on the composite endpoint of in-hospital major bleeds, death, cerebrovascular accident or re-infarction. Boxes denote odds ratios (OR) with lines representing 95% confidence intervals (CI). CABG – coronary artery bypass grafting; GPI – glycoprotein IIb/IIIa inhibitor; TIMI – thrombolysis in myocardial infarction.

Table 1. Baseline Characteristics

	Bivalirudin (n=748)	GPI (n=699)	Heparin (n=676)	p-value
Age – years (SD)	61.9 (13.2)	60.3 (13.0)	62.9 (13.7)	<0.001
Males – no. (%)	539 (72.1)	523 (74.8)	473 (70.0)	0.13
Hypertension – no. (%)	344 (46.5)	328 (47.7)	325 (49.0)	0.65
Diabetes – no. (%)	112 (15.0)	135 (19.3)	119 (17.6)	0.10
Dyslipidemia – no. (%)	305 (42.0)	272 (40.2)	251 (38.6)	0.44
Smoking – no. (%)	313 (42.1)	306 (44.3)	255 (38.4)	0.09
Cardiovascular History – no (%)				
MI	87 (11.7)	91 (13.3)	84 (12.7)	0.64
CABG	15 (2.0)	31 (4.4)	25 (3.7)	0.03
PCI	61 (8.2)	69 (10.1)	64 (9.7)	0.44
Stroke	50 (6.7)	29 (4.2)	48 (7.2)	0.05
Killip Class – no (%)				<0.001
I	682 (91.2)	591 (84.5)	572 (84.6)	
II	45 (6.0)	64 (9.2)	68 (10.1)	
III	10 (1.3)	10 (1.4)	11 (1.6)	
IV	11 (1.5)	34 (4.9)	25 (3.7)	
Systolic BP – mmHg (SD)	137.1 (28.0)	132.8 (28.0)	136.4 (29.9)	0.01
Diastolic BP – mmHg (SD)	81.1 (17.7)	78.6 (17.4)	79.7 (17.7)	0.03
Heart rate – beats per minute (SD)	78.8 (20.6)	76.2 (19.7)	77.1 (20.5)	0.05
BMI – kg/m ² (SD)	27.7 (5.1)	28.0 (5.2)	27.2 (5.3)	0.01
Renal Function				
GFR – MDRD (SD)	73.7 (22.3)	74.3 (26.6)	71.3 (23.8)	0.06
Door to balloon time – minute (IQR)	94 (69-133)	97 (69-133)	105 (79-135)	<0.001

GPI – glycoprotein IIb/IIIa inhibitor; MI – myocardial infarction; CABG – coronary artery bypass grafting; PCI – percutaneous coronary intervention; BP – blood pressure; BPM – beats per minute; BMI – body mass index; GFR – glomerular filtration rate; MDRD – modification of diet in renal disease; SD – standard deviation, IQR – inter-quartile range

Table 2. Procedural Characteristics and Complimentary Medical Therapy

	Bivalirudin (n=748)	GPI (n=699)	Heparin (n=676)	p-value
Infarct Artery – no. (%)				0.66
Left main	4 (0.5)	3 (0.4)	3 (0.4)	
Left anterior descending	304 (40.6)	291 (41.6)	270 (39.9)	
Circumflex	99 (13.2)	96 (13.7)	85 (12.6)	
Right coronary artery	331 (44.3)	300 (42.9)	308 (45.6)	
Unknown	3 (0.4)	0 (0)	0 (0)	
Saphenous vein graft	7 (0.9)	9 (1.3)	10 (1.5)	
Revascularization – no. (%)				
Stent	731 (97.7)	680 (97.3)	663 (98.1)	0.62
Balloon angioplasty	17 (2.3)	19 (2.7)	13 (1.9)	0.62
Aspiration device	395 (53.2)	177 (28.0)	122 (20.2)	<0.001
CABG in hospital	19 (2.5)	9 (1.3)	9 (1.3)	0.39
Multivessel disease – no. (%)	429 (57.4)	382 (54.6%)	373 (55.2%)	0.54
TIMI Flow Pre PCI – no. (%)				0.36
0	443 (59.2)	386 (55.2)	368 (54.4)	
1	52 (7.0)	67 (9.6)	54 (8.0)	
2	96 (12.8)	98 (14.0)	104 (15.4)	
3	157 (21.0)	148 (21.2)	150 (22.2)	
TIMI Flow Post PCI – no. (%)				0.22
0	6 (0.8)	6 (0.9)	8 (1.2)	
1	2 (0.3)	9 (1.3)	5 (0.7)	
2	32 (4.3)	36 (5.2)	23 (3.4)	
3	700 (94.6)	644 (92.7)	636 (94.6)	
GPI – no. (%)				
Eptifibatide	23 (3.1)	412 (58.9)	0 (0)	<0.001
Abciximab	33 (4.4)	287 (41.1)	0 (0)	<0.001
Access – no. (%)				
Radial	42 (5.6)	40 (5.7)	177 (26.2)	<0.001
Discharge medications				
Aspirin	713 (99.0)	660 (98.4)	630 (98.4)	0.50
Plavix	708 (98.3)	657 (97.9)	626 (97.8)	0.76
Coumadin	68 (9.4)	93 (13.9)	104 (16.3)	<0.001
β-blocker	690 (96.0)	551 (94.8)	518 (93.2)	0.08
ACE Inhibitor	602 (83.6)	587 (87.5)	540 (84.4)	0.10
ARB	49 (6.8)	22 (3.3)	26 (4.1)	<0.001
Statin	706 (98.1)	643 (95.8)	624 (97.5)	0.04

GPI – glycoprotein IIb/IIIa inhibitor; TIMI – thrombolysis in myocardial infarction;
 CABG – coronary artery bypass grafting; ACE – angiotensin converting enzyme; ARB –
 angiotensin II receptor blocker.

Table 3. Unadjusted clinical outcomes

	Bivalirudin (n=748)	GPI (n=699)	Heparin (n=676)
In Hospital Outcomes – no (%)			
Bleeding			
TIMI Major non CABG	20 (2.7)	50 (7.3)	22 (3.3)
TIMI Major CABG	3 (15.8)	3 (15.8)	2 (22.2)
Any Major Bleed	23 (3.1)	52 (7.4)	24 (3.6)
TIMI Minor	65 (8.9)	91 (13.2)	63 (9.5)
Any Major or Minor Bleed	88 (11.8)	143 (20.5)	87 (12.9)
Transfusion	31 (4.1)	36 (5.2)	32 (4.7)
Clinical outcomes			
Death – in hospital	28 (3.7)	28 (4.0)	36 (5.3)
Stroke	12 (1.6)	4 (0.6)	11 (1.6)
Re-infarction	14 (1.8)	9 (1.3)	5 (0.6)
Composite Endpoint	57 (7.6)	80 (11.4)	64 (9.5)
Stent Thrombosis – no (%)	14 (1.9)	7 (1.0)	4 (0.6)
ARC Definite	12 (85.7)	7 (100.0)	4 (100.0)
ARC Probable	2 (14.3)	0 (0.0)	0 (0.0)
ARC Acute (<24 hours)	12 (85.7)	4 (57.1)	2 (50.0)
ARC Subacute (1 -30 days)	2 (14.3)	3 (42.9)	2(50.0)
Mortality – no (%)			
30 day	27* (3.7)	29 (4.3)	38 (5.7)
180 day	40 (5.9)	42 (6.1)	50 (7.6)

GPI – glycoprotein IIb/IIIa inhibitor; TIMI – thrombolysis in myocardial infarction; CABG – coronary artery bypass grafting; ARC – academic research consortium.

* - one patient died after 30 days but in-hospital

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Author Contributions:

Conceived and designed the study: BH & CC

Performed the experiments: BH, CC, LF

Performed statistical analysis: BH

Wrote the manuscript: CC, BH, CK, EB

Supervised: ERO, JA

Circulating Endothelial Progenitor Cell Levels Are Not Reduced in HIV-Infected Men

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Reduced levels of endothelial progenitor cells (EPCs) have been associated with increased cardiovascular (CV) risk, but limited data are available on EPC levels in the human immunodeficiency virus (HIV)-infected population. EPCs (CD45^{dim}/CD34⁺/kinase domain receptor⁺) from 36 HIV-uninfected and 30 antiretroviral therapy-naïve HIV-infected men without known CV risk factors were enumerated using flow cytometry. The mean EPC levels ($\pm$ standard error of the mean) were 1.4 ± 0.5 cells/ μ L in the HIV-infected group and 3.7 ± 2.2 cells/ μ L in the control group ($P = .92$). EPC levels were not associated with disease parameters, such as CD4 cell count or viral load. Reductions in EPC levels do not seem to explain the increased risk of CV disease among HIV-infected men.

Epidemiologic studies have shown that rates of cardiovascular (CV) events among human immunodeficiency virus (HIV)-infected individuals may be increased by 3-fold, compared with the general population [1], thus making CV disease a significant threat to longevity in HIV-infected individuals. Because endothelial dysfunction is an early marker of atherosclerosis and carries predictive value for CV events, it has been suggested that endothelial dysfunction may provide a link between HIV infection and atherosclerosis. Furthermore, it has already been

demonstrated that endothelial function is impaired at various stages in HIV infection [2].

Endothelial progenitor cells (EPCs) are a circulating progenitor population thought to be involved in vascular homeostasis. For example, they play a role in endothelial repair by homing to sites of injury and directing repair either through direct incorporation into the endothelium or by secreting proangiogenic paracrine factors [3]. Hill et al [4] first demonstrated a correlation between the number of circulating EPCs and patients' combined Framingham risk scores. Specifically, they demonstrated that levels of circulating endothelial cells were a superior predictor of vascular reactivity, compared with the presence or absence of conventional risk factors, and suggested that levels of EPCs may be a surrogate biologic marker for vascular function and cumulative CV risk. These findings have been corroborated by others, and it is well established that patients with reduced levels of EPCs have more frequent CV events [5].

Our objective was to determine whether there is a difference in EPC levels between HIV-infected and uninfected men. If a difference between these 2 populations were to be demonstrated, this may provide new insight into the mechanisms by which HIV infection accelerates atherosclerosis.

METHODS

Participants

Patients were highly active antiretroviral therapy (HAART)-naïve HIV-infected men attending the Ottawa Hospital Immunodeficiency Clinic in Ottawa, Ontario, or the Maple Leaf Medical Clinic in Toronto, Ontario. Control subjects were HIV-uninfected men recruited from the same sites. Patients and control subjects were enrolled on a consecutive basis. Inclusion criteria included age of 20–40 years and either having never smoked or abstinence from smoking for ≥ 3 months before enrollment. Exclusion criteria were selected to exclude medical conditions or medications known to influence EPC levels. These included a history of coronary artery disease, myocardial infarction, symptoms associated with atherosclerosis or active ischemia, stroke or symptoms suggestive of transient ischemic attack, diabetes, hypertension, dyslipidemia, conditions in which neovascularization may be present (cancer or retinopathy), hepatitis B or C, or any other chronic infection or acute illness. Use of medications known to influence EPC levels, including statins, angiotensin converting enzyme (ACE) inhibitors, glitazones, or granulocyte-colony stimulating factor,

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was also an exclusion criteria. We included only men in this small study, because estradiol dose dependently increases levels of EPCs, and circulating progenitor populations vary throughout the menstrual cycle.

All participants underwent a detailed assessment of cardiac risk and physical examination, including blood pressure, height, and weight measurements. All individuals had blood samples collected for complete blood cell count, fasting lipid profile, blood glucose level, erythrocyte sedimentation rate (ESR), and C-reactive protein measurement. In HIV-infected individuals, CD4 cell count and plasma viral load were measured if values obtained within the previous 3 months were not available. Whole blood was collected in ethylenediaminetetraacetic acid for EPC analysis. Approval was obtained from the respective institutional research ethics boards in advance, and all participants provided written informed consent.

EPC Enumeration

Blood samples were analyzed within 24 hours after collection with use of 5-color flow cytometry. In brief, EPCs were pre-defined as CD45^{dim}/CD34⁺/kinase insert domain receptor (KDR)⁺ based on currently accepted definitions [6, 7]. We also chose to examine CD45^{dim}/CD34⁺/CD117⁺ and CD45^{dim}/CD34⁺/CD184⁺ progenitor populations, because CD117⁺ and CD184⁺ are the cognate receptors for stem cell factor and stromal derived factor-1, respectively, both of which have been shown to have important roles in EPC function. Whole blood underwent red blood cell lysis with IOTEST 3 lysing solution (Beckman Coulter). Antibodies for CD45-Pacific Blue (Beckman Coulter; clone J.33), CD117-PC7 (phycoerythrin cyanin 7) (Beckman Coulter; clone 104D2D1), CD184-APC (allophycocyanin) (BD Pharmingen; clone 12G5), CD133-PE (phycoerythrin) (Miltenyi Biotec; clone AC133), and CD34-ECD (Phycoerythrin-Texas Red) (Beckman Coulter; clone 581) were used in this study. All flow cytometry was performed on a Cyan ADP9 cytometer (Beckman Coulter), and data were analyzed using Kaluza (version 1.1; Beckman Coulter). All flow cytometric data collection and analysis were performed in a central laboratory by an investigator blinded to the serostatus of the study participants.

Statistical Analysis

The sample size was calculated on the basis of the results of Vasa et al [8]. With an α of .05 and a power of 90%, we determined that 23 individuals per group would be required to detect a 50% decrease in mean EPC level in HIV-infected individuals. We sought to enroll a minimum of 30 individuals per group to facilitate the study of potential correlates of EPC levels in HIV-infected persons. Baseline characteristics are expressed as means $\pm$ standard deviations and EPC data are expressed as means $\pm$ standard error of the mean. For normally distributed data, comparisons were performed using a 2-tailed *t* test. For

Table 1. Baseline Characteristics of HIV-Infected Patients and Control Subjects

Characteristic	HIV-Infected Patients (n = 30)	Control Subjects (n = 36)	P
Age, y	33 $\pm$ 6	30 $\pm$ 4	.05
BMI	25.56 $\pm$ 3.96	24.61 $\pm$ 2.52	.15
TG, mmol/L	1.54 $\pm$ 0.71	1.13 $\pm$ 0.65	.01
HDL, mmol/L	0.98 $\pm$ 0.27	1.32 $\pm$ 0.25	.01
LDL, mmol/L	2.43 $\pm$ 0.86	2.66 $\pm$ 1.06	.41
Total cholesterol, mmol/L	4.07 $\pm$ 0.92	4.47 $\pm$ 1.14	.15
Fasting glucose, mmol/L	4.49 $\pm$ 0.97	4.18 $\pm$ 0.81	.29
ESR, mm/h	10.32 $\pm$ 17.85	3.89 $\pm$ 6.92	.02
CRP, mg/L	1.96 $\pm$ 2.53	1.94 $\pm$ 4.06	.91
Blood pressure, mm Hg			
Systolic	125 $\pm$ 13	120 $\pm$ 9	.05
Diastolic	70 $\pm$ 7	73 $\pm$ 8	.09
Family history of premature CAD, No. (%)	6 (20)	4 (11)	.42
Race/ethnicity, No. (%)			
White	18 (60)	25 (69)	.59
Other	12 (40)	11 (31)	.59
HIV parameters			
CD4 cell count, cells/ μ L	478 $\pm$ 303	...	...
CD4 cell percentage	25.1 $\pm$ 8.5	...	...
CD4-CD8 cell ratio	0.56 $\pm$ 0.33	...	...
Viral load, log ₁₀ copies/mL	3.86 $\pm$ 1.01	...	...
Time since HIV diagnosis, y	3.0 $\pm$ 2.3	...	...

Data are means $\pm$ standard deviations, unless otherwise indicated.

Abbreviations: BMI, body mass index (measured as weight in kilograms divided by square of height in meters); CAD, coronary artery disease; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HDL, high-density lipoprotein; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; TG, triglyceride.

nonparametric statistical testing, a Mann-Whitney rank sum test was used. For correlational analysis, Pearson correlation coefficients are reported. All statistical testing was performed using Sigma Stat software (version 3.5).

RESULTS

Baseline Clinical and Laboratory Profiles

Samples were obtained from 30 HIV-infected men and 36 healthy control subjects. Twenty-two HIV-infected men and 28 HIV-uninfected control subjects were enrolled from The Ottawa Hospital, and 8 HIV-infected and 8 HIV-uninfected men were enrolled from the Maple Leaf Medical Clinic. HIV-infected men had a significantly higher mean triglyceride level than did control subjects (1.54 $\pm$ 0.71 vs 1.13 $\pm$ 0.65 mmol/L; *P* = .009) and a higher mean ESR than did control subjects (10.32 $\pm$ 17.85 vs 3.89 $\pm$ 6.92 mm/h; *P* = .015). Otherwise, the 2 groups were similar (Table 1). The mean CD4 cell count and CD4 cell percentage in HIV-infected men at the time of study

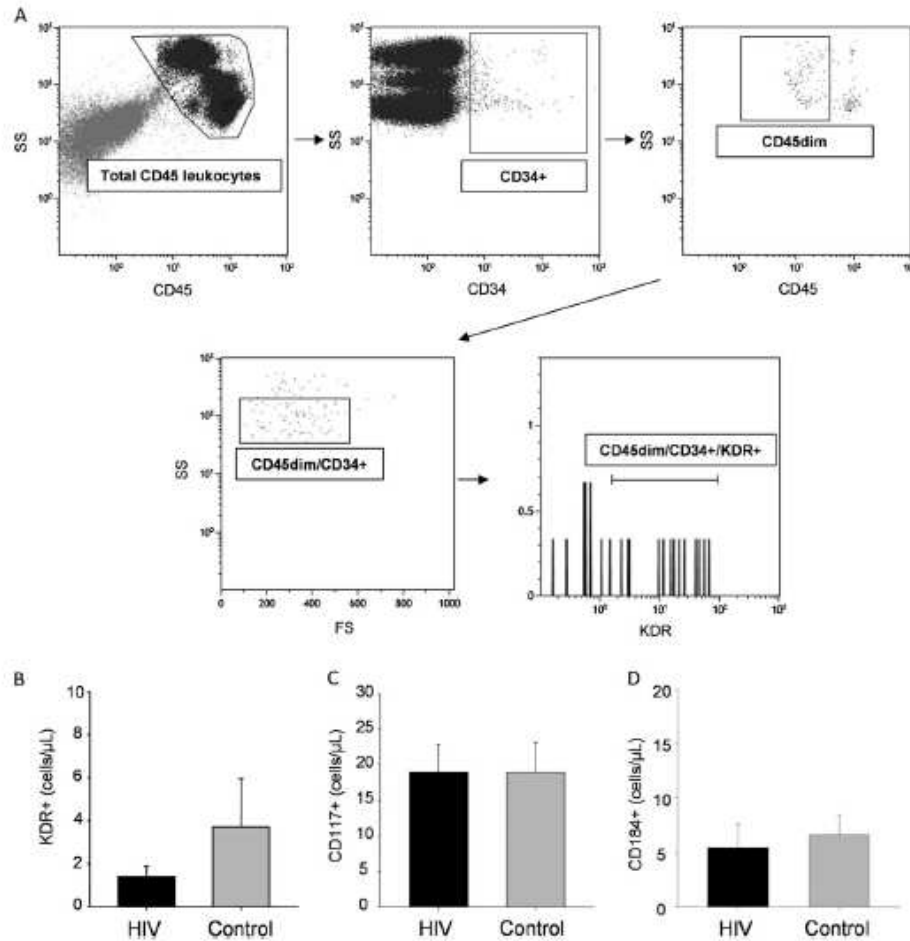


Figure 1. Circulating endothelial progenitor cell (EPC) levels are not reduced in human immunodeficiency virus (HIV) infection. *A*, Representative gating strategy used for rare cell analysis. *B*, CD45^{dim}/CD34⁺/KDR⁺ cells in HIV-infected and healthy men. *C*, CD45^{dim}/CD34⁺/CD117⁺ cells in HIV-infected and healthy men. *D*, CD45^{dim}/CD34⁺/CD184⁺ cells in HIV-infected and healthy men. Data are presented as means $\pm$ standard error of the mean ($n = 30$ for HIV-infected patients and $n = 36$ for control subjects; $P > .05$ for all comparisons).

enrollment were 478 ± 302 cells/ μ L and $25\% \pm 8.5\%$, respectively. The mean CD4-CD8 cell ratio was 0.56 ± 0.33 . The geometric mean viral load at study enrollment was 3.86 ± 1.01 log₁₀ copies/mL, and the mean time from diagnosis of HIV infection to enrollment was 3 ± 2.3 years.

EPC Levels in HIV-Infected Men, Compared With Healthy Control Subjects

We a priori defined EPCs in this study as CD45^{dim}/CD34⁺/KDR⁺ cells and enumerated them using a validated modification of the International Society of Hematotherapy and

Graft Engineering (ISHAGE) protocol (Figure 1A) [7]. With use of this definition, mean EPC levels were 1.4 ± 0.5 cells/ μ L in the HIV-infected group, which was not significantly different from those in the control group (3.7 ± 2.2 cells/ μ L; $P = .92$) (Figure 1B). We also analyzed levels of CD45^{dim}/CD34⁺/CD184⁺ and CD45^{dim}/CD34⁺/CD117⁺ as a secondary end point. Similar to KDR⁺ cells, levels of CD117⁺ progenitors (30.0 ± 7.3 vs 35.1 ± 5.4 cells/ μ L; $P > .05$) (Figure 1C) and CD184⁺ progenitors (5.5 ± 2.2 vs 6.6 ± 1.7 cells/ μ L; $P > .05$) (Figure 1D) did not differ between HIV-infected men and control subjects. In the overall cohort, EPC level did not

correlate with age, C-reactive protein level, ESR, or either CD184⁺ or CD117⁺ progenitor populations. Recently, da Silva et al [9] reported lower numbers of CD34⁺/KDR⁺ cells in HIV-infected HAART-naïve patients. We replicated their method of EPC enumeration by excluding granulocytes (as would be lost with Ficoll isolation) and enumerated CD34⁺/KDR⁺ cells as a percentage of the remaining cells. As with our established definition of EPCs, the proportions of CD34⁺/KDR⁺ cells did not differ between HIV-infected patients (0.006 ± 0.001) and healthy control subjects (0.012 ± 0.003 ; $P = .273$).

EPC Levels and Parameters of HIV Infection

Although no differences existed between healthy control subjects and HIV-infected individuals, we performed an exploratory analysis examining disease-related parameters and EPC levels. Specifically, we wished to determine whether any relationships existed between absolute CD4 cell count, log₁₀ viral load, and time since diagnosis of HIV infection with EPC levels. In our cohorts, no correlation could be established between any of the aforementioned parameters and CD45^{dim}/CD34⁺/KDR⁺ endothelial progenitors.

DISCUSSION

Despite the strong association between EPC levels and CV outcomes in the general population, we did not find a difference in EPC levels between HIV-infected and uninfected men in this case-control study. Furthermore, EPC levels did not appear to be associated with disease parameters, such as CD4 cell count or viral load.

To our knowledge, only 2 studies have been published examining the link between EPC levels and HIV infection. Teofili et al [10] examined 14 antiretroviral therapy-naïve HIV-infected men and women and 15 HIV-uninfected control subjects. They demonstrated that colony-forming unit endothelial cell (CFU-EC) progenitors, but not endothelial colony-forming cell (ECFC) progenitors, were markedly reduced in HIV-infected patients and that proviral HIV DNA was detectable only in CFU-ECs but not in ECFCs. There are many differences between our study and that of Teofili and colleagues that probably explain the divergent results. First, only cell culture techniques were performed in their study, whereas we used flow cytometry, thereby not allowing for a direct comparison between results. Furthermore, the authors noted differences only in CFU-ECs but not ECFCs; of interest, CFU-ECs have been shown to not be true EPCs, whereas ECFCs are clonally distinct and retain the ability to form blood vessels [11]. Thus, in their study, Teofili et al [10] did not demonstrate a difference in the population most important to neoangiogenesis—a finding similar to ours. In addition, our populations differed, because we excluded women, to avoid the confounding effects of estrogen, and included only

younger patients, to avoid the potential influence of subclinical atherosclerosis.

The second study involving HIV-infected individuals by da Silva et al [8] examined the relationship between EPCs, defined as mononuclear cells expressing CD34 and KDR and flow-mediated dilatation, a surrogate marker of endothelial dysfunction. Participants were antiretroviral therapy-naïve men and women. Of note, in contrast to our study, da Silva and colleagues found that individuals with HIV infection had lower EPC levels than did individuals without HIV infection, a result that probably reflects important differences in techniques. Enumeration of rare cell populations requires controls and standardization of protocols to ensure inter- and intra-laboratory reproducibility [7]. Accordingly, the ISHAGE protocol, as used in the present study, was developed and validated for clinical application and, in turn, has been applied to EPC enumeration [6, 7]. Furthermore, in our assay, we used red blood cell lysis enabling determination of accurate reporting of CD45^{dim}/CD34⁺/KDR⁺ cells per volume of blood. In contrast, da Silva et al [8] isolated mononuclear cells with use of Ficoll gradient centrifugation, which allows for the determination of the proportion of specific cell populations but not the absolute number of cells per volume of blood. Nonetheless, we were able to replicate the analysis performed by da Silva and colleagues and did not observe any differences between the populations.

Although we did not observe a difference in EPC levels, it remains possible that HIV infection is associated with altered functional aspects of EPCs. Diabetic adults, whose risk of CV disease is also increased, have EPCs with decreased adhesive and proliferative properties, attenuating the propensity of their EPCs to incorporate into vascular structures. Similarly, functional properties of EPCs are known to be important clinically, with higher rates of in-stent restenosis and delayed arterial healing being linked to lower adhesiveness [12]. Of note, HIV-infected men have been shown to have higher rates of in-stent restenosis [13]. Thus, although levels of EPCs appear to be unaffected by HIV infection, the effects of HIV infection on EPC qualitative properties remain to be established.

Another possible reason for the lack of difference between groups may relate to the fact that the HIV-infected individuals in our cohort were relatively early in the course of disease, with relatively preserved immune function (mean CD4 cell count, 478 cells/ μ L). Previous studies have demonstrated increased intimal medial thickness with CD4 cell counts ≤ 200 cells/ μ L [14], and findings of various studies have suggested an association between lower CD4 cell counts and higher viral loads and incidence of CV events [15]. It therefore remains possible that decreased EPC levels and, thus, increased risk of CV disease are not observed until there is a significant decrease in CD4 cell count.

In conclusion, EPC levels were not observed to be significantly reduced in HIV-infected HAART-naïve men with relatively preserved immune function. The increased risk of CV disease associated with HIV infection therefore cannot be attributed to reductions in EPC levels on the basis of our data. Because current smokers, individuals with established CV risk factors, and individuals receiving HAART were excluded, this conclusion applies to HIV-infected individuals who are HAART-naïve and free of established CV risk factors.

Notes

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Appendix V

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Author Contributions:

Conceived and designed the study: BH, TS, AC, PR EO

Performed the experiments: BH, TS

Collected Data: ML, ML, MF, DS, CG, JM, EO

Performed statistical analysis: BH, TS

Wrote the manuscript: TS

Supervised: EO

Unprotected Left Main Coronary Artery Stenting With Zotarolimus (Endeavor) Drug-Eluting Stents: A Single Center Retrospective Experience

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Objectives: To report the safety and efficacy of zotarolimus eluting stents for treatment of unprotected left main coronary artery disease. **Background:** Percutaneous stent insertion is an increasingly popular alternative to bypass surgery for the management of left main (LM) coronary artery disease. While data support the use of sirolimus- and paclitaxel-coated stents in the LM coronary artery, there are no published series reporting results with Endeavor (zotarolimus) stents, particularly in the context of unprotected left main (ULM) lesions. **Methods:** We retrospectively identified 40 consecutive patients who had ULM disease treated with Endeavor stents (ZES) and who had follow-up angiography. The primary endpoint was the prevalence of major adverse cardiac events (MACE), including cardiac/unexplained death, nonfatal myocardial infarction (MI), and in-stent restenosis (ISR)/target lesion revascularization (TLR). **Results:** Angiographic and procedural success was achieved in all cases. Follow-up angiography occurred on average 5.6 ± 0.9 months after the index procedure. There were three incidences of ISR requiring TLR and another patient who had a NSTEMI in the follow-up period. At late follow-up (12.4 ± 1.8 months) three patients underwent CABG (one for RCA stenosis) and four patients died without knowledge of the status of the ULM stent (two cardiovascular and two deaths related to cancer progression). **Conclusions:** In conclusion, our experience with Endeavor stents for the treatment of ULM disease demonstrates excellent angiographic and clinical outcomes, with a 7.5% ISR/TLR rate and a 15% MACE rate, respectively, at an average clinical follow-up of 12.4 months. © 2012 Wiley Periodicals, Inc.

Key words: left main; unprotected; zotarolimus; endeavor; DES; PCI

INTRODUCTION

Percutaneous coronary intervention (PCI) routinely involves the deployment of stents and is annually performed in more than three million patients worldwide. To a large extent, bare metal stents have been superseded by drug-coated stents that have a lower incidence of in-stent restenosis (ISR) and need for target lesion revascularization (TLR), [1,2]. Traditionally, coronary artery bypass surgery (CABG) has been considered the gold-standard treatment for obstructive disease in the left main (LM) coronary artery [3]. However, recent data demonstrate an acceptable safety profile for both LM and unprotected LM (ULM) PCI, with percutaneous revascularization of ULM lesions becoming common practice, though this data are limited to first generation drug-eluting stents (DESs)—sirolimus (SES) and paclitaxel (PES) eluting stents [4–9]. For example, the recently published SYNTAX trial, a randomized comparison of CABG versus PESs for

revascularization of severe coronary artery disease, reported comparable rates of major adverse cardiac and cerebrovascular events (MACCE) at 1 year in a

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subgroup analysis of LM cases [4]. However, the percutaneous group maintained a higher rate of repeat revascularization relative to the surgical arm, a trend also seen in other meta-analyses specifically addressing ULM cases [4,10,11]. Recently, the American College of Cardiology/American Heart Association (ACC/AHA) changed the recommendation for PCI of LM lesions from class III to class IIb, such that LM PCI is no longer restricted to nonsurgical candidates [12].

Despite evidence supporting first generation DESs for ULM PCI, there is limited data on zotarolimus-eluting stents (ZESs) for ULM PCI [13,14]. Studies in animal models suggests ZESs may provide a more appealing safety profile for ULM interventions, given their diminished inflammatory response and enhanced strut reendothelialization when compared to first generation DESs [15]. Indeed, the more potent inhibition of neointimal formation by SESs may be hazardous—particularly if this leads to positive remodeling—a risk factor for incomplete stent apposition and late stent thrombosis [16–18]. Conversely, some have argued against the use of ZESs because, in some series, they do not limit neointimal formation to the same degree as SESs and may result in greater late lumen loss [14]. Hence, as a high volume PCI center, we elected to review the clinical outcomes of our patients that underwent insertion of ZESs in ULM coronary arteries.

METHODS

We retrospectively identified, from ~28,000 patients who underwent angiography at the University of Ottawa Heart Institute from June 2005 to March 2010, 337 patients who had PCI performed involving their LM artery. From this initial cohort of 337, a further subset of 53 patients was selected with ULM disease treated with Endeavor (zotarolimus) drug eluting stents (ZES). This was then further narrowed to those who received follow-up angiography, to ultimately yield a final cohort of 40 patients who received ZESs to ULM arteries with follow-up angiography, as per Fig. 1. Those who received ZESs for disease in neighboring vessels that extended into the ULM were also included in our analysis, including ostial circumflex (Cx) or ostial left anterior descending (LAD) vessel disease. Presence of other types of ULM stents, shock at presentation, or incomplete medical records were considered exclusion criterion.

Indications for angiography ranged from acute coronary syndrome (ACS), defined as those patients being evaluated for ST-segment elevated myocardial infarction (STEMI), non-ST-segment elevated MI (NSTEMI), or unstable angina (UA), to those with stable angina with or without high risk noninvasive

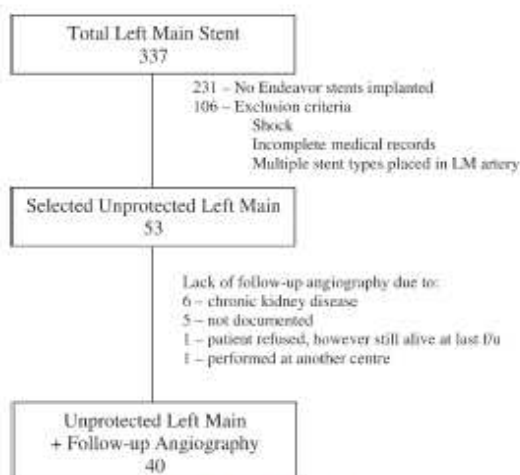


Fig. 1. Study design and methodology.

imaging. Cases were collected from multiple operators utilizing both femoral and radial artery approaches. Interventional techniques and medications were used at the discretion of the individual operators in accordance with current clinical guidelines, including the use of rotational atherectomy, intraaortic balloon pumps (IABPs), intravascular ultrasound (IVUS), and glycoprotein IIb/IIIa inhibitors. ULM lesions were localized as being ostial/shaft or distal/bifurcation lesions, while the presence of multivessel disease versus isolated ULM disease was also reported. Angiographic success was defined as stenosis less than 20% and TIMI (thrombolysis in myocardial infarction) 3 flow in the target vessel [19,20]. Procedural success was defined as the absence of procedural complications or major adverse cardiac events (MACE) in the setting of angiographic success. Procedural time was reported according to the time recorded on the catheterization reports, while postprocedural in-hospital stay lasted from index procedure until discharge from hospital.

Following the index intervention, patients were maintained on dual antiplatelet therapy consisting of enteric-coated acetylsalicylic acid (EC-ASA) indefinitely and clopidogrel (Plavix) for a minimum of 6 months. Review of records did not indicate discontinuation of medications during the follow-up period. Follow-up surveillance angiography was recommended at 4–6 months post index PCI. ULM ISR was defined as stenosis >50% within the ULM stent on follow up angiography, while TLR included any intervention (surgical or percutaneous) for disease of the index lesion in follow up. Patient data and specific procedural information were collected from review of each

TABLE I. Baseline Characteristics

Total patients	40
Age	63.6 ± 1.9
Male	34 (85)
Risk factors	
Smoking	20 (50)
Diabetes mellitus	12 (30)
Hypertension	27 (67.5)
Hyperlipidemia	31 (77.5)
Medications	
ACE inhibitor	21 (52.5)
Beta blocker	34 (85)
Statins	35 (87.5)
Clopidogrel	35 (87.5)
Aspirin	35 (87.5)
Index procedure indication	
Stable elective procedure	20 (50)
Acute coronary syndrome	20 (50)
STEMI	3 (7.5)
NSTEMI	9 (22.5)
UA	7 (17.5)
Rescue PCI	1 (2.5)

Mean ± standard error. Number of patients (percentage).

patient's medical record. The follow-up period extended from the time of the surveillance angiogram to the last available clinical evaluation. Our primary endpoint was the evaluation of MACE, as defined by cardiac or unexplained death, nonfatal myocardial infarction (MI), and TLR due to ISR of the target lesion.

This study was conducted in accordance with the guidelines set out by the Ottawa Hospital Human Research Ethics Board, with all patients providing informed consent.

RESULTS

Patient Characteristics

Forty consecutive patients were identified from the University of Ottawa Heart Institute Interventional Database who met the inclusion criteria (Fig. 1). These patients had a mean age of 63.6 ± 1.9 years and 34 (85%) were males (Table I). Twenty (50%) patients presented with ACS, including STEMI (3, 7.5%), NSTEMI (9, 22.5%), and unstable angina (7, 17.5%), while the remainder had stable angina with or without high-risk noninvasive testing (20, 50%). Only one LM PCI involved the use of fibrinolytic prior to the index procedure (in the context of rescue PCI for a STEMI presentation).

Procedural Characteristics

The majority (32, 80%) of patients were accessed via the radial or brachial artery approach (Table II). We report 16 (40%) high risk cases involving lesions

TABLE II. Procedural Characteristics

Radial/brachial access site	32 (80)
Coronary dominance	
Right	31 (77.5)
Left	6 (15)
Codominant	2 (5)
Unknown	1 (2.5)
Unprotected left main	40 (100)
+Left dominant system	6 (15)
+Right dominant with RCA>70%	10 (25)
Total high risk cases	16 (40)
Isolated LM disease	3 (7.5)
Left main stent characteristics	
Total number	1.2 ± 0.1
Total length (mm)	18.4 ± 1.1
Diameter (mm)	3.7 ± 0.1
Final balloon diameter (mm)	4.4 ± 0.1
Final dilation pressure (atm)	16.9 ± 0.7
Lesion location	
Ostial/Shaft	13 (32.5)
Distal/bifurcation	27 (67.5)
Medina classification	
1,1,1	13 (32.5)
1,1,0	10 (25)
0,1,0	3 (7.5)
0,0,0	1 (2.5)
Bifurcation stenting technique	
Single stent	20 (50)
T-stenting	6 (15)
Culotte	1 (2.5)
Intravascular ultrasound	17 (42.5)
Rotational atherectomy	1 (2.5)
Fractional flow reserve	1 (2.5)
Intraaortic balloon pump	4 (10)
Procedural medications	
Ily/IIIa inhibitors	4 (10)
Thrombin inhibitors	3 (7.5)
Procedural time (min)	107.5 ± 7.5
Fluoroscopy time (min)	21.9 ± 2.0
Contrast volume (mL)	276.1 ± 19.6

Mean ± standard error. Number of patients (percentage).

of ULM arteries serving as the primary supply vessels—10 (25%) had significant concomitant disease (>70% stenosis) of the right coronary artery (RCA) in a right dominant system, while an additional 6 (15%) had left dominant systems. Only 3 (7.5%) patients had isolated ULM disease, with the vast majority showing varying degrees of multivessel disease. The target lesions ranged in location from ostium and/or shaft (13, 32.5%) to distal and/or bifurcation (27, 67.5%). The distal/bifurcation lesions were further characterized according to the Medina Classification system. The majority of our bifurcation cases (13, 32.5%) involved disease of all three vessels (LM, LAD, Cx), with 10 (25%) involving just the LM and LAD, and just 3 (7.5%) of cases having solitary LAD lesions. One case did not have any significant disease of all three vessels (0,0,0) however, due to coronary dissection an Endeavor was inserted into the ULM artery thereby

TABLE III. In-Hospital and Long-Term Outcomes

Postcath length of stay (days)	
Same day	13 (32.5)
One	11 (27.5)
Two	3 (7.5)
Three	3 (7.5)
Four	3 (7.5)
Six or more	7 (17.5)
Follow-up angiography	
Time (months)	5.6 ± 0.9
Indication	
Stable elective	37 (92.5)
ACS	3 (7.5)
STEMI	0 (0)
NSTEMI	1 (2.5)
UA	2 (5)
Restenosis	
Left main restenosis	2 (5)
PCI	1 (2.5)
CABG	1 (2.5)
Re-stenosis in another vessel	8 (20)
RCA	2 (5)
Cx	4 (10)
LAD	2 (5)
Managed by	
PCI	6 (15)
CABG	2 (5)
Final follow-up	
Time (months)	12.4 ± 1.8
CABG	3 (7.5)
MACE	6 (15)
LM ISR/TLR	3 (7.5)
Non-fatal MI	1 (2.5)
Cardiovascular death	2 (5)
Noncardiac death	2 (5)

Mean ± standard error. Number of patients (percentage).

meeting inclusion criteria for our study. Bifurcation stenting techniques included simple single vessel stenting (20, 50%), T-stenting (6, 15%), and one (2.5%) case utilizing the Culotte technique. An average of 1.2 ± 0.1 stents (five patients received two or more) were placed in the ULM with a mean total stent length of 18.4 ± 1.1 millimeters (mm) and mean stent diameter of 3.7 ± 0.1 mm. Stents were postdilated with an average final balloon diameter of 4.4 ± 0.1 mm at a pressure of 16.9 ± 0.7 atm. IVUS was employed in the LM artery for 17 (42.5%) cases, rotational atherectomy once (2.5%) and fractional-flow reserve assessment once (2.5%), while IABP support was required in only 4 (10%) cases. Angiographic success was achieved in all cases with no major complications, and no urgent surgical interventions.

In-Hospital and Long-Term Outcomes

Hospital stay post procedure ranged from same day discharge (13, 32.5%), next day (11, 27.5%), or 2-, 3-, or 4-days post-index procedure (3, 7.5% cases each).

Seven (17.5%) cases required six or more days in hospital prior to discharge but had no stent or procedural-related complications during this interval (Table III). Similarly, there were no deaths, strokes or stent thromboses in the follow-up period leading up to surveillance angiography. On average, patients underwent surveillance angiography at 5.6 ± 0.9 months following their index procedure, with 37 (92.5%) being routinely performed for stable patients and only 3 (7.5%) due to ACS (1 NSTEMI, 2 UA), during which there were no reported complications. Moreover, the single NSTEMI presentation in follow-up was determined to have a patent ULM stent. There were only three (7.5%) cases of ISR requiring TLR of the ULM, one via PCI and the other two via CABG. Overall, three patients underwent CABG—one for revascularization of the RCA and the other two for the left coronary artery system. Of those two, one underwent CABG for severe ISR of their Cx stent in the context of a patent ULM stent, while the other had severe ISR of their Cx and LAD stents combined with moderate ISR of the ULM stent, hence both were included as LM ISR/TLR in our reported results.

Final follow-up was defined as the patient's last available evaluation, angiographic or otherwise, and occurred at a mean of 12.4 ± 1.8 months, during which four patients died. Two patients (5%) died of cardiovascular causes (one chronic congestive heart failure and one cardiac arrest); however, the status of their LM stents was unknown at the time of death and autopsies were not performed. Two (5%) patients died from progression of metastatic cancer. Hence, we report a 15% incidence of MACE in follow up: two cardiovascular deaths, one NSTEMI, and three ULM ISR/TLR.

DISCUSSION

Treatment of ULM coronary lesions by PCI is rapidly being accepted as the standard of care in selected patient populations. While studies support the use of bare metal and first-generation drug eluting stents for ULM PCI, the data supporting ZESs are limited. Hence, our study aimed to elucidate the outcomes of patients receiving Endeavor stents for the treatment of ULM stenoses.

In this series, we report an acceptable safety profile for Endeavor stents in that angiographic success was achieved in all cases, with no patients requiring urgent CABG or suffering (sub)acute thrombosis. Our overall reported rate of MACE at a mean follow-up of 12.4 ± 1.8 months is 15% —2 (5%) cardiovascular deaths, 1 (2.5%) NSTEMI, and 3 (7.5%) TLR for ULM ISR. This is consistent with reports from a recent meta-

analysis by Biondi-Zoccai et al, of first generation DESs used specifically for ULM lesions, yielding overall MACE rates of 16.5% [10]. Indeed, these authors noted that at later follow-up intervals this trend continues (i.e., MACE rates of 14.2% up to 6 months, and 17.7% for >6 months follow-up), while other studies report MACE rates ranging from 14 to 33%, depending on lesion location and number of stents deployed [7,9,10]. Our report is the first to describe outcomes in unprotected left main interventions using ZESs and these results appear similar to those reported in the existing literature.

Given the high-risk profile of the patients selected for ULM PCI in this time period, the reported total MACE was surprisingly low. In terms of events directly related to the ULM stent, there were three cases of ISR (one treated by PCI and two by CABG) and one cardiovascular death. Specifically, of the two cardiovascular deaths reported, only one would have qualified as a "possible stent thrombosis" by the ARC criteria, with the second patient dying as a result of severe progressive LV dysfunction (which predated the intervention) [21]. The remainder of the cases had events related to progression of other underlying illness (i.e., cancer) or disease in other coronary arteries.

Lesion location is thought to influence outcomes following ULM intervention, with distal/bifurcation disease potentially being associated with an increased risk of adverse events [8]. Indeed, such lesions often require challenging interventional techniques, including T-stenting (Fig. 2), to achieve angiographic success in the affected vessels. When assessing outcomes according to lesion location it is important to note that the distribution of patients in the ostial/shaft vs. the distal/bifurcation locations is comparable to other studies [6]. We report a 15% MACE rate (2 cardiovascular deaths) in the smaller ostial shaft group (13, 32.5%), while those in the larger distal/bifurcation group (27, 67.5%) also had a MACE rate of 15% (1 NSTEMI and 3 TLR for ULM ISR). Interestingly, our reported MACE rates are less than those published according to lesion location for first generation DESs, which range from 17 to 24% for ostial/shaft lesions and 19 to 33% for distal/bifurcation lesions [7]. Thus, our data suggest that ZESs can be used in ULM lesions with outcomes similar to those observed with other DESs.

There were only three cases of ULM ISR, one was managed via PCI, the other two by CABG. For the PCI case, we found that the Endeavor stent (3.5-mm diameter) originally placed in the LM was undersized—despite being postdilated to 4.5 mm to produce an excellent angiographic appearance during the index procedure (Fig. 3). On follow-up angiography there was severe ISR, and on IVUS the LM was noted to be



Fig. 2. Left main bifurcation stenting. (A) Significant stenosis located in ostial circumflex artery and ostial left anterior descending artery continuing into the distal LM. (B) Following T-stenting insertion of two Endeavor stents (ZESs)—one 3.5 × 9 mm² to the left circumflex artery and another 3.5 × 18 mm² to the LAD extending back into the LM, with postdilation to 4.5 mm with a noncompliant balloon resulting in excellent angiographic appearance.

more than 6 mm in diameter. Given this size discrepancy, incomplete stent apposition likely played a key role in the development of ISR in this case, a risk factor well known to be associated with ISR in other studies [22,23]. As the patient was having chest pain during follow-up angiography, the ISR lesion was temporarily managed by balloon dilatation. The following day, a larger diameter (6.0 mm) bare metal stent was inserted in the LM, providing excellent angiographic and sonographic results. For the first CABG revascularization case, the asymptomatic patient presented for standard surveillance angiography which revealed severe ISR of the Cx and LAD stents and moderate ISR of the ULM stent. In the second case, the patient

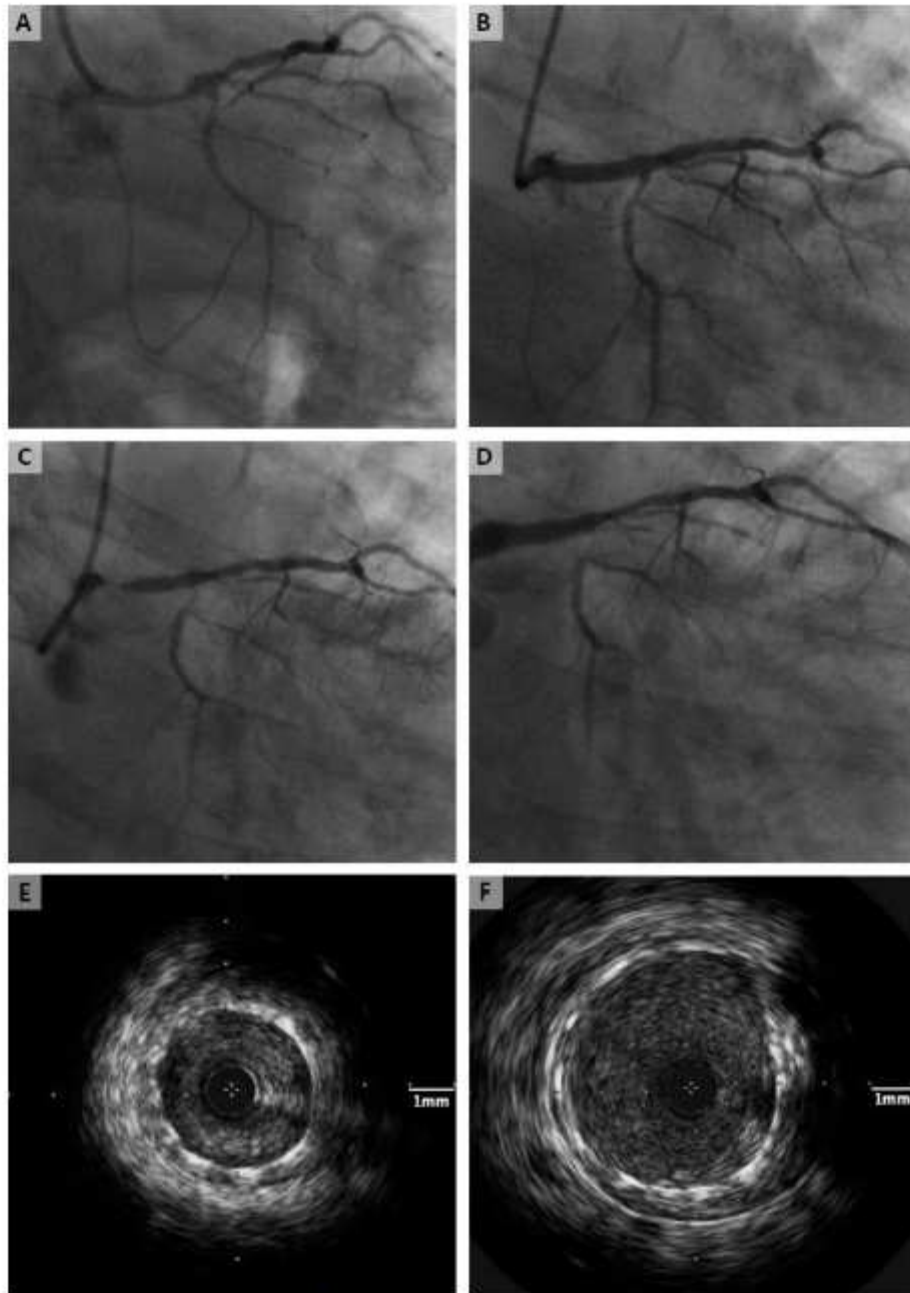


Fig. 3. In-stent restenosis (ISR) of a left main (LM) stent. (A) Index LM lesion displaying ~80% stenosis of distal LM segment extending into the proximal left anterior descending artery prior to intervention. (B) Excellent angiographic appearance following insertion of 30×3.5 mm² Endeavor stent post-dilated to 4.5 mm. (C) Follow-up displaying significant (95%)

ISR of index stent. (D) Following insertion of 6.0-mm bare metal stent with excellent results. (E) Intravascular ultrasound (IVUS) performed at follow-up (corresponds to panel C) displaying incomplete stent apposition to vessel wall with reduced luminal diameter. (F) Final IVUS post restenting of the vessel (corresponds to panel D) with increased luminal diameter.

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presented with recurrent stable angina and so was recatheterized, revealing a patent ULM stent, but severe ISR of the ostial Cx and mild stenosis of the LAD. Hence, these cases were both referred for surgical intervention. The postoperative course was uncomplicated, with excellent outcomes in clinical follow-up.

Our study shares all the limitations of other retrospective case series. First, the cohort is relatively small, and hence we cannot extrapolate the data to make broad practice pattern recommendations. Indeed, many of the patients in this report had a higher risk profile that precluded them from surgical revascularization—hence, our series does not necessarily represent the elective management of LM stenoses. Second, given the lack of randomization it is difficult to compare our results to those reported with first generation DESs as both the complementary medical therapies and PCI strategies continue to evolve. Finally, the relatively small size of this patient series precludes comparison between ostial/shaft and distal/bifurcation lesions, though the results obtained are consistent with those observed by other investigators. Nonetheless, to our knowledge, this is the first report to demonstrate an acceptable safety profile for ZESs for ULM PCI.

CONCLUSIONS

Our real world experience with the insertion of Endeavor (zotarolimus) drug eluting stents for the treatment of LM coronary stenoses demonstrates excellent angiographic and short term clinical results with only a 7.5% ISR/TLR rate and a 15% rate of MACE at an average follow-up of 12.4 months.

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Appendix VI

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Conceived and designed the study: BH, RO LF XM YXC

Performed the experiments: BH, XM AP TS JS KR LF

Performed statistical analysis: BH TS LF ERO

Wrote the manuscript: BH TS LF ERO

Supervised: ERO

Pre-Procedural Atorvastatin Mobilizes Endothelial Progenitor Cells: Clues to the Salutary Effects of Statins on Healing of Stented Human Arteries

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Abstract

Objectives: Recent clinical trials suggest an LDL-independent superiority of intensive statin therapy in reducing target vessel revascularization and peri-procedural myocardial infarctions in patients who undergo percutaneous coronary interventions (PCI). While animal studies demonstrate that statins mobilize endothelial progenitor cells (EPCs) which can enhance arterial repair and attenuate neointimal formation, the precise explanation for the clinical PCI benefits of high dose statin therapy remain elusive. Thus we serially assessed patients undergoing PCI to test the hypothesis that high dose Atorvastatin therapy initiated prior to PCI mobilizes EPCs that may be capable of enhancing arterial repair.

Methods and Results: Statin naïve male patients undergoing angiography for stent placement were randomized to standard therapy without Atorvastatin (n = 10) or treatment with Atorvastatin 80 mg (n = 10) beginning three days prior to stent implantation. EPCs were defined by flow cytometry (e.g., surface marker profile of CD45dim/34+/133+/117+). As well, we also enumerated cultured angiogenic cells (CACs) by standard *in vitro* culture assay. While EPC levels did not fluctuate over time for the patients free of Atorvastatin, there was a 3.5-fold increase in EPC levels with high dose Atorvastatin beginning within 3 days of the first dose (and immediately pre-PCI) which persisted at 4 and 24 hours post-PCI (p < 0.05). There was a similar rise in CAC levels as assessed by *in vitro* culture. CACs cultured in the presence of Atorvastatin failed to show augmented survival or VEGF secretion but displayed a 2-fold increase in adhesion to stent struts (p < 0.05).

Conclusions: High dose Atorvastatin therapy pre-PCI improves EPC number and CAC number and function in humans which may in part explain the benefit in clinical outcomes seen in patients undergoing coronary interventions.

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Introduction

Percutaneous coronary intervention (PCI) is the preferred revascularization strategy for patients with coronary artery disease. Recent developments with drug eluting stents (DESs) have reduced the need for revascularization compared to balloon angioplasty and bare metal stents (BMSs) resulting in less need for repeat revascularization. Current strategies for reducing in-stent neointima formation commonly exploit the anti-proliferative and anti-inflammatory effects of paclitaxel and sirolimus. Unfortunately, the negative effect of these drugs on stent strut endothelialization may be dire due to an increased risk of sudden stent thrombosis.[1] Clinical and animal studies suggest a role for circulating endothelial progenitor cells (EPCs) in reconstituting the endothelium and reducing neointima formation following injury. [2] Indeed, the paucity and/or impaired functional capacity of EPCs are inversely associated with cardiac risk factors, cardiovascular outcomes and restenosis

rates.[3,4] In animal studies, interventions that enhance mobilization of EPCs such as G-CSF, HMG-CoA reductase inhibitors (also known as "statins") or estrogens uniformly result in improved endothelialization and diminished neointimal formation.[5–7]

Statins induce a robust mobilization of murine CD117+/Sca-1+ progenitor cells and EPCs via the PI3K/Akt pathways and enhance endothelialization of injured vessels thereby leading to attenuation of neointimal formation.[7–10] Moreover, recent clinical trials show an LDL-independent superiority of intensive compared to moderate statin therapy in reducing target vessel revascularization in patients who undergo PCI for acute coronary syndromes.[11] However, the precise explanation for the clinical advantage of high dose statin therapy with PCI remains elusive. Therefore, we performed serial assessments of patients undergoing PCI to test the hypothesis that high dose Atorvastatin (80 mg) therapy initiated prior to PCI mobilizes EPCs that may be capable of enhancing arterial repair.

Methods

Ethics Statement

The protocol was approved by the University of Ottawa Heart Institute Research Ethics Committee (protocol #54-122) and Health Canada. All patients gave written informed consent and research was conducted according to the principles expressed in the Declaration of Helsinki.

Patients and Protocol

Our study was designed to longitudinally assess the effects of statin therapy on patients undergoing PCI with stent deployment. Patient inclusion criteria included the following: >18 years of age, no treatment with a HMG-Co-A reductase inhibitor in the preceding 3 months, availability for a 14 day follow-up blood test, and the patient had to be scheduled for a coronary angiogram with possible *ad hoc* PCI and stent implantation. Furthermore, to exclude the confounding factor of estrogens in pre- and post-menopausal women, only males were included in the study. Exclusion criteria included an unstable condition requiring urgent cardiac catheterization, hematological malignancy, therapy with G-CSF or GM-CSF, or previous statin intolerance.

Patients were randomized to Atorvastatin 80 mg per day or medical therapy without a statin beginning three days prior to a scheduled angiogram. Baseline laboratory tests included a complete blood count, HbA1C level, fasting lipid profile, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), assessment of EPCs by flow cytometry (CD45dim/CD34+/CD133+/CD117+), and enumeration of cultured angiogenic cells (CACs). All of the aforementioned tests were repeated at time of arterial catheterization (i.e., at outset of coronary angiographic procedure and prior to commencement of an *ad hoc* PCI), 4 hours post PCI, 24 hours post PCI, and at 14-day follow-up. In addition, troponin-T (TnT) and creatine kinase-MB (CKMB) fractions were measured pre-procedural and at the 4 and 24 hour follow-up time points to identify peri-procedural myocardial infarctions (MI) as defined by $\geq 3\times$ rise above the upper limit of normal.

Statin naïve patients were randomized in blocks of 6; however, after the first 18 patients had been enrolled 8 patients had completed the protocol in the non-statin control arm and only 2 patients had completed the protocol in the statin arm. In the statin arm of the study the following patients did not complete the protocol: 2 patients without CAD, 3 patients who were deemed non-revascularizable and 2 patients who were referred for coronary artery bypass grafting. Therefore, the statin randomization scheme was redone for a total of 40 patients to be enrolled in order to yield 10 patients completing each study arm. As well, 11 healthy controls without clinically evidence of CAD were recruited for comparison of baseline EPC and CAC levels. Four patients initially randomized in the trial whom were found to have normal coronaries were combined with the 11 healthy individuals for a total of 15 healthy controls.

EPC and CAC quantification

EPCs were assessed by flow-cytometry and CACs by *in vitro* culture assay. All blood samples for EPC enumeration were drawn by venous or arterial puncture then anticoagulated with EDTA.

EPCs were enumerated using a standardized flow cytometric protocol. Briefly, five-color flow cytometry was performed for the markers CD34 (clone 581, fluorochrome PC7), CD45 (clone J133, fluorochrome ECD), CD133 (clone AS133, fluorochrome PE), and CD117 (clone 104D2D1, fluorochrome PC5), and CD49e (clone SAM1, fluorochrome FITC). Antibodies were purchased from Beckman Coulter and studies performed on a Beckman Coulter

Cytomics FC 500 cytometer. Red blood cell lysis was performed using IO Test 3 solution (Beckman Coulter) and samples were then incubated with appropriate dilutions of antibodies. As isotype controls are known to mask rare cell populations, [12] none were used in this analysis, and baseline fluorescence was determined using unstained cells. All reported EPC counts were adjusted for total white blood cell counts as determined by standard complete blood counts in our regional hematology laboratory.

We prospectively defined EPCs as CD45dim/CD34+/CD133+/CD117+. Recognizing that circulating EPCs have historically been defined by CD34+/KDR+ [13,14] preliminary studies were performed looking at expression of KDR and CD117 cells which demonstrated that virtually all cells expressing KDR also co-expressed CD117. Thus, KDR was not included in the final panel of markers. It is important to note that human CD45-/CD117+/KDR+ cells can incorporate into the coronary vasculature and display clonal proliferative potential, suggesting an EPC phenotype [13,15]. Moreover, CD45-/CD117+ of cardiovascular origin have recently been shown to co-express CD31, CD34, CXCR4, and KDR [13].

While initially termed EPCs, CACs are now recognized to be involved in regulating angiogenesis without directly contributing to post-natal vasculogenesis and thus are now termed cultured angiogenic cells. CAC have been extensively described in the literature and are known to be inversely associated with risk factors for CAD [16]. Indeed, numerous studies have highlighted the important roles CACs play in regulating the angiogenic and tissue remodeling response in various disease states [17–19]. The methodology used for the CAC culture assay is previously described [20–23]. Briefly, peripheral blood mononuclear cells (5×10^6) isolated by Ficoll centrifugation were cultured in EGM-2 media (Cambrex) before being dispersed on fibronectin coated plates. Cells were washed at 4 days and adherent cells were maintained for 7 days prior to enumeration. For the task of enumeration, CACs were defined as cells dually positive for AcLDL uptake and *Ulex europaeus* agglutinin I (UEAI) binding. While uptake of AcLDL and binding of UEAI may non-specifically identify myeloid and epithelial cells [13,24] we have previously demonstrated the capacity of these cells to facilitate vascular repair *in vivo* [19] and others have demonstrated expression of the endothelial markers KDR, CD31, vWF, and eNOS [13]. DiI-AcLDL (2.5 $\mu\text{g}/\text{ml}$, Molecular Probes) was incubated with cultured CACs for 1 hour in a cell incubator. Subsequently, cells were washed and fixed with Cytifix Buffer (BD) and incubated with FITC-UEAI (5 $\mu\text{g}/\text{ml}$, Sigma) for 30 minutes. Plates of cells were again washed then incubated with a DAPI nuclear counterstain before a coverslip was applied to the well and double positive cells (CAC) were counted in 6 random high power fields ($\times 200$ magnification).

CAC Functional assays

The effects of Atorvastatin on CAC survival, VEGF secretion, and adherence to bare metal stent struts were tested using 7 day old CACs. The culture media for Atorvastatin treated cells was supplemented with 0.1 $\mu\text{mol}/\text{L}$ of Atorvastatin (Pfizer) or DMSO (vehicle) consistent with a previously published study [25].

For cell survival, six high-power fields were enumerated and the media was changed every 4 days. Data are expressed as a percentage of initial cells. Secretion of VEGF was measured using a VEGF ELISA kit (R&D systems). CACs were plated in equal numbers and cultured in VEGF free EGM-2 media for 24 hours. Subsequently, 200 μL of the supernatant was isolated and used in the ELISA assay. Stent adhesion studies were performed using BMSs (Medtronic Micro-Driver) that were cut, flattened, and fixed

to the bottom of 6 well plates using a Type-I collagen solution such that the stent strut projected above the collagen base as previously described.[22] Seven day old CACs (2×10^5) were resuspended and cultured for 48 hours. Cells were stained with DAPI, fixed and attached cells enumerated.

Q-PCR for alpha integrins was performed as previously described.[19] Briefly, PCR was performed utilizing an annealing temperature of 56 degrees Celsius. The Qiagen QuantiTect SYBR PCR system was utilized as per manufacturer instructions. All experiments were performed using a Roche LightCycler 480. The following primer combinations were utilized: GAPDHfwd (CG-CCTGGAGAAAGCTGCTAAGTAT), GAPDH rev (GCTTCA-CAAAGTGGTCATTGAGGG), α -1fwd (ACAAGTGACAGC-GAAGAACCCTCT), α -1rev (TGGGTACAGCACAGGGTA-ACCATT), α -2fwd (AGTTTATCTCCAGCGGTACAAAGT), α -2rev (TGGGCTTATCCCAATCTGACCAA), α -3fwd (CAA-AGACAGGCAACGGCAACGTA), α -3rev (TTATTGGTCCG-CGGTGAGAAGCCTA), α -4fwd (AGGGCAAGGAAGTTCC-AGGTTACA), α -4rev (ACATGAGGACCAAGGTGGTAAG-CA), α -4probe (AGCATTATGCGGAAAGATGTCCGGG), α -5fwd (TGGCTGAGTCTCCCAATTTTCA), α -5rev (ACA-TGAGGAACCAAGGTGGTAAGCA).

Statistical Analysis

For statistical procedures, a p-value of <0.05 was considered significant. All continuous variables are expressed as means $\pm$ standard error of the mean (SEM). Data normality was tested using the Kolmogorov-Smirnov test. Two way comparisons were done by *t* test and the interaction between statin therapy and PCI by two-way repeated measures analysis of variance with pairwise comparisons done with Bonferroni *post hoc* testing. Alternatively, for nonparametric statistical testing a Mann-Whitney Rank Sum test was used. All statistical procedures were performed using the Sigma Stat 3.5 statistical package.

Results

Clinical and laboratory profiles

In each group 10 patients completed the protocol receiving at least one stent. In total, 15 healthy controls were analyzed for comparison – 4 patients who underwent angiography and had normal coronary arteries and 11 healthy individuals without clinical evidence of CAD.

Of the 20 patients completing the protocol, all underwent successful revascularization with deployment of at least one stent. Baseline characteristics were similar between the treatment groups including the number and types of stents (DES vs BMS) used (Table 1). In terms of adverse events, only one post-PCI MI was recorded in the statin group and one patient in the control arm was withdrawn from the study after having a stroke related to the catheterization procedure. At time of discharge patients received evidence-based medical therapy for CAD (eg, aspirin, clopidogrel, β -blocker, and an angiotensin converting enzyme inhibitor or angiotensin II receptor blocker, Table 1). All patients that underwent PCI completed follow-up and no other adverse events were noted to 14 days.

In terms of baseline laboratory values, total white count, HbA1C levels, C-reactive protein levels, and cholesterol profiles were similar at baseline between the groups. However, at follow-up the total white count in the statin arm was higher than controls (8.8 ± 1.4 vs $6.3 \pm 1.9 \times 10^9$ cells/L, $p < 0.01$, Table 2). Furthermore, as expected both total and LDL cholesterol levels were lower in the statin arm (2.9 ± 1.0 vs

Table 1. Patient Characteristics.

	Control Group (n = 10)	Statin Group (n = 10)	Healthy (n = 15)
Characteristics			
Males	10 (100%)	10 (100%)	15 (100%)
Age in years (SD)	59.5 (6.9)	63.0 (10.5)	34.4 (12.6)
CCS class I-III	5 (50%)	7 (70%)	0 (0%)
CCS class III-IV	5 (50%)	3 (30%)	0 (0%)
Previous PCI	2 (20%)	0 (0%)	0 (0%)
Cardiac Risk Factors			
Hypertension	3 (30%)	4 (40%)	0 (0%)
Smoking	2 (20%)	2 (20%)	1 (6.7%)
Family History	3 (30%)	3 (30%)	2 (13.3%)
Hyperlipidemia	3 (30%)	3 (30%)	1 (6.7%)
Diabetes mellitus	2 (20%)	0 (0%)	0 (0%)
eGFR (SEM)	86 (7)	75 (6)	107 (9)
Angiogram/PCI			
# of stents deployed (SD)	1.2 (0.4)	1.6 (0.8)	
Drug-eluting stents used	4 (40%)	5 (50%)	
Severe CAD	0 (0%)	2 (20%)	
Medical Therapy			
Clopidogrel	10 (100%)	10 (100%)	
Aspirin	10 (100%)	10 (100%)	
β -blocker	8 (80%)	9 (90%)	
ACE/ARB	9 (90%)	10 (100%)	
Atorvastatin	0 (0%)	10 (100%)	

CCS Class – Canadian Cardiovascular Society Angina Class.

PCI – percutaneous coronary intervention.

CAD – coronary artery disease.

MI – myocardial infarction.

ACE/ARB – angiotensin converting enzyme/angiotensin II receptor blocker.

eGFR – estimated glomerular filtration rate mL/min/1.73 m².

SD – standard deviation.

SEM – standard error of the mean.

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4.6 ± 1.0 and 1.3 ± 0.7 vs. 3.0 ± 1.0 mmol/L, respectively, $p < 0.01$).

Effect of statin therapy on circulating EPC and CAC levels

a) Baseline Flow Characteristics. CD45dim EPC were enumerated at baseline in a total of 47 individuals, 32 with CAD and 15 healthy controls (Figure 1A). While healthy controls had nearly three fold more EPC than CAD patients the difference did not reach significance (12.7 ± 2.7 vs 5.4 ± 0.6 cells/ μ L, $p = 0.07$). Patients who had been referred for angiography after presenting with an acute coronary syndrome (ACS) in the previous 14 days did not have more EPC at baseline than non-ACS patients (5.0 ± 1.0 vs 5.6 ± 0.6 cells/ μ L, $p > 0.05$).

b) Serial Flow Cytometric Assessment of Cell Profiles. When looking at the effects of PCI on mobilization of EPC we compared the abundance of this cell type at the following intervals: three days prior to catheterization, pre-catheterization, 4 hrs, 24 hrs and 14 days. While EPC levels did not fluctuate over time for the patients who did not receive Atorvastatin, there was a 3.5-fold increase in EPC levels with high dose Atorvastatin within 3 days of the first dose (immediately pre-

Table 2. Laboratory characteristics of patients.

	Control Group (n = 10)	Statin Group (n = 10)	Healthy (n = 15)	p-value
Baseline				
WBC (SEM)	7.2 (1.5)	8.0 (2.4)	6.9 (1.5)	NS
HbA1C	0.052 (0.005)	0.051 (0.003)	0.053 (0.003)	NS
C-reactive protein	6.8 (11.4)	8.3 (9.0)	1.9 (1.1)	p<0.05
Total cholesterol	5.3 (0.7)	4.8 (1.0)	5.0 (1.0)	NS
HDL	1.0 (0.3)	1.0 (0.1)	1.2 (0.3)	NS
LDL	3.3 (0.6)	2.9 (0.9)	3.3 (1.0)	NS
TG	2.1 (1.0)	1.9 (1.2)	1.3 (0.9)	p<0.05
Follow up				
WBC	6.3 (1.9)	8.8 (1.4)		p<0.01
HbA1C	0.053 (0.001)	0.054 (0.001)		NS
C-reactive protein	6.5 (8.2)	21.7 (6.9)		NS
Total cholesterol	4.6 (1.0)	2.9 (1.0)		p<0.01
HDL	1.0 (0.2)	0.9 (0.2)		NS
LDL	3.0 (1.0)	1.3 (0.7)		p<0.01
TG	1.6 (0.5)	1.5 (0.7)		NS

WBC = white blood cell 10^9 cells/L
HbA1C = hemoglobin A1C
HDL = high density lipoprotein mmol/L
LDL = low density lipoprotein mmol/L
TG = triglycerides mmol/L
SEM = standard error of the mean
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PCI) that persisted at 4 and 24 hours post-PCI ($p<0.05$). There was no effect seen on EPC levels caused by PCI (Figure 1B). Additionally, we serially assessed CD49e expression on circulating EPCs and found low levels of basal expression and both the number of CD49e+ cells nor the relative CD49e expression was affected by statin therapy or PCI (data not shown).

c) In vitro CAC Assessment. CAC were enumerated by culture assay and yielded similar results to those obtained by flow cytometry. Baseline CAC levels were 2-fold higher in healthy controls compared to patients with CAD at baseline (e.g., 38.8 ± 2.3 vs. 17.1 ± 1.5 , $p<0.01$, Figure 2A). Again, there were no differences in CAC levels between patients with a recent ACS compared to those with clinically stable symptoms ($p<0.05$, data not shown). Interestingly, a significant interaction was seen between Atorvastatin therapy and PCI when precatheterization, 4 hour and 24 hour levels were assessed (2 way ANOVA: $p=0.006$ for Atorvastatin and Time from PCI interaction). However, in those patients receiving Atorvastatin, there was an early increase in CAC levels until 24 hours post-PCI followed by a tapering off effect – although 14 day levels remained approximately 2-fold higher than baseline (e.g., 4 h: 2.7 ± 1.5 , 24 h: 3.4 ± 0.6 , 14 d: 1.9 ± 0.3 , Figure 2B).

Effect of statin therapy on function of CAC

Statin supplementation of the culture media at a concentration of $0.1 \mu\text{M}$ failed to alter survival of CAC *in vitro* (Figure 3D, $p>0.05$). Similarly, secretion of the angiogenic cytokine VEGF was unaffected by culture in the presence of Atorvastatin in the culture media (e.g., 28.5 ± 3.5 vs 32.9 ± 3.6 ng/mL, Figure 3C, $p>0.05$). Finally, CAC adhesion to BMSs was tested

in vitro by co-culture of CAC in the presence of a bare metal coronary stent immobilized in collagen. After 48 hours *in vitro*, co-culture with Atorvastatin augmented CAC attachment to stents by 2-fold compared to cells co-cultured in the absence of Atorvastatin (e.g., 9.7 ± 0.9 vs. 4.0 ± 0.6 , $n=10$, $p<0.01$, Figure 3A,B). As integrins are known to be important modulators of CAC adhesion [19,26] we assayed by RT-qPCR the expression of the $\alpha 1-5$ subunits of the integrin family and found no significant regulation of mRNA levels ($p>0.05$, data not shown).

Discussion

While it is empirically recognized that statins may increase EPC levels in patients, the temporal course and profile of the cells mobilized by statins at time of PCI had not previously been characterized. Nonetheless, patients undergoing the implantation of stents designed to trap CD34+ cells (e.g., Genous endothelial progenitor cell capturing stent) are routinely pre-treated with high dose statin therapy with the hope of augmented circulating EPC levels [27]. This study is the first attempt to prospectively randomize patients undergoing stent insertion to high dose statin therapy and describe in a serial fashion the time course and extent of EPC and CAC mobilization. Our data demonstrates that 80 milligrams of Atorvastatin per day, beginning three days prior to PCI, is associated with a mobilization of both EPC and CAC. As well, this is the first study to enumerate the highly selective population of CD34+/CD117+ (c-kit) progenitors by flow cytometry in patients undergoing revascularization. Recently c-kit has been found to define a population of human coronary vascular progenitor cells that are capable of regenerating competent coronary vessels and improving coronary blood flow [15]. Finally, we demonstrate that statin treatment improves the functional capacity of CAC, by augmenting attachment to stent struts using a novel *in vitro* model. It is important to note that this increase in EPC adhesion was not due modulation to member of the integrin family and similar to *in vivo* observations by Banerjee *et al.* [28] occurred without a change in VEGF levels.

Results from the studies of EPCs and PCI continue to yield equivocal or discordant results [29]. This likely reflects both the varying manner in which EPCs are quantified as well as limitations in study design. For example, the largest study to date was performed by Inoue *et al.* who looked at 40 patients undergoing PCI [30]. They studied both CD34+ cells by flow cytometry and outgrowth of either endothelial or smooth muscle cells (SMCs) from PBMCs at multiple time points out to one month post PCI. They noticed an association between mobilization of CD34+ cells and outgrowth of SMCs with ISR but by limiting their flow cytometry to a single marker they most certainly included all early bone marrow derived cells including hematopoietic progenitors cells. Mills *et al.* examined circulating EPC number and colony forming unit mobilization in 24 patients undergoing elective PCI [31]. Similar to our data, they observed a 3-fold increase in cultured cells in the first 24 hours following PCI with the majority of their patients being concomitantly treated with statin medications. Finally, Egan *et al.* [32] compared 10 patients undergoing PCI with 13 patients having angiogram only and found that CXCR4 positive cells were mobilized in response to PCI when compared to angiography alone. In contrast, our study looked at multiple time points out to two weeks after PCI in a total of 20 patients between both arms. More importantly, our data is the first to randomize patients to statin medications and to serially assess their EPC levels – providing a novel mechanistic link between the observed clinical outcomes and early statin therapy in patients undergoing PCI.

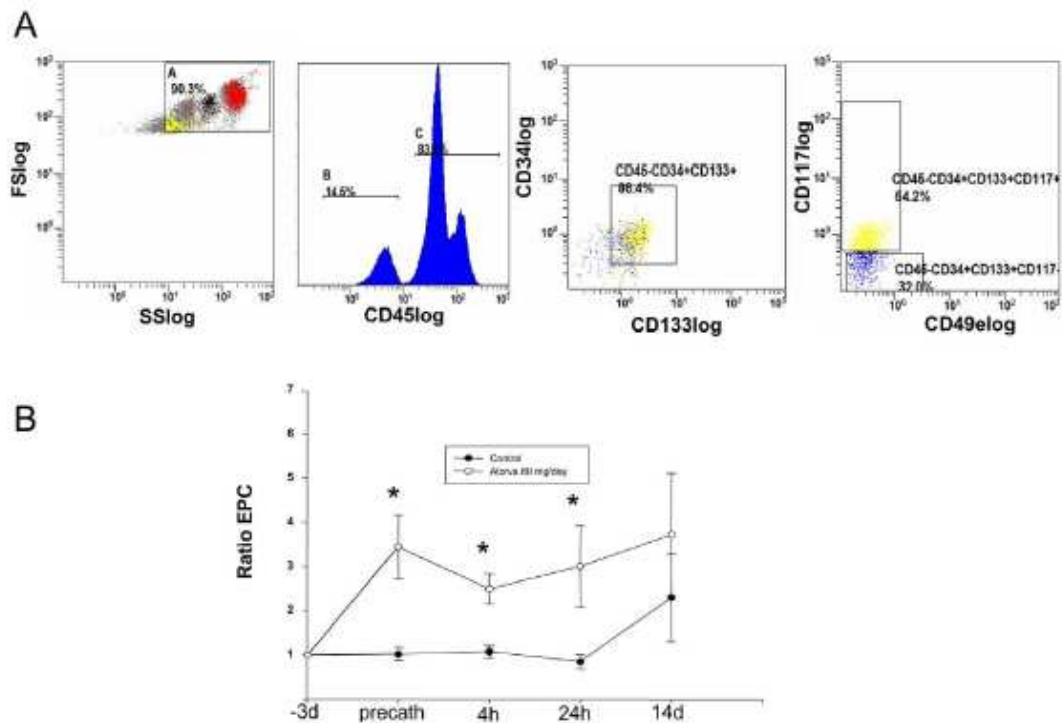


Figure 1. Flow cytometric analysis of CD45dimCD34+CD133+CD117+ endothelial progenitor cells (EPC). (A) Sample gating strategy. (B) EPC levels, expressed at each interval as a ratio to baseline levels (taken three days prior to PCI) for patients receiving and not receiving Atorvastatin therapy (n = 10 per group). * indicates p < 0.05. doi:10.1371/journal.pone.0016413.g001

As in the literature regarding PCI and EPCs, the effect of statin therapy on both initial mobilization and maintenance of EPC levels has yielded sometimes conflicting results. Vasa and colleagues first reported an increase in EPCs (CD34+/KDR+) and CACs out to 28 days in 40 patients with CAD.[33] Most recently, these results were replicated in a small cohort of patients initiated on Atorvastatin therapy, yielding a doubling of CD45dim/CD34+/KDR+ cells at four weeks.[34] While neither of these studies were randomized or had control groups for comparison, their results are very similar to those observed in the current study. Paradoxically, in patients with CAD Hristov *et al.* [35] quantified both EPCs and CACs in a large non-randomized cohort and noted a decrease in EPCs during chronic statin therapy (especially at higher doses). Similarly, Deschêaux *et al.* [36] derived fewer colony forming units from patients on chronic statin therapy than a matched cohort, suggesting the initial mobilization observed may not be sustained over longer periods. However, the non-randomized nature of these studies and the lack of standard dosing certainly justifies trepidation in drawing firm conclusions. Data from large randomized studies of both early and long-term effects of statin therapy on EPCs is clearly warranted.

Perhaps the most intriguing finding of our study was the early difference in EPC levels seen with Atorvastatin loading – an effect that was observed with both flow cytometry and CAC culture which persisted for at least 24 hours post-PCI. However, by 14 days EPC levels had tapered off somewhat – yet remained elevated

relative to baseline levels. The cause of the diminution of EPCs by day 14 is unclear, but perhaps suggests that the mobilization of EPCs by statins is a time-limited, transient effect. Alternatively, there may be either no further EPCs available to be mobilized or some other rate-limiting process is preventing EPCs from being generated and/or mobilized by statins. Interestingly, there was a noticeable rise in EPC levels in the control arm at 14 days. This likely in part reflects more aggressive management of patients atherosclerotic disease and risk factors in patients newly identified with CAD. For example, smoking cessation has been associated with an early and robust rise in EPC levels.[37] Similarly complementary pharmacotherapy such as angiotensin converting enzymes[38] or thiazolidinediones (glitazones)[39] are also known to increase EPC levels. Thus, this late rise in the control patients most likely represents improved risk factor modification.

It is important to realize that our protocol is similar to those used in the ARMYDA,[40] ARMYDA-ACS,[41] and the more recently completed ARMYDA-RECAPTURE[42] studies. Both ARMYDA and ARMYDA-ACS demonstrated a clear reduction in peri-procedural myocardial infarctions in statin naïve patients undergoing PCI for stable coronary artery disease or acute coronary syndromes respectively when high dose Atorvastatin was initiated prior to the procedure. Perhaps more clinically relevant is the ARMYDA-RECAPTURE study in which the investigators reloaded patients already on statins with 80 mg of Atorvastatin and showed reductions in peri-procedural myocar-

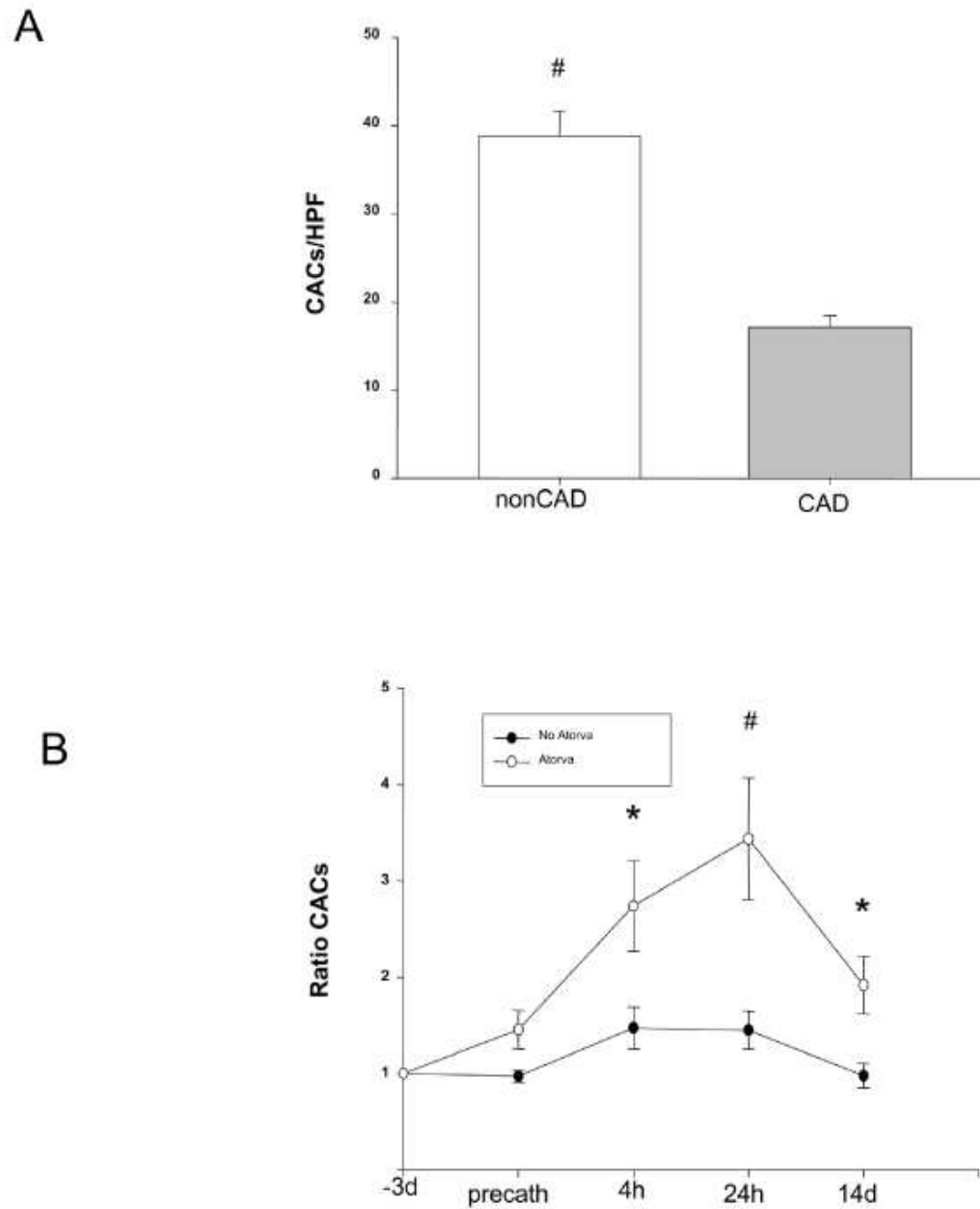


Figure 2. *In vitro* assessment of cultured angiogenic cells (CAC). (A) Comparison of baseline CAC levels in patients with CAD ($n=32$) vs. healthy controls (non-CAD). (B) CAC levels expressed at each interval as a ratio to baseline levels (taken three days prior to the procedure) for patients receiving and not receiving Atorvastatin therapy ($n=10$ per group). * indicates $p<0.05$, # indicates $p<0.01$. doi:10.1371/journal.pone.0016413.g002

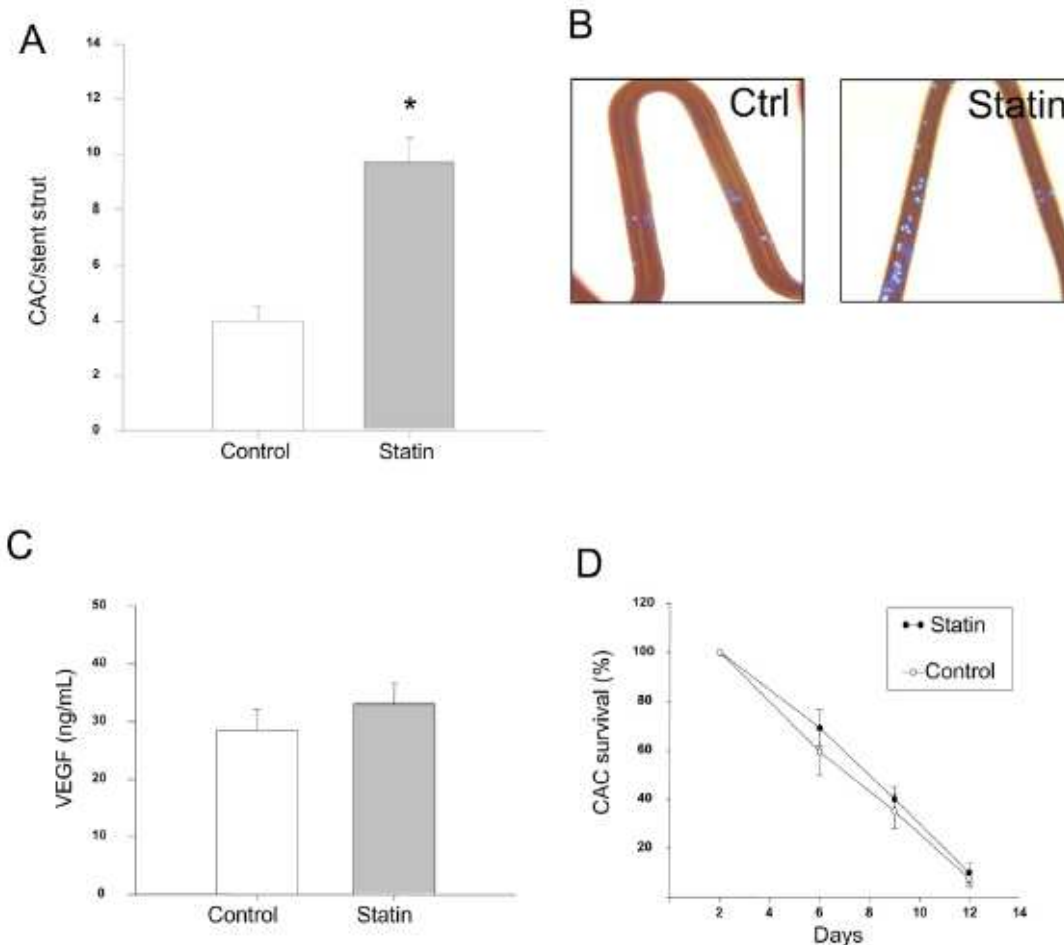


Figure 3. Effect of statin on cultured angiogenic cell (CAC) function *in vitro*. (A) Atorvastatin supplementation of the media (0.1 mM). Statin treatment improves CAC attachment to bare metal stent struts *in vitro* ($n=10$). * indicates $p<0.01$. (B) Representative photos showing DAPI nuclear staining of CAC attachment to stent struts. (C) Atorvastatin supplementation of the media at 0.1 mM does not effect VEGF secretion by EPCs ($n=6$, $p>0.05$). (D) Statin does not ameliorate survival of CACs in culture ($n=6$, $p>0.05$). doi:10.1371/journal.pone.0016413.g003

dial infarctions. Finally, in the recently published PCI-PROVE IT study that compared the frequency of major adverse cardiac events in patients undergoing PCI and treated with either moderate (Pravastatin 40 mg/day) or intensive (Atorvastatin 80 mg/day) statin therapy there was a clear cut advantage for the patients treated in the intensive therapy arm.[11] For example, the primary composite end point (death from any cause, myocardial infarction, documented unstable angina requiring hospitalization and revascularization at least 30 days after randomization, and stroke) showed a 22% relative risk reduction with intensive statin therapy ($p=0.001$). Moreover, the intensive statin therapy reduced target vessel revascularization ($p<0.001$)—an effect that persisted ($p=0.015$) after adjusting for 30-day on treatment serum LDL-C and CRP concentrations. Clearly the rapid clinical benefits seen in these studies involving intensive statin therapy suggests a mechanism of action that is independent

of LDL-lowering. Given the similarities of these studies to our current protocol, it is attractive to hypothesize that the benefit of acute Atorvastatin loading prior to PCI may in part be derived from the off-target effects of statins on EPC mobilization and function.

Certainly, our study is not without limitations. For example, given the widespread use of statins in patients with risk factors for established CAD, it proved difficult to recruit statin naïve patients for this study. Therefore, while our sample size allows us to confidentially address questions specific to serial changes in EPCs, it lacks sufficient power to address the relationship between EPC mobilization profiles and clinical outcomes (e.g., ISR or periprocedural infarctions). Furthermore, given the relatively small patient population, it is unclear if this observation is applicable in populations in whom statin therapy has failed to show a benefit—such as patients on hemodialysis.[43,44]

In summary, our data demonstrate early benefit of statin therapy on EPC number and CAC number and function in humans undergoing PCI. Studies aimed to understand mechanisms by which EPCs are mobilized during PCI and the potential benefits that these cells confer will likely provide novel targets and therapies for improving clinical outcomes in patients undergoing revascularization.

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Author Contributions

Conceived and designed the experiments: BH ERO LF XM YXC. Performed the experiments: BH XM APT JS KR LF. Analyzed the data: BH TS LF ERO. Wrote the paper: BH TS LF ERO.

Appendix VII

Citation Information:

Pourdjabbar, A., **Hibbert, B* (shared first author)**, Simard, T., Ma, X., O'Brien, E.R.
(2011) Pathogenesis of neointima formation following vascular injury. *CHDDT*.
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Wrote the manuscript: AP BH TS XM ERO

Supervised: ERO

Abstract

Revascularization remains the cornerstone of managing obstructive coronary artery disease. Although percutaneous coronary interventions have emerged as the preferred method of restoring vessel patency, as many as 30% of patients will experience a gradual re-narrowing of the lumen caused by neointima (NI) formation, resulting in a condition known as in-stent restenosis (ISR). ISR represents a significant limitation to percutaneous revascularization – however, abrogating NI formation following stent implantation has been hampered by an incomplete understanding of the pathogenesis of in-stent lesions. While numerous mechanisms have been proposed to explain the pathogenesis of ISR, data from human and animal models have yielded conflicting results. Herein, we review key studies of NI development following vascular injury with a focus on the origin of cells participating in NI formation.

Introduction

Ischemic heart disease is a leading cause of mortality and morbidity in industrialized nations, including Canada [1]. Clinically, the mainstay of coronary artery disease (CAD) therapy continues to be optimal medical management in addition to coronary revascularization for symptomatically refractory and acute ischemic events. Currently revascularization is achieved by 2 approaches, coronary artery bypass grafting (CABG), initially the preferred method of coronary revascularization, and percutaneous coronary interventions (PCI). PCIs initially involved the insertion of a balloon tipped catheter which upon inflation would re-open an occluded vessel. Following studies demonstrating improved clinical outcomes in both stable CAD [2] and patients with ST elevation myocardial infarctions [3], this procedure is now almost exclusively performed with the insertion of a metallic scaffolding device known as a stent [4]. The popularity of percutaneous approach continues to increase with over 1.2 million in-patient angioplasty procedures being performed in the United States in 2005 [5, 6]. In Canada, it is estimated that over 40,000 patients will be hospitalized with an acute myocardial infarction [7] of which more than 50% will undergo revascularizations [8].

Although the success rates of PCI in achieving target vessel patency over the long term are excellent, angioplasty with or without stenting induces vessel wall changes, which limit their long-term efficacy. Neointima (NI) formation or recurrent intimal thickening following PCI can result in the formation of obstructive lesions, ultimately leading to ischemic symptoms. In-stent restenosis (ISR) is commonly defined as a re-narrowing of the target lesion greater than 50% of the vessel diameter [9]. Previous reports have shown that between 30% and 50% of patients who undergo conventional

balloon angioplasty require repeat interventions [10-12] and 10%–30% of patients who receive a bare-metal stent (BMS) experience ISR [13, 14]. Thus, given the prevalence of coronary stenting, ISR will continue to affect a significant number of patients.

The introduction of drug-eluting stents (DES), such as sirolimus, paclitaxel, and zotarolimus coated stents, has greatly decreased late luminal loss and the rates of ISR [15-17]. Early clinical results with these agents were encouraging, demonstrating significantly improved rates of ISR and target vessel revascularization. However long-term follow-up studies have shown higher rates of late stent thrombosis with DESs in which incompletely re-endothelialized segments predispose to *in situ* thrombus formation[18]. Due to this potentially catastrophic complication, DESs currently require long-term dual antiplatelet therapy to prevent re-infarction from stent thrombosis which is associated with increased bleeding rates [19]. Thus, development of new drug eluting stents, those that prevent neointimal formation but with improved re-endothelialization kinetics, remains the goal of ongoing research [20-23].

Pathogenesis of neointima formation

While numerous molecular mechanisms have been postulated in initiating and propagating intimal hyperplasia, results from both human studies and animal models have yielded conflicting results, making the etiology of these lesions the subject of intensive investigations and ongoing debate. It is clear from histological descriptive studies that there is an initial influx of inflammatory cells followed by the appearance of synthetic smooth muscle cells (SMCs) and myofibroblasts which produce an abundant extracellular matrix (ECM) [24, 25]. Chronically, the hyperplasia of the neointima results in

obstructive lesions or ISR, which in some cases results in the need for repeat revascularization.

The “response-to-injury” paradigm, proposed by Ross [26] in 1993, has been adapted to the post PCI vessel and widely accepted as the most likely mechanism for the development of NI. In this model, mechanical disruption of the endothelial cells (ECs) denudes the native vessel thus initiating the remodeling process. At a paracrine level, numerous inflammatory cytokines and growth factors produced by activated local ECs and circulating inflammatory cells, such as macrophages and T-cells, stimulates the migration, infiltration and proliferation of medial vascular SMCs into the sub-endothelial space, ultimately resulting in the generation of the NI lesion. The local production of various growth factors such as transforming growth factor- β (TGF- β) and platelet derived growth factor (PDGF) [27] are also thought to shift the phenotype of the SMC and myofibroblasts, such that they produce abundant of ECM proteins, which not only contribute the generation of the space occupying lesions but also serve to further facilitate the migration and proliferation of these cells. Over time, this process leads to the gradual growth of the intimal layer of the injured blood vessel resulting in the loss of lumen diameter and effective vessel flow (**Figure 1, Panel B**).

Even though Ross’s response to injury paradigm has been very helpful in terms of highlighting the importance of injury and subsequent inflammation in initiating NI formation, numerous reports over the last few years have challenged the central tenets of this model – specifically that the smooth muscle cells composing the lesion are derived from the media. More recently, additional mechanisms have been proposed based on the discovery of vascular progenitor cells which are capable of differentiating into both

endothelial and SMC phenotypes [28-30]. These circulating stem or progenitor cells are believed to home to the site of vascular injury and, in response to growth factors and the inflammatory milieu, contribute to NI lesions by both direct cellular contribution, secretion of ECM, and through secretion of cytokines which exert a paracrine effect (**Figure 1, Panel I**) [31, 32]. Rather than the circulation, others have hypothesized that fibroblasts/myofibroblasts and progenitor cells located within the adventitial layer of the damaged vessel, are responsible for generation NI lesions by trans-differentiating in to SMC-like cells and migrating to the intima (**Figure 1, Panel III**). Herein, we will review key studies and some of the conflicting data with regards to the pathogenesis of NI following vascular injury. Moreover we will highlight the pathogenesis of NI formation following percutaneous stent implantation and the origin of the cells which comprise this tissue.

Role of Medial SMCs

The SMCs of the tunica media are the central mediators of NI formation within Ross' response to injury paradigm. Indeed, initial studies into the pathogenesis of NI formation supported the hypothesis that medial SMC proliferation and migration into the intima is a key step. Clowes et al. reported that in rats, following balloon mediated carotid artery injury, SMC proliferation peaked in the media and intima at 48 hours and 96 hours, respectively and this proliferative phase returned to baseline following the re-endothelialization of the artery, within 8 weeks post injury[33]. This group also observed that the development of NI was strongly associated with augmented connective tissue synthesis and accumulation [34]. Further evidence of a medial origin of NI SMC has

been derived from isolated plaques and their cellular components. In these studies, several groups have demonstrated a monoclonal expansion of cells within the lesions [35-37], suggesting that SMC proliferation is initiated early by a small number of cells. However despite these elegant animal models, pathologic studies in humans have repeatedly failed to document significant proliferation [38-40].

In addition to proliferation, a key component of Ross' paradigm is the migration of SMCs towards the tunica intima. Supporting this theory, a number adhesion molecules and integrin proteins, mostly located within the tunica media, have been identified which are believed to be important regulators of SMC migration [41-44]. Osteopontin, a ligand for integrins $\alpha_v\beta_3$ [45] and $\alpha_4\beta_1$ [46], is expressed and synthesized by a variety of cells including SMCs [47] and is generally thought to be a key mediator in cell adhesion and migration [48]. Studies have demonstrated that following balloon angioplasty and cuff-induced vascular injury, the expression and synthesis of osteopontin and its integrin receptors are increased [49-51]. More importantly, the blockade of osteopontin signaling with the use neutralizing antibodies [52, 53] or its receptor integrins via specific antagonists has been shown to limit NI formation [54-57]. Similarly, resistin, a protein linked to inflammation, obesity and insulin resistance, has recently been implicated in NI development in a balloon carotid injury rat model [58]. However, these studies are hampered in that the molecules are also known to be key regulators of inflammatory cell trafficking in the developing lesion and the effects are clearly not specific to inhibition of SMC migration alone.

Clinically, studies have also provided evidence implicating the role of medial SMC in the formation of NI lesions. In 1993, Hruban et al.[59], using fluorescence *in situ*

hybridization (FISH) for the Y chromosome, reported that in sex mismatched heart transplants ECs, SMCs and myocytes were all derived from the donor. Hybridization did identify some recipient derived cells within the explanted hearts, however these cells were identified as lymphocytes, macrophages and mast cells that the authors concluded were likely involved in the host immune response. Similarly, Atkinson et al. reported near identical findings when examining sex-mismatched transplanted human coronary arteries following transplant coronary artery vasculopathy [60]. Using FISH for the Y chromosome and anti-SMC antibodies, the authors reported that in human coronary artery vasculopathy the SMCs within the NI lesions were derived from the tunica media of the donor vessel. Lastly, Glaser et al. [61] also reported that within small to medium arteries of sex-mismatched cardiac transplants in humans, 94.4% to 99.2% of the SMC were derived from the donor cells with only a small percentage of SMCs being derived from the host. A caveat to these findings is that others have noted severe limitations in terms of accurate detection with FISH [62] and analysis of clinical samples with more definitive techniques such as PCR has yet to be done [63]. Finally, all of these studies, by necessity, are performed in transplanted vessels and intima formation in transplant vasculopathy may not recapitulate the process that follows mechanical injury as reflected by higher rates of ISR in transplant vessels [64, 65].

Role of Circulating Progenitor Cells

The concept of the existence of blood borne EC and VSMC progenitors has been postulated as early as 1963 [66, 67], however, their potential contribution to the development of NI lesions has only recently enjoyed a renaissance. Over the last 20 years

a gradual accumulation of evidence has challenged the simple model of medial SMC proliferation and migration into the intimal lesion. Careful review of these studies suggests that the medial SMC may not be the only source of the cells present within NI lesions and that possibly circulating vascular progenitor cells, from either bone marrow (BM) or non-bone marrow sources, almost certainly play a key role in lesion formation.

The earliest studies that strongly inferred a progenitor contribution used Dacron grafts in primates, dogs, pigs and sheep [66-72]. These investigators noted that SMCs in organizing arterial thrombi, first appearing on the luminal surface. Stump et al.[66], was the first to demonstrate the existence of blood borne SMC progenitors following implantation of Dacron grafts in the aorta of pigs. After 7 days, these grafts became covered with ECs and VSMC-like cells despite the barrier of the graft precluding the migration of medial cells. Similarly, Feigl et al.[72], observed the presence of fibroblast-like and myofibroblast like cells within organizing thrombus on the luminal surface of the graft a finding that has been replicated [71]. Finally Campbell et al. [73] also demonstrated that circulating progenitor cells were present in rabbits and rats, capable of generating the various layers of an artery within transplanted silastic tubing. Within 2 weeks the tubes resembled blood vessels with an inner lining of nonthrombotic mesothelial cells, which they regarded as the "intima", a "media" layer consisting of smooth muscle-like cells (myofibroblasts), collagen, and elastin, and finally an outer collagenous "adventitia". These early studies unequivocally demonstrate the ability of SMCs to arise independent of medial input, but were largely overlooked in initial models of intimal hyperplasia.

The ability of circulating progenitor cells to generate various cell lines resembling the different layers of an artery has also been demonstrated in clinical context by Wu et al. [74]. Following removal of a 26 month-old Dacron axillo-femoral bypass graft from a patient, they identified smooth muscle like cells within a “pseudointima” in addition to a layer of endothelial like cells on the luminal surface of the graft. These descriptive studies are the earliest to demonstrate the capacity of blood borne cells to form the most rudimentary components of a vessel wall and provide irrefutable evidence regarding the potential of circulating cells to give rise to synthetic SMCs.

More recently, experimental studies have further implicated progenitor cells in NI generation. Campbell and Campbell [75] reported that SMCs of the normal adult vessel wall display a more differentiated and contractile phenotype, while the SMC within the NI contain larger cell bodies that contains synthetic and secretory organelles. Moreover these "synthetic" SMCs were shown to produce and secrete extra-cellular matrix components and express lower levels of the smooth muscle-specific contractile proteins. Interestingly, synthetic SMCs have also been shown to be more responsive to growth factors, such as PDGF[76, 77], and TGF- β [78]. This distinct phenotypic difference between native medial SMC and those present within the NI was believed to be to the result of genotypic change in response to the vascular injury and the subsequent inflammatory response [79]. However, recent investigations have actually identified progenitor cells capable of differentiating into both EC and SMC in response to specific growth factors [80, 81], thus providing direct evidence of a role for progenitor cells in both NI formation and vascular repair. Yamashita et al. [82] first identified a population of embryonic derived stem cells, capable of differentiating into EC and SMC, which they

labeled as vascular progenitors. These cells were capable of generating vascular cells in response to VEGF and PDGF. Moreover, Simper et al. [83] subsequently identified a population of cells within the circulation of adults, capable of differentiating into SMC like cells. The authors isolated this group of mononuclear cells from the buffy-coat of blood and reported that in response to PDGF these cells expressed smooth muscle cell-specific α -actin, myosin heavy chain, and calponin. To date, it remains unclear if these cells represent a distinct progenitor population from circulating endothelial progenitor cells which are thought to reduce NI formation [84, 85] or if they simply represent alternative differentiation of a common progenitor.

Subsequently, there have been numerous experimental studies that have supported the hypothesis that progenitor cells directly contribute to NI. Perhaps the most compelling evidence that intimal lesions can form in the absence of medial SMCs comes from transplant arteriosclerosis (TA) models. In 1995 Plissonnier et al. [86] demonstrated that in rat aortic TA, NI lesion formation was the final pathological process preceded by the sequential, immune mediated destruction of endothelial and medial SMCs. This was one of the earliest studies to demonstrate the formation of NI lesions despite immune mediated destruction of medial cells. Bigaud et al. [87] also demonstrated that within 7-days of aortic allograft transplantation in rats there is a significant decrease in medial SMC function and that by day 28 there is a marked loss of medial SMCs (up to 75%). This loss of medial SMCs is coupled with a severe inflammatory response, both occurring prior to NI lesion development in the grafts. Thus, the proliferation of existing medial SMC fails to explain the formation NI lesion in transplanted vessels and these observations require an alternative source of cells for lesion development. Other studies

have since confirmed that in models of vascular injury using wire or balloon injury in mice, rats and rabbits, NI and luminal narrowing are preceded by a significant degree of apoptosis within the medial layer [88, 89]. The presence of apoptosis and not proliferation within the tunica media following injury also argues against a medial source of cells.

Saiura et al. [90] were the first group to provide direct evidence implicating that recipient cells were the major cellular source in TA following heterotopic cardiac transplantation between wild type mice and ROSA26 mice, which constitutively express the *LacZ* gene. They reported that 30 days post-transplant of wild type hearts in to ROSA26 mice, 86.2% of the cells within the NI expressed *LacZ*, while only 10.6% of medial cells were *LacZ* positive. In contrast, when ROSA26 hearts were transplanted into wild type mice, only 2.3% of the NI cells and nearly 65% of the medial cells stained positive for *LacZ*. The authors concluded that it is recipient cells and, not the donor medial SMCs, that are mostly responsible for generating lesions. Hillebrands et al. using sex-mismatched cardiac and aortic transplantation demonstrated that as much as 86% to 90% of the SMC within the arteriosclerotic lesion were derived from the male recipient and not from the female donor [91, 92]. As well, the involvement of recipient derived cells has also been confirmed by numerous other groups in rat models [93-95]. More recently, in a rabbit model of allograft transplantation with and without stent implantation, our group, using quantitative polymerase chain reaction (Q-PCR) assessment of Y chromosome, demonstrated that 72% of the cells within the NI lesion of the allograft body were recipient derived; notably, this percentage increased to 81.5% following implantation of a stent in the allograft artery [63]. These studies provide

qualitative and quantitative evidence supporting the role of circulatory derived cells in the development of NI and suggest that therapies targeting these cells may be important for improving clinical endpoints.

Origin of Circulating Progenitor Cells

Given the emerging evidence that circulating progenitor cells are important in NI development, considerable effort has been spent to identify the repositories of these cells within the body. Numerous studies using various models of vascular injury suggest that the BM is an important reservoir. Han et al. initially demonstrated that the following vascular injury, the BM can serve as a complementary source of smooth muscle like cells, which contribute to vascular healing and the development of NI lesions [96]. Using female mice transplanted with BM from male donors, they reported that nearly 56% of the α -smooth muscle actin positive cells within the NI were derived from the BM. Sata et al. [97] have also demonstrated that BM derived cells were mostly responsible for the development of NI lesion in vascular remodeling after injury, graft vasculopathy and atherosclerotic plaques, using wild type mice who had undergone BM transplantation from ROSA26 or transgenic mice that ubiquitously express green fluorescent protein. They reported that following injury, graft vasculopathy and atherosclerosis, that nearly 63%, 88% and 42-58% of the cells within the NI lesion were derived from the recipient's BM, respectively. Furthermore these cells also expressed various smooth muscle cell markers such as smooth muscle myosin heavy chain, α -SMA, calponin and caldesmon. Diao et al. [93] have reported that BM derived cells, SMCs and ECs contributed to NI hyperplasia following vein grafting in mice. They demonstrated that BM-derived cells

incorporated into the endothelial layer of the graft and that by 16 weeks the endothelial integrity was fully restored. More importantly, the authors noted that ~20% of SMCs within the NI lesion at 16 weeks were derived from the BM, since these cells expressed both GFP and smooth muscle myosin heavy chain. Contribution of BM derived cells to neointimal SMCs has also been reported by numerous other groups, using different animal models of vascular injury [98-100]. In all, this body of literature using *in vivo* animal models supports that circulating cells can, at least in part, contribute to lesion formation following vascular injury.

Perhaps most importantly, the role of BM derived cells in the development of NI lesions has also been demonstrated in humans. Caplice et al. [101] examined diseased and non-diseased coronary arteries from deceased patients with CAD who had previously undergone sex-mismatched BM transplantation. The authors, using immuno-histochemistry and *in-situ* hybridization, reported that within diseased vessels nearly 10% of the SMC within the NI lesion were derived from the BM, while in non-diseased arteries BM derived cells made up less than 0.2%. More recently, Inoue et al. [102] established that stent implantation in patients with CAD was associated with a significant increase in the mobilization of BM derived CD34-positive cells, which reached a maximum circulating level 7 days following the procedure. As well, they noted that cultured mononuclear cells differentiated into both endothelial-like and smooth-muscle like cells, with a higher rate of smooth-muscle like cells in patients who developed restenosis. Interestingly, Schober et al. [103] have also previously reported that an elevated CD34-positive cell count following elective PCI was associated with a higher rate of ISR and that elevated CD34-positive cells after coronary stenting constitutes an

independent risk, a finding which suggests a putative role for their involvement in neointimal hyperplasia.

Although there is a significant amount of published evidence supporting the role of the BM and BM derived cells in the generation of NI lesions, a significant portion of the host derived cells within the NI (~15-90%) are derived from non-bone marrow compartment. These findings raise the possibility of an as yet undetermined source of circulating progenitor cell population and a number of experimental studies have suggested non-marrow derived progenitors play a significant role in the development of NI [104-106]. For example, Hu et al. [104], in a mouse model of TA following aortic allografting, demonstrated that although recipient derived cells were the source of SMC within the NI, there was no BM involvement in lesion formation. Hillebrands et al. [105] have also previously reported a very limited involvement of BM derived cells in endothelial replacement in TA. In fact, they reported only a 1-3% contribution from the BM to ECs in their model. More recently, this group also demonstrated that non-bone marrow derived cells were important in the formation NI following experimental stenting in a rat model of ISR [106]. Using BM transplantation between human Placental Alkaline Phosphatase (hPAP) transgenic rats and wild type (WT) rats, they reported that 4 weeks post-stent implantation only a small number of hPAP positive cells within the NI lesion. Moreover, none of the hPAP positive cells within the NI expressed smooth muscle actin and hPAP positive cells were found to express CD45 and identified as infiltrating leukocytes.

Although a large body evidence exists supporting the role of non-bone marrow derived progenitor cells in NI formation; the exact location of these cells is currently

unknown. Nonetheless several possible sources have been investigated such as vascular pericytes, the liver, the spleen, the intestines and the vascular system. Pericytes, which partially surround EC within the microvasculature are derived from a mesenchymal origin and contain smooth muscle specific proteins such smooth muscle specific isoforms of actin and myosin [107, 108]. Moreover, these cells have also been shown to be capable of differentiating into a variety of cell types, such as SMC [109-111]. The liver has also been shown to contain a population of non-myogenic, clonal stem cells which could possibly give rise to SMC like cells and cardiomyocytes in rats [112]. More recently, Aicher et al. [113] reported that the intestinal system and liver represent a rich source for mobilized tissue-residing progenitor cells capable of generating various vascular cell lines in response to appropriate stimuli. Finally, the spleen has been reported to contain a population of mononuclear cells that under appropriate stimuli, demonstrate an EPC phenotype and are capable of regenerating ECs, restoring normal vascular function and reducing NI lesion formation following arterial injury [113, 114]. These studies confirm that in addition to the BM, a variety of other sources of vascular progenitors exist. With the development of double transplant models and more sophisticated methods for tracking cells *in vivo* we will undoubtedly gain greater insight into the diverse origins of cells that comprise neointima following vascular injury.

Role of Adventitial Cells:

The final proposed mechanism of NI formation involves fibroblasts and progenitor populations residing within the adventitia. The adventitial layer had long been considered just a supporting layer within the blood vessel, whose main function was to

provide nourishment to the muscles of the medial layer. However, the role of adventitia as a source of cells, specifically adventitial fibroblasts and progenitor cells, as well as cytokines which can influence NI formation is receiving greater attention [115]. Within the tunica adventitia, fibroblasts are the predominant population. They are a heterogeneous population, capable of adapting both structurally and functionally to a changing environment such as the inflammatory response that occurs following vascular injury [116]. Additionally, these cells can develop synthetic and contractile phenotypes, like SMCs, which are referred to as myofibroblasts. Various growth factors such as TGF- β , PDGF, tumor necrosis factor α (TNF- α) and basic factor growth factor (bFGF) have been shown to stimulate proliferation and differentiation of these cells [115]. These newly generated myofibroblasts can express SM α -actin, similar to SMCs within the intimal layer [117].

Thus, given this potential for adventitial fibroblast/myofibroblasts to contribute to vascular remodeling following injury, a number of studies have investigated their role in the context of vascular injury [118-122]. Shi et al. [118] reported that within 3 days of porcine coronary artery injury, the adventitia undergoes a rapid hyper-proliferative response as measured by BrdU uptake, exceeding that observed in the injured medial layer. This rapid proliferative response was also associated with increased expression of the SMC marker, α -SM actin within adventitial cells – a finding which peaked at 7-14 days post injury. Finally, the authors also noted increased transmigration of adventitial cells towards the medial layer and to the NI, where these cells made up a significant portion of the cells within the newly formed lesion. Similarly Scott et al. [119] demonstrated that following balloon injury to the left anterior descending and circumflex

artery in swine, significant early cellular proliferation was identified within the adventitial layer as compared to the medial layer. This group further demonstrated the migratory potential of adventitial cells by administering BrdU between 2-3 days post balloon induced coronary injury, when proliferation was highest within the adventitia. They reported that >40% of the cells within the NI were BrdU positive and likely of adventitial origin. The migratory capabilities of adventitial cells were further highlighted by Li et al. [120] who reported that Lac-z expressing fibroblasts, introduced into the adventitia of rat carotid arteries immediately following balloon injury were detected within the media and the NI 7 days post injury. Finally, Siow et al. [121] reported that Lac-Z expressing fibroblast can transmigrate through the media, express α -SM actin and participate in the formation of NI lesions. These findings have been confirmed by other groups using adenoviral beta-galactosidase tagged myofibroblasts in the adventitia which could then be traced to the NI, within 2 weeks of epigastric vein grafts to femoral arteries in rats and autologous saphenous vein grafts in a porcine model [122].

Several important studies have also identified a population of resident progenitor cells within the adventitia layer which demonstrate features of SMCs and can contribute to NI lesions. Hu et al. [123] reported that the adventitia contains a large population of stem like cells, expressing various progenitor markers such as Sca-1, c-kit, CD 34, and Flk-1. Notably, in response to PDGF these cell populations are able to differentiate into SMC like cells and when Lac-Z expressing cells were transplanted to the adventitial side of vein grafts, B-Gal + cells were identified within the NI lesions. Zhang et al., [124] using a mouse model of vascular injury, demonstrated a significant increase in the number of Sca-1-positive cells in the adventitia and the peripheral circulation 1-week

following injury. This increase in the levels of Sca-1 positive cells was associated with increases in intimal cell proliferation which may have contributed to vascular remodeling and SMC-rich neointimal lesion formation. Thus, populations of fibroblasts and progenitor cells reside in the adventitia which in various models have been shown to contribute to NI formation; however, whether or not a similar mechanism exists in humans and their overall contribution to NI lesion formation remains to be elucidated.

Conclusion:

ISR complicates coronary artery stenting in up to 30% of cases and represents a major limitation in the treatment of patients with obstructive CAD. Here we have reviewed three proposed mechanisms of NI generation following vascular injury and the evidence supporting each. The question of whether or not ISR in humans is similar to that observed in experimental models remains controversial. However, what is clear is that a number of cell types exhibit the potential to differentiate into a SMC phenotype and at least in some models, each shows the potential to partially contribute to the NI. Certainly, NI formation is a complex process and rather than any one source of cells, the pathogenesis likely involves numerous sources which result in the final lesion. Moreover, this complexity has undoubtedly contributed to divergent results and obviates any single model from fully explaining the pathogenesis of NI formation. More than anything, this review highlights the need for further research and development of new models to better understand the processes ongoing in the post injury vessel wall to help develop new therapeutic targets and improve clinical outcomes.

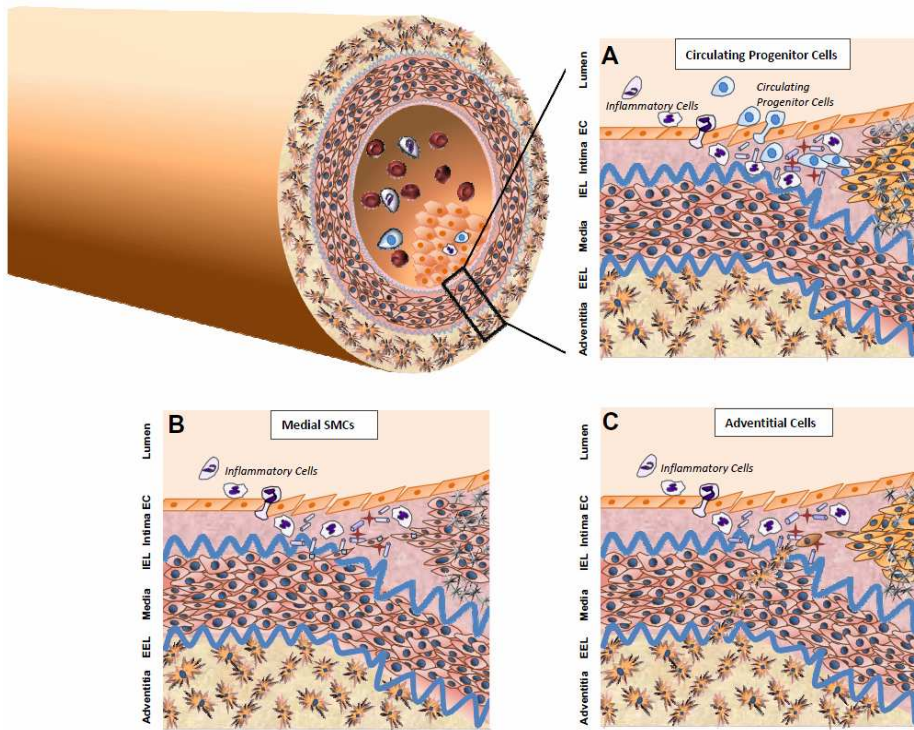


Figure 1: Models of neointima formation in coronary arteries. Damage to vascular endothelial cells (EC) leads to infiltration of inflammatory cells and subsequent pro-inflammatory cytokine release (blue rods), resulting in neointima formation via three postulated mechanisms. **(A)** Circulating Progenitor Cells –Cytokine release stimulates influx of circulating progenitor cells then differentiate under the influence of cytokines and growth factors (red stars) into a smooth muscle cell (SMC)/myofibroblast phenotype with a secretory phenotype. Production of extracellular cellular matrix (ECM, grey stars) contributes to NI volume. **(B).** Medial Smooth Muscle Cells Inflammatory cell influx and cytokine release stimulates proliferation and migration of medial SMCs the internal elastic lamina (IEL) towards the intima. Once in the subendothelial space SMCs acquire a synthetic phenotype producing an abundant ECM. **(C)** Adventitial Cells – Cytokine release stimulates migration of advential fibroblast cells across the external elastic lamina (EEL), through the tunica media, and across the IEL to the intima. These migratory fibroblasts then differentiate into SMC-like cells known as myofibroblasts with subsequent ECM secretion under the influence of pro-inflammatory cytokines and growth factors.

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Appendix VIII

Citation Information:

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Contributed Reagents: SW

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Contribution of Recipient-Derived Cells in Allograft Neointima Formation and the Response to Stent Implantation

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Abstract

Allograft coronary disease is the dominant cause of increased risk of death after cardiac transplantation. While the percutaneous insertion of stents is the most efficacious revascularization strategy for allograft coronary disease there is a high incidence of stent restenosis. We developed a novel rabbit model of sex-mismatched allograft vascular disease as well as the response to stent implantation. *In situ* hybridization for the Y-chromosome was employed to detect male cells in the neointima of stented allograft, and the population of recipient derived neointimal cells was measured by quantitative polymerase chain reaction and characterized by immunohistochemistry. To demonstrate the participation of circulatory derived cells in stent neointima formation we infused *ex vivo* labeled peripheral blood mononuclear cells into native rabbit carotid arteries immediately after stenting. Fourteen days after stenting the neointima area was 58% greater in the stented vs. non-stented allograft segments ($p = 0.02$). Male cells were detected in the neointima of stented female-to-male allografts. Recipient-derived cells constituted $72.1 \pm 5.7\%$ and $81.5 \pm 4.2\%$ of neointimal cell population in the non-stented and stented segments, respectively and the corresponding proliferation rates were only $2.7 \pm 0.5\%$ and $2.3 \pm 0.2\%$. Some of the recipient-derived neointimal cells were of endothelial lineage. The *ex vivo* tagged cells constituted $9.0 \pm 0.4\%$ of the cells per high power field in the stent neointima 14 days after stenting. These experiments provide important quantitative data regarding the degree to which host-derived blood-borne cells contribute to neointima formation in allograft vasculopathy and the early response to stent implantation.

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Introduction

The artery wall consists of three layers; the (inner) intima, the media and the (outer) adventitia. Non-diseased human coronary arteries normally have a modest layer of intimal thickening (or neointima, NI) that consists of an accumulation of smooth muscle cells (SMCs) and extracellular matrix[1]. Currently, it is believed that medial SMC proliferation and inward migration, as well as the transmigration and retention of blood borne inflammatory cells play key roles in transforming a benign NI into the obstructive lesions that are seen in atherosclerotic coronary artery disease (CAD), as well as allograft coronary disease (ACD) that occurs after heart transplantation[2,3]. Like garden variety CAD, ACD can ultimately lead to life threatening clinical sequelae, and the most common revascularization strategy for either of these entities involves the insertion of metallic scaffolding devices known as stents. While stent restenosis due to recurrent NI formation (also known as in-stent restenosis or ISR) occurs in up to 5–30% of CAD lesions (depending on the stent and/or drug coating on the stent)[4,5], ISR is a more frequent and serious problem for ACD lesions[3,6–9]. For example, Simpson and colleagues report 6 and 12 month ISR rates of 41% and 53%, respectively, with 39% of stented patients dying or undergoing repeat cardiac transplan-

tion within approximately 2 years of their first intervention[9]. The histopathology of ACD is characterized by inflammatory cell infiltrates with SMC accumulation and hence is more akin to ISR than the complex atherosclerotic lesions. Indeed, Jonas and colleagues recently demonstrated that angiographic progression of ACD and ISR lesions in allograft coronary arteries correlate and share histological features[3].

The pathogenesis of ACD is incompletely understood, and debate lingers over the relative importance of immune vs. non-immune factors, as well as the origin of NI cells. Unfortunately, experimental studies involving a variety of animal models of transplantation have produced discordant results (see review by Hillebrands *et al.*[10]). Indeed, there is critical concern when high-resolution confocal or deconvolution microscopy is not used to track cell lineage in allografts (see editorial by Hoofnagle *et al.*[11]). Regardless of these experimental studies, one cannot ignore observations from a handful of important human studies that suggest that host cells participate in allograft disease. For example, in 1971 Kennedy and Weissman[12] used anti-sera directed against donor and host HL-A antigens to suggest host cells were at least partially responsible for NI formation in a human cardiac allograft coronary artery. Together with at least five larger and more sophisticated studies of human transplantation, there is

already irrefutable evidence that extracardiac cells engraft both non-diseased or diseased arteries of allografts[13–17].

Hence, while there is information available about the involvement of host-derived cells in allograft NI formation, many questions remain. For example, the precise degree to which host cells populate the allograft NI has only been assessed semi-quantitatively by sampling a relatively small number of cells in just a handful of arteries. Certainly, a more comprehensive quantitative assessment of the degree to which blood borne host cells participate in NI formation is required before we can assign a level of significance to these cells in the pathogenesis of allograft vascular disease, and determine the need to develop therapeutic strategies to inhibit their participation. Therefore, the goals of the current study are to address the following questions using a rabbit model of allograft vasculopathy and the response to stent implantation: i) what is the origin of NI cells in allografts with or without stents? ii) to what (precise) degree do recipient cells contribute to the NI formation? and iii) what is the identity of the NI cells that populate the allograft NI?

Results

Model of Allograft Vascular Disease and Stent NI Formation

New Zealand White (NZW) rabbits were used for these experiments because their carotid artery is similar in calibre to

human coronary arteries and hence suitable for both surgical transplantation and stent deployment. Allografting of a female carotid artery to a male recipient ($n = 6$), and vice versa ($n = 6$), was performed (**Figure 1A–F, Movie S1 and S2** available online). Two weeks after stent deployment rabbits were euthanized and vascular tissues were harvested for analyses. Total plasma cholesterol levels increased almost fourfold after 5 weeks of ingesting a cholesterol-enriched diet (e.g., baseline: 56.7 ± 2.0 mg/dL and at euthanasia: 200.2 ± 13.2 mg/dL).

Immunocytochemical and Histomorphological Characterization of NI

A modest NI was present in all non-stented and stented allograft segments as well as stented native carotid arteries (**Figure 2A & B**). In non-stented native carotid arteries a NI was absent (data not shown). In non-stented allograft segments the NI largely consisted of an almost equal mix of SMCs and macrophages (**Figure 2C**). As well, a minor infiltration of lymphocytes was present in the allograft NI—consistent with previous descriptions of the early NI phase of ACD[10]. The allograft stent NI was distinct from non-stented allograft segments as well as the stent NI of native carotid arteries. Specifically, in the stent NI of 7/12 allografts more than 50% of the cells failed to label with SMC, macrophage or lymphocyte markers. In the NI of

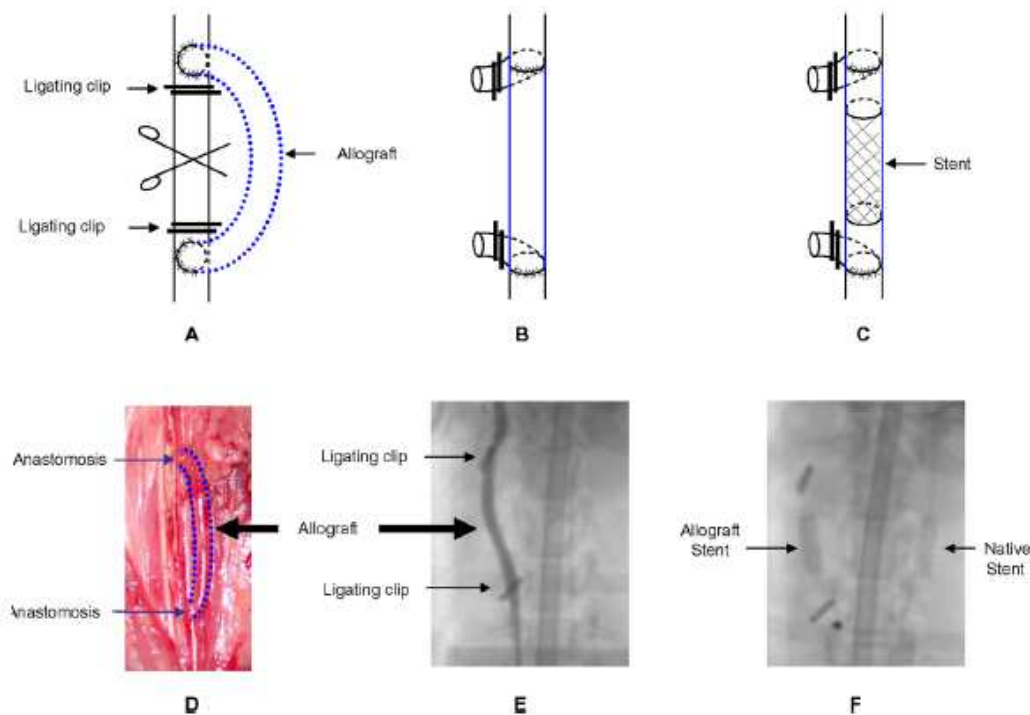


Figure 1. Sex-mismatched rabbit common carotid allograft model. (A–C) Donor carotid artery acting as an allograft to the recipient's common carotid artery. Titanium clips were used to ligate recipient artery. After transplantation the recipient carotid was cut in the middle and the cerebral circulation was restored in the allograft. Three weeks later, a S670 stent (3.0 diameter×12 mm length) was deployed in the middle of the allograft. (D) Photo of microsurgery showing the bypass allograft anastomosed to native common carotid. (E) Angiogram of the allograft after stent implantation. (F) Patent allograft (without contrast dye) showing the radiopaque stent located in the middle of the graft. A stent is also present in the contralateral native carotid artery.
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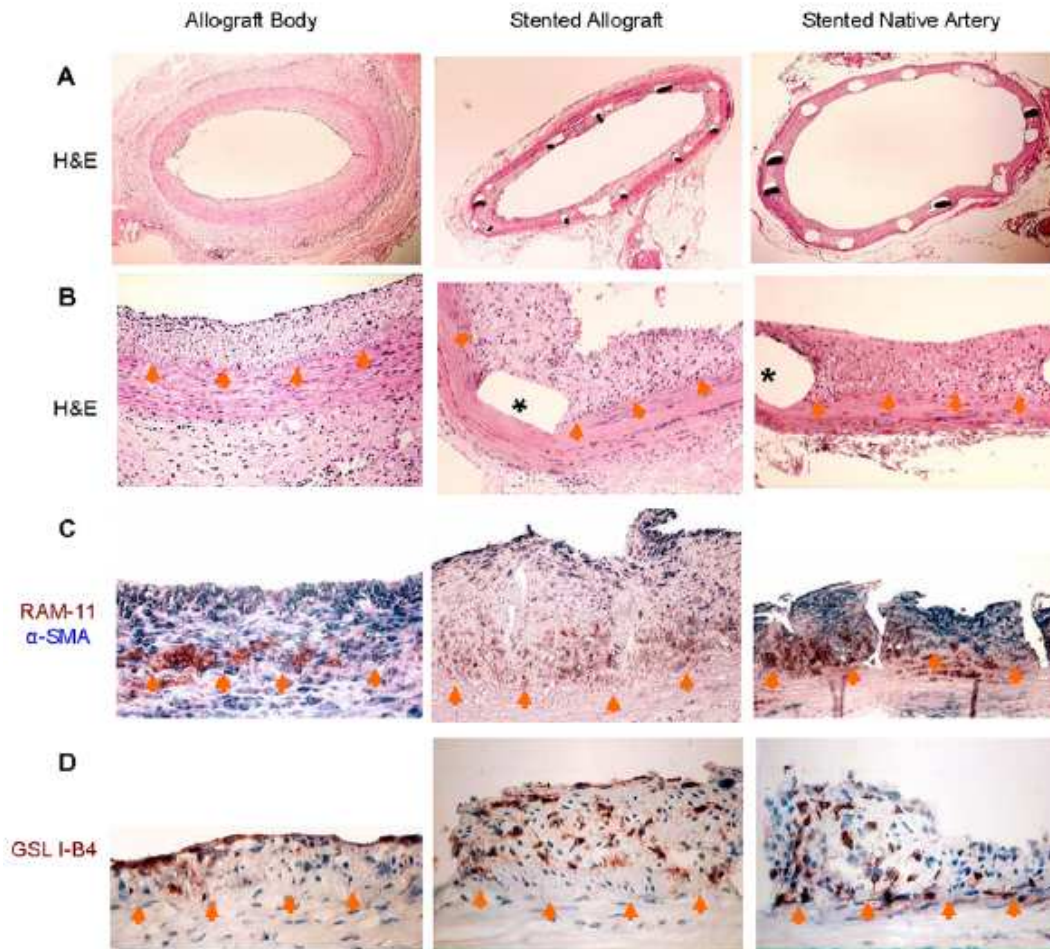


Figure 2. Morphologic and immunohistochemical study of allograft, stented allograft and native artery. A & B: H&E stained tissue section shown at $\times 20$ – 40 (top row) and $\times 200$ (second row) magnifications with * denoting location of stent struts removed post-mortem. C: Dual labeling for the macrophage marker RAM-11 (brown) and the SMC marker α -SMA (blue) demonstrates similar basal NI location of macrophages with overlying SMCs. Note that larger allograft stent NI has a large number of cells that do not label with either of these cell markers. D: Labeling for the endothelial specific marker GSL I-B4 (brown) shows immunopositive cells within the NI, particularly in the stent NIs of the allograft and native artery. Orange arrow heads delineate the internal elastic lamina.
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the remaining stented allografts 4 were dominated by macrophages and 1 by SMCs. In contrast, the NI of stented native arteries largely consisted of SMCs with macrophages that were confined to the peri-strut tissue-consistent with our previous description of stent NI formation in native rabbit carotid arteries [18]. Griffonia simplicifolia Lectin I-isolectin B4 (GSL I-B4) was used to identify cells of endothelial lineage as it is well recognized as a reliable endothelial marker [19,20]. As expected, the luminal endothelium of non-stented allograft segments was intact and immunolabeled for the endothelial marker GSL I-B4 (Figure 2D). However, we were surprised to find scattered within the NI of the non-stented and stented allograft segments, as well as stented native arteries multiple cells that were immunopositive for

this endothelial marker—yet did not appear to be part of vasa vasorum (i.e., no organized cellular network encompassing red blood cells).

While a modest NI formed in the allografts NI area varied according to the location within the allograft (Figure 3A). At the allograft anastomotic sites (the ends of the allograft that were sutured to the carotid artery) the NI area was 59% larger than in the non-stented body of the allograft ($1.05 \pm 0.13 \text{ mm}^2$ vs. $0.66 \pm 0.07 \text{ mm}^2$, respectively, $p < 0.05$). Interestingly, the area of the NI at the site of stent implantation was $1.04 \pm 0.14 \text{ mm}^2$ and approximately 58% larger than the adjacent non-stented allograft segment ($p < 0.05$). In contrast, the NI generated in response to stenting the contralateral (native) carotid artery was

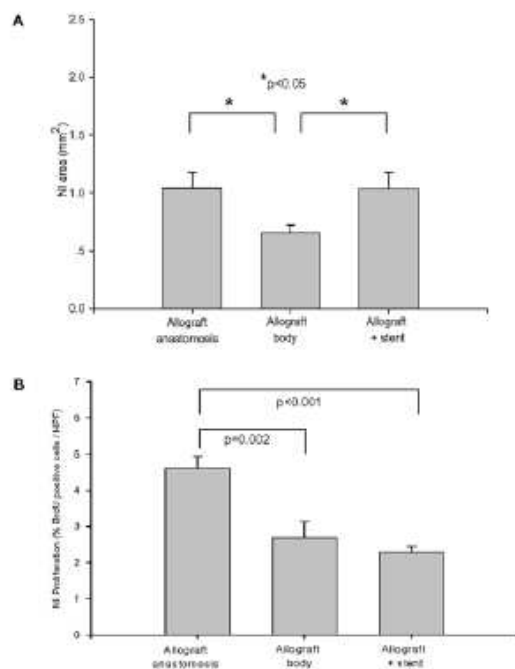


Figure 3. Characterization of NI formation in stented allograft. A: The NI area of the allograft at the site of stent implantation, anastomoses, and the adjacent non-stented allograft body segment, $n=12$, $p<0.05$. B: Low level of proliferation in the non-stented body of the allograft and stented allograft segments. Proliferation at the anastomoses was higher at the non-stented body of the allograft ($n=12$, $p=0.002$) or stented allograft ($n=12$, $p<0.001$). doi:10.1371/journal.pone.0001894.g003

$0.56\pm0.04\text{ mm}^2$ or only 54% of the allograft stent NI area ($p<0.01$) and similar in magnitude to the non-stented allograft NI found at a distance from anastomotic sites.

As a separate control for this allograft model, we studied a cohort of three rabbits that underwent sex-mismatched carotid artery transplantation but were euthanized 3-weeks post-transplantation (i.e., without stent insertion). A NI was present in all three 3 week old allografts, but was much smaller than that of the 5 week old stented allografts ($0.087\pm0.017\text{ mm}^2$ vs. $1.04\pm0.14\text{ mm}^2$, $p<0.01$). The NI cells of the 3 week old allografts consisted mainly of SMCs and macrophages.

To determine the role of proliferation in the NI BrdU immunolabeling was performed (Figure 3B). In the non-stented ($n=10$) and stented ($n=9$) allograft segments the prevalence of proliferating cells was low ($2.7\pm0.5\%$ and $2.3\pm0.2\%$; respectively). The proliferation profile at the anastomoses ($4.5\pm0.3\%$, $n=10$) was higher than that of the non-stented and stented allograft sites ($p=0.002$ and $p<0.001$; respectively). In the native carotid artery (no stent) proliferation was virtually absent, but at the site of stent insertion $1.6\pm0.3\%$ of cells were proliferating—a frequency similar to that found in non-stented or stented allografts and certainly lower than that detected at the allograft anastomoses ($p<0.001$).

Origin of Stent NI Cells

To assess the contribution of the recipient's cells to stent NI formation we employed two techniques. First, by using Fluorescent *in situ* hybridization (FISH) for the Y chromosome male recipient cells were detected in the NI of the non-stented segment of a female-to-male allograft (Figure 4A), the NI in the allograft stent (Figure 4B), and in the media of the proximal native artery (Figure 4C). Similar to other reports[17,21] the sensitivity of FISH to detect male cells was limited (e.g., we detected only $20.0\pm2.6\%$ of positive cells in pure male tissue)—despite adequate hybridization as reflected by parallel use of a control probe specific for histone-H3 (data not shown).

Second, Laser capture microdissected (LCM) was used to precisely isolate stent NI tissue (Figure 4D) and quantitative polymerase chain reaction (Q-PCR) assessment of Y chromosome copy number relative to that of an autosomal gene was employed to determine the percentage of host or donor cells involved in NI formation. The NIs ranged in area from approximately $0.48\text{--}1.48\text{ mm}^2$, and had an average cell density of $6,085\text{ cells/mm}^2$; hence between 2,434 and 9,005 cells were sampled on each tissue slide using LCM. Figure 4E shows the linearity of a sample standard curve for GAPDH. Both the sex-determining region Y (SRY) and GAPDH reactions were linear over a one million fold range for the concentration of the standard plasmid. Furthermore, the correlation coefficient for SRY and GAPDH were excellent (r^2 value range: $0.99\text{--}1.00$ and $0.98\text{--}1.00$; respectively). Hence, using this technique and the appropriate calculations we were able to determine the origin of cells in the NI of both male and female arteries. Examination of nine native carotid artery samples (5 female and 4 male) revealed that $96.5\pm6.6\%$ of the cells were of recipient origin (Figure 4F). The contribution of recipient cells to the allograft NI in non-stented segments ($n=9$) was $72.1\pm5.7\%$ of cells. In the larger NI of the allograft stents ($n=10$) $81.5\pm4.2\%$ of the cells were of recipient origin. Therefore, by inference approximately 27.9% of the non-stented allograft NI cells originated from the allograft, while only 18.5% of the stent NI cells in the allografts were derived from the vessel wall. Given the relatively low proliferation profile in the allograft NI, these data suggest that host cell engraftment plays a major role in allograft NI formation.

Differentiation of Circulatory Cells: In Vitro and In Vivo

To test whether the GSL I-B4 immunopositive NI cells (described above) may be host-derived cells that differentiate from an endothelial to a SMC or monocyte/macrophage lineage we cultured rabbit peripheral blood mononuclear cells (PBMCs) on fibronectin-coated plates in EGM-2 media. Similar to human PBMCs maintained in culture for seven days[22] the adherent rabbit PBMCs on day 7 in culture were positive for both DiI-acLDL uptake and GSL I-B4 labeling but negative for both α -smooth muscle actin (SMA) and RAM-11 immunolabeling (Figure 5A). However after 28 days the majority of cells expressed α -SMA or RAM-11 but did not show DiI-acLDL uptake or GSL I-B4 immunolabeling (Figure 5B). Hence, these *in vitro* data suggest that PBMCs are capable of transdifferentiating from an EC lineage to either SMCs or monocyte/macrophages.

Similarly, to directly determine if circulatory cells engraft stent NI, rabbit PBMCs tagged *ex-vivo* with the fluorescent dye PKH26 (Figure 6A) were selectively injected into native carotid arteries immediately after stent implantation. Fourteen days after stent implantation PKH26-tagged cells constituted $9.0\pm0.4\%$ of stent NI cells per high power field (HPP) (Figure 6B–D).

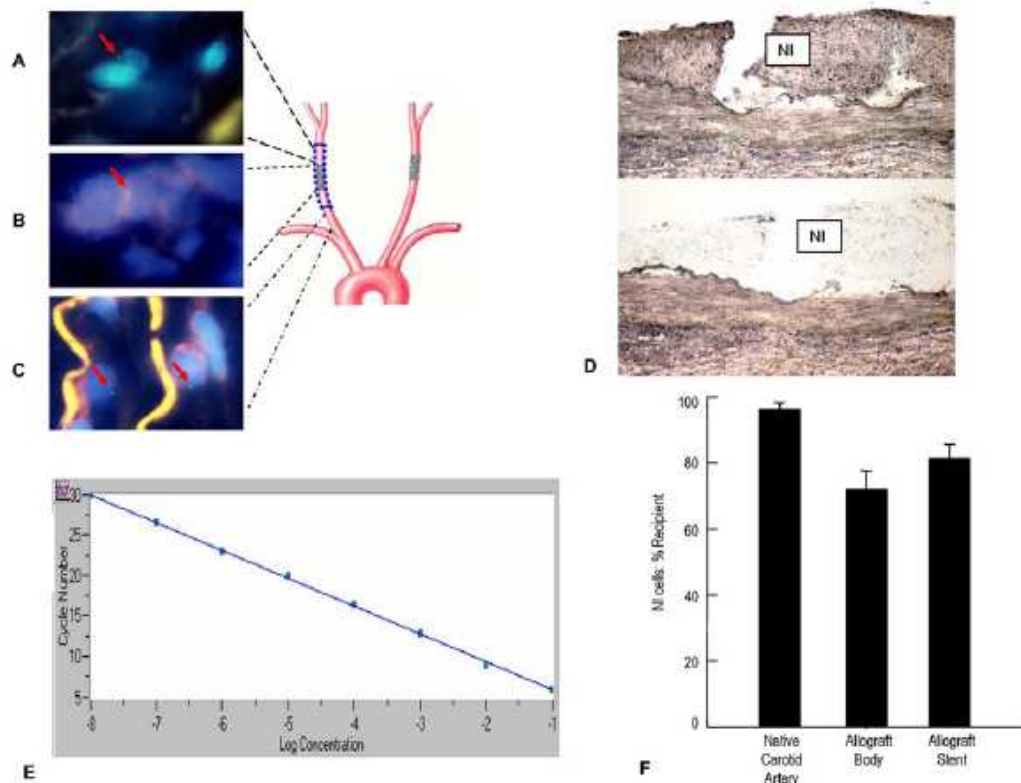


Figure 4. A–C: FISH hybridization with an SRY specific probe in the NI of a female-to-male allograft with or without stent. Blue areas show DAPI staining of nuclei with green labeling of Y chromosomes (highlighted with red arrows). The yellow color is due to auto fluorescence of elastic fibers. Male cells (recipient origin) were detected in NI of female donor allograft without (A) or with stent (B). Male native carotid artery medial cells are SRY positive (C) and were used as positive controls. D: Arcturus HistoGene stained allograft arterial wall before and after NI microdissected by LCM. NI = neointima. E: Sample Q-PCR standard curve for GAPDH. F: Percent contribution of recipient cells to the NI in allograft body, allograft stent, and native vessels. Bars represent means $\pm$ SEM.
doi:10.1371/journal.pone.0001894.g004

Discussion

ACD ultimately leads to failure of cardiac transplants and is difficult to manage due to its relative refractoriness to immune modulation and/or a variety of revascularization procedures, including percutaneous coronary stent insertion. Distinct from plaque rupture in atherosclerotic native coronaries, progressive NI accumulation is the ultimate cause of ACD as well as allograft ISR [1]. In order to improve our understanding and treatment of ACD and allograft ISR, we developed a novel sex-mismatched rabbit model of carotid artery grafting in order to quantitatively study the contribution of host vs. donor cells during NI formation. Using both *in situ* hybridization for the Y chromosome and Q-PCR for the Y chromosome on NI tissue isolated with the aid of LCM we show that host derived cells play a dominant role in the genesis of allograft NI formation. Specifically, 5 weeks after allografting 72.1% of allograft NI cells are of host origin. Moreover, when a stent is implanted in a 3 week old allograft and the resulting NI is examined 2 weeks later we note that 81.5% of the allograft stent NI cells are host derived.

While a number of human studies unquestionably demonstrate the presence of host derived cells in allograft NI formation [12–17], considerable debate lingers in the experimental literature regarding this phenomenon. In part, it can be argued that the experimental models are poor mimics of the human condition, and while undoubtedly true, this sweeping statement does not do justice to the plethora of excellent experimental studies in this field. Space does not permit a balanced discussion of all of these studies, hence interested readers are referred to two excellent reviews by Hillebrands *et al.* [10,23].

In the current study we used two techniques to detect the Y chromosome: FISH on arterial cross sections and Q-PCR on sections of NI carefully isolated with LCM. While the sensitivity of FISH for the Y chromosome is widely recognized to have limited sensitivity (e.g., varying between approximately 20–50%) [17,21] this technique is useful for localization reasons. Quite the opposite, Q-PCR is exquisitely sensitive for quantifying the percentage of male tissue in chimeric systems using Y chromosome specific amplicons (e.g., Y chromosome detection thresholds as low as 0.125%—equivalent to 6 copies of the Y chromosome) [24–27]. We

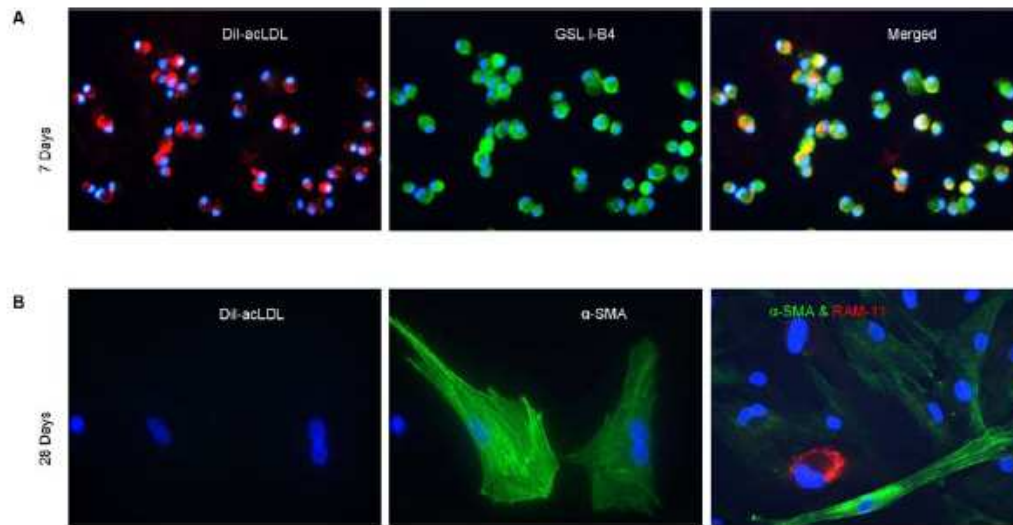


Figure 5. Differentiation of circulatory cells *in vitro*. A: Rabbit PBMCs maintained in culture for 7 days: DII-acLDL labeling (red, left panel); GSL I-B4 labeling (green, middle panel); Merged image showing both DII-acLDL uptake and GSL I-B4 labeling in individual cells (yellow, right panel). B: Rabbit PBMCs after 28 days in culture: DII-acLDL labeling is absent (red, left panel), α -SMA expression is evident (green, middle panel), and some cells that do not express α -SMA express the macrophage marker, RAM-11 (red, right panel). For both A and B: blue DAPI nuclear counterstain and $\times 400$ magnification.
doi:10.1371/journal.pone.0001894.g005

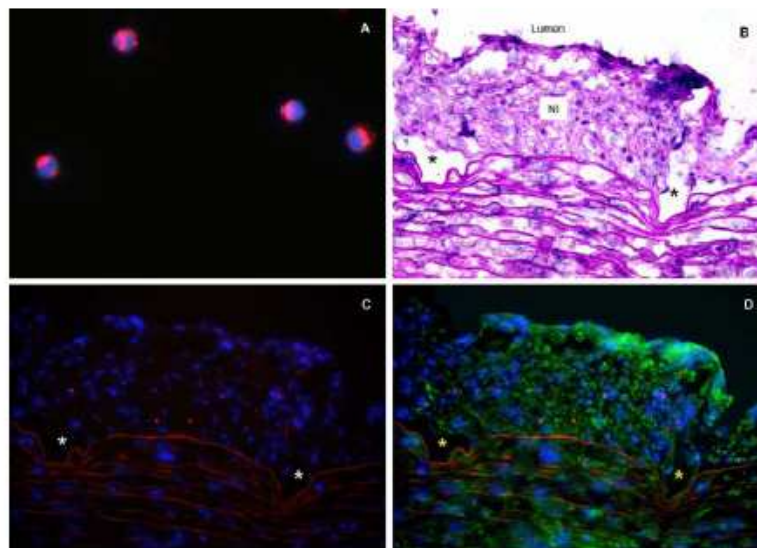


Figure 6. Differentiation of circulatory cells *in vivo*. A: PBMCs labeled *in vitro* with PKH26 dye (red) and DAPI nuclear counterstain (blue); magnification: $\times 1000$. These cells were injected into the carotid artery immediately after stent insertion. B: Cross section of native rabbit carotid artery 14 days post-stent implantation (H&E stain). C: PKH26-labelled PBMCs (red) were detected in the NI of stented artery. D: Some PKH26-labelled PBMCs (red) were also immunopositive for α -SMA (green). For panels B–D: arrows or autofluorescence delineate the internal elastic lamina; *denote sites of manually removed stent struts; magnification: $\times 400$.
doi:10.1371/journal.pone.0001894.g006

examined a far larger number of allografts than the most relevant previous study whereby the investigators limited their cell sampling protocol to cells that were α -SMA immunopositive and derived their conclusions from the analyses of only 21 nuclei harvested from 3 allografts [28]. In contrast, we performed LCM on a total of 12 allografts (6 male to female, and 6 female to male), with analyses performed at 2 specific subsegments within each graft (i.e., unstented body of graft, and stented mid-segment). As well, using Q-PCR protocol with remarkable linearity for the amplification reaction, we do not rely simply on the presence or absence of an amplicon. Thus, while we acknowledge the pioneering work by other investigators in this field, particularly Hillebrands and colleagues, our work expands upon the initial studies and provides a large data set to draw quantitative conclusions from.

Undoubtedly, the NI of the allografts in our study is the result of both the response to stent implantation and the alloimmune features of vasculopathy; however, this is no different from the cardiac transplant recipient with ACD who undergoes coronary stenting. Stenting ultimately results in increased NI formation that cannot be explained by excessive proliferation. Actually, the proliferation rates in stented or non-stented allografts were similarly low and comparable to that of the stented native arteries—observations that are consistent with our previous study of the response to stent implantation in rabbits [18].

To further demonstrate that rabbit circulatory cells can populate and differentiate into vascular cells within the NI of stents we performed two additional experiments. First, we demonstrated that PBMCs from rabbits can differentiate *in vitro* and at day 7 express markers consistent with an endothelial lineage (DiI-acLDL uptake, GSL I-B4 labeling). By 4 weeks *in vitro* the same cells no longer express these endothelial cell markers, but instead showed features consistent with either SMCs or macrophages (e.g., immunolabeled for α -SMA or RAM-11; respectively). Second, when PBMCs are tagged with the fluorescent PKH26 dye and injected at time of native carotid artery stent insertion, they constitute a surprisingly high percentage (9%) of the stent NI cell population 14 days later.

Perhaps the most intriguing observation from the current study is the detection of cells that express the endothelial lineage marker GSL I-B4 [19,20] within the NI of both non-stented and stented allograft segments, as well as stented native arteries. *In situ*, these cells are morphologically similar to NI SMCs, as they are surrounded by an extracellular matrix and do not appear to be part of an organized vasa vasorum. Certainly, in the porcine model of stent neointimal formation, SMCs almost exclusively populate the neointima, thereby raising the possibility that these GSL I-B4 cells may be unique to the rabbit model [29]. While the involvement of cells expressing other endothelial markers in experimental lesions formation is not novel [30,31] to our knowledge, this is the first report of GSL I-B4 positive cells within the NI of stented arteries. Given our current data as well as that from other laboratories [32–34] showing the differentiation of PBMCs to either endothelial cells, SMCs or macrophage phenotypes, it is tempting to speculate that GSL I-B4 positive NI cells are a form of circulatory progenitor cells that later differentiates *in vivo* into a SMC or macrophage phenotype. It is less likely that these cells eventually constitute vasa vasorum within the stent NI because although neovascularization of atherosclerotic lesions is common, vasa vasorum are exceedingly rare in ISR lesions [35].

Finally, there are limitations to our studies. First, it must be remembered that we detected host-derived NI cells at a relatively early interval in lesion development and it is unclear if these host-derived cells survive and participate in mature lesion formation.

While Religa and colleagues [36] noted a similar frequency of host-derived SMCs in 8 week old allografts, Bentzon and colleagues [37] found only locally derived cells at 20 and 32 week old after allografting. Hence, it is possible that the early engraftment of host-derived cells (perhaps facilitated by apoptosis of local vessel wall cells) is later replaced by a regrowth of local cells that are stimulated by immune-mediated inflammation. A second question relates to the prospect that host cells from the native carotid artery anastomoses migrated inward to colonize the allografts. Our rabbit model used isografted carotid segments that are quite long (30 mm). Given that each stent was 12 mm long and placed in the middle of the allograft, vascular cells from the adjacent native carotid artery would have to migrate at least 9 mm in a period of two weeks to reach the margins of the stent. It is unlikely that two weeks is sufficient time for inward migration of cells from the anastomoses to populate the stent NI. Moreover, in our study the NI of the allograft body between either anastomosis and the stent is approximately 50% smaller than that of the stent itself. Therefore, if inward migration of mature carotid artery cells contributed to stent NI formation, it is surprising to note that the intervening allograft body shows a “gap” in the NI area relative to that of the more centrally located stented segment. Third, the engraftment of *ex vivo* PKH26-labeled cells into the stent NI was remarkably high, and perhaps reflects modifications of these cells that occurred while in culture (e.g., upregulated expression of integrins). Hence, we must cautiously interpret the magnitude of this observation, yet can acknowledge that this information is supportive of the concept that blood derived cells can directly participate in stent NI formation. Finally, one must ask why were all of the NI cells not blood borne? Perhaps one clue might come from elegant studies performed decades ago that clearly demonstrated the role of tissue wall hypoxia in the genesis of the NI. Essentially, the vasa vasorum of carotid arteries arise from terminal branches of adjacent arteries (e.g., ophthalmic) [38]. Hence, the harvesting of these arterial allografts ultimately results in disruption of the vasa vasorum and leads to vessel wall hypoxia—a phenomenon previously shown to induce atherosclerotic lesions [39]. Therefore, it begs the question could this hypoxic stimulus be a confounding factor that prompts NI expansion by endogenous SMCs that, in effective, partially minimize the involvement of blood borne (circulatory) cells?

In summary, our sex-mismatched carotid allograft rabbit model provides a novel method to study NI formation in ACD and the response stent implantation in allografts. Our findings suggest that blood-borne, host-derived progenitor cells—including cells of endothelial lineage—are important contributors to NI formation in both these lesions. Hence, modulating the mobilization, homing, and differentiation of host derived circulating cells may provide a new therapeutic target in prevention of ACD and stent NI formation in allografts.

Materials and Methods

A detailed description of the methodology (Materials and Methods S1) is provided as supporting information.

Sex-Mismatched Carotid Allograft Model

Animal procedures were carried out with the approval of the University of Ottawa Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. Carotid artery transplantations were performed between gender-mismatched NZW rabbit siblings (2.5–3.0 kg, Charles River Laboratories, Quebec). Six male and six female rabbits were recipients of sex-mismatched carotid allografts. Beginning one

week prior to transplantation the rabbits were fed a 0.3% cholesterol diet (Harlan Teklad, Madison, WI) that was continued till euthanasia. Under general anesthesia, the donor carotid arterial segment (2.5–3.0 cm long) was harvested and the donor allowed to recover for subsequent transplantation or euthanized if donating a second carotid artery. The recipient rabbit was heparinized (175 IU/kg), the right carotid artery exposed, cross-clamped over a 3–3.5 cm segment and incised longitudinally. The harvested donor carotid arterial allograft was anastomosed to the segment end-to-side with interrupted 8-0 prolene sutures. Circulation was restored and vigorous pulsations in the allografted carotid artery without leaking confirmed successful transplantation. Recipients were then allowed to recover for three weeks before a cobalt chromium stent (S670, 3.0×12 mm, Medtronic AVE, Inc., Santa Rosa, CA) was deployed at 6 atmospheres in the middle of the allograft (experimental). As well, a second (control) stent was deployed in the contra-lateral native carotid artery. Immunohistochemistry was performed on tissue sections in order to detect the presence of macrophages, SMCs, T cells, cells of endothelial lineage (GSL I-B4 immunopositive), as well as proliferating cells. FISH was performed with a specific probe for the Y chromosome (SRY). LCM intimal tissue was collected, genomic DNA was isolated and subjected to Q-PCR for SRY and GAPDH genes in order to assess Y chromosome copy number relative to that of an autosomal gene, and therefore, determine the percentage of host or donor cells involved in NI formation.

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Differentiation of PBMCs *in vitro* and *in vivo*

Differentiation of rabbit PBMCs was examined *in vitro* using previously described methodologies[40]. To track the *in vivo* fate of circulatory cells, 1.75×10^7 of the rabbit's own PKH26 labeled PBMCs (Sigma)[41–43] were selectively re-infused into each carotid artery immediately post-stent insertion. One S670 stent was deployed per native carotid artery and 14 days later the rabbits were euthanized and the stented arteries (n=8) were harvested. The maximum percentage of PKH26 labeled cells per HPF was determined.

Supporting Information

Movie S1 Angiogram of stented carotid allograft

Found at: doi:10.1371/journal.pone.0001894.s001 (2.42 MB MPG)

Movie S2 Angiogram of stented native carotid artery

Found at: doi:10.1371/journal.pone.0001894.s002 (2.00 MB MPG)

Materials and Methods S1

Found at: doi:10.1371/journal.pone.0001894.s003 (0.07 MB DOC)

Author Contributions

Conceived and designed the experiments: XM EO BH. Performed the experiments: XM BH DW RS. Analyzed the data: XM BH. Contributed reagents/materials/analysis tools: SW. Wrote the paper: XM EO BH.

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Appendix IX

As presented in Chapter 2

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Appendix X

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Appendix XI

Book Chapter

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Hibbert, B., Simard, T., O'Brien, E.R. Chapter 8. Endothelial progenitors and repair of cardiovascular disease **(2011)** Regenerative therapy using blood-derived stem cells.

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Abstract

Since their initial description in 1997(Asahara et al 1997), considerable effort has been expended defining subsets of endothelial or vascular progenitor cells with the capacity to modulate a host of cardiovascular diseases. Indeed, the expansion of regenerative medicine as a field has led to a paradigm shift, from pharmaceutical or surgical intervention, to the potential use of cell based therapies. While preliminary clinical studies have shown promise, conflicting results from both preclinical animal models and small clinical trials reflect, in part, a lack of consensus regarding the characteristics, isolation methods, and definitions of what constitutes an endothelial progenitor cell (EPC). Moreover, as our understanding of the mechanisms by which EPCs modulate cardiovascular repair progress, novel strategies are emerging to either enhance the function of transplanted cells *or* modulate endogenous progenitor mediated repair. Herein we will review highlights of the preclinical and clinical data underlying the therapeutic potential of EPCs for repair following arterial injury.

6.1 Defining Endothelial Progenitor Cells

The earliest studies to suggest the existence of vascular progenitor cells described implantation of Dacron grafts or silastic tubing into the vasculature of various animal models (Stump et al 1963; Florey et al 1961; Jordan, Jr. et al 1962; Stump et al 1962; Pasquinelli et al 1987; Feigl et al 1985; Campbell, Efendy and Campbell 1999; Shi et al 1998). These experiments demonstrated the formation of a rudimentary vessel wall or “pseudo-neointima”, comprised of mature endothelial (ECs) and vascular smooth muscle cells (SMCs), independent of input from the native vasculature – leading the authors to postulate a blood derived source of mature vascular cells. However, it wasn’t until the isolation and characterization of endothelial progenitor cells (EPCs) in 1997 (Asahara et al 1997) that an explosion in the study of vascular progenitors as both markers and mediators of cardiovascular disease truly began. Consequently, the last decade has seen a rapid progression in our understanding of the fundamental biology that underlies this endogenous repair mechanism, with results from early small trials showing significant promise for therapeutic potential.

Paradoxically, as research in the field progresses at an exponential rate, the very definition of what constitutes a true EPC continues to evolve (Urbich and Dimmeler 2004a; Urbich and Dimmeler 2004b; Hirschi, Ingram and Yoder 2008; Yoder 2009). The lack of a uniform understanding of the most fundamental question – what is an EPC – has hampered both pre-clinical and clinical studies as conflicting results, at least in part, reflect a lack of consensus regarding definitions. While it is beyond the scope of this chapter to review all of the literature regarding the definition of an EPC (see Chapter 2), for the purpose of this chapter we will discuss the topic using *in vitro* culture definitions

ascribed in a recent review by Hirschi and colleagues (Hirschi, Ingram and Yoder 2008). Briefly, fibronectin adherence depleted colony forming cells (CFU-Hill), adherent early cultured angiogenic cells (CACs), and late outgrowth proliferative endothelial colony forming cells (ECFCs) will be specifically referenced.

Similarly, the characterization of an EPC by FACS analysis of peripheral blood has evolved with an ongoing debate regarding the appropriate gating strategy and panel of markers needed to ensure both reproducibility and specificity (Schmidt-Lucke et al 2010; Schmidt-Lucke et al 2005; Fadini et al 2010; Bearzi et al 2009). While CD45 negative/dim, CD34+, and VEGFR2/KDR+ markers are generally accepted to be present on EPCs, numerous authors have suggested inclusion of CD133, CD117 (c-kit), CXCR4, and CD31 as additional markers of putative EPCs (Bearzi et al 2009; Brehm et al 2009; Grundmann et al 2007; Wojakowski et al 2004; Estes et al 2010; Peichev et al 2000; Duda et al 2007). In the absence of clear evidence favoring the use of any one panel of markers, we have elected to use the term circulating EPC (cEPC) to describe vascular progenitors enumerated by flow cytometry. While cultured and cEPC populations undoubtedly represent distinct lineages of cells, each has been termed “EPC” in the literature and as such will be discussed in the context of cardiovascular repair.

6.2 Endothelial Progenitor Cells and the Coronary Artery

Atherosclerosis and development of obstructive coronary artery disease (CAD) remains a leading cause of mortality and morbidity in developed nations (Manuel et al 2003). In addition to pharmacotherapy, the mainstay of treatment for ischemic heart disease includes revascularization whereby blood flow is restored to ischemic

myocardium. With well over one million procedures being performed annually, percutaneous coronary interventions (PCI) with deployment of stents has become the preferred revascularization strategy for patients with CAD (Froeschl et al 2004) (**Figure 1**). While coronary stenting has greatly reduced the rates of arterial re-narrowing at the site of intervention, in-stent restenosis (ISR) still occurs in 10-30% of patients receiving bare metal stents (Serruys, Kutryk and Ong 2006). As the development of an intima was initially thought to arise through proliferation of local SMCs, current therapies target SMC proliferation and have achieved reduced rates of ISR ranging from 5-12.5% (Marx et al 1995; Marx and Marks 2001; Wessely 2010; Stone et al 2007; Moses et al 2006; Kirtane et al 2008). However, while these therapeutic devices have improved outcomes dramatically, they may increase the risk of late stent thrombosis – a catastrophic acute closure of the stent due to clot formation on exposed stent struts – by delaying re-growth of the endothelium (Mauri et al 2007; Luscher et al 2007). Thus, the ongoing challenge remains to devise therapies that enable vascular homeostasis, maintaining endothelial integrity and function, at sites of arterial injury while simultaneously inhibiting neointimal formation.

6.2.1. Pre-Clinical Models of Vascular Injury & EPC Mediated Arterial Repair

Interventions designed to increase vessel lumen patency with either balloon angioplasty or PCI result in focal denudation of the endothelium and subsequent development of a neointima (NI) (**Figure 1**). Rapid reconstitution of the endothelium is not only necessary for reducing the risk of thrombosis, but evidence suggests that an intact endothelium abrogates SMC and extra cellular matrix (ECM) accumulation in the

NI (Kirton and Xu 2010). The first evidence that EPCs may play a role in repairing an injured artery originated from studies on the effects of statins in rodent models. Both cEPCs and CACs were independently reported to home and incorporate into sites of injury, mitigating NI formation, when animals were treated with statins (Werner et al 2002; Walter et al 2002). However, because of the concomitant statin therapy, improvements in re-endothelialization could not be attributed to the EPCs alone, and thus subsequent studies were performed using isolated spleen derived CACs (Werner et al 2003). The authors isolated CACs from mice and following carotid wire injury injected 1×10^6 cells at the time of injury and one day later resulting in a 30% reduction in the intima to media ratio. Interestingly, these cells homed only to the injured vessel and were not found in the contralateral uninjured artery suggesting a specific homing mechanism. Recently, our group replicated the findings of this seminal paper using CACs isolated from patients with CAD and demonstrated that improving CAC function could further improve re-endothelialization (Hibbert et al 2009). Finally, estrogens (Strehlow et al 2003), G-CSF therapy (Kong et al 2004), and leptin (Schroeter et al 2008) can mobilize cEPCs or CACs resulting in enhanced arterial repair in various models of vascular injury. These findings have supported the hypothesis that interventions which improve either EPC number or function may enhance vascular repair and ultimately clinical outcomes. Thus, results from these pre-clinical models have provided the impetus to investigate clinical interventions specifically designed to modulate EPC biology.

6.2.2. Clinical Interventions and EPC Mediated Arterial Repair

6.2.2.1. Statin Therapy

Early studies in patients with CAD noted both higher levels of cEPCs and CACs with statin treatment as well as a robust mobilization during initiation of therapy (Llevadot et al 2001; Vasa et al 2001). Since then, increased numbers of EPCs have been observed with statin therapy in patients with stable CAD (Schmidt-Lucke et al 2010), pre-PCI, in the setting of acute myocardial infarction (Leone et al 2008), and post coronary arterial bypass grafting (Spadaccio et al 2010). In addition to simply increasing the number of cEPCs and CACs isolated from patients, statin therapy also appears to regulate qualitative properties of circulating progenitors. Notably, members of the integrin family of extra cellular matrix receptors have been shown to be upregulated by statin therapy which may in part improve adhesion at the site of arterial injury (Walter and Dimmeler 2002; Chavakis et al 2005). This is of particular importance as cEPCs and CACs from patients with CAD can have impaired functional capacity which may bear importance on their ability to maintain vessel wall homeostasis. For example, it is known that not only are fewer CFU-Hill (George et al 2003) and CACs (Hibbert, Chen and O'Brien 2004) found in patients with ISR, but also that a decrease in their adhesive properties is an independent risk factor for development of neointima following stent implantation (George et al 2003). Overall, these small observational studies demonstrate convincingly that statin therapy is associated with both improved number and function of EPCs in patients undergoing PCI but all have lacked the power to address meaningful clinical outcomes.

Statins have a number of effects independent of LDL lowering, including improving endothelial function, enhancing atherosclerotic plaque stability, and modulating inflammation (Liao and Laufs 2005). These pleiotropic effects may in part

explain benefits above and beyond what might be expected by LDL cholesterol lowering alone. For example, in the PCI sub-study of the PROVE IT trial, there was a marked reduction of target vessel revascularization in patients treated with high dose Atorvastatin, an effect which remained after adjusting for on treatment LDL cholesterol (Gibson et al 2009). Similarly, the Atorvastatin for Reduction of MYocardial Damage during Angioplasty (ARMYDA) study group has demonstrated important early reductions in peri-procedural infarction by either initiating (Pasceri et al 2004) or reloading patients with high dose statin immediately prior to performing PCI (Di et al 2009). While these larger trials do not specifically test the hypothesis that EPCs can improve clinical outcomes following PCI, it is attractive to postulate a link between the early and robust mobilization of EPCs with statin therapy and the observed clinical benefit.

6.2.2.3. Stent Design

Coronary stents have evolved from mere scaffolds designed to prevent elastic recoil of the artery into highly sophisticated drug delivery systems in which every aspect – from the alloy used to the polymer coating – is designed to facilitate arterial healing (Garg and Serruys 2010b; Garg and Serruys 2010a). Thus, it is only natural that as the potential impact EPCs play in restoring an intact endothelium following PCI became apparent, researchers have attempted to enhance attachment and proliferation of EPCs at the site of deployment (Padfield et al 2010).

The first stent designed to exploit endogenous EPC reparative mechanisms was the Genous CD34 antibody coated stent (Aoki et al 2005). In this first in man registry, 16

patients underwent successful coronary stenting with only a single patient requiring target vessel revascularization at 9 months of follow-up. To date, this stent has been studied in randomized studies of ST elevation myocardial infarction patients (Co et al 2008; Lee et al 2010) and in a prospective registries of stable CAD patients (Miglione et al 2008; Duckers et al 2007b) with satisfactory efficacy and safety profiles out to one year. However, most recently (Beijk et al 2010) data comparing the Genous EPC capture stent to the Taxus Liberte paclitaxel eluting stent showed more than double the late lumen loss (1.14 vs 0.55 mm). In these 193 patients at high risk of restenosis, there was also a non-significant trend towards an increased need for target vessel revascularization with the CD34 antibody coated stent. Thus, while the use of an EPC capture stent is promising, data from larger randomized control trials will be needed before use of this device is widely adopted.

Perhaps the most interesting results from the clinical studies of the Genous stent is the post-hoc analyses in which patients whom appear to benefit most are those with higher circulating EPC levels (Duckers et al 2007a). In this study, cEPCs defined as CD45+/CD34+/KDR+/7AAD- were approximately two fold higher in patients treated with a statin and inversely correlated with late lumen loss. As patients with CAD have fewer EPCs and decreased EPC function (Werner et al 2005; Werner et al 2007; Hill et al 2003), this has led researchers to mandate statin therapy in studies looking at the Genous EPC capture stent in an attempt to increase re-endothelialization (Beijk et al 2010). However, these findings may under-score a major limitation in therapies targeting EPCs. That is, in the patient populations in whom these therapies are designed to function, EPC number may be too low or dysfunctional to derive the benefits observed in pre-clinical

models. Further studies into molecules which mobilize EPCs, such as statins, or targets which improve survival and paracrine function of the recruited cells, such as glycogen synthase kinase 3 α (Ma et al 2010; Hibbert et al 2009), may ultimately improve the performance of stents designed to improve EPC mediated arterial repair.

6.2.2.4. Cell Based Therapies

To date, there are no clinical studies investigating either systemic or intra-coronary injection of EPCs for reduction in rates of ISR or late lumen loss following coronary stenting. Moreover, the relationship between EPCs, PCI and ISR continues to yield discordant and sometimes confusing results likely reflecting both the inconsistencies by which EPCs are defined as well as limitations in study design. Banerjee *et al.* (Banerjee et al 2006) looked at 38 patients and enumerated ECFCs. While they observed a mobilization of ECFCs induced by PCI, the authors noted that this seemed to be independent of serum VEGF levels and was restricted to those patients undergoing elective procedures. In contrast, Garg *et al.* noted a 37% increase in Hill-CFUs in 20 patients having PCI with all of their patients having had a recent non-ST elevation myocardial infarction (Garg et al 2008). Finally, Egan *et al.* (Egan et al 2009) compared 10 patients undergoing PCI with 13 patients having only angiography and found that CXCR4 positive cEPCs were mobilized in response to PCI alone, suggesting that vascular injury is necessary to induce mobilization. While these findings strongly support the notion that the vascular injury induced by PCI mobilizes EPCs, none of the aforementioned studies were adequately powered nor designed to assess the association between EPCs and ISR.

The first study to examine the relationship between EPCs and ISR was performed by Inoue *et al.* (Inoue et al 2007). They enumerated cEPCs (defined as CD34+ cells), CACs and outgrowth of smooth muscle progenitor cells and noted an association between mobilization of CD34+ cells, outgrowth of SMCs and the ultimate development of ISR. Interestingly, others had previously noted a strong correlation between CD34+ cell levels and late lumen loss following PCI (Schober et al 2005). However by limiting their flow cytometry to a single marker these investigators included all early bone marrow derived cells including hematopoietic progenitors making it difficult to draw conclusions. The largest study to date was conducted by Pelliccia *et al.* in 155 patients undergoing PCI for stable angina (Pelliccia et al 2010). Paradoxically they too noted an association between elevated cEPCs (defined as CD45-/CD34+/KDR+) and clinical ISR which developed in 30 patients. Thus, while animal models purport regenerative benefit with regards to the vascular endothelium, in humans when either CACs or cEPCs are mobilized following vascular injury there seems to be at least an association between mobilization of CD34+ cells and development of ISR. Thankfully, clinical studies using intracoronary injection of cultured cells have not demonstrated the adverse events that may have been predicted by these observational trials. Specifically, the ASTAMI trial (Lunde et al 2006; Beitnes et al 2009), the REPAIR-AMI trial (Schachinger et al 2006), and the study by Janssens *et al.* (Janssens et al 2006) all performed intra-coronary injection of mononuclear derived cells without increased rates of ISR. While these studies do not directly address the potential benefit of a cell based therapy, the experimental data provides a rationale while these clinical trials demonstrate an acceptable safety profile in patients undergoing PCI.

6.3. Future Directions

Therapies which augment re-endothelialization following vascular injury, such as in the context of PCI, are necessary to improve arterial healing and ultimately enhance clinical outcomes. Clearly, we are only beginning to better define the involved cell populations and the underlying mechanisms which promote their mobilization, homing, and incorporation into the arterial wall. While it is conceivable that cell based therapies may show benefit, it stands to reason that as our understanding of the biology of EPCs improves, pharmacologic interventions or devices which modulate this endogenous repair mechanism represent the most likely manner in which EPCs will make the leap from bench to bedside.

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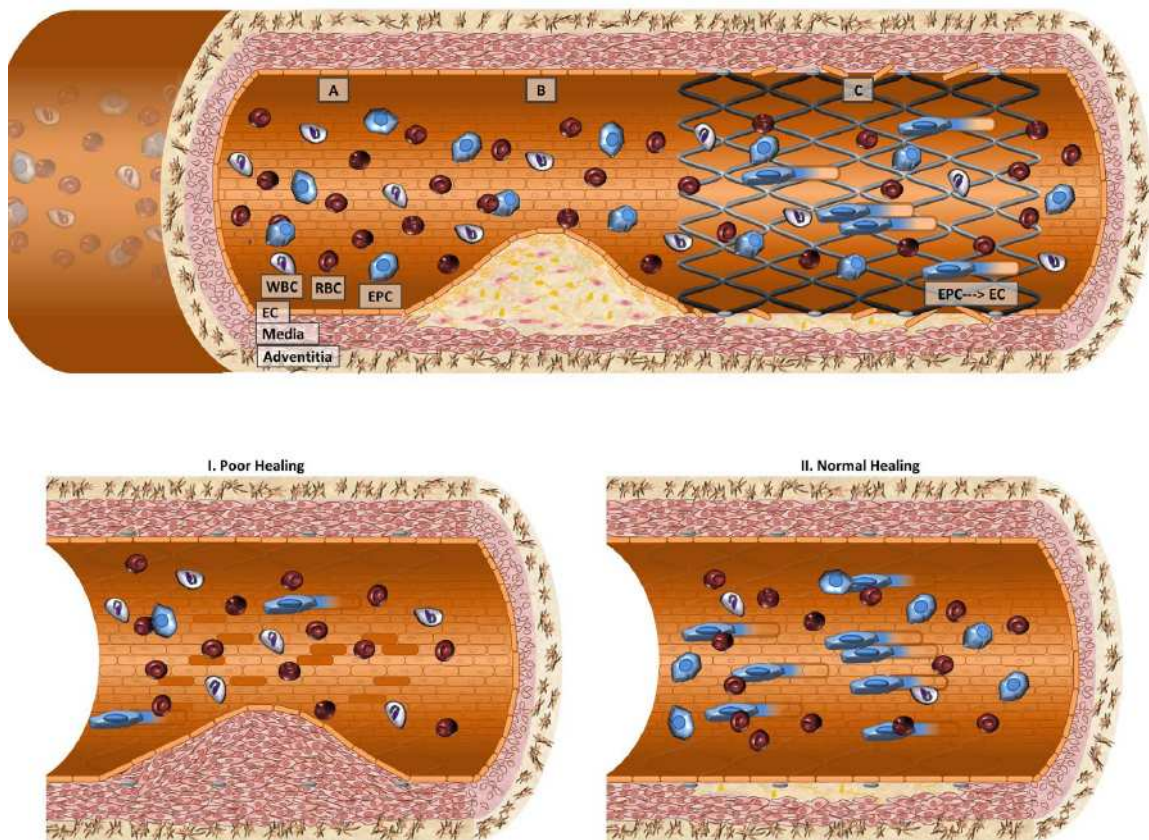


Figure 1. Role of EPCs in vascular healing. Arterial cross section (upper panel) displaying vascular anatomy composed of outer adventitial layer with fibroblasts and extracellular matrix (ECM), medial layer with smooth muscle cells, and inner endothelial cell (EC) layer. Erythrocytes (RBCs), leukocytes (WBCs), and endothelial progenitor cells (EPCs) circulate within the vessel's lumen. **Region (A)** Normal arterial vessel with intact endothelium. **Region (B)** Atherosclerotic lesion containing a fibrous cap and cholesterol crystals lined by endothelium. **Region (C)** Atherosclerotic lesion treated via insertion of metal stent, compressing the plaque while denuding the endothelial layer in this region. Re-endothelialization then occurs via EPCs adhering to the vessel wall where they differentiate into endothelial cells (EPC---> EC) or secrete paracrine factors which promote re-endothelialization by local mature ECs. Lower panels illustrate time lapse healing of stented region (C). **I. Poor Healing** due to diminished EPC numbers and/or function results in incomplete re-endothelialization with exposed stent struts and neointima formation. This delayed arterial homeostasis results in in-stent restenosis. **II. Normal Healing** displays completely re-endothelialized stented segment with no exposed struts or neointima formation owing to EPC numbers and/or function stimulating normal arterial homeostasis. Adapted from Padfield et al. 2010.

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Appendix XII

Book Chapter

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Chapter 6

Coronary Physiology and Atherosclerosis

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KEY POINTS

1. To safely care for patients with coronary artery disease in the perioperative period, the clinician must understand how the coronary circulation functions in health and disease.
 2. Coronary endothelium modulates myocardial blood flow by producing factors that relax or contract the underlying vascular smooth muscle.
 3. Vascular endothelial cells help maintain the fluidity of blood by elaborating anticoagulant, fibrinolytic, and antiplatelet substances.
 4. One of the earliest changes in coronary artery disease, preceding the appearance of stenoses, is the loss of the vasoregulatory and antithrombotic functions of the endothelium.
 5. The mean systemic arterial pressure and not the diastolic pressure may be the most useful and reliable measure of coronary perfusion pressure in the clinical setting.
 6. Although sympathetic activation increases myocardial oxygen demand, activation of α -adrenergic receptors causes coronary vasoconstriction.
 7. It is unlikely that one substance alone (e.g., adenosine) provides the link between myocardial metabolism and myocardial blood flow under a variety of conditions.
 8. As coronary perfusion pressure decreases, the inner layers of myocardium nearest the left ventricular cavity are the first to become ischemic and display impaired relaxation and contraction.
 9. The progression of an atherosclerotic lesion is similar to the process of wound healing.
 10. Lipid-lowering therapy can help restore endothelial function and prevent coronary events.
-

When caring for patients with coronary artery disease, the anesthesiologist must prevent or minimize myocardial ischemia by maintaining optimal conditions for perfusion of the heart. This goal can be achieved only with an understanding of the many factors that determine myocardial blood flow in both health and disease. This chapter begins with an overview of the structure and function of coronary arteries. Rapid progress has been made in the last several decades in the understanding of the physiology of blood vessels, particularly the role of the endothelium in maintaining flow. Following this overview is an analysis of the major determinants of coronary blood flow. Physiologic or pharmacologic interventions alter myocardial flow by their effects on these factors. The section on coronary pressure-flow relationships explains the important concepts of autoregulation and coronary reserve. Studies of the coronary circulation are sometimes misinterpreted because of an inadequate understanding of the complex interrelationships among the heart, the coronary circulation, and the peripheral circulation. The discussion of pathophysiology begins with a description of the process of atherosclerosis and the current understanding of how this disease evolves and causes clinical events. Next, the anatomy and hemodynamic effects of a coronary stenosis are explained. Coronary collateral function and development are reviewed here. These concepts are the basis of predicting how significantly the stenoses seen on angiography impair myocardial perfusion. The topic of the final section is the pathophysiology of myocardial ischemia. Here the concepts learned in the preceding sections are applied in an analysis of clinical ischemic syndromes. The final section highlights future directions in the treatment of coronary artery disease.

ANATOMY AND PHYSIOLOGY OF BLOOD VESSELS

The coronary vasculature has been traditionally divided into three functional groups: large conductance vessels visible on coronary angiography, which offer little resistance to blood flow; small resistance vessels ranging in size from about 250 to 10 μm in diameter; and veins. Although it has been taught that arterioles (precapillary vessels less than 50 μm in size) account for most of the coronary resistance, recent studies indicate that under resting conditions 45 to 50 percent of total coronary vascular resistance resides in vessels larger than 100 μm in diameter^{1,2,3} (Fig. 6–1). This may be due, in part, to the relatively great length of the small arteries. During intense pharmacologic dilation, the proportion of total coronary vascular resistance due to larger arteries and veins is even greater.² The regulation of tone in coronary arteries larger than 100 μm in diameter plays an important role in delivering adequate myocardial perfusion.⁴ One of the early changes in coronary artery disease is a diminished ability of the endothelium of epicardial coronary arteries to dilate in response to increased flow (see Endothelium-Derived Relaxing Factors, below). Advances in technology have enabled measurement, in the beating heart, of diameters of coronary vessels as small as 15 μm . It is becoming evident that, in response to a given intervention, different size classes of coronary vessels can change diameter with different intensity or even in opposite directions.^{5,6} This heterogeneity of response according to vessel size would be an important consideration in predicting the effects of vasoactive agents on myocardial perfusion. For example, a drug that dilated large vessels and collaterals but not arterioles would be beneficial to patients with coronary disease (see Coronary Steal).

The Normal Artery Wall

The arterial lumen is lined by a monolayer of endothelial cells that overlies smooth muscle cells (Fig. 6–2). The inner layer of smooth muscle cells, known as the intima, is circumscribed by the internal elastic lamina. Between the internal elastic lamina and external elastic lamina is another layer of smooth muscle cells, the media. Outside the external elastic lamina is an adventitia that is sparsely populated by cells and microvessels of the vasa vasorum.

The Intima

Traditionally, the intima has been considered the most important layer of the artery wall.⁷ The intima can vary from a single endothelial layer to a more complex structure of an endothelium overlying a patchwork of extracellular matrix and vascular smooth muscle cells. As part of the normal development of many large arteries, smooth muscle cells populate this space and form a neointima. This diffuse form of intimal thickening consists of layers of smooth muscle cells and connective tissue the thickness of which may vary considerably. For convenience, the intima:media ratio is often measured, and the normal range is 0.1 to 1.0. How this benign intima forms is not well understood. Presumably, the intima represents a physiologic adaptation to changes in arterial flow and wall tension. The intima is made up of two distinct layers.⁸ As seen by electron microscopy, the inner layer

subjacent to the luminal endothelium contains an abundance of proteoglycan ground substance. Smooth muscle cells found in this layer are usually distributed as isolated cells in a sea of matrix, rather than in contiguous layers. A few macrophages may also be found in this layer underneath the endothelial monolayer. The outer, musculo-elastic layer of the intima is adjacent to the internal elastic lamina and contains smooth muscle cells and elastic fibers.

The Media

In normal adult arteries, several smooth muscle cell subpopulations with distinct lineages exist within the media.⁹ These diverse cell populations likely fulfill different functions in order to maintain homeostasis in the artery wall. For example, in response to pressure elevations, an increase in smooth muscle cell mass and extracellular matrix may be required. Alternatively, for arteries to be able to stretch both longitudinally and circumferentially, smooth muscle cells with variable orientations of cytoskeletal fibers must be present. These distinct cell types may be important not only in health but also in disease. In certain experimental models of neointimal formation, proliferation and inward migration of subpopulations of medial smooth muscle cells occur.¹⁰ The biologic determinants of medial smooth muscle cell diversity are unknown.¹¹

The Adventitia

The adventitia, the outermost layer of the artery wall, normally consists of a sparse collection of fibroblasts, microvessels (vasa vasorum), nerves, and few inflammatory cells. The majority of the vasa vasorum that nourish the inner layers of the artery wall originate in the adventitia. Traditionally, the adventitia has been ignored and is not thought to play a role in vascular lesion formation. However, more recent studies have elucidated the role of the adventitia as not only a source of inflammatory cells in the development of atherosclerosis, but also a hub for paracrine signaling that can maintain vascular homeostasis in a variety of vascular diseases.¹²

Transmembrane and Transcellular Communication

Blood vessels respond to a multitude of neural, humoral, and mechanical stimuli in fulfilling their role in homeostasis. When norepinephrine, released from adrenergic nerve terminals in the adventitia, binds to receptors on the vascular smooth muscle cell membrane, a series of events take place, culminating in a change in vessel diameter. Much progress has been made in understanding this transmembrane signaling since the discovery of cyclic adenosine monophosphate (cAMP) in the late 1950s. Hormones circulating in the blood must interact with receptors on endothelial cells before the message reaches the vascular smooth muscle cell. The mechanism of communication between cells has been one of the central themes of biologic research in the last decades. Future understanding of cardiovascular disease will likely be based on identification of abnormalities of the molecules involved in transmembrane and transcellular communication. A brief introduction to these topics is provided here.

Figure 6–3 illustrates examples of pathways of transmembrane signaling. Up to five components can be involved: receptor, G protein, effector producing a second messenger, phosphorylation of regulator protein, and the consequent change in cell behavior. G proteins (guanine nucleotide binding regulatory proteins) are made up of 3 subunits (α , β , γ) and float in the cell membrane. On contact with a ligand-receptor complex, guanosine diphosphate (GDP) on the α subunit is replaced by guanosine triphosphate (GTP). The activated α subunit then dissociates from the β - γ complex and can interact with several membrane targets (Fig. 6–3B). For example, β -receptor activation results in the activation of G_s (s = stimulate), which will stimulate the synthesis of cAMP by adenylyl cyclase. Muscarinic receptor activation activates a G_i (i = inhibit) protein that inhibits adenylyl cyclase. A single G protein can interact with more than one effector. In this way the G protein can be a branch point for the regulation of multiple effectors in response to a single signal. These proteins have already been implicated in human disease: Cholera toxin covalently modifies G_s so that it becomes persistently active in stimulating adenylyl cyclase in intestinal epithelial cells, likely causing the severe diarrhea of cholera.

Several second-messenger systems have been characterized. G_s can directly enhance conductance through calcium channels, with the increased intracellular calcium acting as second messenger. The cyclic nucleotides, cAMP and guanosine monophosphate (GMP), act as second messengers. Their intracellular action is terminated when they are cleaved by phosphodiesterase enzymes, which, in turn, are also regulated by stimuli and second messengers. The breakdown products of membrane phosphoinositide constitute another, more recently recognized, set of second messengers.¹³ In response to agonists such as vasopressin, G protein is activated, leading to activation of the membrane-associated enzyme phospholipase C. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate on the inner leaflet of the plasma membrane, producing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Both are second messengers. IP3 diffuses through the cytoplasm and mobilizes calcium from intracellular stores. DAG remains within the plasma membrane and activates protein kinase C, which modulates cellular activity by phosphorylating intracellular proteins. In many cell types activation of the same receptors that control phosphoinositide breakdown also results in the liberation of arachidonate and/or eicosanoids (prostaglandins, leukotrienes, and thromboxanes). The resultant change in cell behavior can be the opening of an ion channel, contraction or relaxation of smooth muscle, secretory activity, or initiation of cell division (see Chap. 7).

Endothelium

Although the vascular endothelium was once thought of as an inert lining for blood vessels, it is more accurately characterized as a very active, distributed organ with many biologic functions. It has synthetic (Table 6–1), and metabolic (Table 6–2) capabilities and contains receptors for a variety of vasoactive substances (Table 6–3). Functions of the endothelium that may play an important role in the pathophysiology of ischemic heart disease are discussed.

Endothelium-Derived Relaxing Factors

The first vasoactive endothelial substance to be discovered was prostacyclin (PGI₂), a product of the cyclooxygenase pathway of arachidonic acid metabolism (Fig. 6-4 and Box 6-1).¹⁴ The production of PGI₂ is activated by shear stress, pulsatility of flow, hypoxia, and a variety of vasoactive mediators. Upon production it leaves the endothelial cell and acts in the local environment to cause relaxation of the underlying smooth muscle or to inhibit platelet aggregation. Both actions are mediated by the stimulation of adenylyl cyclase in the target cell to produce cAMP.

BOX 6-1 *Endothelium-Derived Relaxing and Contracting Factors*

Healthy endothelial cells have an important role in modulating coronary tone by producing

Vascular Muscle Relaxing Factors

- Prostacyclin
- Nitric oxide
- Hyperpolarizing factor

Vascular Muscle Contracting Factors

- Prostaglandin H₂
- Thromboxane A₂
- Endothelin

In 1980, Furchgott and Zawadzki observed that the presence of an intact endothelium was necessary for acetylcholine-induced vasodilation.¹⁵ Since that time it has been shown that many physiologic stimuli cause vasodilation by stimulating the release of a labile, diffusible, nonprostanoid molecule termed "endothelium-derived relaxing factor" (EDRF) (see Fig. 6-4) now known to be nitric oxide (NO). Nitric oxide is the basis of a widespread paracrine signal transduction mechanism whereby one cell type can modulate the behavior of adjacent cells of different type.^{16,17} NO is a very small lipophilic molecule that can readily diffuse across biologic membranes and into the cytosol of nearby cells. The half-life of the molecule is less than 5 seconds so that only the local environment can be affected. NO is synthesized from the amino acid L-arginine by NO synthase. In vascular endothelium the enzyme (eNOS or NOS3) is always present (constitutive) and resides in the cytoplasm. Its function depends on the presence of Ca⁺⁺ and calmodulin as well as tetrahydrobiopterin. Serine phosphorylation is important for prolonged activity. The enzyme is activated in response to receptor occupancy or physical stimulation (Table 6-3). When NO diffuses into the cytosol of the target cell, it binds with the heme group of soluble guanylate cyclase, resulting in a 50- to 200-fold increase in production of cyclic GMP, its second messenger. If the target cells are vascular smooth muscle cells, vasodilation occurs; if the target cells are platelets, adhesion and aggregation are inhibited. In vascular smooth muscle cGMP leads to activation of protein kinase G which phosphorylates various intracellular target proteins, including the myosin light-chain regulatory subunit and proteins that control intracellular calcium.¹⁸

It is likely that NO is the final common effector molecule of nitrovasodilators (including sodium nitroprusside and organic nitrates such as nitroglycerin). The cardiovascular system is in a constant state of active vasodilation that is dependent on the generation of NO. The molecule is more important in controlling vascular tone in veins and

arteries compared to arterioles. When the microcirculation dilates in response to metabolic myocardial demand (e.g. exercise) increased flow through epicardial coronary arteries increases shear stress at the endothelium. This leads to release of NO which causes vascular smooth muscle relaxation and dilation of the conductance vessels, thereby facilitating the increase in flow. The importance of the loss of this mechanism in atherosclerosis is underlined by the fact that in this situation more than 50% of the resistance to flow in the coronary circulation resides in vessels larger than 100 μm in diameter (Fig. 6-1). Abnormalities in the ability of the endothelium to produce NO likely plays a role in diseases such as diabetes, atherosclerosis, and hypertension.^{19,20} The venous circulation of humans seems to have a lower basal release of NO and an increased sensitivity to nitrovasodilators when compared with the arterial side of the circulation.²¹

Many agents, such as acetylcholine and norepinephrine, can cause contraction when applied directly to the vascular smooth muscle membrane instead of relaxation, which occurs when it is applied to the intact endothelium (Fig. 6-5). The net effect of neural or humoral stimuli depends on a combination of direct effects mediated by binding to vascular smooth muscle receptors and indirect effects due to the ligand binding to endothelial receptors causing NO release from the endothelium. In the presence of healthy endothelium vasodilation usually predominates. When the endothelium is absent (injured vessel) or diseased (atherosclerosis) vasoconstriction may be the net effect. NO has important roles in neurohumoral regulation of vascular tone, in preventing intravascular platelet aggregation, and in the structural adaptation of blood vessels to the demands of blood flow and pressure. Knowledge of its role in inflammation and atherosclerosis is rapidly expanding.

In addition to prostacyclin and NO another, less well understood, pathway for receptor mediated or mechanically induced endothelium-derived vasodilation exists that is associated with smooth muscle hyperpolarization. Both epoxyeicosatrienoic acid (a metabolite of cytochrome p450) and H_2O_2 have been suggested as possible endothelium-derived hyperpolarizing factor(s) (EDHFs).^{22,23} Smooth muscle relaxation is a result of hyperpolarization of the myocyte which leads to decreased intracellular calcium concentration. EDHF mediated vasodilation can be blocked by inhibition of calcium dependent potassium channels. EDHF may have an important vasodilator role in the human coronary microcirculation.²⁴

Endothelium-Derived Contracting Factors

Contracting factors produced by the endothelium include prostaglandin H_2 , thromboxane A_2 (via cyclooxygenase), and the peptide endothelin. Endothelin is a potent vasoconstrictor peptide (100-fold more potent than norepinephrine)²⁵ with remarkable similarities to the toxin of the burrowing asp. Both have potent coronary constrictor activity to which the strong cardiac toxicity and lethality of the toxin are attributed.²⁶ Three closely related 21 amino-acid peptides have been identified: endothelin-1 (ET-1), ET-2, and ET-3. The primary product of vascular endothelium is ET-1 which is synthesized from prepro-endothelin-1 within vascular endothelial cells by the action of endothelin converting enzyme. It is not stored, but rapidly synthesized in response to stimuli such as ischemia, hypoxia, and shear stress and released predominantly abluminally (toward the underlying smooth muscle).²⁷ In vascular smooth muscle cells, ET-1 binds to specific membrane receptors (ET_A) and, via phospholipase C, induces an increase in intracellular calcium

resulting in long-lasting contractions.²⁸ It is also linked via a G_i protein to voltage-operated calcium channels. This peptide has a greater vasoconstricting potency than any other cardiovascular hormone, and in pharmacologic doses can abolish coronary flow, leading to ventricular fibrillation and death.²⁹ Another receptor subtype, ET_B , is expressed by both smooth muscle and endothelium and binds ET-1 and ET-3 equally well (Fig. 6–6). When isolated vessels are perfused with ET-1 there is an initial NO mediated vasodilation due to binding with ET_B receptors on the endothelial cells followed by contraction due to binding of ET-1 to ET_A receptors on the vascular smooth muscle membrane. Studies utilizing bosentan, a combined ET_A and ET_B receptor antagonist, have demonstrated that endothelin exerts a basal coronary vasoconstrictor tone in humans.³⁰ There is evidence that endothelin may play a role in the pathophysiology of pulmonary and arterial hypertension, atherosclerosis, myocardial ischemic syndromes, and heart failure.³¹ Clinical trials of bosentan in patients with congestive heart failure³² and hypertension have shown promise but hepatic side effects have limited the dose to less than 500mg daily, with the primary indication being severe pulmonary hypertension.³³

Endothelial Inhibition of Platelets

A primary function of endothelium is to maintain the fluidity of blood. This is achieved by the synthesis and release of anticoagulant (e.g., thrombomodulin, protein C), fibrinolytic (e.g., tissue-type plasminogen activator), and platelet inhibitory (e.g., PGI_2 , NO) substances (Box 6-2).³⁴ Mediators released from aggregating platelets stimulate the release of NO and PGI_2 from intact endothelium, which act together to increase blood flow and decrease platelet adhesion and aggregation (Fig. 6–7), thereby flushing away microthrombi and maintaining the patency of the vessel.

BOX 6–2 Endothelial Inhibition of Platelets

Healthy endothelial cells have a role in maintaining the fluidity of blood by producing

- Anticoagulant factors: protein C and thrombomodulin
- Fibrinolytic factor: tissue-type plasminogen activator
- Platelet inhibitory substances: prostacyclin and nitric oxide

With vital roles in modulating the tone of vascular smooth muscle, inhibiting platelets, and processing circulating chemicals, it seems clear that endothelial cell dysfunction would cause or contribute to ischemic syndromes. There is evidence of endothelial dysfunction in atherosclerosis, hyperlipidemia, diabetes, and hypertension.³⁵ Procedures such as coronary artery surgery and angioplasty disrupt the endothelium. The role of endothelium in the pathophysiology of myocardial ischemia is discussed below (see Dynamic Stenosis).

DETERMINANTS OF CORONARY BLOOD FLOW

Under normal conditions there are four major determinants of coronary blood flow: perfusion pressure, myocardial extravascular compression, myocardial metabolism, and neurohumoral control. Changes in myocardial perfusion caused by different interventions can be explained by analyzing the effects of those interventions on these four factors.

BOX 6-3 *Determinants of Coronary Blood Flow*

The primary determinants of coronary blood flow are

- Perfusion pressure
- Myocardial extravascular compression
- Myocardial metabolism
- Neurohumoral control

Perfusion Pressure and Myocardial Compression

Coronary blood flow is proportional to the pressure gradient across the coronary circulation (Box 6-3). This gradient is calculated by subtracting downstream coronary pressure from the pressure in the root of the aorta. The determination of downstream pressure is complicated because the intramural coronary vessels are compressed with each heart beat.

During systole the heart throttles its own blood supply. The force of systolic myocardial compression is greatest in the subendocardial layers, where it approximates intraventricular pressure. Resistance due to extravascular compression increases with blood pressure, heart rate, contractility, and preload. Because it is difficult to measure intramyocardial pressure, the relative importance of these factors is controversial.^{36,37} Flow is impeded both by direct compression and by shear caused by twisting of vessels as the heart contracts. Myocardial extravascular compression is less in the right ventricle, where pressures are lower and coronary perfusion persists during systole (Fig. 6-8). In pathologic conditions associated with pulmonary hypertension, right coronary flow assumes a phasic pattern similar to left coronary flow. Under normal conditions extravascular compression contributes only a small component (10% to 25%) to total coronary vascular resistance. When the coronary vessels are dilated by pharmacologic agents such as dipyridamole or during ischemia, the effects of extravascular compression on myocardial perfusion become more important (see Transmural Blood Flow).

With each contraction the intramural vessels are squeezed and blood is expelled forward into the coronary sinus and retrograde into the epicardial arteries. The large coronary arteries on the epicardial surface act as capacitors, charging with blood during systole and expelling blood into the coronary circulation during diastole.³⁸ Coronary capacitance likely explains the findings of Bellamy, who reported that flow in the proximal left anterior descending coronary artery of the dog ceased when arterial pressure decreased below 45 mmHg.³⁹ It was suggested that flow throughout the coronary circulation stopped at pressures far in excess of the pressure at the coronary sinus. This pressure at which flow stopped was termed *critical closing pressure* or *zero-flow pressure* (P_{zf}). This had important implications in the calculation of coronary resistance because the effective downstream pressure would be P_{zf} and not the much lower coronary venous pressure. This is analogous to a stream with a waterfall, where flow rate over the waterfall depends on the drop from the source to the waterfall edge and is unaffected by the distance to the bottom of the falls. It was later suggested that flow through the intramural coronary vessels continues after coronary inflow near the ostia (measured by Bellamy) has ceased.^{40,41} There is evidence that antegrade movement of red blood cells in 20- μ m arterioles continues until coronary pressure is only a few mmHg higher than coronary sinus pressure.⁴² The concept of a critical closing pressure greatly in excess of coronary sinus pressure is probably not valid in the coronary circulation.

Although the true downstream pressure of the coronary circulation is likely close to the coronary sinus pressure, other choices may be more appropriate in clinical circumstances. In patients with coronary artery disease, the subendocardial layers of the left ventricle are at greatest risk of ischemia and necrosis (see Transmural Blood Flow). Because this layer is perfused mostly when the aortic valve is closed, the most appropriate measure of the driving pressure for flow here is the average pressure in the aortic root during diastole. This can be approximated by aortic diastolic or mean pressure. Pressures monitored in peripheral arteries by routine methods in clinical settings can differ from central aortic readings. This is due to distortion of the pressure waveform as it is propagated through the arterial tree and inaccuracies associated with the hydraulic and electronic components of the monitoring system. Under these conditions the mean arterial pressure may be the most reliable measure of coronary driving pressure. The true downstream pressure of the left ventricular subendocardium is the left ventricular diastolic pressure, which can be estimated by pulmonary artery occlusion pressure. When the right ventricle is at risk of ischemia (e.g., severe pulmonary hypertension), right ventricular diastolic pressure or central venous pressure may be more appropriate choices for downstream pressure.

Myocardial Metabolism

Myocardial blood flow, like flow in the brain and skeletal muscle, is primarily under metabolic control. Even when the heart is cut off from external control mechanisms (neural and humoral factors), its ability to match blood flow to its metabolic requirements is almost unaffected.³⁵ Because coronary venous oxygen tension is normally 15 to 20 mmHg, there is only a small amount of oxygen available through increased extraction. A major increase in cardiac oxygen consumption (MVO_2) can occur only if oxygen delivery is increased by augmentation of coronary blood flow. Normally flow and metabolism are closely matched so that over a wide range of oxygen consumption coronary sinus oxygen saturation changes little.⁴³

Despite intensive research over the past several decades, the mediator or mediators linking myocardial metabolism so effectively to myocardial blood flow is still unknown. Hypotheses of metabolic control propose that vascular tone is linked either to a substrate that is depleted, such as oxygen or adenosine triphosphate (ATP), or to the accumulation of a metabolite such as CO_2 or hydrogen ion (Box 6-4). Adenosine has been proposed in both categories. Feigl⁴³ has proposed six criteria for a chemical transmitter between the cardiac myocyte and the coronary vascular smooth muscle cell:

1. The transmitter is released under appropriate conditions and can be recovered from the tissue under those conditions.
2. Transmitter substance infused into the target tissue should faithfully mimic physiologic activation.
3. The biochemical apparatus for production of the proposed transmitter is present in the tissue in an appropriate location.

4. A mechanism for inactivation and/or uptake of the transmitter is present at an appropriate location in the tissue.
5. The action of various inhibitors and blocking agents on synthesis, release, target-organ receptor function, or transmitter inactivation should have effects consistent with the hypothesis. Blocking agents should give the same effect whether the transmitter is released physiologically or artificially applied.
6. Quantitative studies should indicate that the amount and time course of transmitter release under physiologic conditions are appropriate to give the indicated effect.

Many potential mediators of metabolic regulation have been proposed.⁴⁴ Although NO has a role in many coronary vasoregulatory pathways it does not fulfill the role of metabolic regulator as blockade of NO synthase does not alter the increase in myocardial blood flow associated with an increase in myocardial oxygen demand.⁴⁵ The arguments for oxygen, carbon dioxide, and adenosine are briefly examined.

BOX 6-4 Myocardial Metabolism

Several molecules have been proposed as the link between myocardial metabolism and myocardial blood flow including

- Oxygen
- Carbon dioxide
- Adenosine

Current evidence suggests that a combination of local factors act together, each with differing importance during rest, exercise, and ischemia, to match myocardial oxygen delivery to demand.

Oxygen

In order for oxygen to regulate coronary flow through a direct vascular action, the coronary smooth muscle would have to be more sensitive to lack of oxygen than the working cardiocytes. Coronary microvessels in vitro do not relax until PO_2 is below 5 mmHg, a level well below the average PO_2 of 20 mmHg in cardiac muscle cytosol.^{46,47} With myocardial oxygen consumption held constant, increases in arterial oxygen content cause coronary flow to decrease while decreases in arterial oxygen content cause flow to increase. These changes could explain only 40 percent of the increase in flow observed with tachycardia.⁴⁸ It is undecided whether the constancy of myocardial oxygen tension is the cause or the consequence of the excellent match between myocardial metabolism and myocardial blood flow.⁴⁹

Carbon Dioxide

The end product of substrate oxidation is CO_2 , the formation of which is directly related to the level of cardiac work. Carbon dioxide is highly diffusible and can easily reach coronary smooth muscle cells. Unfortunately, it is very difficult to separate the effects on coronary tone of increasing CO_2 from concomitant increases in other metabolites. Broten et al

pump-perfused the left main coronary artery of dogs and used an oxygenator in the perfusion circuit to alter coronary arterial PCO₂ and PO₂ at a constant level of myocardial metabolism.⁴⁸ Increases in arterial and coronary sinus PCO₂ caused increases in coronary blood flow in the absence of changes in myocardial oxygen consumption. Interestingly, there was a synergistic action of PCO₂ and PO₂: The increase in flow with elevation of PCO₂ was much greater at low PO₂ and vice versa. The effect of increasing CO₂, however, could not completely account for flow changes associated with an increase in myocardial oxygen consumption.

Adenosine

Adenosine is a powerful coronary vasodilator via its activation of receptors on vascular endothelium and smooth muscle. In 1963 both Berne and Gerlach independently demonstrated the production of adenosine in ischemic heart muscle.^{50,51} They hypothesized that the release of adenosine may serve as a feedback signal inducing coronary vasodilation and augmenting coronary blood flow in proportion to myocardial metabolic needs. Initially it was suggested that adenosine formation was coupled to myocardial oxygen tension.⁵⁰ To explain metabolic regulation by adenosine under both normoxic and ischemic conditions, a substrate theory has been proposed whereby adenosine production is linked to the cardiac energy state by the regulation of cytosolic AMP concentration.⁵² According to this theory, increases in cardiac work lead to a fall in ATP potential, which results in a quantitatively appropriate change in cytosolic AMP concentration, leading to increased adenosine release. In this way the rate of adenosine production is determined by the myocardial oxygen supply/demand ratio. It is likely that adenosine causes coronary arteriolar dilation through stimulation of A₁ receptors directly coupled to ATP-sensitive K⁺ (K⁺_{ATP}) channels and A₂ receptor-mediated elevation of cyclic AMP / protein kinase A which leads to vasodilation in part by opening of K⁺_{ATP} channels.^{53,54}

Evidence against the adenosine hypothesis is accumulating. Adenosine deaminase is an enzyme that, when introduced in sufficient quantity into the myocardium, can significantly reduce the interstitial concentration of adenosine. Aminophylline and theophylline interfere with the coronary dilating effects of adenosine by acting on the receptor on vascular smooth muscle. Experiments using these agents to inhibit adenosine effect have shown that resting coronary blood flow, exercise-induced coronary dilation, autoregulation, and reactive hyperemia are largely unrelated to adenosine.^{55,56,57,58} Measuring coronary microvessel diameters in beating hearts in situ, Kanatsuka et al found that when MVO₂ was doubled by pacing, vessels between 40 and 380 μm dilated, whereas when a similar increase in flow was induced by the infusion of adenosine or dipyridamole at constant MVO₂, only vessels smaller than 150 μm dilated.⁵ Although adenosine does not seem to have an important role in metabolic regulation in the normal heart, adenosine blockade has been shown to cause a decrease in blood flow to hypoperfused myocardium sufficient to decrease systolic segment shortening.⁵⁹ Adenosine may have other important roles in ischemia where there is evidence of a cardioprotective action.^{60,61}

Current evidence suggests that a combination of local factors act together, perhaps with differing importance in different situations, to match myocardial oxygen delivery to

demand. The extreme difficulty of designing an experiment that can distinguish the effects of individual factors on coronary blood flow suggests that the exact mechanism of metabolic coronary regulation will not soon be elucidated.

Neural and Humoral Control

Neural Control

The role of neural control in the regulation of myocardial blood flow is difficult to study because sympathetic or parasympathetic activation can cause profound changes in heart rate, blood pressure, and contractility. The resulting changes in coronary tone, mediated by metabolic regulation, can mask the concomitant direct effects of autonomic nerves on coronary smooth muscle. Studies of isolated vessels have given results that contradict in vivo studies in part because of damage to the endothelium during preparation. Despite these difficulties, there is much interest in exploring the role of autonomic control, because it is implicated in the pathogenesis of myocardial ischemia.

Coronary Innervation.

The heart is supplied with branches of the sympathetic and parasympathetic divisions of the autonomic nervous system. Thicker vagal fibers end in the adventitia of coronary vessels, while fine nonmedullated sympathetic fibers end on vascular smooth muscle cells.⁶² Large and small coronary arteries as well as veins are richly innervated. The sympathetic nerves to the heart and coronary vessels arise from the superior, middle, and inferior cervical sympathetic ganglia and the first four thoracic ganglia. The stellate ganglion (formed when the inferior cervical and first thoracic ganglia merge) is a major source of cardiac sympathetic innervation. The vagi supply the heart with efferent cholinergic nerves.

Parasympathetic Control.

Vagal stimulation causes bradycardia, decreased contractility, and lower blood pressure. The resultant fall in MVO_2 causes a metabolically mediated coronary vasoconstriction. When myocardial metabolism is held constant, however, cholinergic coronary dilation is consistently observed in response to exogenous acetylcholine, electrical vagal stimulation, and reflex activation through baroreceptors, chemoreceptors, and ventricular receptors.^{35,43,63} These effects can be abolished by atropine.

In patients with angiographically normal coronary arteries the response to intracoronary acetylcholine injection is predominantly dilation; whereas in atherosclerotic segments of epicardial arteries, constriction is observed.^{64,65,66} Acetylcholine injected intraluminally binds to muscarinic receptors on the endothelium and stimulates the release of NO, which causes smooth muscle dilation. Acetylcholine is not normally found circulating in the blood but is released from vagal fibers and reaches the coronary smooth muscle from the adventitial side. Surprisingly, activation of muscarinic receptors on vascular smooth muscle cells causes constriction. Parasympathetic stimulation normally causes coronary vasodilation. This response depends on the ability of the coronary endothelium to elaborate

NO and perhaps also EDHF (see earlier).^{67,68} Parasympathetic control has not been shown to be important in the initiation of myocardial ischemia.

β -Adrenergic Coronary Dilation.

β -Receptor activation causes dilation of both large and small coronary vessels even in the absence of changes in blood flow.^{35,43} Studies in animals indicate that both β_1 and β_2 receptors are present throughout the coronary circulation, but β_1 receptors predominate in the conductance vessels whereas β_2 receptors predominate in the resistance vessels. Mature canine coronary collaterals respond similarly to the conductance vessels.^{69,70} β -Adrenergic coronary dilation may improve the speed and accuracy of coronary blood flow regulation during exercise.⁷¹

α -Adrenergic Coronary Constriction.

Activation of the sympathetic nerves to the heart results in increases in heart rate, contractility, and blood pressure, which lead to a marked, metabolically mediated, increase in coronary blood flow (Box 6-5). This suggested to early investigators that the effect of sympathetic coronary innervation is vasodilation. More recent investigation has revealed that the direct effect of sympathetic stimulation is coronary vasoconstriction, which is in competition with the metabolically mediated dilation of exercise or excitement. Whether adrenergic coronary constriction is powerful enough to further diminish blood flow in ischemic myocardium or if it can have some beneficial effect in the distribution of myocardial blood flow is controversial.

BOX 6-5 α -Adrenergic Coronary Constriction

Sympathetic activation causes increased heart rate, contractility, and blood pressure, leading to a marked metabolically mediated increase in coronary blood flow. Surprisingly, the direct effect of sympathetic stimulation on the coronary vessels is vasoconstriction, sufficient to restrict the increase in blood flow and increase oxygen extraction.

Classification.

α -Adrenergic receptors can be classified anatomically as pre- or postsynaptic and also according to their pharmacologic properties as α_1 and α_2 (Table 6-4). The receptors can be further divided into subtypes according to their signal transduction mechanism (G-protein subtype) and second messenger (adenylyl cyclase, phospholipase C, etc.).⁷²

Presynaptic α -Receptors.

α -Receptors on cardiac sympathetic nerve terminals mediate feedback inhibition of neuronal norepinephrine release. Both α_1 - and α_2 -receptors seem to be involved because exercise-induced increases in heart rate and contractility can be potentiated by either idazoxan (α_2 -blockade) or prazosin (α_1 -blockade).⁷³

Cardiac Muscle Cells.

Activation of myocardial α_1 -receptors results in a positive inotropic effect that, in contrast to β -receptor activation, is associated with prolongation of contraction. Although normally of minor functional importance, this effect may serve as an inotropic reserve mechanism when β -receptor-mediated inotropy is impaired (e.g., hypothyroidism, cardiac failure, chronic propranolol treatment).⁷⁴ The importance of this mechanism in humans is uncertain. An increase in inotropy due to stimulation of myocardial α -receptors would result in increased MVO_2 and a metabolically mediated coronary dilation.

Coronary Endothelium.

Binding of norepinephrine to α_2 -receptors on vascular endothelium stimulates the release of NO, which acts to relax vascular smooth muscle. The endothelium can also act to limit the effect of norepinephrine by metabolizing it. In these ways the endothelium modulates the direct constrictive effects of α -adrenergic activation. Abnormal endothelial function in atherosclerosis may predispose to excessive α -adrenergic constriction and is implicated in the pathogenesis of myocardial ischemia (see Dynamic Stenosis).

Coronary Resistance.

The magnitude of α -adrenergic vasoconstriction that occurs in the coronary bed is small compared with that which occurs in the skin and skeletal muscle. In the presence of β -blockade, intense sympathetic stimulation results in only a 20% to 30% increase in coronary resistance.⁷⁵ Mohrman and Feigl examined the effect of sympathetic activation on coronary flow in the absence of β -blockade.⁷⁶ The net effect of α -receptor vasoconstriction was to restrict the metabolically related flow increase by 30%, thereby increasing oxygen extraction and decreasing coronary sinus oxygen content.

Epicardial coronary diameter changes little during sympathetic stimulation.⁷⁷ α_1 -Adrenergic and α_2 -adrenergic receptors are found throughout the coronary circulation; however, α_1 seem to be more important in the large epicardial vessels whereas α_2 predominate in small coronary vessels less than 100 μm in diameter.⁷⁸ Studies of mature coronary collateral vessels in dogs have generally failed to provide evidence of α -receptor-mediated vasoconstriction.⁷⁹ Following heart transplant, patients demonstrated a lesser increase in myocardial blood flow following a cold pressor test in denervated regions of the heart.⁸⁰ The authors argue that this was not due to increased myocardial metabolism secondary to myocardial β -receptor activation. They suggest that sympathetic innervation has an important role in coronary vessel dilation during stress.

Exercise.

α -Adrenergic coronary constrictor tone during exercise is exerted predominantly by circulating catecholamines.⁸¹ Numerous studies indicate that myocardial blood flow during exercise is limited by α -vasoconstriction.³⁵ In a study of exercising dogs, Huang and Feigl found that despite an increase in total coronary flow in an α -blocked region of myocardium, flow to the inner, subendocardial layer was diminished.⁸² These results suggest a beneficial

effect of α -adrenergic coronary constriction on the distribution of blood flow within the myocardium.

Myocardial Ischemia.

Buffington and Feigl demonstrated the persistence of α -adrenergic coronary vasoconstriction distal to a moderate coronary stenosis during norepinephrine infusion.⁸³ Investigations in dogs have demonstrated that, as coronary reserve is depleted by increasing stenosis severity, the response to sympathetic stimulation shifts from a metabolically induced coronary dilation to coronary constriction.^{84,85} These observations suggest that sympathetic coronary vasoconstriction limits coronary blood flow even during myocardial ischemia, when autoregulatory reserve is exhausted (see Coronary Reserve, below). There is no consensus as to the importance of α_1 - vs α_2 -receptors in ischemic myocardium.³⁵ Using constant flow coronary perfusion in anesthetized dogs, Nathan and Feigl compared the transmural distribution of myocardial blood flow in α -blocked and intact regions of myocardium during hypoperfusion.⁸⁶ Surprisingly, α -blockade diverted blood flow from the subendocardium to the subepicardium. This suggests that α -vasoconstriction had limited flow more in the subepicardium, thereby producing an anti-steal effect and improved perfusion of the more vulnerable inner layers of the left ventricle. Chilian and Ackell found similar results in exercising dogs with an artificial coronary stenosis.⁸⁷ On the other hand, work from Heusch and colleagues demonstrated improved subendocardial perfusion distal to a severe coronary stenosis with α_2 -receptor blockade.^{88,89} This controversy is unresolved.⁹⁰ α -receptor blockers have not been shown to have a role in the treatment of myocardial ischemia in patients with coronary artery disease.

Studies in Humans.

Studies indicate that there is little α -adrenoceptor-mediated tone in resting humans.⁹¹ Clinical studies have failed to provide convincing evidence that α -adrenergic coronary constriction plays an important role in Prinzmetal's variant angina (angina with ST-segment elevation at rest).⁹² During sympathetic activation, however, there is evidence that α -vasoconstriction can precipitate myocardial ischemia by further narrowing diseased coronary arteries. This has been shown during isometric exercise, dynamic exercise, and with the cold pressor test^{93,94,95,96,97,98} (see Dynamic Stenosis).

Humoral Control

A complete understanding of the effects of circulating substances on the coronary vessels would require determining their effects on large versus small coronary vessels while separating direct effects on coronary vessels from changes in tone mediated by changes in myocardial metabolism. This is further complicated by the critical role of an intact vascular endothelium in modulating these responses (see Endothelium, above). Some of the better-studied agents are discussed briefly below.

The peptide hormones include vasopressin (AVP or ADH), atrial natriuretic peptide (ANP), vasoactive intestinal peptide, neuropeptide Y, and calcitonin gene-related peptide.⁴⁴

Of these, AVP and ANP have been the most studied. It has been demonstrated in dogs that AVP, in concentrations 3 to 30 times those found in stressed patients, can cause vasoconstriction sufficient to produce myocardial ischemia.⁹⁹ In large coronary arteries the dilator response (via NO) likely exceeded the constrictor response.³⁵ This was due to constriction of the small-resistance vessels. In physiologic concentrations, AVP acts primarily as an antidiuretic hormone with little effect on the coronary circulation. ANP can cause endothelium-dependent coronary dilation, but is not known to have significant vascular effects in physiologic concentrations.¹⁰⁰

Angiotensin-converting enzyme (ACE) is present on vascular endothelium and converts angiotensin I to angiotensin II (AII), which causes coronary vasoconstriction. AII also facilitates release of norepinephrine from presynaptic adrenergic nerve terminals. ACE inactivates bradykinin, which can attenuate vasoconstriction via NO stimulation. Thus, ACE inhibition can reduce coronary tone by suppressing AII formation and degrading bradykinin and perhaps also by decreasing norepinephrine release. Despite these theoretical considerations, ACE inhibition has not been shown to be of benefit in human myocardial ischemia other than through control of afterload.¹⁰¹

Prostacyclin (PGI₂) and thromboxane (TxA₂) are synthesized from arachidonic acid in a reaction catalyzed by cyclooxygenase. PGI₂ is synthesized in the vascular endothelium and, in addition to inhibiting platelet aggregation, induces vasodilation (see Endothelium-Derived Relaxing Factors). TxA₂ is mainly synthesized in platelets and causes platelet aggregation and vasoconstriction in the presence of damaged vascular endothelium. In response to TxA₂ the intact endothelium releases NO causing both vasodilation and platelet disaggregation, a mechanism to maintain patency of normal vessels (see Endothelial Inhibition of Platelets, above). Unlike platelets, the vascular endothelium can synthesize proteins *de novo*, and thus cyclooxygenase acetylation by aspirin administration has a lesser effect in reducing vascular PGI₂ than platelet TxA₂. Other than in platelet-vessel interactions and inflammation, prostaglandins are not known to have an important role in the regulation of coronary blood flow.³⁵ Serotonin (5-HT) is another platelet product that can cause endothelium dependent dilation of coronary arterial vessels smaller than 100 μm but causes constriction of larger epicardial coronary arteries.¹⁰²

Histamine receptors are present in the coronary vessels. H₁ receptors are located on vascular smooth muscle cells of large and small coronary arteries and mediate vasoconstriction. H₂ receptors are located on smooth muscle cells of arterioles and mediate vasodilation. H₁ receptors are also located on vascular endothelium and can mediate vasodilation via stimulation of NO release. In patients with vasospastic angina and endothelial dysfunction administration of exogenous histamine can cause vasospasm.¹⁰³

CORONARY PRESSURE-FLOW RELATIONS

Autoregulation

Autoregulation is the tendency for organ blood flow to remain constant despite changes in arterial perfusion pressure.¹⁰⁴ Autoregulation can maintain flow to myocardium served by stenotic coronary arteries despite low perfusion pressure distal to the obstruction. This is a local mechanism of control and can be observed in isolated, denervated hearts. If myocardial oxygen consumption (MVO_2) is fixed, coronary blood flow will remain relatively constant between mean arterial pressures of 60 to 140 mmHg. Figure 6–9 illustrates that at a given cardiac workload the level of flow (determined by metabolic regulation) is maintained constant over a broad range of pressure by autoregulation.

To study autoregulation, coronary perfusion pressure must be varied while holding MVO_2 constant. This is difficult in the heart since changing aortic pressure changes both the perfusion pressure for the coronary arteries and the afterload of the left ventricle. Thus, changes in aortic pressure inevitably change MVO_2 . This problem is overcome by cannulating the coronary arteries and perfusing them with a pump. However, even when heart rate and aortic pressure are held constant, MVO_2 changes with changing coronary pressure. This is because myocardial contractility and metabolism increase when coronary pressure is raised above the normal autoperfused level. This phenomenon is known as the Gregg effect and may be explained by the "garden hose" hypothesis of Lochner, whereby engorgement of the coronary vasculature elongates the myocardial sarcomere length during diastole and contractile strength is increased due to the Frank-Starling mechanism (for a detailed review see Feigl⁴³ and Gregg¹⁰⁵).

In addition to the Gregg effect, two other issues complicate studies of autoregulation: collateral flow and myocardial oxygen extraction. If pressure is lowered in the left coronary artery and not in the right, there will be a pressure gradient for flow from the right to left coronary artery via collateral vessels. Flow measured proximally in the left coronary artery will then underestimate flow reaching the myocardium. Normal coronary sinus oxygen tension (CSO_2) is below 20 mmHg. Dole observed that autoregulation was effective when CSO_2 was below 25 mmHg, but was completely lost when CSO_2 exceeded 32 mmHg.¹⁰⁶ Autoregulation can be intensified by vasoconstriction (increased oxygen extraction) and attenuated by vasodilation (decreased oxygen extraction).¹⁰⁷ The degradation of autoregulation with α -receptor blockade suggests a benefit of adrenergic coronary vasoconstriction.¹⁰⁸

Early reports indicated that autoregulation is less effective in the right ventricle than the left. More recently it has been suggested that increases in right coronary pressures may produce large changes in MVO_2 , perhaps due to an exaggerated Gregg effect. When changes in myocardial metabolism are taken into account, autoregulation in the right and left ventricle is similar.^{109,110}

Quantitation of the degree of autoregulation must involve a comparison of the observed change in vascular resistance to the change in resistance that would have occurred in the absence of flow autoregulation. Some degree of autoregulation exists when the relative change in flow ($\Delta F/F$) is less than the relative change in pressure ($\Delta P/P$). From these definitions, Dole has derived an autoregulation index that can be used to quantify the effects of different agents on coronary autoregulation.^{107,111}

Three theories have been proposed to explain coronary autoregulation: the tissue pressure theory, the myogenic theory, and the metabolic theory.¹¹² The tissue pressure hypothesis proposes that changes in perfusion pressure result in directionally similar changes in capillary filtration and therefore tissue pressure. In this way extravascular resistance would oppose changes in flow with changes in perfusion pressure. Experimental evidence has shown, however, that there is no relationship between the degree of autoregulation and the magnitude of change in tissue pressure. Arterial smooth muscle contracts in response to augmented intraluminal pressure; this is known as the myogenic response. Recently, this response has been demonstrated in coronary arterioles in the presence and absence of functioning endothelium.¹¹³ The argument for myogenic regulation of coronary flow is that myocardial metabolic changes are not rapid enough to explain large decreases in resistance following coronary occlusions for one or two heartbeats. However, myocardial metabolic events have been shown to occur during the course of a single cardiac contraction.¹¹⁴ The metabolic theory of autoregulation proposes that coronary arteriolar tone is determined by the balance of myocardial oxygen supply and demand. An increase in flow above the requirements of metabolism would wash out metabolites or cause accumulation of substrates, and this would be the signal for an appropriate change in coronary tone. Although metabolic regulation and autoregulation are separate phenomena, they may therefore have a common underlying mechanism. Metabolic regulation is discussed above (see Myocardial Metabolism). For an instructive, three-dimensional, graphical analysis of the interrelations among coronary artery pressure, myocardial metabolism, and coronary blood flow, see Feigl et al.¹¹⁵

Coronary Reserve

Myocardial ischemia causes intense coronary vasodilation. Following a 10- to 30-second coronary occlusion, restoration of perfusion pressure is accompanied by a marked increase in coronary flow. This large increase in flow, which can be five or six times resting flow in the dog, is termed *reactive hyperemia*. Figure 6–10 illustrates that the repayment volume is greater than the debt volume. There is, however, no overpayment of the oxygen debt because oxygen extraction falls during the hyperemia.¹¹⁶ The presence of high coronary flows when coronary venous oxygen content is high suggests that mediators other than oxygen are responsible for this metabolically induced vasodilation.⁴³ The difference between resting coronary blood flow and peak flow during reactive hyperemia represents the autoregulatory coronary flow reserve: the further capacity of the arteriolar bed to dilate in response to ischemia. In Figure 6–9 the flow reserve is the vertical distance from the autoregulating pressure-flow curve (open or closed circles) to the non-autoregulating curve (triangles). The reserve is greater at higher perfusing pressure and lower MVO₂. Unlike cannula-perfused preparations in which these data are obtained, in the clinical setting increases in pressure increase both perfusing pressure and MVO₂. Reactive hyperemia responses have been used in animals and humans to estimate coronary reserve in conditions such as obstructive coronary disease, aortic stenosis, and left ventricular hypertrophy^{117,118,119} (see Critical Stenosis). The myocardial fractional flow reserve is calculated by dividing the pressure in a coronary vessel distal to a stenosis during maximal pharmacological dilation by the aortic root pressure. This ratio (FFR) can be easily

measured in the angiography suite and has been recommended as a useful index of the functional severity of coronary stenoses of intermediate morphologic severity on angiography as well as a measure of residual obstruction following interventions.¹²⁰ Indeed, the relevance of a reduction in the FFR is highlighted in a recent randomized control study which demonstrated improved clinical outcomes in FFR guided percutaneous coronary interventions as opposed to angiography alone.¹²¹

It has been generally accepted that the coronary resistance vessels are maximally dilated when coronary perfusion pressure is reduced sufficiently to cause myocardial ischemia. In fact, agents such as adenosine, carbochromen, and dipyridamole can cause further increases in coronary flow in the presence of intense ischemia, when autoregulatory reserve is believed to be exhausted. This pharmacologic vasodilator reserve is greater than the autoregulatory vasodilator reserve. If flow to ischemic myocardium can be increased by pharmacologic dilation of resistance vessels, the use of these agents should reverse ischemic dysfunction and metabolism. Arteriolar dilators have, in general, not been found to be beneficial during myocardial ischemia. Coronary blood flow in the different layers of the ventricle must be reviewed to understand why (Box 6-6).

BOX 6-6 Transmural Blood Flow

- When coronary perfusion pressure is inadequate, the inner third of the left ventricular wall is the first region to become ischemic or necrotic.
- During systole, intramyocardial pressure is highest in the inner layers of the ventricle, and this restricts perfusion to that region.
- In eccentric hypertrophy, this effect is exaggerated, and the subendocardium is at increased risk of ischemia.

Transmural Blood Flow

It is well known that, when coronary perfusion pressure is inadequate, the inner one third to one quarter of the left ventricular wall is the first region to become ischemic or necrotic.¹²² This increased vulnerability of the subendocardium may be due to an increased demand for perfusion or a decreased supply, compared with the outer layers. There has been extensive study of the transmural distribution of: oxygen consumption, utilization of oxidizable substrates, activity of glycolytic and mitochondrial enzymes, tissue contents of endogenous substrates, high-energy phosphates, lactate, isoforms of contractile proteins, and fiber stress and fiber shortening. In general, these studies indicate that if such differences exist between the layers of the left ventricle, they are unlikely to exceed 10% to 20%.^{43,123} It is likely that preferential underperfusion of the subendocardium is the primary determinant of its increased vulnerability.

Regional blood flow in the myocardium is usually determined using radioactive microspheres. These plastic beads, labeled with a radioisotope, are injected into the blood stream. The assumption is that they will mix uniformly with blood and be distributed in proportion to blood flow, as if they were red blood cells.¹²⁴ Because they are rigid and larger than red cells (9- or 15-micron diameters are usually chosen) they are trapped in the microcirculation. At the end of an experiment the heart can be divided into small blocks and the amount of radioactivity in each piece measured in a gamma counter. The blood flow to each block of tissue will be proportional to the number of microspheres in each piece, which

can be determined from its radioactivity. By using different radioisotopes as labels, several sets of microspheres can be injected during an experiment, giving "snapshots" of what flow was at the time of each injection. It is difficult to reduce the variability of the technique below 10%.^{43,125} Using this technique, subendocardial blood flow is found to be about 10% greater than subepicardial blood flow under normal circumstances. This gives a normal subendocardial/subepicardial or inner/outer (I/O) blood flow ratio of 1.10. This ratio is maintained at normal perfusing pressures even at heart rates above 200 beats/min.

If coronary pressure is gradually reduced, autoregulation is exhausted and flow decreases in the inner layers of the left ventricle before it begins to decrease in the outer layers (Fig. 6–11). This indicates that there is less flow reserve in the subendocardium than in the subepicardium. The limits of autoregulation will depend on the level of cardiac work (see Autoregulation) and on the experimental conditions. In conscious dogs the mean coronary pressure at which evidence of subendocardial ischemia appeared was 38 mmHg at a heart rate of 100 beats/min, and increased to 61 mmHg at 200 beats/min. Subepicardial flow during tachycardia did not fall even at pressures as low as 33 mmHg.¹²⁶ Because subepicardial flow is rarely inadequate, a subendocardial/subepicardial blood flow ratio close to 1.0 indicates adequate subendocardial flow and an appropriate matching of myocardial oxygen supply to oxygen demand. For this reason the I/O ratio is often used as a measure of the adequacy of myocardial blood flow.

Three mechanisms have been proposed to explain the decreased coronary reserve in the subendocardium: differential systolic intramyocardial pressure, differential diastolic intramyocardial pressure, and interactions between systole and diastole. Because the force of systolic myocardial compression is greatest in the inner layers of the ventricle and is low at the subepicardium, it was believed that the outer layers of the heart were perfused throughout the cardiac cycle while the subendocardium was perfused only during diastole. The subendocardium would have to obtain its entire flow during only a portion of the cycle and, therefore, would have to have a lower resistance. Recent studies, suggesting that there may be very little systolic flow even to the outer layers, argue against this explanation.¹²⁷ The second mechanism is based on the high coronary pressures observed when coronary flow has ceased during a long diastole, P_{zf} (see Perfusion Pressure).³⁹ The shape of the pressure-flow relation during a long diastole suggests that P_{zf} is higher in the subendocardium. This would mean that perfusion pressure for the subendocardium is lower in diastole compared with the outer layers of myocardium. Available evidence suggests that P_{zf} is not high in any layer and unlikely to be more than 2 to 3 mmHg higher in the subendocardium than in the subepicardium.¹²⁷ Hoffman proposed an interaction between systole and diastole as the explanation for the increased vulnerability of the subendocardium to ischemia.^{109,127} During systole, intramyocardial pressure is high enough throughout most of the ventricular wall to squeeze blood out of the intramural vessels and into the extramural coronary veins and arteries. Because the compressive force is greatest in the subendocardium, vessels here are the narrowest at end-systole. At the beginning of diastole blood will be directed first to vessels with the lowest resistance, the larger vessels in the subepicardium, and last to the most narrowed vessels in the subendocardium. In this way, should the duration of diastole or the diastolic perfusion pressure be reduced, the subendocardial muscle would receive the least flow. Spaan presents an interesting analysis

of the interaction between arterial pressure and force of contraction as an intramyocardial pump.³⁶ Although this theory is compatible with existing evidence, support for it will remain indirect until it becomes possible to measure phasic pressures and flows in separate layers of myocardium.

When the left ventricle hypertrophies in response to a pressure load (aortic stenosis, hypertension), myofibrillar growth outstrips the capillary network, resulting in decreased capillary density and increased diffusion distances. The net effect is to reduce coronary autoregulatory reserve.¹²⁸ The transmural gradient of reserve is exaggerated as well, so that the subendocardium is at increased risk of ischemia in the hypertrophied heart compared with normal.¹²⁹

In addition to the transmural gradient of coronary reserve from outer to inner layer of the left ventricle, there is also marked variation of reserve between small regions of myocardium within a layer.¹³⁰ This heterogeneity of flow reserve may explain why pharmacologic reserve exceeds autoregulatory reserve (see Coronary Reserve). During hypoperfusion regional myocardial blood flow is decreased, but in all layers some small pieces of muscle will have no flow reserve left while adjacent pieces can have substantial reserve. Fewer pieces will retain reserve in the subendocardium than in the subepicardium. The increase in flow in response to an infusion of adenosine is due to increased flow in the small regions with reserve, with no change in the adjacent fully dilated regions.^{131,132,133} These findings suggest that ischemia causes maximal coronary vasodilation and that increases in flow with adenosine or dipyridamole is due to dilation of vessels in nonischemic regions. Contrasting evidence is provided by Duncker and Bache,⁵⁴ who used a balloon occluder to simulate a coronary stenosis in exercising dogs. The occluder was adjusted to maintain distal coronary pressure constant at 43 mmHg. During exercise an intracoronary infusion of adenosine increased blood flow to all myocardial layers and improved regional systolic segment shortening. Although this is evidence of vasodilator reserve in ischemic myocardium the constant distal pressure preparation does not faithfully mimic a coronary stenosis because it makes transmural steal impossible (see Coronary Steal). In general pharmacologic dilation of resistance vessels has the potential to worsen ischemia by producing coronary steal. Dilation of larger penetrating vessels (50 – 500 μ m in diameter) with nitrovasodilators could preferentially decrease resistance to blood flow to the subendocardium, and this may, in addition to favourable effects on the systemic circulation, explain the usefulness of nitrates in the treatment of angina.⁵⁴

ATHEROSCLEROSIS

The atherosclerotic lesion consists of an excessive accumulation of smooth muscle cells in the intima, with quantitative and qualitative changes in the noncellular connective tissue components of the artery wall and intracellular and extracellular deposition of lipoproteins and mineral components (e.g., calcium) (Box 6-7). By definition, atherosclerosis is a combination of atherosis and sclerosis. The latter term, sclerosis, refers to the hard collagenous material that accumulates in lesions and is usually more voluminous than the pulsatous "gruel" of the atheroma (Fig. 6-12).

Stary and colleagues noted that the earliest detectable change in the evolution of coronary atherosclerosis in young people was the accumulation of intracellular lipid in the subendothelial region, giving rise to lipid-filled macrophages or "foam cells."¹³⁴ Grossly, a collection of foam cells may give the artery wall the appearance of a "fatty streak." In general, fatty streaks are covered by a layer of intact endothelium, and are not characterized by excessive smooth muscle cell accumulation. At later stages of atherogenesis, extracellular lipoproteins accumulate in the musculo-elastic layer of the intima, eventually forming an avascular core of lipid-rich debris that is separated from the central arterial lumen by a fibrous cap of collagenous material. Foam cells are not usually seen deep within the atheromatous core, but are frequently found at the periphery of the lipid core.

Atherogenesis

Certain human arteries are more prone to develop atherosclerosis than others. For example, the coronary, renal, and internal carotid arteries, as well as some areas of the aorta, are known to be common sites for lesion formation.¹³⁴ In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, the aorta and right coronary arteries of 1378 young people aged 15 to 34 years who died as a result of trauma were studied.¹³⁵ Two-dimensional maps of lipid-laden fatty streaks as well as fibrous plaques were made for each vessel. Although atherosclerosis is usually clinically silent until middle age or later, these investigators found that the disease process begins in adolescence or childhood. Moreover, fatty streaks and fibrous plaques do not occur randomly in the circulation, but follow a well-defined distribution pattern. For example, in the right coronary artery fatty streaks were found with the highest probability in the proximal 2 cm of this vessel, which closely parallels the distribution of raised fibrous lesions. However, in the abdominal aorta, where aortic lesions are commonly found, the high prevalence of fatty streaks did not always correlate with the prevalence of raised fibrous lesions. Therefore, at least in the aorta, the role of childhood fatty streaks in the development of adult fibrous lesions is uncertain.

BOX 6-7 Atherosclerosis

- The atherosclerotic process begins in childhood and adolescence.
- The progression of an atherosclerotic lesion has many similarities to the process of wound healing.
- Inflammation, lipid infiltration, and smooth muscle proliferation have important roles in atherogenesis.
- Impairment of endothelial function is an early consequence of atherosclerosis.
- Statin therapy has been shown to improve endothelial function, impede development of atherosclerosis, and in some cases may reverse established disease .

The atherogenic stimuli that promote the progression of early lesions to clinically relevant stenoses are not known. Currently, the development of coronary artery disease is associated with various risk factors. Dyslipidemias, hypertension, diabetes mellitus, cigarette smoking, and a family history of premature coronary artery disease are known to correlate with premature vascular disease. The association between lipid disorders and atherogenesis is best understood, and will be discussed below. Unfortunately, little is known about how the remaining risk factors may contribute to lesion development.

Historically, there are two classical theories of atherogenesis. According to von Rokitansky's thrombogenic (or encrustation) theory, fibrin is the initiating factor in lesion development.¹³⁶ Later, Duguid expanded on this theory by suggesting that atherosclerosis is the result of altered fibrinolysis; whereas more recent studies have documented the overexpression of prothrombotic factors, such as plasminogen activator inhibitor-1, in atherosclerotic plaques.^{137,138} Alternatively, in 1856, Virchow's imbibition (or insudation) theory proposed that atherosclerotic lesions were the result of altered vessel wall permeability.¹³⁹ Variations of this theory have been suggested by others and all support the concept that the accumulation of various plasma components, including lipoproteins, may be important during lesion formation. For example, Ross and Glomset blended the concepts of these original hypotheses into the "response-to-injury" hypothesis in which both lipid infiltration and thrombus formation play important roles in atherogenesis.¹⁴⁰ Similarly, Schwartz and colleagues compared arterial narrowing in atherosclerotic arteries with the process of wound healing.¹⁴¹ This perspective has advantages, as it allows a multifactorial process such as atherosclerosis to be broken down into components of a more completely understood process such as the biology of a skin wound. For example, wound healing of any form begins with the formation of a clot (fibrin- and fibronectin-containing gel) that fills the wound and provides a provisional matrix for inflammatory cells, fibroblasts, and newly formed microvessels.^{142,143} This is followed by the proliferation and migration of fibroblasts into the wound.¹⁴⁴ By day 7 after injury, microvessels grow into the base of the wound and form granulation tissue. As the wound matures and undergoes contracture, these blood vessels regress and fibroblasts disappear. Following resorption of microvessels, tissue hypoxia develops and likely plays a role in the completion of the final scarring process.¹⁴⁵ As discussed below, there is now ample evidence to suggest that many similar events take place during arterial wound healing; however, as atherosclerosis is a chronic process, it is likely that vascular lesion formation involves indolent levels of inflammation with ongoing cycles of injury and repair over many years.^{142,146,147}

Arterial Wall Inflammation

A number of studies have demonstrated the presence of monocytes/macrophages and T lymphocytes in the arteries of not only advanced lesions but also early atherosclerotic lesions of young adults.^{148,149} Moreover, in experimental atherosclerosis, leukocyte infiltration into the vascular wall is known to precede smooth muscle cell hyperplasia.¹⁵⁰ Once inside the artery wall, mononuclear cells may play several important roles in lesion development. For example, monocytes may transform into macrophages and become involved in the local oxidation of low-density lipoproteins and accumulation of oxidized low-density lipoproteins. Alternatively, macrophages in the artery wall may act as a rich source of factors that, for example, promote cell proliferation, migration, or the breakdown of local tissue barriers. The latter process of local tissue degradation may be very important for the initiation of acute coronary artery syndromes as loss of arterial wall integrity may lead to plaque fissuring or rupture.¹⁵¹

Normally, the endothelium exhibits a low affinity for circulating leukocytes. Therefore, the transmigration of leukocytes into the artery wall must occur as a facilitated process. The

release of proinflammatory cytokines such as interleukin-1 may promote the expression of leukocyte adhesive molecules.¹⁵² For simplicity, the interaction between leukocytes and the endothelium can be considered to involve three steps.¹⁵³ First, leukocytes in the blood stream must loosely associate and roll along the endothelium—a process that is mediated by selectins expressed on endothelial cells.¹⁵² Second, firm adhesion of these leukocytes to endothelial cells occurs via the interaction between integrins, such as $\alpha_4\beta_1$ (also known as very late antigen-4 or VLA-4), expressed on leukocytes, and counter-receptors, such as vascular cell adhesion molecule-1 or VCAM-1, on endothelial cells.¹⁵⁴ Finally, the transmigration of leukocytes into the subendothelial space is mediated by various migration-inducing factors, such as monocyte chemoattractant protein-1 or MCP-1.¹⁵⁵

Dysfunction, discontinuity, or injury of the endothelial cell monolayer has been postulated to play a significant role in facilitating the transmigration of leukocytes into the intima and the development of intimal hyperplasia. However, the premise that regrowth of a healthy endothelium will limit neointimal accumulation is inconsistent with the results of several independent lines of investigation. For example, in experimental models smooth muscle cell proliferation is not increased in arterial regions devoid of an endothelium.¹⁵⁶ Moreover, restoration of the endothelium, as might be achieved by seeding endothelial cells back into a denuded artery, does not decrease neointimal accumulation after vascular interventions.¹⁵⁷ Therefore, the presence of an endothelium in the central lumen of an artery and resistance to intimal growth do not appear to be inextricably linked. Finally, it is important to note that the endothelium is not restricted to the central lumen, as the artery wall is also invested with a rich supply of microvessels (i.e., vasa vasorum).^{158,159,160,161} The vasa vasorum are likely another portal of entry for inflammatory cells into the artery wall, particularly since the expression of certain adhesion molecules is more abundant in the endothelium lining these microvessels than that of the central arterial lumen.¹⁶²

Role of Lipoproteins in Lesion Formation

The clinical and experimental evidence linking dyslipidemias with atherogenesis is well established and need not be reviewed here. However, the exact mechanisms by which lipid moieties contribute to the pathogenesis of atherosclerosis remain elusive. Although the simple concept of cholesterol accumulating in artery walls until flow is obstructed may be correct in certain animal models, this theory is not correct for human arteries.

Much of the pioneering work in understanding cholesterol metabolism is based on seminal observations by Goldstein and Brown.¹⁶³ The work of these two investigators focused on low-density lipoprotein (LDL), the so-called bad form of cholesterol, and the absence (or deficient forms) of the LDL receptor that are seen in familial hypercholesterolemia (FH). Patients with FH have high levels of LDL cholesterol and suffer from accelerated forms of atherosclerosis as cholesterol moieties enter the cell via an alternate route. In the absence of a functional LDL receptor, LDL cholesterol is oxidized and taken up by scavenger receptors of monocytes and macrophages resident within the artery wall. Steinberg and others have integrated this data into a theory of atherogenesis that highlights the central role of LDL oxidation and the formation of lipid-laden monocytes in fatty streaks.¹⁶⁴

One of the major consequences of cholesterol accumulation in the artery wall is thought to be the impairment of endothelial function. The endothelium is more than a physical barrier between the blood stream and the artery wall. Under normal conditions, the endothelium is capable of modulating vascular tone (e.g., via nitrous oxide), thrombogenicity, fibrinolysis, platelet function, and inflammation. In the presence of traditional risk factors, particularly dyslipidemias, these protective endothelial functions are reduced or lost. It is important to note that the loss of these endothelial-derived functions may occur in the presence or absence of an underlying atherosclerotic plaque, and may simply imply that atherogenesis has begun. Aggressive attempts to normalize atherosclerotic risk factors (e.g., diet and lipid-lowering therapies) may markedly attenuate endothelial dysfunction—even in the presence of extensive atherosclerosis. A number of clinical studies now demonstrate dramatic improvements in endothelial function, as well as cardiovascular morbidity and mortality, with the use of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, or "statins."^{165,166,167} Future studies may help clarify the exact mechanisms by which dyslipidemias (and other risk factors) alter endothelial function.

Smooth Muscle Cell Proliferation, Migration, and Arterial Remodeling

The dominant cell type in atherosclerotic lesions is the smooth muscle cell, and as lesions progress the number of smooth muscle cells in the artery wall tends to increase. Therefore, smooth muscle replication must occur at some time during atherogenesis. Perhaps the first line of evidence that cell replication occurs in human arteries is from the observation that atherosclerotic plaques contain monoclonal cell populations. Elegant studies by Benditt and Benditt demonstrated that groups (or clones) of cells that arise from a single progenitor cell are present in tissue from atherosclerotic coronary arteries of women who were deficient in glucose-6-phosphate dehydrogenase (G-6-PD).¹⁶⁸ As G-6-PD is an X-chromosome-linked enzyme that has two isoforms, cells would express only one isoform, with the other isoform being suppressed on the inactivated X chromosome. Therefore, groups of cells in an atherosclerotic plaque that contain only one isoform of G-6-PD are likely the result of proliferation of a single progenitor cell. More recently, Murry and colleagues studied the monoclonality of atherosclerotic plaques using X chromosome inactivation patterns.¹⁶⁹ Using the polymerase chain reaction they examined the monoclonality of plaques according to the methylation pattern of the human androgen receptor gene, a highly polymorphic locus on the X chromosome for which 90% of women are heterozygous. These investigators noted that diseased as well as normal arteries contain monoclonal populations (or patches) of cells. Therefore, they speculated that the monoclonality of plaques might be due to expansion of a pre-existing monoclonal patch of cells, rather than mutation or selection of individual cells in the artery wall.

Little is known about when and why cells proliferate in the artery wall. However, it is known that early in life there is a rapid expansion in neointimal smooth muscle cell mass. Sims and Gavin described the accumulation of intimal smooth muscle cells in the left anterior descending coronary artery of neonates.¹⁷⁰ Using electron microscopy, these investigators demonstrated interruptions in the internal elastic lamina in coronary arteries where a neointima had formed.¹⁷¹ These interruptions in the internal elastic lamina are not present in all human arteries. Indeed, the internal mammary artery, which typically is devoid

of atherosclerosis, has an intact internal elastic lamina. Therefore, it has been suggested that medial smooth muscle cells migrate inward through breaks in the internal elastic lamina to expand and form a neointima. The frequency and degree of smooth muscle cell replication in adult coronary arteries have been examined by various investigators. The majority of these studies have demonstrated very low replication rates in tissue from both normal and diseased arteries.^{172,173,174,175} Whether these low cell replication rates are sufficient to gradually result in advanced lesions, or if sporadic bursts of replication occur in response to injury, is unknown. Finally, it is recognized that programmed cell death, or apoptosis, occurs in the artery wall.¹⁷⁶ Therefore, the accumulation of cells in the artery wall is a function of not only cell proliferation but also apoptosis.

The role of smooth muscle cell migration in adult coronary artery disease is poorly understood. It has been suggested, however, that like fibroblasts that migrate into the base of a wound, arterial wall smooth muscle cells migrate inward to expand plaque mass. Smooth muscle cell migration into the intima has been studied in various animal models of neointimal formation (e.g., rat carotid artery model).¹¹ The majority of these models demonstrate the inward migration of medial smooth muscle cells after normal arteries are subjected to balloon injury. A number of growth factors (e.g., platelet-derived growth factor) have been shown to play an important role in facilitating smooth muscle cell migration in these models.^{177,178,179} Unfortunately, the clinical relevance of these experimental observations remains to be clarified, as the milieu for cell migration in complex human lesions appears to be very different from that of normal animal arteries that are subjected to injury. More information is required regarding the factors that regulate smooth muscle cell migration, as well as why smooth muscle cells differ in their propensity to migrate after injury.

Finally, it is important to point out that the buildup of atherosclerotic plaque does not always translate into the formation of arterial obstructions.¹⁴² For example, Glagov noted that human vessels can accumulate massive amounts of atherosclerotic plaque without encroaching on the central arterial lumen. Instead, abluminal expansion of the artery wall may occur until 40% of the area encompassed by the internal elastic lamina is occupied by plaque, thereafter no further enlargement may occur, and luminal narrowing may ensue. While this form of compensatory enlargement is referred to as remodeling the term is confusing as it holds different meaning in different contexts (Fig. 6–13 and 6-14).^{180,181,182} For example, remodeling has also been used to describe the arterial response to changes in blood flow (e.g., during pregnancy or in the neonatal period) or pressure (e.g., hypertension).¹⁸³ In addition, remodeling has been invoked as a key component of the response to arterial injury – however, with quite a different meaning.¹⁸⁴ In animal models of arterial injury as well as studies of human coronary arteries that have undergone angioplasty (or percutaneous transluminal coronary angioplasty, PTCA), “shrinkage” or constrictive remodeling of the artery wall is a major determinant of luminal narrowing, while neointimal formation plays a minor role in this process.^{185,186,187,188,189,190,191,192}

How arterial wall constriction is accomplished or why some but not all arteries undergo compensatory dilatation in order to preserve lumen area is incompletely understood.^{193,194,195} Blood flow and shear stress are known to play a critical role in

remodeling. The response of arteries to chronic alterations in blood flow are endothelium dependent.^{196,197} For example, Langille and O'Donnell demonstrated in rabbit carotid arteries that decreased blood flow results in narrowing of the vessel diameter that is unchanged with papaverine and likely due to structural changes in the artery wall. However, when the endothelium is removed from these vessels the response to reduced blood flow is abolished. In atherosclerotic arteries that contain a rich network of endothelial cell-lined microvessels or vasa vasorum the role of the endothelium in regulating remodeling may be very important.^{198,199,200}

Assessment of Atherosclerosis by Intravascular Ultrasonography

A detailed description of both diagnostic and therapeutic procedures performed in the cardiac catheterization laboratory is given elsewhere in this text. However, given that an integral understanding of the anatomy of the coronary artery and the atherosclerotic lesion is necessary for the appropriate interpretation and use of these technologies, a brief review of new developments in invasive assessment of atherosclerosis by intravascular ultrasonography (IVUS) will be included here.

Standard coronary angiography gives operators a two-dimensional representation of the lumen. By examining arteries in multiple views the operator estimates coronary stenoses by comparison of the lumen diameter at the point of maximal narrowing to adjacent disease free segments. However, as discussed earlier, development of the atherosclerotic plaque results not only in luminal encroachment but also arterial remodeling²⁰¹ – meaning significant disease may be overlooked on traditional angiography. Thus, technologies, such as intravascular ultrasonography, are becoming more prevalent in the assessment of coronary disease.

Intravascular ultrasonography of coronary arteries was first popularized in the 1990's²⁰² with subsequent refinement of catheter delivery systems and commercialization making it now common place in the modern catheterization laboratory. Compatible with most guiding catheters, IVUS probes are delivered to the coronary arteries via standard angiography techniques and both manual and mechanical pullback of the IVUS probe allows operators to assess real-time cross-sectional images. Complementary software can then allow users to generate either longitudinal or three dimensional reconstructions of the interrogated vessel.

IVUS images demonstrate remarkable fidelity to cross-sectional histologic specimens and permit accurate visualization and measurement of the intima, media, and in some instances the adventitia (Figure 6-15). Arterial remodeling with significant intimal hyperplasia but relatively intact lumen diameter can thus identify occult disease not otherwise appreciated on standard angiography. One landmark paper has noted that even if only minor luminal irregularities exist, atherosclerotic disease can be demonstrated throughout most other vessels in the coronary circulation suggesting luminograms may be simply the tip of the atherosclerotic iceberg.²⁰³ Indeed, IVUS is now commonly used to more accurately quantify lesions in cases of intermediate severity lesions²⁰⁴ or in regions that are otherwise difficult to assess on

standard angiography such as left main disease. As well, IVUS allows operators to assess arteries following percutaneous intervention for not only adequate stent deployment but also for complications, such as arterial dissection, which can be missed on standard angiography.²⁰⁵ However, IVUS is not limited to simply documenting and quantifying atherosclerotic burden. Plaque composition can also be assessed qualitatively and classified based on acoustic impedance allowing differentiation between fibromuscular “soft” lesions, dense “fibrous” lesions, and “calcified” hyperechoic lesions.²⁰⁶ Although not yet reliably predicted, ongoing studies are aimed at identifying which plaques are “vulnerable” or susceptible to rupture, thus causing acute vessel closure and myocardial infarction.^{207,208}

PATHOPHYSIOLOGY OF CORONARY BLOOD FLOW

Coronary Artery Stenoses and Plaque Rupture

Coronary atherosclerosis is a chronic disease that develops over decades, remaining clinically silent for prolonged periods of time (Box 6-8). Clinical manifestations of coronary artery disease occur when the atherosclerotic plaque mass encroaches on the vessel lumen and obstructs coronary blood flow causing angina. Alternatively, cracks or fissures may develop in the atherosclerotic lesions and result in acute thromboses that cause unstable angina or myocardial infarction.

BOX 6-8 Pathophysiology of Coronary Blood Flow

- In the majority of patients experiencing a myocardial infarction, the coronary occlusion occurs at the site of <50% stenosis.
- Plaque rupture leads to incremental growth of coronary stenoses and can cause coronary events.
- Plaque rupture occurs at the shoulder of the plaque where inflammatory cells are found.

Patients with stable angina typically have lesions with smooth borders on angiography. Only a minority of coronary lesions are concentric, most having a complex geometry varying in shape over their length. Eccentric stenoses, with a remaining pliable, musculoelastic arc of normal wall, can vary in diameter and resistance in response to changes in vasomotor tone or intraluminal pressure. The majority of human coronary stenoses are compliant.²⁰⁹ The intima of the normal portion of the vessel wall is often thickened, making endothelial dysfunction probable (see Dynamic Stenosis, below). In contrast, patients with unstable angina usually have lesions characterized by overhanging edges, scalloped or irregular borders, or multiple irregularities. These complicated stenoses likely represent ruptured plaque or partially occlusive thrombus or both.²¹⁰ On angiography these lesions may appear segmental, confined to a short segment of an otherwise normal proximal coronary artery. At autopsy, however, the most common pathologic finding is *diffuse* vessel involvement with superimposed segmental obstruction of greater severity.²¹¹ In a diffusely narrowed vessel, even modest progression of luminal narrowing can be significant. In such a patient, rating the significance of the obstruction by the percentage of diameter reduction relative to adjacent vessel segments will underestimate its physiologic importance.^{212,213} Therefore, understanding the characteristics of atherosclerotic plaques is of central importance to the management of acute coronary artery syndromes.

The intuitive notion that the severity of coronary artery stenoses should correlate with the risk of complications from coronary artery disease has been disproved by several key investigations. Ambrose and colleagues reviewed the coronary angiograms of 38 patients who had had a Q-wave myocardial infarct in the interval between serial studies.²¹⁴ On the pre-infarct angiograms, the mean percentage stenosis at the coronary segment that was later responsible for infarction was only 34 percent. Similarly, Little and colleagues reviewed the coronary angiograms of 42 patients who also had this procedure performed at an interval prior to and after myocardial infarction.²¹⁵ Total occlusion of a previously patent artery was observed in 29 patients, yet, for 19 of these occluded arteries, the degree of stenosis was less than 50% on the initial angiogram. Therefore, although the revascularization of arteries with critical stenoses in target lesions is appropriately indicated in order to reduce symptoms and myocardial ischemia, the risk of further cardiac events remains because atherosclerosis is a diffuse process and mild or modest angiographic stenoses are more likely to result in subsequent myocardial infarction than are severe stenoses.

With this background comes the question of predicting which arterial segments with minimal angiographic disease will later develop new critical stenoses? Clues to the answer for this question are emerging from careful pathologic studies of lesions by Davies and Thomas.²¹⁶ Superficial intimal injury (plaque erosions) and intimal tears of variable depth (plaque fissures) with overlying microscopic mural thrombosis are commonly found in atherosclerotic plaques. In the absence of obstructive luminal thrombosis, these intimal injuries do not cause clinical events. However, disruption of the fibrous cap, or plaque rupture, is a more serious event that typically results in the formation of clinically significant arterial thromboses. From autopsy studies it is known that rupture-prone plaques tend to have a thin, friable fibrous cap.²¹⁷ The site of plaque rupture is thought to be the shoulder of the plaque, where substantial numbers of mononuclear inflammatory cells are commonly found.²¹⁸ The mechanisms responsible for the local accumulation of these cells at this location in the plaque are unknown; presumably, monocyte chemotactic factors, the expression of leukocyte cell adhesion molecules, and specific cytokines are involved.^{162,219} Moreover, macrophages in plaques have been shown to express factors such as stromelysin, which promote the breakdown of the extracellular matrix, and thereby weaken the structural integrity of the plaque.¹⁵¹ Currently, no effective strategies have been designed to limit the possibility of plaque rupture; however, as discussed below, aggressive lipid-lowering therapy may be a helpful preventative measure.

Hemodynamics

If accurate angiographic assessment of the geometry of a coronary stenosis is made, hydrodynamic principles can be used to estimate the physiologic significance of the obstruction.²²⁰ Energy is lost when blood flows through a stenosis because of entrance effects, frictional losses in the stenotic segment, and separation losses due to turbulence as blood exits the stenosis (Fig. 6-16). The equation relating stenosis geometry to hemodynamic severity is:

$$\Delta P = fQ + sQ^2$$

where ΔP is the pressure drop across the stenosis, Q is the volume flow of blood, f is a factor accounting for frictional effects, and s accounts for separation effects. Based on the Poiseuille law for laminar flow:

$$f = 8 \pi \eta L A_n / A_s^2$$

where π is the blood viscosity, L is stenosis length, A_n is the cross-sectional area of the normal vessel and A_s is the cross-sectional area of the stenosis. The separation or turbulence factor

$$s = \rho k / 2 (A_n / A_s - 1)^2$$

where ρ is blood density and k is an experimentally determined coefficient. Thus, frictional losses are directly proportional to the first power of stenosis length but are inversely proportional to the square of the area (or fourth power of diameter). Separation losses are particularly prominent because they increase with the square of flow. Even at resting flows, more than 75% of energy loss is due to this turbulence when blood exits the stenosis. Except for very long stenoses the frictional term can be neglected.²⁰⁹ Thus, the amount of energy loss or pressure drop across the obstruction increases exponentially as flow rate increases. For this reason, exercise, anemia, and arteriolar vasodilator drugs (e.g., dipyridamole) are poorly tolerated in the presence of a severe stenosis. Figure 8–17 illustrates that although resting flow is unaffected until coronary diameter is reduced by more than 80%, maximal flow begins to fall when diameter is reduced by 50%.

Resting flow in Figure 6-17 remains constant as lumen diameter decreases because the coronary arterioles progressively dilate, thereby reducing the resistance of the distal coronary bed sufficiently to compensate for the resistance of the stenosis. As the severity of the stenosis increases further, the arteriolar bed can no longer compensate and flow begins to fall. This is an example of autoregulation: as stenosis severity increases, distal perfusion pressure falls, arterioles dilate to maintain flow until autoregulation is exhausted (in the subendocardium first) and flow becomes pressure dependent. As illustrated in Figure 6–9, the distal pressure (or stenosis diameter) at which flow becomes pressure dependent is lower at low levels of myocardial metabolism (MVO_2). The interpretation of normal resting flow can be difficult in the presence of coronary artery disease. A coronary artery supplying blood through collaterals to a large mass of myocardium will require high resting flow rates and even a mild stenosis may be flow limiting.

The term *critical stenosis* is frequently used. This is usually defined as a coronary constriction sufficient to prevent an increase in flow over resting values in response to increased myocardial oxygen demands.²²¹ This is a greater degree of obstruction than an angiographically significant stenosis, which is usually defined as a reduction in cross-sectional area of 75%, which is equivalent to a 50% decrease in the diameter of a concentric stenosis.²¹² A critical stenosis is demonstrated experimentally by blunting or abolishing reactive hyperemia (see Autoregulation). This is evidence that autoregulation has been exhausted in at least the inner layer of myocardium (see Transmural Blood Flow). It

should be noted that the critical nature of the stenosis is relative to the resting MVO_2 . If oxygen demand decreases, some coronary autoregulatory reserve will be recovered and the stenosis will no longer be critical. The failure to recognize this fact has led to misinterpretation of studies designed to demonstrate coronary steal (see later).

Coronary Collaterals

Coronary collaterals are anastomotic connections, without an intervening capillary bed, between different coronary arteries or between branches of the same artery. In the normal human heart these vessels are small and have little or no functional role. In patients with coronary artery disease well-developed coronary collateral vessels may play a critical role in preventing death and myocardial infarction. Individual differences in the capability of developing a sufficient collateral circulation is a determinant of the vulnerability of the myocardium to coronary occlusive disease.²²² There is great interspecies variation in the ability of the collateral circulation to support myocardial perfusion after acute coronary occlusion: Pigs and rats have very little collateral circulation and infarct almost all the area at risk whereas dogs and cats with better collateralization will infarct less than 75% of the area at risk.²²³ In the guinea pig, collaterals are so well developed that coronary occlusion does not even decrease myocardial blood flow. There are also differences in the location of collateral vessels: in dogs collaterals develop in a narrow subepicardial zone, at the border of the potentially ischemic region, whereas in pigs a dense subendocardial plexus develops in response to coronary occlusion. In the presence of coronary disease, humans exhibit a small number of large epicardial collateral vessels and numerous small subendocardial vessels.

In response to coronary occlusion, native coronary collateral vessels do not passively stretch but undergo an active growth process that within 8 weeks, in the dog, can restore perfusion sufficient to support normal myocardial function even during exercise. Human collaterals have a tortuous corkscrew-like pattern visible on angiography. This may be due to an embryonal pattern of vascular development where longitudinal growth of smooth muscle cells occurs at the same time as radial growth. In the non-growing adult heart this increase in length results in tortuosity.²²⁴ There is much interest in discovering the factors that control collateral vessel growth in the hope of providing therapy for patients who cannot be revascularized otherwise. Arteriogenesis refers to the transformation of preexisting collateral arterioles into functional arteries with a thick muscular coat and the acquisition of viscoelastic and vasomotor properties.²²⁵ Fujita and Tambara²²⁶ provide an overview of the process: a high-grade coronary stenosis decreases distal intra-arterial pressure resulting in an increased pressure gradient across the pre-existing collateral network. Increased collateral blood flow results in increased shear stress at the endothelium which upregulates cell adhesion molecules. This leads to adherence of monocytes which transform into macrophage and the production and release of growth factors such as granulocyte macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), and basic fibroblast growth factor (bFGF). Angiogenesis is not directly related to collateral vessel development but refers to the proliferation, migration, and tube formation of capillaries in the central area of ischemic regions.²²⁷ The development of a treatment to promote collateral growth in patients with intractable coronary artery disease is currently a subject of intense investigation.

Evidence in dogs suggests that mature coronary collaterals respond differently to neurohumoral stimulation than normal coronary arteries. Collaterals do not constrict in response to α -receptor activation, but do dilate in response to β_1 - or β_2 -agonists. They constrict in response to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and angiotensin II, but less so than normal vessels. Remarkably, collateral vessels constrict in response to vasopressin to a much greater extent than normal vessels. In vivo studies in dogs indicate that levels of vasopressin present during stress (hemorrhage, cardiopulmonary bypass) can diminish flow to collateral-dependent myocardium. This is likely due both to constriction of collateral vessels as well as enhanced vasoconstriction of the resistance vessels in the collateral dependent myocardium.⁷⁹ It is possible that the endothelial cells of both types of vessel are dysfunctional.²²⁸ Relaxation in response to nitroglycerin was enhanced. This is a further mechanism for the beneficial effects of nitroglycerin in coronary artery disease. The deleterious effects of coronary arteriolar dilators such as adenosine and dipyridamole are discussed below (see Coronary Steal).

It has been estimated that, in humans, perfusion via collaterals can equal perfusion via a vessel with a 90% diameter obstruction.²²⁹ Although coronary collateral flow can be sufficient to preserve structure and resting myocardial function, muscle dependent on collateral flow usually becomes ischemic when oxygen demand rises above resting levels.²³⁰ It is possible that evidence from patients with angina underestimates collateral function of the population of all patients with coronary artery disease. Perhaps individuals with coronary obstructions but excellent collateralization remain asymptomatic and are not studied.

Pathogenesis of Myocardial Ischemia

Ischemia is the condition of oxygen deprivation accompanied by inadequate removal of metabolites consequent to reduced perfusion.²³¹ Clinically, myocardial ischemia is a decline in the blood flow supply/demand ratio resulting in impaired function. There is no universally accepted "gold standard" for the presence of myocardial ischemia. In practice, symptoms, anatomic findings, and evidence of myocardial dysfunction must be combined before concluding that myocardial ischemia is present.²³² Conclusive evidence of anaerobic metabolism in the setting of reduced coronary blood flow (relative to demand) would be convincing. Such evidence is extremely difficult to obtain, even in experimental preparations.

Determinants of Myocardial Oxygen Supply/Demand Ratio

An increase in myocardial oxygen requirement beyond the capacity of the coronary circulation to deliver oxygen results in myocardial ischemia (Box 6-9). This is the most common mechanism leading to ischemic episodes in chronic stable angina and during exercise testing. Intraoperatively the anesthesiologist must measure and control the determinants of myocardial oxygen consumption and protect the patient from "demand" ischemia. The major determinants of myocardial oxygen consumption are heart rate, myocardial contractility, and wall stress (chamber pressure $\times$ radius/wall thickness).

Shortening, activation, and basal metabolic requirements are minor determinants of MVO_2 (Fig. 6-18).

An increase in heart rate can reduce subendocardial perfusion by shortening diastole. Coronary perfusion pressure may fall due to reduced systemic pressure or increased left ventricular end-diastolic pressure. With the onset of ischemia, perfusion may be further compromised by delayed ventricular relaxation (decreased subendocardial perfusion time) and decreased diastolic compliance (increased left ventricular end-diastolic pressure). Anemia and hypoxia can also compromise delivery of oxygen to the myocardium. Several indices of myocardial oxygen supply/demand ratio have been proposed to guide therapy. The rate-pressure product (heart rate $\times$ systolic blood pressure) gives a good estimate of MVO_2 but does not correlate well with ischemia. A patient with a systolic pressure of 160 and heart rate of 70 has a much lower likelihood of ischemia than a patient with a pressure of 70 and rate of 160 although both have a rate-pressure product of 11,200. The ratio of the diastolic pressure–time index (DPTI) to the systolic pressure–time index (SPTI) was devised to estimate subendocardial perfusion and takes into account determinants of oxygen delivery (Fig. 6-19).^{233,234,235} When blood oxygen content was included, the index became a good predictor of endocardial flow in animals with normal coronary arteries. More recently the ratio of mean arterial pressure/heart rate has been proposed as a correlate of myocardial ischemia.²³⁶ In dogs with moderate to severe coronary stenoses, systolic shortening was best with high pressures and low heart rate and worst with low pressure and high heart rate. None of these indices has proven to be reliable in the clinical setting. Their major value is to bring attention to the important variables determining the supply/demand ratio. These variables should be measured (or estimated) and controlled individually.

BOX 6–9 Determinants of Myocardial Oxygen Supply/Demand Ratio

The major determinants of myocardial oxygen consumption are

- Heart rate
- Myocardial contractility
- Wall stress (chamber pressure $\times$ radius/wall thickness)

Dynamic Stenosis

Patients with coronary artery disease can have variable exercise tolerance during the day and between days. Ambulatory monitoring of the electrocardiogram has demonstrated that ST-segment changes indicative of myocardial ischemia, in the absence of changes in oxygen demand, are common.²³⁷ These findings are explained by variations over time in the severity of the obstruction to blood flow imposed by coronary stenoses.

Although the term *hardening of the arteries* suggests rigid, narrowed vessels, in fact most stenoses are eccentric and have a remaining arc of compliant tissue (Fig. 6-20). A modest amount (10%) of shortening of the muscle in the compliant region of the vessel can cause dramatic changes in lumen caliber.²⁰⁹ This was part of Prinzmetal's original proposal to explain coronary spasm. Maseri suggests that the term *spasm* should be reserved for "situations where coronary constriction is both focal, sufficiently profound to cause transient coronary occlusion, and is responsible for reversible attacks of angina at rest" (i.e., variant

angina).²³⁸ Although this syndrome is rare, lesser degrees of obstruction in response to vasoconstrictor stimuli are very common among patients with coronary artery disease.

Sympathetic tone can be increased by the cold pressor test (immersing the arm in ice water), or isometric handgrip testing. In response to this maneuver coronary resistance decreased in normal subjects but increased in patients with coronary disease, some of whom experienced angina.²³⁹ This increase in resistance appears to be mediated by α -receptors because it can be prevented by phentolamine. Studies using quantitative coronary angiography have documented reductions in caliber in diseased vessels in contrast to dilation of vessels in normal subjects.^{98,240} Zeiher et al showed that the same vessel segments that constricted with the cold pressor test also constricted in response to an infusion of acetylcholine.²⁴⁰ Because the normal, dilatory, response to acetylcholine is dependent on intact endothelium, these findings suggest that the abnormal response of stenotic coronary arteries is due to endothelial dysfunction.

Animal models of coronary vasospasm demonstrate that enhanced vascular smooth muscle reactivity may also underly vasospasm.²⁴¹ Rho, a GTP-binding protein, sensitizes vascular smooth muscle cells to calcium by inhibiting myosin phosphatase activity through an effector protein called Rho-kinase. Upregulation of this pathway may be a mechanism of coronary vasospasm. Interestingly, Rho-kinase inhibitors have been shown to block agonist-induced vasoconstriction of internal thoracic artery segments from patients undergoing coronary artery surgery.²⁴²

It has also been noted that, in patients with coronary disease, some of the angiographically normal appearing segments also respond abnormally.²⁴³ It seems likely that, during the development of coronary atherosclerosis, endothelial dysfunction precedes the appearance of visible stenoses. In patients with angiographically smooth coronary arteries, Vita et al. found that an abnormal response to acetylcholine was correlated with serum cholesterol, male gender, age, and family history of coronary disease.²⁴⁴ The normal dilation of epicardial coronary arteries in response to increased blood flow (shear stress) has been shown to be absent in atherosclerotic vessels.²⁴⁵ As well, it has been demonstrated that patients with coronary disease respond to serotonin with coronary vasoconstriction instead of the normal vasodilatory response.^{246,247} The concentrations of serotonin used were within the range found in coronary sinus blood of patients with coronary disease. Very high concentrations of serotonin may be found on the endothelium at the site of aggregating platelets.²⁴⁶ All these findings point to the central role of endothelial dysfunction in the abnormal coronary vasomotion of patients with atherosclerosis.²⁴⁸ (See Endothelium).

Coronary Steal

Steal occurs when the perfusion pressure for a vasodilated vascular bed (in which flow is pressure dependent) is lowered by vasodilation in a parallel vascular bed, both beds usually being distal to a stenosis. Two kinds of coronary steal are illustrated: collateral and transmural (Fig. 6-21).

Collateral steal in which one vascular bed (R_3), distal to an occluded vessel, is dependent on collateral flow from a vascular bed (R_2) supplied by a stenotic artery is shown (Fig 6-21A). Because collateral resistance is high, the R_3 arterioles are dilated to maintain flow in the resting condition (autoregulation). Dilation of the R_2 arterioles will increase flow across the stenosis R_1 and decrease pressure P_2 . If R_3 resistance cannot further decrease sufficiently, flow there will fall, producing or worsening ischemia in the collateral-dependent bed. The values of all the resistances, including collaterals, and the baseline myocardial metabolic state will determine how powerful the vasodilator stimulus must be to produce ischemia in the collateral bed. Failure to recognize this has confounded studies of vasodilator drugs. If collateral vessels are very well developed or MVO_2 is low, sufficient autoregulatory reserve may remain in the collateral-dependent bed to maintain adequate myocardial blood flow even with the administration of a moderately powerful vasodilator.

Transmural steal is also illustrated (Fig 6-21B). Normally, vasodilator reserve is less in the subendocardium (see Transmural Blood Flow). In the presence of a stenosis, flow may become pressure dependent in the subendocardium while autoregulation is maintained in the subepicardium. This is illustrated in Figure 6-11, where at a perfusion pressure of 50 mmHg, flow has fallen in the subendocardium while the subepicardium retains autoregulatory reserve. Dilation of the subepicardial arterioles, R_2 , will then increase flow across the stenosis (R_1) causing P_2 to fall and resulting in decreased flow to the subendocardium as subepicardial flow increases.

The term *steal* is most appropriate when the vasodilation is caused by a pharmacologic agent (adenosine, dipyridamole) producing "luxury" flow (beyond metabolic requirements) in the vascular bed with coronary reserve (R_2). The same redistribution of blood flow also occurs during exercise in response to metabolically mediated vasodilation. The study of coronary steal demonstrates well the complex interrelationships among the determinants of myocardial blood flow.

FUTURE DIRECTIONS

There is a major need to not only identify but also treat the vulnerable nonstenotic plaque that is prone to rupture. Although angiography is ideal for imaging the lumen of arteries, it provides little information about the atherosclerotic process within the vessel wall. Unfortunately, newer imaging techniques, such as intravascular ultrasound and electron beam computer tomography, have yet to evolve as practical predictive modalities that can be used to manage patients at risk for acute coronary syndromes secondary to plaque rupture.

Interestingly, clues to the biology of plaque rupture are indirectly emerging from clinical trials with statins, in that these trials show very modest reductions in the angiographic degree of vessel stenoses but consistently demonstrate a significant decrease in acute ischemic events well before the effects of LDL cholesterol lowering should appear. For example, the MIRACL trial demonstrated that treating only 38 patients with acute coronary syndromes with high dose Atorvastatin could prevent one

recurrent infarction, refractory angina, or death as early as thirty days following the index event.²⁴⁹ Similarly, the ARMYDA²⁵⁰, ARMYDA-ACS²⁵¹, and ARMYDA RECAPTURE²⁵² studies have demonstrated that acute treatment with Atorvastatin in patients undergoing percutaneous coronary interventions results in reductions in periprocedural myocardial infarctions when started just days before the procedure. Certainly given the impressive clinical benefits there is much interest in exploring the pleiotrophic effects of statins that extend beyond lipid lowering and understanding how plaque stabilization is occurring.^{253,254}

Most recently there has become a renewed focus on the adventitia of the coronary artery and its role in initiation of inflammation in the vessel wall and subsequent development of atherosclerosis.^{255,12} While much effort has been devoted to studying the intima and media in development of vascular lesions, the adventitia is just now garnering the focus it may deserve. The adventitia is unique in that it houses the vaso vasorum which provides nutrients, vasoactive factors and acts as a portal of entry for inflammatory cells into the media and intima of epicardial coronary arteries. As well, the adventitia is unique in that it alone supplies all neural input to the vessel wall – an input that has been implicated in plaque progression and destabilization.²⁵⁶ Supporting this notion of an “outside-in” hypothesis to the development of atherosclerosis is that changes in the adventitial vaso vasorum often precede intimal changes.²⁵⁷ In addition to atherosclerosis, the adventitia has also been implicated as a source of cells in neointimal development following vascular injury such as balloon angioplasty.²⁵⁸ Given the dynamic nature of the adventitia in disease and the unique role it serves in vessel homeostasis our understanding of adventitial biology is lacking.

There is also a growing need to better understand the role of vascular progenitor cells in both arterial repair²⁵⁹ and lesion formation following vascular injury.²⁶⁰ Studies suggest that endothelial and smooth muscle cells appear to be derived from multiple sources such as circulating stem and progenitor cells, as well as tissue-resident progenitor cell populations.^{259,260,261} A number of observational clinical studies have inversely correlated endothelial progenitor cell (EPC) number and cardiovascular risk fueling the hypothesis that impaired progenitor cell mediated repair of arteries is a risk factor for atherosclerosis and clinical events.^{262,263} Moreover, reports of the involvement of vascular progenitor cells in the pathogenesis of other vascular lesions are also emerging. For example, observations from organ transplantations highlight the involvement of a blood-borne population of human vascular cells in development of transplant arteriosclerosis.²⁶⁴ Circulating EPC levels have also been found to be fewer or have impaired adhesion capacity in patients who develop in-stent restenosis compared with patients with patent stents or an absence of CAD.^{265,266} Hence, taken together, the emerging data suggest that vascular progenitor cells may play a critical role, not only in maintaining the artery wall but also in ensuring that appropriate repair mechanisms occur in the face of injury. Therapeutic strategies that target EPC mobilization, homing, and differentiation may prove beneficial. Already early clinical trials have suggested potential benefit of progenitor cell transplantation following myocardial infarction in improving left ventricular ejection fraction²⁶⁷ and a combined clinical endpoint of death, recurrent myocardial infarction or revascularization.²⁶⁸ While these early clinical studies are promising, significant progress in our understanding the role vascular progenitors play in

the pathogenesis of a wide variety of vascular lesions remains hampered by the absence of a clear definition of what constitutes a true vascular stem cell.²⁶⁹

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Figure 6–1 Pressure drop through the hamster cheek pouch circulation illustrates the resistance and nomenclature of various portions of the vascular bed. The important contribution of small arteries to vascular resistance is clearly shown here. Similar observations have been made in the coronary circulation. MAP = mean arterial pressure; VP = venous pressure. Error bars indicate SE. (From Davis MJ, Ferrer PN, Gore RW: Vascular anatomy and hydrostatic pressure profile in the hamster cheek pouch. *Am J Physiol* 250:H291, 1986.)

Figure 6–2 Normal human coronary artery of a 32-year-old woman. The intima (i) and media (m) are composed of smooth muscle cells. The adventitia (a) consists of a loose collection of adipocytes, fibroblasts, vasa vasorum, and nerves. The media is separated from the intima by the internal elastic lamina (*open arrow*) and the adventitia by the external elastic lamina (*closed arrow*). (Movat's pentachrome-stained slide, original magnification $\times 6.6$.)

Figure 6–3 Steps in the process whereby hormone-receptor binding results in a change in cell behavior. In this example, the final result is the opening of an ion channel. (A) A hormone or ligand (L) binds to a receptor (R) embedded in the cell membrane. The receptor-ligand complex interacts with G protein (G) floating in the membrane, resulting in activation of the α subunit (G_α). The activated α subunit can then follow different pathways (B). Effector enzymes in the membrane (E), such as adenylyl cyclase, cyclic guanosine monophosphate (cGMP), phospholipase C, or phospholipase A₂, change the cytoplasmic concentration of their “messengers”: cyclic adenosine monophosphate (cAMP), cGMP, diacylglycerol (DAG), and inositol-1,4,5-triphosphate (IP₃). These soluble molecules activate protein kinase A or C (PKA, PKC), or release Ca^{++} from sarcoplasmic reticulum (SR). Subsequently, cell behavior is changed by phosphorylation of an ionic channel on the cell membrane (CHAN) or by release of Ca^{++} from SR. (B) Several pathways coupling receptor activation to final effect are illustrated. It is likely that multiple pathways are activated concomitantly, both facilitatory and inhibitory. In this way, the final response can be determined by the sum of the effects of several stimuli. (A, B, From Brown AM, Birnbaumer L: Ionic channels and their regulation by G-protein subunits. *Annu Rev Physiol* 52:197, 1990.)

Figure 6–4 The production of endothelium-derived vasodilator substances. Prostacyclin (PGI₂) is produced via the cyclooxygenase pathway of arachidonic acid (AA) metabolism, which can be blocked by indomethacin (Indo) and aspirin. PGI₂ stimulates smooth muscle adenylyl cyclase and increases cyclic adenosine monophosphate (cAMP) production, which cause relaxation. Endothelium-derived relaxing factor (EDRF), now known to be nitric oxide (NO), is produced by the action of NO synthase on L-arginine in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxygen (O₂), and calcium and calmodulin. This process can be blocked by arginine analogs like NG-monomethyl-L-arginine (LNMA). NO combines with guanylate cyclase in the smooth muscle cell to stimulate production of cyclic guanosine monophosphate (cGMP), which results in relaxation. Less well characterized is an endothelium-derived factor, which hyperpolarizes the smooth muscle membrane (EDHF) and probably acts via activation of potassium (K⁺) channels. ACh = acetylcholine; M = muscarinic receptor; 5-HT = serotonin; ADP = adenosine diphosphate; P = purinergic receptor; T = thrombin receptor. (From Rubanyi GM: Endothelium, platelets, and coronary vasospasm. *Coron Artery Dis* 1:645, 1990.)

Figure 6–5 The role of endothelium in the control of coronary tone. Intact endothelium has an important modulatory role in the effect of numerous factors on vascular smooth muscle. In the absence of a functional endothelium (mechanical trauma, atherosclerosis), many factors act directly on smooth muscle to cause constriction (*left side*). Under normal conditions (*right side*), the release of NO (endothelium-derived relaxing factor [EDRF]) and prostacyclin (PGI₂) stimulated by these same factors can attenuate constriction or cause dilation. PGI₂ release is predominantly into the lumen, whereas EDRF release is similar on both the luminal and abluminal sides. Substances in parentheses elicit only vasodilation. ADP = adenosine monophosphate; ATP = adenosine triphosphate; 5-HT = serotonin; PAF = platelet-activating factor; ACh = acetylcholine; BK = bradykinin; SP = substance P; VIP = vasoactive intestinal polypeptide; CGRP = calcitonin gene-related peptide; AII = angiotensin II; VP = vasopressin; NA =

norepinephrine; A = adenosine; ET = endothelin. (From Bassenge E, Heusch G: Endothelial and neurohumoral control of coronary blood flow in health and disease. *Rev Physiol Biochem Pharmacol* 116:77, 1990.)

Figure 6–6 Endothelin (ET) released abluminally interacts with ET_A and ET_B receptors on vascular smooth muscle to cause contraction. Activators of ET_B receptors on endothelial cells cause vasodilation. ECE = endothelin-converting enzyme; PGI₂ = prostacyclin; cGMP = cyclic guanosine monophosphate; cAMP = cyclic adenosine monophosphate. (Reprinted from Luscher TF: Do we need endothelin antagonists? *Cardiovasc Res* 29:2089, 1997. Reproduced with permission of Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, the Netherlands.)

Figure 6–7 Inhibition of platelet adhesion and aggregation by intact endothelium. Aggregating platelets release adenosine diphosphate (ADP) and serotonin (5-HT), which stimulate the synthesis and release of prostacyclin (PGI₂) and endothelium-derived relaxing factor (EDRF; nitric oxide [NO]), which diffuse back to the platelets and inhibit further adhesion and aggregation and can cause disaggregation. PGI₂ and EDRF act synergistically by increasing platelet cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively. By inhibiting platelets and also increasing blood flow by causing vasodilation, PGI₂ and EDRF can flush away microthrombi and prevent thrombosis of intact vessels. P_{2y} = purinergic receptor. (From Rubanyi GM: Endothelium, platelets, and coronary vasospasm. *Coron Artery Dis* 1:645, 1990.)

Figure 6–8 Blood flow in the left and right coronary arteries. The right ventricle is perfused throughout the cardiac cycle. Flow to the left ventricle is largely confined to diastole. (From Berne RM, Levy MN: Special circulations. *In* Berne RM, Levy MN [eds]: *Physiology*. St. Louis, CV Mosby, 1988, pp 540–560.)

Figure 6–9 Autoregulation at two levels of myocardial oxygen consumption. Pressure in the cannulated left circumflex artery was varied independently of aortic pressure. When pressures were suddenly raised or lowered from 40mmHg, flow instantaneously increased with pressure (*steep line, open triangles*). With time, flow decreases to the steady-state level determined by oxygen consumption (*open and closed circles*). The vertical distance from the steady-state (autoregulating) line to the instantaneous pressure-flow line is the autoregulatory flow reserve (see text). (From Mosher P, Ross J Jr, McFate PA, Shaw RF: Control of coronary blood flow by an autoregulatory mechanism. *Circ Res* 14:250, 1964.)

Figure 6–10 Schematic diagram of the reactive hyperemic response to a 10-second coronary occlusion. (From Marcus ML: Metabolic regulation of coronary blood flow. *In* Marcus ML [ed]: *The Coronary Circulation in Health and Disease*. New York, McGraw-Hill, 1983, pp 65–92. Reproduced with permission of The McGraw-Hill Companies.)

Figure 6–11 Pressure-flow relationships of the subepicardial and subendocardial thirds of the left ventricle in anesthetized dogs. In the subendocardium, autoregulation is exhausted and flow becomes pressure dependent when pressure distal to a stenosis falls below 70mmHg. In the subepicardium, autoregulation persists until perfusion pressure falls below 40mmHg. Autoregulatory coronary reserve is less in the subendocardium. (Redrawn from Guyton RA, McClenathan JH, Newman GE, Michaelis LL: Significance

of subendocardial ST segment elevation caused by coronary stenosis in the dog. *Am J Cardiol* 40:373, 1977.)

Figure 6–12 Atherosclerotic human coronary artery of an 80-year-old man. There is severe narrowing of the central arterial lumen (L). The intima consists of a complex collection of cells, extracellular matrix (M), and a necrotic core with cholesterol (C) deposits. Rupture of plaque microvessels has resulted in intraplaque hemorrhage (*arrow*) at the base of the necrotic core. (Movat's pentachrome-stained slide, original magnification $\times 40$.)

Figure 6–13 Arterial remodeling. Serial sections, proximal (A), mid (B), and distal (C), of an atherosclerotic human left circumflex coronary artery (Movat's pentachrome-stained slide, original magnification $\times 40$). There is narrowing of the central arterial lumen in the mid and distal sections; however, the total arterial area of these sections is also larger than that of the proximal section. The ability of arteries to undergo compensatory enlargement is referred to as *arterial remodeling*.

Figure 6–14 A model of atherogenesis in human coronary arteries. **A** – Normal coronary artery **B** – Infiltration of the intima by particles containing low-density lipoproteins (LDL) stimulates expression of adhesion molecules on the luminal surface of the endothelium **C** – Monocyte/macrophage translocation into the intima. Once translocation is complete, uptake of LDL via scavenger receptors on macrophages gives rise to foam cells which secrete proinflammatory cytokines, such as interleukin -1 **D** – Further inflammation promotes division and migration of medial and/or adventitial smooth muscle cells with ongoing accumulation of foam cells and extracellular matrix resulting in intimal thickening. **E** – Local cell signaling via paracrine factors can lead to regional apoptosis and development of a necrotic core with accumulation of cholesterol deposits.

Figure 6–15 Intravascular ultrasonography (IVUS) for assessment of human coronary arteries. **A** – A right anterior oblique projection of the left coronary circulation. The left main artery (LM), left anterior descending artery (LAD), circumflex artery (Cx), and obtuse marginal artery (OM) are seen. A severe distal LM stenosis is seen bifurcating into the LAD and Cx. **B** – Post percutaneous intervention angiogram demonstrates no residual stenosis. Drug eluting stents were placed in the left main, LAD, and Cx. The tip of an aortic balloon pump can be seen (*arrow*) Letters indicate location of IVUS images for correlating panels. **C,D,E** IVUS images of LM, LM bifurcation and LAD respectively. IP – IVUS probe, In – intima, and M - media. * indicates guide wire. Arrows indicate stent struts.

Figure 6–16 Sources of energy loss across a stenosis. Equations that (accurately) predict the pressure gradient across a stenosis usually ignore entrance effects. Frictional losses are proportional to blood velocity but are usually not important except in very long stenoses. Separation losses, due to turbulence as blood exits the stenosis, increase with the square of blood velocity and account for more than 75% of energy loss. V = blood velocity; F = friction coefficient (Poiseuille); S = separation coefficient (see text). (From Marcus ML: The physiologic effects of a coronary stenosis. *In* Marcus ML [ed]: The Coronary Circulation in Health and Disease. New York, McGraw-Hill, 1983, pp 242-269. Reproduced with permission of The McGraw-Hill Companies.)

Figure 6–17 Effect of increasing stenosis severity at resting and maximal coronary flows. At rest, lumen diameter must be reduced by more than 80% before flow decreases. Because pressure drop across a stenosis increases exponentially with blood velocity,

maximal coronary flow is restricted by a 50% diameter reduction. (From Gould KL, Lipscomb K: Effect of coronary stenoses on coronary flow reserve and resistance. *Am J Cardiol* 34:48, 1974.)

Figure 6–18 Relative importance of variables that determine myocardial oxygen consumption (MVO_2). Each line roughly approximates the effect of manipulating one variable without changing the others. Most interventions cause changes in several of the variables at the same time. The importance of contractility, which is difficult to monitor in practice, is apparent. (From Marcus ML: Metabolic regulation of coronary blood flow. *In* Marcus ML [ed]: *The Coronary Circulation in Health and Disease*. New York, McGraw-Hill, 1983, pp 6592. Reproduced with permission of The McGraw-Hill Companies.)

Figure 6–19 Three indices, proposed to predict the adequacy of subendocardial perfusion in normal dogs, illustrate the variables determining myocardial oxygen supply and demand. The systolic pressure-time index (SPTI) relates to oxygen demand. The diastolic pressure-time index (DPTI) relates to the supply of coronary blood flow (CBF) to the inner layers of the left ventricle. Arterial oxygen content (O_2 content) is important when there are large changes in hematocrit. Ao = aortic pressure; LV = left ventricular pressure; ENDO = subendocardial layer of left ventricle; EPI = subepicardial layer of the left ventricle. (From Hoffman JIE, Buckberg GD: Transmural variations in myocardial perfusion. *In* Yu PN, Goodwin JF [eds]: *Progress in Cardiology*. Philadelphia, Lea & Febiger, 1976, p 37.)

Figure 6–20 Drawings and incidence of the various types of structure of stenoses observed in human coronary artery specimens. In almost three quarters of vessels with greater than 50% narrowing, the residual arterial lumen was eccentric and partially circumscribed by an arc of normal arterial wall. In such lesions, a fall in intraluminal pressure or an increase in vasomotor tone can cause lumen diameter to decrease further, sufficiently to precipitate myocardial ischemia. (From Brown BG, Bolson EL, Dodge HT: Dynamic mechanisms in human coronary stenosis. *Circulation* 70:917, 1984; redrawn from Freudenberg H, Lichtlen PR: The normal wall segment in coronary stenoses—a postmortem study. *Z Kardiol* 70:863, 1981.)

Figure 6–21 Conditions for coronary steal between different areas of the heart (collateral steal [A]) and between the subendocardial and the subepicardial layers of the left ventricle (transmural steal [B]). See text for detailed description. R_1 = stenosis resistance; P_1 = aortic pressure; P_2 = pressure distal to the stenosis; R_2 and R_3 = resistance of autoregulating and pressure-dependent vascular beds, respectively. (From Epstein SE, Cannon RO, Talbot TL: Hemodynamic principles in the control of coronary blood flow. *Am J Cardiol* 56:4E, 1985.)

Table 6–1 Substances Produced by Vascular Endothelium

Antithrombotic Substances	Procoagulants
Prostacyclin	von Willebrand factor
Antithrombin III	Collagen
Plasminogen activator	Fibronectin

Protein C	Thromboplastin
α_2 -Macroglobulin	Thrombospondin
Glycosaminoglycans (heparin)	Plasminogen inhibitors Platelet-activating factor Thromboxane A ₂

From Bassenge E, Busse R: Endothelial modulation of coronary tone. Prog Cardiovasc Dis 30:349, 1988.

Table 6–2 Vasoactive Substances Processed by Vascular Endothelium

Uptake and Metabolism	Enzymatic Conversion or Degradation
Norepinephrine	Angiotensin I to angiotensin II (ACE)
Serotonin	Angiotensin II to angiotensin III (angiotensinase)
Prostaglandins (E ₁ , E ₂ , E ₂ α)	Bradykinin degradation (ACE)
Leukotrienes	Substance P degradation
Adenosine	

ACE = angiotensin-converting enzyme.

From Bassenge E, Busse R: Endothelial modulation of coronary tone. Prog Cardiovasc Dis 30:349, 1988.

Table 6–3 Stimulators of Endothelium-Mediated Vasodilation

Transmitters	ADP
Acetylcholine	Adenosine
Norepinephrine	Serotonin
Peptides	Thrombin
Angiotensin	Trypsin
Bradykinin	Local Hormones
Vasopressin	Histamine
Oxytocin	Platelet-activating factor
Substance P	Physicochemical Stimuli
Vasoactive intestinal peptide	Shear stress (flow)
Calcitonin gene-related peptide	Mechanical stress (pulsatility)
Platelet or Blood Components	Hypoxia
ATP	

ATP = adenosine triphosphate; ADP = adenosine diphosphate.

Modified from Bassenge E, Busse R: Endothelial modulation of coronary tone. Prog Cardiovasc Dis 30:349, 1988.

Table 6–4 Classification of α -Adrenergic Receptor Subtypes in the Heart*

Selective Agonists	Selective Antagonists	Effects of Activation
α_1		
Phenylephrine release	Prazosin	<i>Presynaptic:</i> feedback inhibition of norepinephrine
Methoxamine myocardial inotropism,		<i>Postsynaptic:</i> coronary vasoconstriction, increase in arrhythmias
α_2		
Clonidine release	Yohimbine	<i>Presynaptic:</i> feedback inhibition of norepinephrine

Azepexole
BHT 920
UK 14, 304

Rauwolscine
Idazoxan

Postsynaptic: coronary vasoconstriction, arrhythmias (?)

Modified from Heusch G: Alpha-adrenergic mechanisms in myocardial ischemia. Circulation 81:1, 1990, by permission of the American Heart Association.

*Norepinephrine is a nonselective agonist. Phentolamine and phenoxybenzamine are nonselective antagonists. Phenylephrine also causes β -receptor activation.

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9.1 Curriculum Vitae



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PUBLICATIONS

1. Le May, M.R., Wells, G.A., So, D.Y., Glover, C.A., Froeschl, M., Marquis, J.F., O'Brien, E.R., **Hibbert, B.**, Chong, A.Y., Dick, A., Weaver, J., Labinaz, M. (2013) Platelet Function Monitoring in Patients Treated with Clopidogrel at the Time of Primary Percutaneous Coronary Angioplasty: The CARdiovascular Percutaneous Intervention TriAL Group (CAPITAL) LOAD Study. *Submitted*
2. **Hibbert, B.**, MacDougall, A., Blondeau, M., O'Brien, E.R., So, D.Y.F., Labinaz, M., Dick, A., Glover, C., Froeschl, M., Marquis, J., Wells, G.A., Le May, M.R. (2013) Letter re: "Bivalirudin for primary percutaneous coronary interventions: outcome assessment in the Ottawa STEMI registry." *Circulation Cardiovascular Interventions (In press)*

3. **Hibbert, B.**, Maze, R., Pourdjabbar, A., Simard, T., Ramirez, F.D., Blondeau, M., , Labinaz, M., Dick, A., Glover, C., Froeschl, M., Marquis, J.F., So, D.Y.F., Le May, M.R. (2013) A Comparative Pharmacodynamic Study of Ticagrelor versus Clopidogrel and Ticagrelor in Patients Undergoing Primary PCI: the CAPITAL RELOAD Study. *Submitted*.
4. **Hibbert, B.**, Lavoie, J., Ma, X., Seibert, T., Raizman, J., Simard, T., Chen, Y.X., Filion, L., Stewart, D.J., O'Brien, E.R. (2013) Glycogen Synthase Kinase-3 β Inhibition Decreases Endothelial Progenitor Cell Apoptosis via Cathepsin B to Enhance Therapeutic Progenitor Cell Therapy in Patients with Diabetes. *Submitted*.
5. Ma, X., **Hibbert, B. (shared first author)**, McNulty, M., Hu, T., Zhao, X., Ramirez, F.D., Simard, T., Seibert, T., Wells, D.J., de Belleruche, J.S., O'Brien, E.R. (2013) Heat shock protein 27 improves arterial repair: a potential role for vascular endothelial growth factor-mediated enhancement of endothelial progenitor cells. *Submitted*.
6. Mylonas, I., Alam, M., Ahmed-Muhsin, N., Small, G., Chen, L., Yam, Y., **Hibbert, B.**, Chow, B.J.W. (2013) Quantifying Coronary Artery Calcification from a Contrast Enhanced Cardiac Computed Tomography Angiography Study. *Submitted*.
7. Maze, R., **Hibbert, B.**, Le May, M.R. (2013) Response to letter "Role of Intra-aortic balloon pumping on cerebral perfusion after cardiac arrest" *Resuscitation* 84(1):e7.
8. Costiniuk, C.T., **Hibbert, B.**, Simard, T., Ghazawi, F.M., Angel, J., O'Brien, E.R. (2013) Circulating Endothelial Progenitor Cells in HIV Infections: A Systematic Review. *Trends Cardiovasc Med* 12: S1050
9. **Hibbert, B.**, Simard, T., Ramirez, D., Maze, R., Wilson, K.R., Hawken, S., O'Brien, E.R. (2013) Effect of Statin Therapy on Circulating Endothelial Progenitor Cells: a Systematic Review of Randomized Clinical Trials. (*Submitted*)
10. Raizman, J.E., Chen, Y.X., **Hibbert, B.**, Cuerrier, C.M., Salari, S., Seibert, T., Zhao, X.L., Hu, T., Shi, S., Ma, X., Simard, T., Caravaggio, J., Rayner, K., Bowdish, D., Moore, K., O'Brien, E.R. (2013) HSP27 attenuates foam cell formation and atherogenesis by down regulating SR-A expression via NF- κ B Signaling. (*Submitted*)
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12. Simard, T., **Hibbert, B. (shared first author)**, Raizman, J., Ramirez, D., Pourdjabbar, A., O'Brien, E.R. (2013) Current Understanding of In-stent Restenosis and the Benefits of Drug Eluting Stents. *Canadian Journal of Cardiology* (*In press*)
13. Giles, K., **Hibbert, B.**, Dryzmala, L., Armstrong, So, D.Y.F., Le May, M.R., Glover, C. (2013) Three year clinical outcomes with zotarolimus (Endeavor) drug-eluting stents in an unrestricted contemporary practice. *IJC* (*In Press*).
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15. Costiniuk, C.T., **Hibbert, B.M.**, Filion, L., O'Brien, E., Angel, J.B. Reply to: Cardiovascular disease risk in HIV infection and endothelial progenitor cells. (2012) *Journal of Infectious Diseases* 206(9): 1480-1.

16. Ramirez, F.D., **Hibbert, B.* (shared first author)**, Simard, T., Pourdjabbbar, A., Wilson, K., Hibbert, R., Kazmi, M., Hawken, S., Ruel, M., Labinaz, M., O'Brien, E.R. (2012) Natural history and management of aortocoronary saphenous vein graft aneurysms: a systematic review of published cases. *Circulation* 126(18): 2248-56.
17. **Hibbert, B.**, MacDougall, A., Blondeau, M., O'Brien, E.R., So, D.Y.F., Labinaz, M., Dick, A., Glover, C., Froeschl, M., Marquis, J., Wells, G.A., Le May, M.R. (2012) Bivalirudin for primary percutaneous coronary interventions: outcome assessment in the Ottawa STEMI registry. *Circulation Cardiovascular Interventions* 5(6): 805-12.
18. **Hibbert, B.**, Kazmi, M., Veinot, J., O'Brien, E., Glover, C. (2012) Infective endocarditis presenting as STEMI: an angiographic diagnosis. *Canadian Journal of Cardiology* 28(4):515.
19. Costiniuk, C.T., **Hibbert, B.**, Filion, L., Kovacs, C.M., Benko, E., O'Brien, E.R., Angel, J. (2012) Circulating Endothelial Progenitor Cells are not reduced in HAART naïve HIV Positive Men. *Journal of Infectious Diseases*. 205(5): 713-7.
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22. **Hibbert, B.**, Simard, T. Wilson, K.R., Hawken, S., Wells, G.A., Le May, M.R., So, D.Y., Glover, C.A., Froeschl, M., Marquis, J., Labinaz, M., Dick, A., O'Brien, E.R. (2012) Radial versus Femoral Artery Approach for Coronary Angiography in the Extremely Obese. *Journal of the American College of Cardiology: Cardiovascular Interventions* 5(8): 819-26.
23. Simard, T., **Hibbert, B* (shared first author)**., Chong, A.Y., Ruchin, P., Le May, M., Labinaz, M., Froeschl, M., So, D., Glover, C., Marquis, J.F., O'Brien, E.R. (2012) Unprotected Left Main Coronary Artery Stenting with Zotarolimus (Endeavor) Drug-Eluting Stents: A Single Center Experience. *CCI* 80(2): e15-22.
24. Pourdjabbbar, A., **Hibbert, B.**, Parfrey, B., Ramirez, D., Simard, T., O'Brien, E.R. (2012) RACER Renal Stents for Percutaneous Coronary Intervention in Large Left Main Coronary Arteries. *Int Journal Cardiol* 156(3): e68-70.
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29. **Hibbert, B.**, Lim, T.W, Hibbert, R., Gollob, M.H., Green, M., Davis, D.R. (2010) Refractory Ventricular Tachycardia following Tube Thoracotomy. *Europace*. 12(10):1504-6.
30. **Hibbert, B.**, Costiniuk, C., Hibbert, R., Joseph, P., Alanazi, H., Simard, T., Dennie, C., Angel, J., O'Brien, E.R. (2010) Cardiovascular complications of non-typhii Salmonella infections. *Canadian Journal of Cardiology*. 26(8):323-5.
31. Ma, X.*, **Hibbert, B*(shared first author)**, Dhaliwal, B., Seibert, T., Chen, Y.X., Zhao, X., O'Brien, E.R. (2010) Delayed Re-endothelialization with Rapamycin-coated Stents is Rescued by the Addition of a Glycogen Synthase Kinase 3 Beta Inhibitor: Novel Strategy for Augmentation of Endothelial Progenitor Cell-Mediated Healing. *Cardiovascular Research*. 86(2):338-345.
32. **Hibbert, B.**, Hibbert, R., Simard, T., Dennie, C., O'Brien, E.R. (2010) Pulmonary Hypertension. *Canadian Journal of Cardiology*. 26(5):e176.
33. **Hibbert, B.**, Ma, X., Pourdjabbar, A., Holm, E., Rayner, K., Chen, Y.X., Sun, J., Filion, L., O'Brien, E.R. (2009) Inhibition of endothelial progenitor cell glycogen synthase kinase-3beta results in attenuated neointima formation and enhanced re-endothelialization after arterial injury. *Cardiovascular Research*. 83(1): 16-23.
34. **Hibbert, B.**, Whitman, S.C., O'Brien, E.R. (2008) Letter by Hibbert et al. regarding article, "Smooth Muscle cells healing atherosclerotic plaque disruptions are of local, not blood, origin in apolipoprotein E knockout mice". *Circulation* 117(16):e317.
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37. Rayner, K., Chen, Y.X., **Hibbert, B.**, Miller, H., White, D., Postel, E.H., O'Brien, E.R. (2007) nm23-H2, an estrogen receptor beta associated protein facilitates estrogen mediated transcriptional signalling and shows diminished expression with progression of atherosclerosis: potential role in mediating the vasculoprotective effects of estrogen therapy *Am J Physiol Regul Integr Comp Physiol* 292(2):R743-50
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<http://www.athero.org/comm-index.asp>
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45. Doyon, C., Trudeau, V.L., **Hibbert, B.M.**, Howes, L.A., Moon, T.W. (2003) mRNA analysis in flattened fauna: Obtaining gene-sequence information from road-kill and game-hunting samples. *Can J Zool.* 81(4):692-698.

SCIENTIFIC MEETINGS

1. So, D.Y.F., Wells, G., McPherson, R., Labinaz, M., Glover, C., Le May, M., Dick, A., Froeschl, M., Marquis, J.F., Garg, N., Bernick, J., Tran, L., **Hibbert, B.**, Roberts, J. (2013) A Pharmacogenomic Approach to Antiplatelet Therapy in STEMI patients: ReAssessment of Anti-Platelet Therapy Using an InDividualized Strategy in Patients with ST-elevation Myocardial Infarction (The RAPID STEMI study). American College of Cardiology, Oral Presentation, San Francisco, USA.
2. **Hibbert, B.**, Maze, R., Pourdjabbar, A., Simard, T., Ramirez, F.D., Froeschl, M., Dick, A., Glover, C., Marquis, J.F., Labinaz, M., So, D., Le May, M. (2013) Ticagrelor versus Ticagrelor and Clopidogrel in Patients undergoing Primary PCI: A Platelet Reactivity Pharmacodynamic Study. American College of Cardiology, Oral Presentation, San Francisco, USA.
3. **Hibbert, B.**, Lavoie, J., Seibert, T., Simard, T., Stewart, D., O'Brien, E. (2013). Glycogen Synthase Kinase 3B Inhibition Decreases Endothelial Progenitor Cell Apoptosis via Cathepsin B to Enhance Therapeutic Progenitor Cell Therapy in Patients with Diabetes. American College of Cardiology, Poster, San Francisco, USA.
4. Simard, T., Ma, X., Hibbert, B., Ramirez, F.D., Seibert, T., O'Brien, E. (2013) Heat Shock Protein 27 Improves Arterial Repair following Arterial Injury by Regulation of Vascular Endothelial Growth Factor. American College of Cardiology, Poster, San Francisco, USA.
5. Seibert, T.A., **Hibbert, B.**, Simard, T., Chen, Y.X., Hu, T., Zhao, X., Rayner, K., Chow, B., Wilson, K.R., Hawken, S., O'Brien, E.R. (2012) Low serum HSP27 levels are predictive of future cardiovascular events and experimental vascular lesions are prevented or stabilized with recombinant HSP27 therapy. American Heart Association, Los Angeles, USA.

6. Ramirez, F.D., **Hibbert, B.**, Simard, T., Pourdjabbbar, A., Wilson, K., Hibbert, R., Kazmi, M., Hawken, S., Ruel, M., Labinaz, M., O'Brien, E.R. **(2012)** Natural history and management of aortocoronary saphenous vein graft aneurysms: a systematic review of published cases. Canadian Cardiovascular Congress, Poster Presentation, Toronto, Canada.
7. **Hibbert, B.**, MacDougall, A., Blondeau, M., O'Brien, E.R., So, D.Y.F., Labinaz, M., Dick, A., Glover, C., Froeschl, M., Marquis, J., Wells, G.A., Le May, M.R. **(2012)** Bivalirudin for primary percutaneous coronary interventions: outcome assessment in the Ottawa STEMI registry. Canadian Cardiovascular Congress, Poster Presentation, Toronto, Canada.
8. Raizman, J.E., Chen, Y.X., Hu, T., Seibert, T., Salari, S., **Hibbert, B.**, Simard, T., Cuerrier, C., Zhao, X., Ma, X., Rayner, K., Moore, K., O'Brien, E.R. **(2012)** Extracellular HSP27 Inhibits Foam Cell Formation by Targeting Scavenger Receptor-A in the Vessel Wall. ATVB, Poster Presentation, Chicago, United States of America.
9. Raizman, J.E., Hu, T.H., Chen, Y.X., Salari, S., Seibert, T., Ma, X., **Hibbert, B.**, Simard, T., Zhao, X., Rayner, K., O'Brien, E.R. Heat Shock Protein 27 mediated atheroprotection requires scavenger receptor-A: mechanistic insight into a novel therapeutic. **(2011)** American Heart Association, Oral Presentation, Orlando, Florida, United States.
10. Simard, T., **Hibbert, B.**, Pourdjabbbar, A., Wilson, K.R., Hawken, S., O'Brien, E.R. Percutaneous Coronary Intervention With or Without On-Site Coronary Artery Bypass Surgery: a Systematic Review and Meta-Analysis. **(2011)** Canadian Cardiovascular Congress, Oral Presentation, Vancouver, British Columbia, Canada.
11. Pourdjabbbar, A., **Hibbert, B.**, Parfrey, B., Ramirez, D., Simard, T., O'Brien, E.R. RACER Renal Stents for Percutaneous Coronary Intervention in Large Left Main Coronary Arteries. **(2011)** Canadian Cardiovascular Congress, Oral Presentation, Vancouver, British Columbia, Canada.
12. Giles, K., Drzymala, L., So, D., **Hibbert, B.**, Armstrong, M., O'Brien, E.R., Labinaz, M., Le May, M.R., Froeschl, M., Dick, A., Glover, C. Three year outcomes associated with the use of Zotarolimus-eluting stents in an unrestricted contemporary practice. **(2011)** Canadian Cardiovascular Congress, Poster Presentation, Vancouver, British Columbia, Canada.
13. Mylonas, I., Ziadi, M.C., deKemp R.A., Chow, B., Guo, A., **Hibbert, B.**, Renaud, J., Ruddy, T., Davies, R.A., Hessien, R., Dennie, C., Brennan, J., Etele, J., Beanlands, R. In patients with Normal Relative Perfusion Imaging, is there a Relationship Between Calicium Score and Myocardial Flow Reserve? **(2011)** Canadian Cardiovascular Congress, Oral Presentation, Vancouver, British Columbia, Canada.
14. **Hibbert, B.**, Simard, T., Le May, M., So, D.Y., Glover, C., Froeschl, M., Marquis, J.F., Labinaz, M., Dick, A., O'Brien, E.R. **(2011)** Coronary Angiography/Intervention in Patients with Extreme Obesity (BMI>40): Radial versus Femoral Approach. American College of Cardiology, Poster Presentation, New Orleans, USA.
15. Simard T., **Hibbert, B.**, O'Brien, E.R. **(2010)** Coronary Angiography in Patients with a BMI over 40: A Preliminary Assessment of the Efficacy and Safety of the Radial Artery Approach. American Heart Association, Oral Presentation, Chicago, USA.
16. **Hibbert, B.**, Pourdjabbbar, A., Ma, X. Simard, T., Holm, E., Rayner, K., Sun, K, Chen, Y.X., Filion, L., O'Brien, E.R. **(2009)** Mobilization of Vascular Progenitor Cells in Patients Undergoing PCI who are Randomized to receive Atorvastatin: Serial Assessment

and Delineation of Timeline. American Heart Association, Oral Presentation, Orlando, USA.

17. Sun, K., Ma, X., Rayner, K., McNulty, M., Ramirez, D., Simard, T., Dhaliwal, B., Chen, Y.X., **Hibbert, B.**, O'Brien, E.R. (2008) Selective Activation of Estrogen Receptor Beta Attenuates Atherogenesis and Represses Cholesterol-associated Immune-inflammatory Responses. American Heart Association, Poster Presentation, New Orleans, USA.
18. Ma, X., **Hibbert, B.**, Dhaliwal, B., Rayner, K., Chen, Y.X., Sun, J, Zhao, X, Seibert, T, Raizman, J, O'Brien, E.R. (2008) Glycogen Synthase Kinase 3 Beta Inhibitor Coated Stents Inhibit Neointimal Formation and Enhance Re-endothelialization through improved EPC Recruitment and Function. American Heart Association, Oral Presentation, New Orleans, USA.
19. **Hibbert, B.**, Ma, X., Pourdjabbar, A., Holm, E., Rayner, K., Chen, Y.X., Sun, J., Filion, L., O'Brien, E.R. (2007) Inhibition of Glycogen Synthase Kinase 3 Beta Improves Endothelial Progenitor Cell Mediated Arterial Repair and Stent Re-endothelialization. – American Heart Association, Poster Presentation, Orlando, Florida, USA.
20. Sun K., Ma, X., Chen, Y.X., Ramirez, D., **Hibbert, B.**, Rayner, K., McNulty, M., O'Brien, E.R. (2007) Selective Estrogen Receptor Beta Activation Attenuates Atherogenesis: Potential Role in Regulating the Survival of Vascular Progenitor Cells. American Heart Association, Oral Presentation, Orlando, Florida, USA.
21. Ma, X., **Hibbert, B.**, White, D., Seymour, R., Whitman, S.C., O'Brien, E.R. (2006) Circulatory origin of stent neointima demonstrated in a novel sex-mismatched carotid allograft rabbit model. – American Heart Association, Oral Presentation, Chicago, Illinois, USA.
22. Ma, X., **Hibbert, B.**, White, D., Seymour, R., Whitman, S.C., O'Brien, E.R. (2005) Contribution of Circulatory Cells to Stent Neointima Formation in a Novel Sex-Mismatched Rabbit Carotid Allograft Model. 16th Great Wall International Congress of Cardiology and American College of Cardiology Symposium. Beijing, China.
23. **Hibbert, B.**, Ma, X., White, D., Seymour, R., Whitman, S.C., O'Brien, E.R. (2005) Contribution of circulatory cells to stent neointima formation assessed by quantitative PCR analysis of laser captured microdissected tissue derived from a novel sex-mismatched rabbit carotid allograft model. Presentation – Ottawa Heart Institute Research Day, University of Ottawa, Ottawa, Canada.
24. Miller, H., Poon, S., **Hibbert, B.**, Rayner, K., Chen, Y.X., O'Brien, E.R. (2005) Modulation of estrogen signalling by the novel interaction of HSP27, a biomarker of atherosclerosis, and estrogen receptor beta: mechanistic insight into the vascular effects of estrogen. Poster – Keystone Meeting – Hormonal regulation of tumorigenesis, Monterey, USA.
25. Rayner, K., Chen, Y.X., **Hibbert, B.**, Miller, H., White, D., O'Brien, E.R. (2005) Discovery of the association of nm23-H2, a candidate metastasis suppressor protein, and estrogen receptor beta: potential protective role in atherosclerosis. Poster – Keystone Meeting – Hormonal regulation of tumorigenesis, Monterey, USA.
26. **Hibbert, B.**, Chen, Y.X., O'Brien, E.R. (2004) c-kit-immunopositive vascular progenitor cells populate human coronary in-stent restenosis but not primary atherosclerotic lesions. Poster – Medical Student Research Day, University of Ottawa, Ottawa, Canada.

27. **Hibbert, B.**, Fung, I., McAuley, R., Samia, M., Trudeau, V.L. (2002) Catecholaminergic regulation of GAD67 and GABA synthesis in the goldfish brain. Poster – 2002 Society for the Study of Reproduction Annual Conference.

BOOK CHAPTERS

- O'Brien, E.R., **Hibbert, B.**, Nathan, H.J. Chapter 6: Coronary Physiology and Atherosclerosis (2011) Kaplan's Cardiac Anesthesia: The Echo Era. 6th edition.
- **Hibbert, B.**, Simard, T., O'Brien, E.R. Chapter 8. Endothelial progenitors and repair of cardiovascular disease (2011) Regenerative therapy using blood-derived stem cells.

AWARDS & SCHOLARSHIPS

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| • William Keon Trainee Award
Professional competency and commitment | 2013 |
| • UOHI Case Report Competition
Best presentation | 2011&2012 |
| • UOHI Research Day – Clinical Research
Runner-up | 2011& 2012 |
| • CanMED Award – Communicator
Internal Medicine program award for best exemplifying role of Communicator as defined by the RCPSC | 2009 |
| • Internal Medicine Research Day
PGY-3 award for top rated project | 2009 |
| • CanMED Award – Scholar
Internal Medicine program award for best exemplifying role of Scholar as defined by the RCPSC | 2008 |
| • Department of Biochemistry Research Day
Best presentation | 2008 |
| • Internal Medicine Research Day
PGY-2 award for top rated project | 2008 |
| • University of Ottawa Graduate Admission Scholarship
Tuition Award | 2007 |
| • Deryck Stone Scholarship in Cardiology | 2006 |
| • Merck Frost Award
OHI Research Day – Best Basic Science Presentation | 2005 |
| • Heart and Stroke Foundation Research Scholarship
Salary award for summer research project | 2003 & 2004 |

- **University of Ottawa Medicine Merit Scholarship** 2003
Academic standing in first year Medicine
- **Susan Tolnai Scholarship** 2003
Academic achievement
- **University of Ottawa Medicine Admission Award** 2003
Admission scholarship to Faculty of Medicine
- **NSERC PGS-M** 2002
Salary award for graduate studies – declined
- **Gold Medal, Faculty of Science** 2002
Top standing in an Honours program, Faculty of Science
- **Department of Biology Plaque** 2002
Top standing in Honours Biology program
- **Thomas F. Lapierre Award** 2002
Top standing in a Bachelor program, Faculty of Science
- **John B. Armstrong Award** 2002
Top Honours research project in Biology
- **NSERC Undergraduate Research Award** (Three Awards) 2000-2002
Summer salary research awards
- **University of Ottawa Academic Achievement Award** 1998 - 2002
- **University of Ottawa Dean's Honour List** 1998 - 2002
- **University of Ottawa Admission Scholarship** 1998 - 2002

PROFESSIONAL MEMBERSHIPS

- Canadian Medical Association (#125840)
- Ontario Medical Association (#0876458)
- American Heart Association (#146344650)
- American College of Cardiology (#928760)
- Canadian Society for Clinical Investigators
- Canadian Cardiovascular Society (#130106)
- College of Physicians and Surgeons of Ontario (#84452)
- Canadian Medical Protective Association (#20059837)
- Licentiate of the Medical Council of Canada (#104941)
- Fellow of the Royal College of Physicians and Surgeons of Canada (#744937)
- Ontario Health Insurance Plan Billing Number (#0000-025600-13)
- Canada GCP (CITI # 1980109, ref # 5165178)