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Molecular Identification of Novel Genes Associated with Atherosclerosis

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MOLECULAR IDENTIFICATION OF NOVEL GENES

ASSOCIATED WITH ATHEROSCLEROSIS

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DEDICATION

This thesis is dedicated to Dr. Christine S. Moravec on April 26, 2011.

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ABSTRACT

Despite the identification of the risk factors which lead to CAD, we hypothesize there are novel genes to be identified that cause dysfunctional mechanisms leading to disease. For these discoveries, we utilized the genechip also referred to as a microarray.

We did expression profiling of coronary arteries from human patients with and without disease and identified 56 genes. The association of 49 genes with CAD appears to be novel, and they included genes: ICAM-2, PIM-2, ECGF1, fusin, B cell activator (BL34, GOS8), Rho GTPase activating protein-4, retinoic acid receptor responder, ß2-arrestin, membrane aminopeptidase, cathepsins K and H, MIR-7, TNF-α-induced protein 2 (B94), and flavocytochrome 558.

It is well-known that IMAs are resistant to the development of atherosclerosis, whereas the coronary arteries are athero-prone. The contrasting properties of these arteries provides an innovative strategy to identify the gene(s) that may play important roles in the development of CAD.

We found 29 genes with a significant difference in their expression levels between IMA and the coronary artery which included the TES gene encoding Testin. Using assays relevant to atherosclerosis, we showed that the knockdown of TES expression by siRNA promoted oxLDL-mediated monocyte adhesion to endothelial cells (EC), EC migration, and the transendothelial migration of monocytes, while the over-expression of TES in ECs blunted these processes. These results define a new molecular determinant (TES) for CAD and establish a novel role for TES.

We also investigated the gene expression of the ECs harvested from IMA and Cor to further distinguish these arteries' differential sensitivities to the develop atherosclerosis. The most statistically significant gene identified was the adenosine A2B receptor (A2B). Consequently, A2B was selected for follow-up functional studies to define its role(s) in ECs and how it may promote a resistance to atherosclerosis. We showed that the over-expression of the A2B receptor blunted processes relevant to the atherosclerosis.

In sum, expression profiling offered us the opportunity to identify novel genes that may be related to atherosclerosis. The identification of genes associated with this disease may lead to more aggressive interventions to halt the disease process of atherosclerosis by understanding the underlying genetic processes.

TABLE OF CONTENTS

4   GLOBAL GENE EXPRESSION STUDIES OF ATHEROSCLEROSIS-
     RESISTANT INTERNAL MAMMARY ARTERIES AND ATHEROSCLEROSIS-
     SENSITIVE CORONARY ARTERIES IDENTIFIES AN ANTI-
     ATHEROSCLEROSIS ROLE FOR TES IN ATHEROSCLEROSIS

LIST OF TABLES

Chapter 1

No tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

No tables

LIST OF FIGURES

8. APPENDIX

CHAPTER 1

INTRODUCTION

1.1 The Development of Atherosclerosis.

The heart is one of the most important organs in the entire human body. It is really nothing more than a pump and composed of four muscular chambers and beats an average of 70 times per minute. The coronary arteries are the heart's own system to deliver oxygen-rich blood to itself. There are two main coronary arteries (the left and the right), and they are the first branches off of the aorta. They are called the coronary arteries because they encircle the heart in the manner of a crown. In fact, the word "coronary" comes from the Latin "corona" and Greek "koron" meaning crown.

Based on the American Heart Association 2010 Statistical Update, the death rate from CAD was 262.5 per 100,000. Specifically, the rates are 306.6 per 100,000 for white males, 422.8 per 100,000 for black males, 215.5 per 100,000 for white females, and 298.2 per 100,000 for black females. Interestingly, 1 death occurs every 38 seconds. The expense of CAD management and intervention on the U.S. health care system is immense with direct and indirect costs totaling approximately $111.8 billion for 2002 (1).

The coronary artery consists of 3 layers, the (1) intima, (2) media, and (3) adventitia. The intima is an inner monolayer of endothelial cells (ECs) lining the lumen

and is bound by the internal elastic lamina, a fenestrated sheet of elastin fibers. The media consists of the thin subendothelial space in between these two areas contains thin elastin and collagen fibers along with smooth muscle cells (SMCs). And, the adventitia consists of the outer covering of the area consisting of collagen, connective tissue, and fibroblasts.

There have been four theories on how atherosclerosis develops (2). The Encrustation Theory was proposed by Karl Rokitansky in 1851. He suggested that atherosclerosis begins in the intima with deposition of thrombus and its subsequent organization by the infiltration of fibroblasts and a secondary deposition of lipid. In 1856, Rudolf Virchow proposed the Lipid Theory in which he thought atherosclerosis started with the diffusion of lipid into the arterial wall and its interaction with cellular and extracellular elements causing intimal proliferation leading to the gradual narrowing of the arterial lumen. Russell Ross, in 1999 then proposed a more unifying theory called the Response-to-Endothelial Injury Theory. He postulated that atherosclerosis begins with endothelial injury, making the endothelium susceptible to the accumulation of lipids and the deposition of thrombus. Yet currently, Fuster and colleagues proposed in 1996 the now accepted theory which is the Response-to-Vascular Injury theory in which the main cause of atherosclerosis is the injury to the endothelium by local disturbances of blood flow at angulated or branch points, along with systemic risk factors (eg, hyperglycemia, dyslipidemia, cigarette smoking, possibly infection) which perpetuates a series of events that involve the entire artery.

Consistent with the Response-to-Vascular Injury theory, CAD has a multifactorial etiology and is the response to many genetic and environmental factors and their

interactions (3-7). Long-term prospective clinical and epidemiological studies have identified several types of risk factors for the development of CAD: smoking history, older age, male gender, high fat diet, low antioxidant level, infectious agents, personal history of angina pectoris, family history of MI, obesity, diabetes mellitus, hypertension, hyperlipidemia (specifically low density lipoproprotein (LDL) cholesterol), increased plasma triglycerides, and low high-density lipoprotein (HDL) cholesterol (2-8).

New risk factors have been proposed for CAD and include elevated levels of lipoprotein(a), homocysteine, C-reactive protein (CRP), apolipoprotein E isoforms, fibrinogen, and plasminogen activator (3-8). Most of these risk factors, old and new, are likely to be impacted by the underlying genetic factors. Genetic-epidemiological studies suggest that family history is the most significant independent risk factor for CAD (8-13). Twin studies also provide an estimate of the genetic component of disease (12). Using monozygotic and dizygotic twins, the relative risk of death from CAD among male twins was 8.1 in monozygotic twins in comparison to 3.8 in dizygotic twins (12-13). This supports the hypothesis that genetic factors contribute to the development of CAD.

1.2 The Molecular Mechanisms of Atherosclerosis

Atherosclerosis leads to the gradual occlusion of the coronary artery lumen causing myocardial ischemia (7, 14) (Fig. 1). This process starts with the leukocytes initially tethering and rolling on the surface of activated endothelial cells (ECs), their arrest and firm adhesion, and then their subsequent transmigration into the media of the artery. Of note, a very specific class of monocytes containing the gene Lys-6 and Gr-1

3

have been found to infiltrate coronary arteries (14). Yet, the gene expression of each step of this process has been defined.

The initial rolling of leukocytes is mediated mainly by the selectins, a family of cell adhesion molecules (CAM), expressed on the ECs (15) as well as on the leukocytes themselves. The main gene expressed here encodes for P-selectin glycoprotein ligand-1 (PSGL-1). Subsequently, the genes that participate in leukocyte arrest and their firm adhesion is mediated primarily by the genes that express vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) on the ECs as well. These two receptors specifically interact with B1 and B2 integrins expressed on leukocytes (16-19). As a result, leukocytes attach to the ECs and start to transmigrate into the media of the artery. At the same time, the leukocytes are also a rich source of pro-inflammatory cytokines and secrete TNF-α, IFN-γ, IL-1, and IL-4 which perpetuate the cycle by promoting the expression of more CAMs, leukocyte adhesion and recruitment (20-23).

The defining process underlying the actual transmigration of leukocytes through the vessel wall by diapedesis is becoming better defined. Six genes are expressed at the lateral borders of ECs have been implicated in this process. These are: PECAM-1, CD99, VE-cadherin, and JAM-A, B, and C (24). The first change that occurs and permits the transmigration of monocytes is the increase in intracellular free calcium in the ECs. This is triggered by the cationic proteins released by stimulated neutrophils or by the cross-linking of E and P-selectin or VCAM-1 (25). As a result, intracellular calcium activates myosin light chain kinase and the unfolding of myosin II. This causes mild EC retraction so a space develops allowing the monocytes to easily pass through leaving the EC layer virtually intact.

A number of genes assist in the transmigration of the monocyte. PECAM-1 is a member of the immunoglobulin gene superfamily that is expressed diffusely on the surface of leukocytes as well as at the borders of ECs (26, 27). The interaction of PECAM-1 on the EC cell and the monocyte, specifically via the amino terminal portions 1 and 2 of PECAM-1 form a homophilic interaction. Blocking this interaction with a domain-specific monoclonal antibody also blocks diapedesis and has been shown to help prevent CAD (26, 27). Similarly, CD99 is a highly O-glycoslyated molecule that is also expressed on leukocytes and the borders of ECs (24). Like PECAM-1, it facilitates monocyte transmigration but at a later stage. For example, blocking CD99 arrests monocyte transmigration when the monocyte is part way across the EC border (24). Consequently, the monocyte initially binds to PECAM-1 and gets further assistance by CD99 as it passes through the EC layer.

VE-cadherin, which belongs to the class of cadherins and is located in the adherens junctions between ECs, maintains the EC permeability barrier. It has been proposed that VE-cadherein must be transiently down-regulated and removed from the junctional complex during diapedesis (28, 29). Interestingly, using a monoclonal antibody against this protein in animal models also promotes the infiltration of monocytes into the artery.

Finally, the junctional adhesion molecules (JAM), namely, JAM-A, B and C also localize to the intercellular borders of ECs as well as on monocytes. These proteins form a homophilic channel between the adjacent ECs allowing the monocytes to pass through and into the media of the artery. In sum, the genetic pathways of endothelial dysfunction in ECs are now becoming better defined.

Upon its arrival in the intima, the monocytes mature to tissue macrophages. They express scavenger receptors that bind lipoprotein particles, specifically ox-LDL that have passively diffused into the area. Over time, the monocytes become described as foam cells, a hallmark of the arterial lesion, so named because of the foamy appearance of the macrophages under the microscope (7, 14). Additionally, the foam cells secrete pro-inflammatory cytokines that amplify the local inflammatory response in the lesion, as well as generate reactive oxygen species. They also produce matrix metalloproteinases (MMPs) that degrade the extracellular matrix (30). If a plaque is present, the MMPs can weaken the strength of the plaque's fibrous cap. When and if the plaque ruptures occurs, it permits the blood to contact another macrophage product, tissue factor, which promotes thrombus formation. Eventually the macrophages congregate in a central core and form plaque. And, the entire cascade of events continues and culminates not only in the gradual occlusion of an arterial lumen, but this process also sets the stage for the plaque to rupture, thrombi to form, and result in myocardial infarction.

As this state in inflammation progresses in the artery, vascular SMCs are in proximity to and physically interact with inflammatory cell types, which play a very important role in further exacerbating the disease (31). By this time, there is a change in the phenotype of the vascular smooth muscle cells (VSMCs) from a "contractile" to a "synthetic" phenotype. Under the influence of the various cytokines that have been secreted by the macrophages, the VSMCs migrate to the intima. Here, the VSMCs perform four functions: 1) they secrete proteinases that break down the medial basement membrane allowing them to migrate to the site of inflammation, 2) they produce various growth factors such as vascular endothelial growth factor and platelet-derived growth

factor that facilitate their proliferation, 3) they secrete a large amount of matrix proteins such as glycosaminoglycans, elastin, collagen isoforms 1 and 3 that are necessary to repair the vessel and form a fibrous cap over the lipid-rich core of the lesion, and 4) their presence provides an element of plaque stability (7, 14). In sum, the entire cascade of events, triggered by the initial response of the ECs to injury ultimately leads to the transmigration of monocytes and their differentiation into macrophages, the uptake of oxLDL leading to foam cells, a perpetual secretion of cytokines that stimulate more changes in the ECs and VSMCs and lead to a cascade of events that not only occlude the arterial lumen but in this process causes myocardial ischemia.
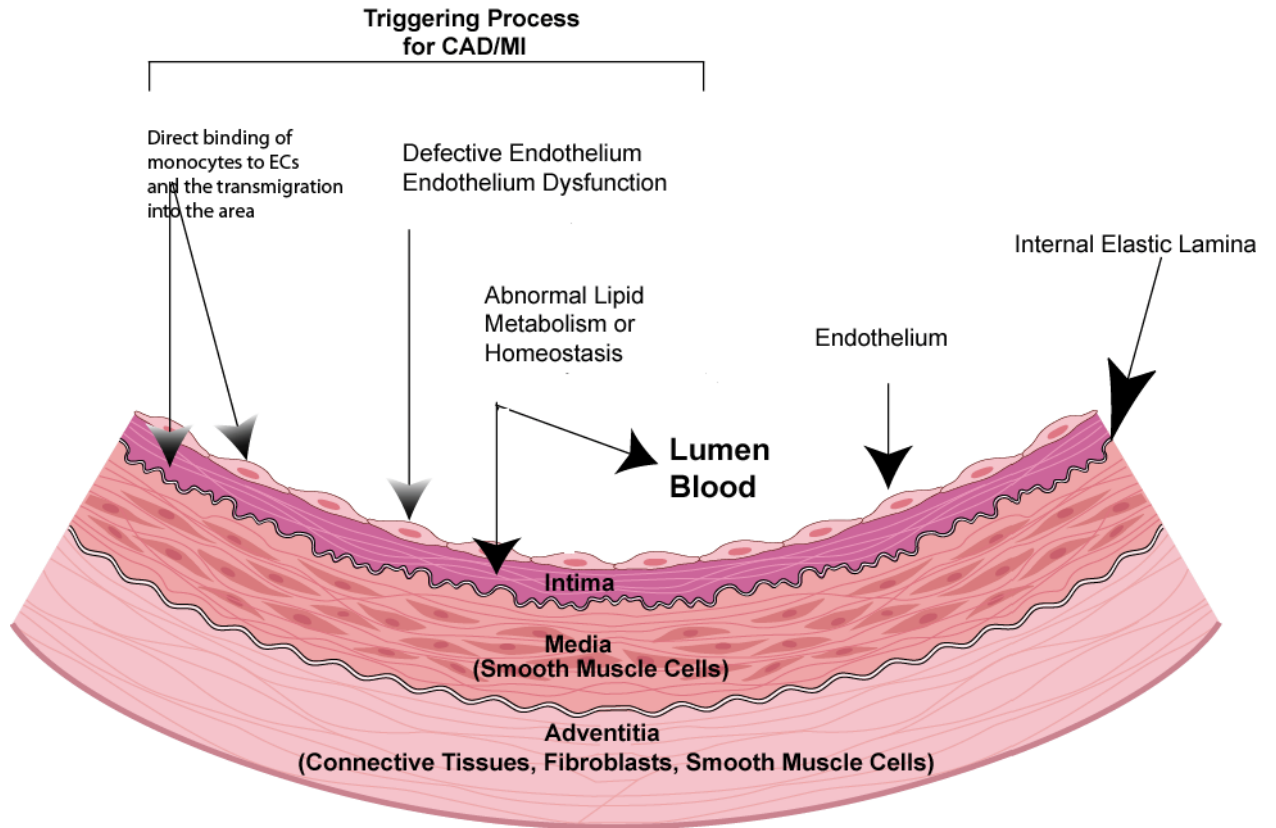
Fig. 1. Schematic drawing of the structure of coronary arteries. The processes important to the development of CAD and MI and their targets are indicated. The coronary artery is composed of three distinct layers, including the intima, media, and adventitia. The intima consists of extracellular connective tissue matrix (collagen and proteoglycans) bounded by the endothelium (endothelial cells) on the luminal side and the internal elastic lamina on the peripheral side. The media contains smooth muscle cells, and the adventitia consists of connective tissues, fibroblasts and smooth muscle cells.

1.3 Identifying Novel Pathways in Atherosclerosis

With the identification of the specific molecular pathways and genes involved in the development of atherosclerosis, we hypothesize that there are novel genes and pathways to be identified and elucidated to halt disease. Technology is now available to document the gene expression underlying disease. Sequencing of the entire human DNA genome has laid the groundwork to study the genetic susceptibility to disease in different populations. All this information is available in various databases (32, 33) and identifying a patient who has these genes (before atherosclerosis develops) may be the only hope to curtail its development and long-term consequences.

Currently, there are several approaches to study gene expression in atherosclerosis. These are: genetic linkage studies, genetic association studies, candidate gene association studies, genomic-wide association studies, and expression profiling. We shall briefly review these genetic approaches.

Being family-based and using DNA markers (only 1,000 are available at this time), genetic linkage analysis studies can correlate inherited regions of DNA with family members who have the disease compared to family members who do not (34). One will see a family-tree or pedigree mapping the gender and presence of disease among the entire family often spanning three generations. This approach and method allows one to determine the regions of the chromosomes that are likely to contain a disease causing gene.

Linkage analysis uses markers, well-characterized regions of DNA, which have been identified by the Human Genome Project (and other studies) to map chromosome regions. When one finds a marker that is found associated with a disease, the marker and the disease-causing gene are said to be linked. This is reflected in LOD score which compares the likelihood of obtaining the test data if the two loci are indeed linked, to the likelihood of observing the same data purely by chance. When a high LOD score is found (usually greater than 3), one does fine mapping of these area to identify candidate(s) genes and then do further studies to define an underlying, dysfunctional mechanism. The goal is to find a single-gene (or genes) defect in common with affected family members and determine its pattern of inheritance. Subsequently, this gene(s) can be studied in the population at large.

For example, linkage analysis has been successful in identifying specific genes that may contribute to diseases such as CAD. Two genes include: Myocyte Enhancer Factor-2 (MEF2A) with myocardial infarction (MI) (34) and Arachidonate 5-Lipoxygenase Activating Protein gene (ALOX5AP) with MI and stroke (35).

The MEF2A gene, a member of the myocyte enhancer factor 2 (MEF2) family of MADS-box transcription factors, has been found to be the gene responsible for an autosomal dominant form of CAD in a single large family (34). A genome-wide linkage scan identified that the family's disease was linked to a single locus on chromosome 15q26. Among the 93 genes found to be significant in this area by fine mapping, MEF2A was studied because of its known effects on myocardial development and vascular morphogenesis (34). Our lab sequenced the MEF2A gene and found a 21-bp deletion in

the last exon of the gene, which was present in all affected members of the analyzed CAD family. The same 21-bp deletion was absent in both the unaffected family members and 119 controls (all of which had normal left heart catherizations). Further studies showed that the in vitro expression of the MEF2A mutant protein indicated that the 7-amino acid deletion blocks MEF2A entry into the nucleus and impairs its transcriptional activity by a dominant-negative mechanism (34).

Subsequent genetic studies by our lab with more than 400 patients with CAD compared to controls identified three additional missense mutations (Asn263Ser, Pro279Leu, and Gly283Asp) in exon 8 of *MEF2A* (often reported as exon 7. These mutations, found in 1.9% of cases but not in controls, were suggested to reduce the transcriptional activity of MEF2A by a loss-of-function mechanism, thus playing a substantial role in the development of CAD (35). With the identification of MEF2A as a candidate gene based on linkage analysis, this now serves as a platform to identify other patient populations to find an underlying cause for CAD.

Additionally, the ALOX5AP gene region was linked with MI in 296 Icelandic families as well as an additional 713 subjects as well as in central European patient (36-41). But most importantly, this gene also identified by linkage analysis has led to the identification of its product, 5-lipoxygenase activating protein (FLAP) which participates in leukotriene synthesis and a potential therapeutic option to half disease (42).

Compared with linkage studies, genetic association studies use single nucleotide polymorphisms (SNPs). The term polymorphism has been used to refer to genetic mutations that occur with a frequency ≥1% in the population. The goal is to identify

differences in the inheritance of particular SNPs among subjects in general, not just families and find a statistically significant association to disease (43, 44,). Ideally, one wants to find at least one or several SNPs within a gene and determine what functional purpose the SNPs may have and how it might cause the disease (such as CAD). Then, the findings can be extrapolated to other patients with the disease to see if it can be used as an identifiable marker. The advantage of this approach is to identify novel pathways associated with a disease process. Yet, the disadvantage is the massive statistical analysis making multiple comparisons among groups and accepting a false positive rate of, for example 0.1%, which would generate 500 false positives. As a result, multiple rounds of replication are needed.

Candidate gene association studies are more focused on the selection of a limited group of genes. A candidate gene is a gene located in a chromosomal region suspected causing a disease. For example, investigators have specifically studied the relationship between SNPs in the C-reactive protein (CRP) gene and levels of CRP which correlates directly with risk for atherosclerosis (45). Using a case control design, four SNPs associated with risk of arrhythmias have been identified in pre-selected genes such as the cytoskeletal protein paladin (a tyrosine kinase), or two G-protein coupled receptors (46).

Currently, the main focus is on genome wide association studies (GWAS). Using this approach, one uses over two million identified SNPs that may be separated by merely 1,000 base pairs. This is in contrast to using the DNA markers located approximately 1cM over entire chromosomes. As a result, the chances of identifying a candidate gene associated or a group of SNPs with a disease such as CAD is more likely.

One final approach to identify novel genes associated with CAD is expression profiling (47-48). DNA microarrays are an orderly, high-density arrangement of nucleic acid spots on a glass which represents a unique sequence of a gene (48). The target for these probes come from messenger RNA harvested from a specific tissue, for example, coronary arteries which are then labeled, incubated on the chip to allow hybridization to occur, washed and scanned to identify genes that are present. Subsequently, statistical programs are available (such as GeneSpring from Aligent Genetics) to determine statistically significant genes between samples (49).

The purpose of our research was to not only identify novel genes associated with coronary artery but understand the molecular mechanisms that may lead to or prevent this disease. We started our approach with expression profiling with the microarray using intact coronary arteries. After identifying 56 novel genes associated with CAD, we generated our first list of candidate genes to study with the option of looking for SNPs, mutations, and other associations with CAD. Subsequently, we continued our expression profiling examining gene expression not only in the internal mammary artery, an artery resistant to atherosclerosis, but also in pure cell lines of ECs. Using these techniques, we generated even more candidate genes to study. We ultimately chose two candidate genes, Testin-2 and the Adenosine A2B receptor. Using in vitro techniques which reflect processes related to CAD, we invested how these genes (when over-expressed or silenced) affected monocyte adhesion, cell migration, and the transmigration of monocytes.

1.4 Future work.

The most important research questions to the field of CAD are: What are the specific genes expressed in atherosclerosis? How does their alteration impact the process of disease? Who expresses these genes? And, can patients without CAD be identified on the basis of genes alone in an effort to aggressively alter the disease process. In my research, I hypothesized that there are novel genes yet to be identified that cause CAD. Using human coronary arteries, I used expression profiling to identify novels genes and identified novel mechanisms related to the molecular genetics of CAD.   In the future, I will continue using basic genetic techniques to search for mutations, SNPs, and follow up association studies to observe the impact of these genes on CAD in the general population.   It is my goal to not only identified novel genes, but make clinical correlations to alter the process of atherosclerosis and make a contribution to lower its morbidity and mortality.

CHAPTER 2

EXPRESSION PROFILING OF CARDIOVASCULAR DISEASE

2.1 Introduction. Gene expression is thought to be central to the pathogenesis or progression of coronary artery disease (CAD)/atherosclerosis, congestive heart failure (CHF) and common congenital heart disease (CHD). Microarray analysis is a powerful technique for high-throughput, global transcriptonomic profiling of gene expression. It holds great promise for analyzing the genomic basis of various complex diseases and permits the analysis of thousands of genes simultaneously, both in diseased and non-diseased tissues and/or cell lines.

Microarrays are made by depositing spots of DNA on a solid support like a coated glass surface (2). A flat glass surface makes it possible (i) to array molecules in a parallel fashion, (ii) to miniaturize the procedure and (iii) to use fluorescent dyes for detection and thus avoid radioactivity. This differs in several ways from conventional methods such as filter-based supports of charged nylon and nitrocellulose for studying mRNA expression.

A variety of technologies have been used to produce microarrays. Spotted cDNA arrays, produced by deposition of polymerase chain reaction (PCR) products, and GeneChip oligonucleotide arrays (Affymetrix; Santa Clara, CA), produced by in situ synthesis of oligonucleotides, have both been used successfully. Microarrays can now be tailored to focus on a specific set of genes (e.g. the Cardiochip, with genes related to the cardiovascular system only). The advantages of cDNA arrays are that they are focused to a specific pathway or functional class of genes and will identify the gene(s) of particular interest in the tissue studied. Use of this smaller chip is less expensive, and the same sample can be used to compare similar genes in several species. The limitation of cDNA arrays, however, is that only a very limited set of genes can be evaluated. Oligonucleotide arrays have the advantage of allowing one to monitor every gene in a genome at the same time, and not to focus on a particular subset of genes. The disadvantage is that this method is expensive and must be made commercially. At this time, only a few organisms (human, mouse and rat) are available for studies.

Microarrays can serve to complement other genetic and genomic tools, including positional cloning (2) and proteomics (3), to understand the underlying biological pathways that trigger or facilitate the development of CAD/atherosclerosis, CHF or common CHD. In this paper, we review the current status of microarray studies in profiling gene expression in cardiovascular disease, with a particular focus on human tissues and cells.

2.2 Expression profiling of coronary artery disease

Coronary artery disease (CAD) occurs due to an accumulation of atherosclerotic plaques (atherosclerosis) in the walls of the coronary arteries. Atherosclerosis leads to symptoms of stable angina if no clinically apparent plaque rupture or significant breach of the arterial wall occurs, but it will result in unstable angina, acute myocardial infarction (MI) or sudden cardiac death once the arterial wall integrity is lost and thrombosis has occurred.

In the USA and most other Western countries, CAD is the leading cause of morbidity and mortality. In the USA alone, it caused almost one million deaths in 1999 — twice the number caused by cancer and ten times the number caused by accidents (4). Despite significant medical advances, MI due to CAD (and strokes) is responsible for more deaths than all other causes combined.

There are two main theories that explain the development of atherosclerosis. First, high levels of cholesterol in the blood injure the endothelial cells of a coronary artery, causing an inflammatory reaction and enabling cholesterol and other fatty materials to accumulate there. Secondly, repeated injury to the wall of the artery may occur through various mechanisms involving the immune system or by direct toxicity with substances such as nicotine or homocysteine, leading to the recruitment and accumulation of inflammatory cells (5). In both cases, there are changes that can lead to the formation of atheromas and plaques. These two theories probably overlap and are not mutually exclusive. To date, several microarray studies have been reported to profile gene expression in CAD using human tissues; one study from the authors' group focused on the identification of genes differentially expressed in CAD and non-CAD coronary

arteries, and the other studies have characterized gene expression in atherosclerotic plaques and plaque rupture.

Four microarray studies have so far been reported for the characterization of atherosclerosis, one of which focused on restenosis after coronary interventions. The current authors used oligonucleotide arrays to study >12,000 genes for their expression in human coronary arteries, the target organ of CAD (Table 1) (6). Fifty-six genes showed differential expression. In the atherosclerotic coronary artery tissues, the expression of 55 genes was increased, whereas only one gene, encoding glutathione-S-transferase (GST), a reducing agent, showed down-regulated expression. This study detected the expression of genes previously linked to the generation of CAD, such as osteopontin expressed in smooth muscle cells, vascular cell adhesion molecule (VCAM)-1 expressed in endothelial cells and matrix metalloprotease (MMP)-9 expressed in macrophages. This lends credibility to expression profiling as a valid approach for studying gene expression in CAD.

The associations of 49 genes appeared to be novel and had not previously been shown to be associated with CAD. These included genes encoding retinoic acid responder binding (RAR) protein, butyrophilin, steroidogenic acute regulatory protein (STAR), PIM-2, STAT-91 and cathepsins K and H. The RAR gene is regulated by vitamin A signalling pathways and upregulates the expression of the scavenger receptor CD36 (7); butyrophilin has receptor functions that mediate the transfer of lipid (8); and cathepsins K and H are lysosomal proteases involved in protein degradation (9, 10). Acute steroidogenic regulatory protein (STAR) is the rate-limiting step in steroidogenesis for the transfer of cholesterol into the mitochondria (11). The PIM-2 gene is present in

18

mitogenically stimulated hematopoietic cells (12) and is expressed widely in the media of coronary arteries (6). The STAT-91 gene is a signal transducer and activator of transcription in haematopoietic cells (13). Intercellular adhesion molecule 2 (ICAM-2) is another novel gene whose expression is increased in CAD tissues, and it is expressed mainly in endothelial cells (6). GST is the only gene whose expression is reduced in CAD tissues, and it is a reducing enzyme (14). The most significant group of genes identified in the study were involved in inflammatory processes and consisted of genes expressed by B and T lymphocytes, as well as macrophages. Other genes included five genes involved in lipid transfer, oxidation and metabolism; nine genes involved in cell migration, adhesion and matrix degradation; 12 genes involved in cell necrosis, apoptosis and proliferation; and several genes with currently unknown functions.

A cDNA microarray study was used to analyze 18,376 genes for changes in expression in whole-mount human atherosclerotic plaques from the internal and external iliac arteries, aorta, popliteal artery, posterior tibial artery and the tibio-fibular trunk with advanced lesions as compared with normal arteries (15). Similarly to the earlier study by Archacki et al., (6) 17 genes previously connected to atherosclerosis, e.g. actin, cathepsin S, apoE and P-glycoprotein, were

Table 1. Expression profiling of coronary arteries.

Target Tissue	Experimental Treatment	Number of Genes Affected
Intact coronary arties (6)	Severe atherosclerosis versus non-atherosclerotic arteries	56
Diffuse survey of several arteries (15)	Documentation of gene expression in atherosclerotic tissue	75
Coronary plaques form patients with stable or unstable angina (16)	Ruptured plaque material	5
Atherosclerotic plaque from human coronary arteries (17)	Specimen retrieve by novel helix cutter	201
Human atherosclerotic plaque (23)	Specimens from ruptured plaque	Focus on perilipin; consistently unregulated.

identified, which validates microarray analysis as a valid approach for studying gene expression in atherosclerosis (15). The study identified 75 new differentially expressed genes, 44 of which were unregulated in advanced lesions. Differential expression of Janus kinase 1(JAK-1), vascular endothelial growth factor (VEGF) receptor-2 and CD31 in atherosclerotic plaques was confirmed with reverse-transcription PCR and immunocytochemistry. JAK-1 is a protein kinase which may activate platelet-derived growth factor-b (PDGF) signaling (15). VEGF receptor-2 is a receptor for angiogenic factor VEGF, and acts as an early marker of vasculature in embryogenesis (15). CD31 is also an endothelial cell marker that is involved in monocyte and T-cell adhesion to endothelial cells (15). These studies support the current authors' hypothesis that endothelial dysfunction is the earlier triggering event for the development of CAD (2). Thirty-one genes were downregulated in advanced lesions, whereas the Archacki study identified only one downregulated gene in diseased coronary artery tissues (6). A limitation of this study is that various arteries were utilized, and the interpretation of the data may be challenging as different arteries may have unique characteristics. This study did not share any genes in common with those found in the current authors' study, discussed above, which appears to be a common finding in microarray analysis of atherosclerosis. The potential explanations for this incongruity may be that different arteries (coronary artery versus other atherosclerotic tissues) were used in different studies, and that the arteries used were at different stages of disease.

The third of the afore-mentioned microarray analyses was a small-scale study with cDNA arrays, in which cDNA probes were hybridized to nylon arrays containing only 482 genes involved in haemostasis, inflammation and cell adhesion (16). The study

investigated gene expression in human coronary plaques from patients with stable or unstable angina. One gene, encoding tissue factor, showed increased expression in unstable angina samples, whereas five genes, including anticoagulant protein S, cyclooxygenase-1 (COX-1), interleukin (IL)-7 and the chemokines monocyte chemotactic protein (MCP)-1 and -2, were expressed at lower levels in unstable angina samples.

In the fourth microarray study, rather than investigating coronary artery disease per se, the authors focused on restenosis, the most important limitation of percutaneous angioplasty procedures (a common surgical treatment for patients with coronary artery disease) (17). Zohlnhofer et al. retrieved tissue specimens from 16 patients with symptomatic in-stent restenosis using a novel helix cutter. The control samples included media specimens from seven gastrointestinal arteries and seven coronary arteries from cardiac transplantation. Atlas cDNA arrays (human cancer 1.2, human 1.2 cardiovascular, and stress arrays, Clontech) were used, and a total of 2,435 genes were examined. The study identified several genes that had previously been reported to be expressed in smooth muscle cells and regulated in neointima and restenosis. These included upregulation of thrombospondin-1 (TSP-1), 70-kDa heat shock protein B, COX-1 and downregulation of desmin. The study also identified two new genes that are connected to restenosis, FKBP-12 (upregulated) and the gene encoding the mammary-derived growth inhibitor (MDGI) (downregulated). MDGI is a potent tumour suppressor (18, 19), and downregulation of MDGI may lead to proliferation and migration of smooth muscle cells, resulting in restenosis after coronary interventions (20). Upregulation of FKBP-12 is significant because it is involved in controlling transforming growth factor-b (TGF-b)

signalling and may prevent cycle arrest, leading to the reocclusion of coronary arteries by the proliferation of smooth muscle cells (18). This latter study lent support to the testing of Rapamycin (sirolimus) as a novel pharmacological approach to preventing restenosis, as it binds to FKBP-12 and down regulates TGF-b inhibitory activity (21). The potential use of Rapamycin to prevent restenosis is also supported by the report that it inhibits smooth muscle cell (SMC) migration and proliferation and intimal thickening after balloon angioplasty in a porcine model of restenosis (22).

In addition to microarray analysis, suppression subtractive hybridisation (SSH) technology was successfully used to study gene expression patterns in whole-mount human stable and ruptured plaques (23, 24). The details of the procedures involved in SSH have been described by Faber et al. (23, 24). Compared with microarray analysis, SSH is more time-consuming, but it can lead to the identification of low-abundant sequences which may be missed by microarray technology. Perilipin, a phosphoprotein present on the surface of intracellular lipid droplets in adipocytes and steroidogenic cells (25, 26), was found to have a twofold difference in expression and was upregulated in ruptured plaques. As a result of this finding, it was hypothesised that the overexpression of perilipin increases triacylglycerol storage by reducing triacylglycerol hydrolysis (27, 28). Consequently, this increase in lipid retention may lead to plaque stabilization and rupture. Perilipin was found to be consistently expressed in eight ruptured plaques and was completely absent in stable plaques, identifying it as a unique marker. In addition, fibronectin and immunoglobulin l chain were downregulated in ruptured plaques.

In summary, the studies discussed above highlight the importance of using microarrays to identify genes with potential roles in the generation of atherosclerosis,

including the evolution of plaque and plaque rupture. Novel genes have been identified which may serve as focal points of future functional and pharmacological studies in an effort to alter the process of atherosclerosis. Furthermore, the combination of microarray analysis and positional cloning may provide a powerful synergy in the characterisation of the molecular bases of human diseases. First, the genes identified by microarray analysis become the strong candidate genes if they are located in a previously mapped genetic susceptibility locus for the disease. Secondly, the results from microarray analysis may provide independent supportive evidence for the new signaling pathway for the pathogenesis of the disease identified by positional cloning. An excellent example is the authors' recent identification of the first non-lipid-related disease-causing gene, MEF2A, for CAD and MI (2). Using a large family with 13 patients who displayed an autosomal dominant inheritance pattern of CAD and MI, Wang et al. successfully identified a significant linkage to CAD/MI on chromosome 15q26 (LOD score 4.19), the first locus for autosomal dominant CAD and MI (adCAD1). The adCAD1 disease locus contains 93 known or putative genes, and mutational analysis established one of the genes in the region, MEF2A, as the gene responsible for CAD and MI. A 21 base pair deletion of MEF2A results in the deletion of seven amino acids in the MEF2A protein (D7aa), and co-segregates with the disease in the family. It reduces the transcriptional activation activity of MEF2A by approximately threefold and abolishes the synergistic activation of transcription by MEF2A in combination with the transcription factor GATA-1 through a dominant-negative mechanism. Using immunostaining, a strong MEF2A protein signal was detected in cultured endothelial cells and also within the endothelium of coronary arteries, which is a barrier between vessels and blood elements such as the platelets and

macrophages that are central to the pathophysiology of CAD. The authors' studies suggest that an early step in the development of CAD/MI may involve deregulation of specific transcriptional programs in the endothelium. Defective or abnormally developed vascular endothelium will be more susceptible to inflammation and the diapedesis of monocytes, and expose the subendothelial matrix to the genesis of atherosclerotic plaque or thrombosis. Consistent with the authors' hypothesis, microarray analysis of CAD, atherosclerosis and restenosis revealed differential expression patterns of several genes involved in vasculogenesis, angiogenesis and vascular remodeling, including ECGF1and MMP-9 in the study by Archacki et al. (6), VEGF receptor 2, JAK-1 and CD31 in the study by Hiltunen et al. (15), TSP-1 in the study by Zohlnhofer et al. (17), and MCP-1 and MCP-2 in the study by Randi et al.(16). It is anticipated that microarray analysis will be increasingly used in the positional cloning studies for identification of susceptibility genes for complex human diseases.

2.3 Expression profiling of ischaemic myocardium in animal models

A limitation of using human tissues for microarray analysis is that there is a tremendous amount of biological variability. A study of human tissues from the population usually contains patients with several medical problems, taking several medications and with prior risk factors for atherosclerosis. A study using animal models can overcome the problem of biological variability. Although very limited to date, gene expression profiling has been performed on myocardial tissues in several animal models that have been used to provide information on the process of MI in a controlled setting, enabling the study of the gene expression profile of MI. The precise mechanisms of ischaemic injury to the myocardium secondary to atherosclerosis have not been fully

elucidated. With microarray technology, however, one can start to have a comprehensive overview of the gene expression patterns occurring in the ischaemic heart (Table 2).

Rabbit hearts that were subjected to two five-minute episodes of ischaemia (followed by five minutes of reperfusion) and then up to five hours of additional reperfusion identified 35 genes that were differentially expressed (29). A strong upregulation of mitogen-activated protein kinase-activated protein kinase-3 (MAPKAPK-3) was identified. It was inferred that this gene has a protective mechanism against cell injury, as a related gene, MAPKAPK-2, is known to be atheroprotective (30). Similarly, the same group studied the same model of ischaemia in rat hearts.

Table 2. Expression profiling of ischaemic myocardium in animal models.

Target Tissue	Experimental Treatment	Number of gene affected
Rabbit heart (29)	Two five-minute episodes of ischaemia followed by five hours of reperfusion	Strong up-regulation of MAPKAPK-3
Rat Hearts (30,31)	20-minute episode of ischaemia followed by four hours of reperfusion	Significant up-regulation of HSP-27 and 70
Rat in vivo myocardial infarction model (32)	Permanent coronary occlusion	731 differentially expressed genes
Rat hearts (33)	Administration of angiotensin-converting enzyme (ACE) inhibitor	37 genes clustered into 11 functional groups. ACE inhibition after myocardial infarction inhibits cardiac hypertrophy
Rat hearts (34)	Ligation of the left anterior descending coronary artery	Expression of genes associated with a fetal transcription program

subjected to a 20-minute transient episode of ischaemia followed by four hours of reperfusion. The purpose of the study was to determine whether activation of protective genes takes place within four hours following a brief episode of ischaemia, mimicking angina pectoris (31). Ischaemia led to strong upregulation of mRNA transcripts for heat shock proteins 70, 27, 105, 86 and 40 kDa, vascular endothelial growth factor, brain-derived neurotrophic factor, plasminogen activator inhibitor-1, activating transcription factor 3, B-cell translocation gene 2 and growth arrest and DNA damage inducible 45 alpha protein. The study demonstrated that a rapid onset 'genetic reprogramming' occurs during a brief episode of ischaemia and can be characterised as protective in nature.

Stanton et al. studied an in vivo rat MI model by causing a permanent coronary occlusion (32). On a microarray with 4,000 probes, they found a total of 731 genes differentially expressed during the 16-week experimental period from two regions of the heart, the left ventricular free wall and the interventricular septum. Upregulated genes included those encoding atrial natriuretic peptide (ANP), sarcoplasmic/endoplasmic reticulum Ca2þ-ATPase, collagen, fibronectin, decorin, fibulin, tissue inhibitor of metalloproteinase-3, fibrillin, laminin, secreted protein acidic and rich in cysteine (SPARC) and osteoblast-specific factor-2. These genes were classified as belonging to a remodelling pathway in the post-MI period. The functional clustering of the gene list showed that the majority of the upregulated genes were classified as cytoskeletal and extracellular matrix (ECM) proteins, while downregulated genes were classified as contractile proteins or fatty acid metabolism-related genes, suggesting a profound change in the energy-generating processes in the ischaemic and then injured myocardial tissue.

In the setting of MI, the angiotensin-converting enzyme inhibitor, captopril, was administered to rats for eight weeks after ligation of their left coronary artery (33). In control animals, there was an increased expression of collagen I and III, thrombospondin-4, lysyl oxidase, fibronectin and biglycan eight weeks after MI. There was also the upregulation of components of the complement pathways and lipopolysaccharide binding proteins, and VCAM-1. The downregulation of fatty acid metabolising enzymes was also documented. With captopril treatment, there was a downregulation of hypertrophy-related genes such as TGF-b3 and insulin-like growth factor binding protein-6. These results indicate that pharmacological treatment in the post-MI period can have an impact on gene expression. Many genes were not affected, however, suggesting the need for more novel forms of therapy to alter ischaemic damage after an MI.

Finally, another rat model employed to study gene expression in the myocardium, induced with the ligation of the left anterior descending (LAD) coronary artery, documented gene expression after 24 hours (34). Several genes differentially expressed included genes associated with cardiac muscle development such as the cell cycle regulator p18ink4 and the structural proteins, a-myosin heavy chain (a-MHC) and fetal myosin alkali light chain(MLC). Expression of early growth response factors Egr-1 and Egr-3 were upregulated by ischaemia, whereas a there was a downregulation of GST. Other stress responses to ischaemia included an induction in the expression of the apoptosis regulator Bax, which may contribute to cell death. This gene list may represent a balance between the cardioprotective and degenerative processes that accompany myocardial ischaemia.

In summary, microarrays have been utilised to understand the consequences of atherosclerosis, which can abruptly cut off the supply of blood and oxygen to the myocardium. The microarray enables investigators to look at a comprehensive overview of genetic changes and may stimulate the development of pharmacological therapy to alter the processes involved in the consequences of MI. It is interesting to note that several genes were identified by both the human and animal model studies (genes for 70 kDa heat shock protein, fibronectin, VCAM-1 and GST), suggesting that microarray analysis of animal models is appropriate for some aspects of the characterisation of human diseases.

2.4 Expression profiling of endothelial cells

The endothelial cell layer of an artery is the first strategic location for the initiation of atherosclerosis. Dysfunction and/ or injury of endothelial cells play an important role in atherogenesis (2). The endothelium has many functions — for example, regulation of tone, coagulation and fibrinolysis, and it secretes several substances. Expression profiling has been performed for endothelial cell lines to study the impact of oxidised low-density lipoprotein (LDL), regulators of inflammation [tumour necrosis factor-a (TNF-a), IL-1b], infection with Chlamydia pneumoniae (CP), antioxidants, homocysteine and differential patterns of blood flow.

It is hypothesised that areas of the vascular system with turbulent flow are more likely to develop atherosclerotic plaques than are regions with laminar blood flow (35). Expression profiling was used to examine the differential expression of genes in human aortic endothelial cells (HAEC) that were exposed to these two conditions (Table 3) (36).

In total, more than 130 genes were induced by turbulent flow as compared with laminar flow. Approximately 50 per cent of the genes surveyed did have a detectable expression at baseline, but at two hours 3–8 per cent of the expressed genes were upregulated, and by 24 hours 14.2 per cent, or 50 genes, were downregulated. The study demonstrated that changes in flow serve as a distinct biomechanical stimulus which has a profound impact upon the gene expression profile of HAEC in vitro.

Table 3. Expression profiling of endothelial cells (EC).

Target Tissue	Experimental Treatment	Number of genes affected
Aortic EC (36)	Disturbed flow and steady laminar flow	100
Coronary artery (38)	Oxidized low-density lipoprotein	78
Coronary artery (40)	Infection with Chlamydia pneumonia	268
Coronary artery (46)	Homocysteine	600
Coronary artery (48)	Nicotine	4
Umbilical vein (49)	BO-653, probocol and BHQ	17
Smooth muscle cells (SMCs) from plaque (50)	SMC+tumour necrosis factor (TNF)-alpha	5
Umbilical vein (30)	Interleukin-1beta and TNF-alpha	209 EC + 39 in SMCs

LDL is well established as contributing to the generation of atherosclerosis. Oxidation of LDL is a key event associated with endothelial cell injury, by leading to the expression of endothelial cell receptors that bind to inflammatory molecules (37). Primary human umbilical vein endothelial cells (HUVEC) were exposed to oxidised LDL for six hours (38). Of the 588 genes evaluated, 78 genes displayed a greater than twofold change in expression levels: 57 genes were upregulated and 21 genes were downregulated in response to the oxidized LDL. Oxidised LDL significantly affected the expression of genes encoding transcription factors, cell receptors, adhesion molecules, ECM proteins and enzymes involved in cholesterol metabolism. It is interesting to note that expression of retinoic acid receptor-b (RXR- b) was upregulated in both the turbulent flow and oxidised LDL studies.

Gene expression has been documented in endothelial cells infected with pathogens such as human cytomegalovirus (HCMV) or CP. These pathogens are linked to the development of vascular disease, including atherosclerosis (39), yet the role of pathogens in vasculopathies has been controversial. Expression profiling has identified mechanisms by which CP alters coronary artery endothelial cells (40). Twenty of 268 genes on the array were upregulated in human endothelial cells in response to CP, suggesting that CP infection does not lead to a generalised response in gene expression. Genes coding for cytokines (IL-1), chemokines (MCP-1 and IL-8) and cellular growth factors (heparin-binding epidermal-like growth factor, basic fibroblast growth factor (FGF) and PDGF- b-chain) were the most prominently upregulated in response to the atypical bacteria. Additionally, increases in the expression of genes coding for intracellular kinases and cell surface receptors with signal transduction activities were

observed. Time course experiments showed that mRNA levels were upregulated within two hours following infection.

Novel growth factor genes were also identified, and included b-FGF, heparin binding growth factor (HBGF), PDGF- b and activin A. b-FGF suggested a pathway in common with endothelial cells and SMCs. HBGF is a growth factor synthesized by endothelial cells which can act as a potent mitogen for the proliferation of SMCs (41). Similarly, PDGF-b not only has SMC growth-promoting activity but is also associated with neointimal proliferation of SMCs (42). PDGF-b-associated protein is also a growth factor accessory molecule that modulates the activity of specific growth factors (42). Activin A, or erythroid differentiation protein, has been shown to modulate monocyte/macrophage function, including immunological activation of monocytes (43) and the induction of MMP-2 (44). CP induced the expression of E-selectin, ICAM-2 and VCAM-1 two hours after infection, yet returned to basal levels by 24 hour post-infection (45). These genes were also upregulated in response to oxidised LDL, and E-selectin and VCAM-1 were also upregulated in the endothelial cells exposed to laminar or disturbed flow. These results identify the response of endothelial cells to CP by defining the list of CP-inducible genes and provide new insights into potential mechanisms of atherogenesis from these intracellular bacteria.

Several other factors directly alter gene expression in endothelial cells. Elevated homocysteine levels have been identified as a risk factor for CAD, and it has been shown to alter gene expression in endothelial cells (46). Using the cardiovascular cDNA microarray approach, the expression of 600 genes in endothelial cells was screened and a subset was identified and considered to be modulated by homocysteine (47). They were

clustered according to the function of the encoded proteins, such as: endothelial motility, signalling and lipid metabolism. Similarly, oligonucleotide arrays were used to identify gene expression in endothelial cells exposed to nicotine, the major constituent of cigarette smoke (48). The most significant changes in gene expression found were the upregulation of proteins in the inositol phospholipid pathway, namely phosphatidylinositol phosphate kinase and diacylglycerol kinase. Changes were also detected for transcription factors cAMP response element binding protein and nuclear factor (NF)-kB.

Takabe et al. examined gene expression in endothelial cells exposed to BO-653, probucol and BHQ — which act as free radical scavenging antioxidants (49). These agents were initially developed as anti-atherosclerotic medications. Among 6,416 genes, 17 genes, including those encoding mitochondrial proteins and proteins related to oxidative stress responses, were induced more than threefold by these three drugs. By contrast, genes of three subunits of the proteosome (PSMA2, PSMA3, PSMA4) were downregulated. The gene coding for the cytochrome P-450 1A1 isozyme pathway, a drug metabolizing phase I enzyme, was expressed only by BHQ treatment.

Cultures of coronary artery endothelium and SMCs derived from a single coronary artery have been exposed to IL-1b and TNF-a, potent stimulators of inflammation (50). The most noticeable difference between the cell types was a considerable greater magnitude and complexity of the transcriptional response in the endothelial cells. Among the 209 regulated genes in the endothelium, 99 were not previously linked directly to atherosclerosis including those that had been associated with leukocyte function (e.g., IL-7 receptor, EBI-3 receptor) and others related to antiviral and antibacterial defense (e.g., oligoadenylate synthetase, LMP7, toll-like receptor 4,

complement component 3). In addition, 43 genes likely to participate in signal transduction (e.g. IL-18 receptor, STK2 kinase, STAF-50, ANP receptor, VIP receptor, RAC-3, IFP-35) were expressed. This provides evidence that a major effect of TNF-a and IL-1b is to alter the potential of the endothelial cell to respond to various other external stimuli.

In summary, multiple pathways are altered in endothelial dysfunction. Expression profiling has documented the alterations in expression of groups of genes by many common agents including laminar flow, infectious agents such as CP, as well as toxic agents like homocysteine, oxidised LDL and nicotine. Alteration of the function of endothelial cells may serve as the first strategic location for halting the cascade of events that may follow modification of gene expression in these cells. Each experimental protocol discussed above elicited an alteration in the expression of a unique cluster of genes. Although most genes were not found in common among the studies, this may be attributed to the unique response of the endothelial cell under the various conditions. Nevertheless, this may provide further medical strategies and targets for down regulating the inflammatory response which ultimately leads to atherosclerosis.

2.5 Expression profiling of vascular SMCs

The proliferation, migration and invasion of SMCs throughout a coronary artery make a significant contribution to atherosclerosis. Late occlusion of autologous saphenous vein grafts (SVGs) is due to medial and neointimal thickening secondary to migration and proliferation of SMCs and the subsequent formation of atherosclerotic plaques. This process is the main cause of restenosis after percutaneous transluminal

coronary angioplasty (PTCA). Understanding SMC proliferation may provide insights into the pathogenic mechanisms of CAD, as well as restenosis after PTCA. Expression profiling of SMCs has identified alterations in gene expression that may be related to the transition of quiescent, contractile SMCs to proliferative SMCs.

Human vascular SMCs were exposed to iloprost, a prostacyclin (PGI2)analogue which activates cyclic adenosine 3',5' monophosphate signalling, which is associated with maintaining SMCs in a quiescent state (Table 4) (51). PGI2 is known to be atheroprotective, as it has been

Table 4. Expression profiling of smooth muscle cells (SMCs).

Target tissue	Experimental design	Number of genes affected
Human vascular SMCs (50)	Exposure of cells to iloprost	83
SMCs from coronary artery (63)	Quiescent and invasive SMCs	47
Human aortic SMCs (64)	Exposure to tumour necrosis factor-alpha	Focus on eotaxin and CCR3 receptor
Saphen vein grafts (68)	Comparison with ungrafted veins	6
Human aorta (70)	Alpha-Tocopherol treatment	Focus on connective tissue growth factor
Arterial SMCs (75)	Butyrate	58

shown to exert many effects such as vasodilation, inhibition of platelet aggregation, modulation of cholesterol metabolism (52), as well as preventing SMC proliferation and migration (53, 54). A total of 83 genes were differentially expressed six hours after iloprost exposure. Several of these genes account for the atheroprotective response to iloprost. MAP kinase phosphatase-1 (MKP-1) has been regarded as a critical counteracting factor for several transcription factors (p38, JNK, ERK), leading to the downregulation of stimuli inducing the proliferation of SMCs (55). The zinc finger protein, hEZF, is associated with a growth arrest, as it has been shown to inhibit DNA synthesis in fibroblasts overexpressing this gene (56). The gene, Cyr61, was found to be downregulated by iloprost. This gene plays a role in mediating cell adhesion and migration, and augments growth factor-induced DNA synthesis in fibroblasts (57). Other critical genes include: has2 (of uncertain physiological consequence), stanniocalcin (cell protection against ischaemia (58), plasminogen activator inhibitor-1 (its downregulation may enhance fibrinolytic activity (59), MCP-1 (its downregulation may prevent monocyte attachment to SMCs (60), heme oxygenase- 1 (inhibits SMC proliferation (61) and COX-1 (promotes the synthesis of prostaglandins, and thus is a positive autoregulatory mechanism for enhancing prostaglandin levels (62). Downregulation of MCP-1 and COX-1 was identified in atherosclerotic plaques from human patients with unstable angina (16).

Microarray analysis using 558 cardiovascular-associated genes identified 47 genes differentially expressed in proliferating and non-proliferating human arterial SMCs (63). Most of the genes in the study were associated with the ECM and cell motility. The main gene groups identified included matrix organising proteins, ECM proteins, cell

adhesion proteins, and extracellular communication and cytoskeleton motility proteins. Genes previously associated with SMC migration and invasion, such as those coding for tissue inhibitor of metalloproteinases (TIMP)-2, TIMP-3 and MMP-3, were confirmed by the array data. Reduced expression of several cytoskeletal proteins, such as vimentin, fibronectin, cytokeratins and b1- integrin, were shown in the invasive phenotype. Furthermore, angio-associated protein, a-E-catenin and brain ANP receptor were downregulated, whereas TFPI-2 was strongly upregulated in invasive proliferating SMCs. Consequently, the data document the expression profile of essential genes in SMCs involved in the invasive process.

Microarray analysis with 8,600 genes identified .20-fold increases in steady-state eotaxin mRNA, and follow-up immunohistochemical studies with tissue samples from seven non-diseased and 14 atherosclerotic arteries demonstrated overexpression of eotaxin protein and its receptor, CCR3, in cultured human aortic SMCs treated with TNF-a (which induces vascular inflammation), whereas their expression was negligible in normal vessels (64). This was the first study to report the expression of eotaxin by human atheroma. While this was known to be a potent eosinophil chemoattractant and activator (65, 66), no eosinophils were identified in the target tissue. Similarly, CCR3 had previously been observed in macrophages, mast cells, neutrophils and endothelial cells in endobronchial biopsies of the atopic asthmatic lung (67). The implications of the study by Haley et al. are that TNF-a may recruit and activate macrophages and mast cells through the CCR3 receptor (64). This study may lead to the generation of either eotaxin- or CCR3-null mice, which might be resistant to atherogenesis.

In addition to SVGs being used for coronary artery bypass grafting, left and right internal mammary arteries have also been used (68). The SVG is the standard conduit for coronary artery bypass grafting to all areas of the heart except the LAD coronary artery. Accelerated atherosclerosis occurs in venous conduits such that during the first year after bypass surgery, 15 per cent of vein grafts occlude; between one and six years, the graft attrition rate is 1–2 per cent per year; and between six and ten years, it is 4 per cent per year (69). Thus, within the first decade of bypass surgery, most bypass grafts will be occluded or significantly diseased. A major component of this occlusion is the proliferation of SMCs. Differentially expressed genes in the saphenous vein have been documented and correlate with expected gene expression patterns, including upregulation of c-jun and CDK-10. In addition, previously unidentified gene expression patterns were detected, such as the upregulation of HSP-70, fibronectin-1, erbB-3 proto-oncogene and c-myc.

The effect of a-tocopherol treatment on gene expression in human aortic SMCs has been studied because it may accelerate wound repair and tissue regeneration during atherosclerosis (70). This medication is also often recommended because of its antioxidant properties (71). The expression of the connective tissue growth factor (CTGF) gene was induced by a-tocopherol 1.8-fold. It was hypothesised that this gene is involved in normal repair processes and is permanently overexpressed in pathological events (70). a-tocopherol may either stimulate the synthesis of CTGF or interfere with the effect of TNF-a on downregulation of this gene.

cDNA array technology has been utilised to unravel the molecular basis of the antiproliferative effect of butyrate on vascular SMCs. Butyrate is a natural fatty acid

which has been shown to inhibit SMC proliferation in in vitro cell culture systems (72). It is derived from the microbial metabolism of dietary fibre in the colon, where it is produced in high levels (73). It can function as an anti-inflammatory agent by suppressing intestinal inflammation (74), and may alter gene expression by reversibly inhibiting histone deacetylase, causing the hyperacetylation of histones and thereby leading to selective changes in gene expression (73). To assess the involvement of gene expression in butyrate-inhibited vascular SMC proliferation, proliferating vascular SMCs were exposed to butyrate (75). A total of 111 genes exhibiting moderate (two- to fivefold) to strong (fivefold) differential expression were identified.   Analysis of these genes indicates that butyrate treatment mainly alters the expression of four different functional classes of genes, which include: 43 genes implicated in cell growth and differentiation, 13 genes related to stress response, 11 genes associated with vascular function and eight genes normally present in neuronal cells. cDNA expression profiles indicate that butyrate-inhibited vascular SMC proliferation involves a combined action of a proportionally large number of both positive and negative regulators of growth, which ultimately causes growth arrest of vascular SMCs (75). For example, the downregulated genes included pRb (required for G1 to S phase entry), S phase-specific PCAN, G2/M phase-specific cyclinB1 and cdc2, and p55cdc. The downregulation of these critical positive regulators of cell cycle progression indicates that butyrate exerts its antiproliferative effect by altering key positive regulators of all phases of cell cycle progression. Furthermore, butyrate also appeared to block SMC proliferation by upregulating negative regulators of cell growth or differentiation inducers such as p21waf1, p14INK4B/p15INK5B and clusterin. Consequently, the identification of the

molecular mechanisms of butyrate-inhibited proliferation is important, not only to understand the molecular basis of butyrate-induced SMC proliferation, but it may be used as a therapeutic agent to prevent restenosis of arteries in humans.

In summary, smooth muscle cell proliferation contributes to the formation of atherosclerotic lesions and restenosis after angioplasty, and microarray studies have identified multiple genes that are involved in this process. Understanding the molecular mechanisms involved may provide novel insights into pharmacological approaches to preventing SMC migration.

2.6 Expression profiling of inflammatory cells

Inflammatory cells play an important role in the development of CAD. First, monocyte recruitment is a key event in the ormation of the earliest vascular lesion, the fatty streak. Secondly, macrophage uptake of modified LDLs via scavenger receptors gives rise to foam cells in the atheroma, and these secrete growth factors, cytokines and inflammatory mediators that influence the growth and development of other cell types within the atherosclerotic lesion, generating plaques. Microarrays have been used to profile gene expression changes in the inflammatory cells in relation to the formation of atherosclerotic lesions.

Gene expression of macrophages has been analysed using microarrays containing approximately 16,000 human cDNAs (Table 5) (76). The human monocytic leukaemia cell line (THP-1) was treated with oxidised LDL. The cells were then activated by treatment with lipopolysaccharide (LPS), and RNA was harvested at zero, one and six hours after LPS addition. Oxidised LDL treatment affected the expression of 57 of 127

genes in macrophages. Among them are genes that code for potent intracellular and extracellular signaling molecules such as NF-kB, A20 and numerous cytokines and chemokines. Oxidised LDL pretreatment resulted in a significant change in LPS-induced NFkB activation. Some of the oxidised LDL effects were mediated by the nuclear receptors RXR and peroxisomal proliferator-activated receptor-g.

To further define the mechanisms by which LDL particles affect macrophages and foam cells, a large-scale gene expression analysis of cholesterol-loaded macrophages was carried out (77). Oxidised LDL-treated and time-matched control untreated cells were hybridised to microarrays containing 9,808 human genes. Two hundred and sixty-eight genes were found to be differentially expressed at least twofold at one or more time points. These patterns of regulation were classified into seven clusters. Shiffman et al. identified patterns of gene expression of known and novel molecular components of the cellular response that are implicated in the growth, survival, migratory, inflammatory, and matrix remodelling activity of vessel wall macrophages (77). The data indicate that oxidized LDL loading of THP-1 cells did not elicit any measurable immediately transcriptional response from the genes studied until day 4. The novel and most highly upregulated genes identified were: DSCR1, LARC/MIP-3 a, CD73/50-nucleotidase, epithelial membrane protein-1, uridine phosphorylase and two expressed sequence tags (ESTs). The incidence of these genes indicates that the previously published data on oxidised LDL loading of macrophages only partially describes the changes that occur in some inflammatory cells. The most highly downregulated genes included promoters of the anti-microbial potential of macrophages (carbonic anhydrase, cytochrome b-245,

Table 5. Expression profile of inflammatory cells.

Target tissue	Experimental design	Number of genes affected
Macrophages (76)	Oxidized low-density lipoprotein	268
Human monocytic leukaemia cell line (77)	Lipopolysaccharide	57
Macrophages (78)	Exposure to copper	7
Macrophages (80)	Exposure to arsenic	62
THP-1 cells (81)	Biomechanical forces	3
Macrophages (86)	Exposure to androgens	27 genes exclusively upregulated in males

differentially expressed at least twofold at one or more time points. These patterns of regulation were classified into seven clusters. Shiffman et al. identified patterns of gene expression of known and novel molecular components of the cellular response that are implicated in the growth, survival, migratory, inflammatory, and matrix remodelling activity of vessel wall macrophages (77). The data indicate that oxidized LDL loading of THP-1 cells did not elicit any measurable immediately transcriptional response from the genes studied until day 4. The novel and most highly upregulated genes identified were: DSCR1, LARC/MIP-3 a, CD73/50-nucleotidase, epithelial membrane protein-1, uridine phosphorylase and two expressed sequence tags (ESTs). The incidence of these genes indicates that the previously published data on oxidised LDL loading of macrophages only partially describes the changes that occur in some inflammatory cells. The most highly downregulated genes included promoters of the anti-microbial potential of macrophages (carbonic anhydrase, cytochrome b-245, RNase A2, CD64) and three involved in cell cycle progression or synthesis of precursors involved in cell division (GOS2 protein, thymidylate synthetase, lamin B1).

Similarly, DNA microarrays were used to define the changes in gene expression profile in response to copper exposure of human macrophages (78). Serum copper has been shown to be associated with cardiovascular disease (a 3.5-fold increased risk of acute MI) but its mechanism of action has not been identified (79). The results showed that expression of 91 genes were altered. Copper increased the expression of seven cholesterogenic genes (3-hydroxy-3-methylglutaryl coenzyme A synthase, IPP isomerase, squalene synthase, squalene epoxidase, methyl sterol oxidase, H105e3 mRNA and sterol-C5-desaturase) and LDL receptor, and decreased the expression of CD36 and

lipid-binding proteins. Thus, copper activates cholesterogenic genes in macrophages involved in LDL uptake and de novo cholesterol biosynthesis. This leads to lipid accumulation in the inflammatory cells and thus the arterial wall.

Gene expression was also studied in lymphocytes exposed to arsenic, which is associated with an increased risk for vascular disease (80). Patients were exposed to low, intermediate or high levels of arsenic as determined by monitoring serum levels. A total of 62 genes showed a significant difference in the intermediate and high levels of blood arsenic, as compared with low levels. These genes included those encoding IL-1 b, IL-6, chemokine C-C motif ligand2/MCP-1, chemokine C-X-C motif ligand 1/growth-related oncogene-a, chemokine C-X-C motif ligand2/growth-related oncogene- b, CD14 antigen and MMP-1.

The effect of biomechanical forces on macrophage functions in atherosclerosis was determined by microarrays with 1,056 genes (81). Mechanical deformation at 1Hz was applied to a thin transparent membrane on which the cells were cultured. This stress induced only three genes to more than 2.5-fold higher expression at three and six hours: prostate apoptosis response-4 (3.0-fold at three hours, 6.7-fold at six hours), IL-8 (4.3-fold at six hours) and the immediate-early response gene, IEX-1 (2.6-fold at six hours). IL-8 may be important in the initiation and amplification of inflammation and angiogenesis in atherosclerosis, because it is chemotactic for vascular SMCs, T lymphocytes and neutrophils (82). It also triggers the firm adhesion of monocytes to the vascular endothelium (83). IEX-1 is an NF-kB-inducible immediate-early gene which inhibits apoptosis (84). It may also participate in the differentiation of monocytes/macrophages. PAR-4 is known to be a widely expressed protein which

47

confers sensitization to apoptosis induced insults (85). Consequently, this study demonstrated that human monocytic cells respond to mechanical deformation with induction of immediate-early and inflammatory genes. This suggests that mechanical stress in vivo, such as that associated with hypertension, may play an important part in atherogenesis and instability of coronary artery plaques through biomechanical effects on vascular macrophages.

Human monocyte-derived macrophages from healthy male and female donors have been exposed to androgen (dihydrotestosterone) in an effort to detect gender-specific changes in gene expression (86), as it has already been established that there is a gender difference in the severity of disease, with men having more severe coronary artery disease than women (87). In macrophages harvested from males, treatment with androgen resulted in the differential upregulation of 27 genes, whereas none was upregulated in female harvested macrophages. Some of these genes directly related to the process of atherosclerosis, including the upregulation of acyl coenzyme A:cholesterol acyl transferase I and lysosomal acid lipase (LAL) (which plays a role in the delivery of lipoproteins to cells) (88), caveolin-2, CD40, leukotriene B4 receptor and cadherin-19 (the latter three play a role in inducing cell surface receptors for monocyte attachment (89)). Functional studies to complement the microarray results showed a direct clinical correlation with LAL, in that there was a consistent increase in activity with 125I-AcLDL exposure. It can be concluded that androgen exposure as it occurs naturally in the male gender is directly responsible for inducing genes associated with the development of atherosclerosis and may account for gender differences in the incidence of CAD.

2.7 Expression profiling of CHF.

CHF is a term used to describe any condition in which the heart is unable to generate an adequate pressure to pump blood throughout the body. This condition causes symptoms such as shortness of breath (dyspnea), fatigue, weakness and swelling (edema) of the legs and sometimes in the abdomen (ascites). An estimated 4.8 million Americans have CHF (90), and half of all patients diagnosed with CHF will be dead within five years. Each year, there are an estimated 400,000 new cases. Increasing prevalence, hospitalizations and deaths have made CHF a major chronic condition in the USA. It often occurs as the end stage of cardiac disease, referred to as ischemic cardiomyopathy (ICM) due to severe CAD. Expression profiling of human cardiac tissues has been employed to identify genes whose expression is linked to CHF, and these studies are summarized below.

Oligonucleotide microarrays were used to profile seven non-failing (NF) and eight failing (F) human hearts with a diagnosis of end-stage dilated cardiomyopathy (Table 6).91 Of 6,606 genes on the microarray, 103 genes in ten functional groups were differentially expressed between F and NF hearts. In failing hearts, K means clustering revealed two potentially novel pathways associated with up-regulation of atrial natriuretic factor and brain natriuretic peptide and with increased expression of ECM proteins. A dendrogram or genetic profile of the two tissues distinguished two failing hearts with distinct etiologies (familial and alcoholic cardiomyopathy, respectively). The study demonstrated that there is a unique molecular signature in failing hearts.

Similarly, a 'genomic portrait' of heart failure derived with microarrays was obtained from end-stage dilated cardiomyopathy (DCM) using the CardioChip with non-redundant 10,848-element human cardiovascular-based ESTs (92). More than 100 transcripts were consistently differentially expressed in DCM .1.5-fold (versus pooled NF hearts). ANP was also found to be upregulated in DCM (19-fold compared with NF hearts) as well as numerous sarcomeric and cytoskeletal proteins (e.g. cardiac troponin, tropomyosin), stress response proteins (e.g. HSP-40, HSP-70) and transcription/translation regulators (e.g. CCAAT box binding factor, eIF-1AY). Downregulated genes were classified as cell-signaling channels and mediators, particularly those involved in calcium pathways [calcium/calmodulin-dependent kinase, inositol 1,4,5-trisphosphate receptor, and sarcoplasmic reticulum Ca 2þ-ATPase (SERCA)]. Several novel, cardiac-enriched ESTs were also co-expressed. The study demonstrated a common expression pattern among the sets of samples with DCM, and documents a gene list that may serve as possible targets for therapeutic intervention specific to cardiac tissue.

Also using patients with either DCM or hypertrophic cardiomyopathy (HCM) at end-stage CHF, Hwang et al. generated a list of predictive markers for these two diseases (93). Similarly to previous studies, ANP and SERCA were found to be increased and decreased, respectively, and this was consistent in all of the tissues studied. Other genes having the same expression levels in both DCM and HCM tissues included: copper/zinc superoxide dismutase, heat shock protein 90, elongation factor-2, calcium-activated neutral protease, decorin and CD59. Genes found to be differentially expressed between DCM and HCM included atrial myosin alkali light chain, calsequestrin, lipocortinand

lumican, which were upregulated, while myc transcriptional activity and b-dystrobrevin were downregulated. In total, the study documented 192 genes. As in the previous studies reviewed, ANP and SERCA displayed the same expression patterns consistently in both populations of samples.

In a study by Steenman et al., the expression profiles of four specific pathophysiological cardiac situations was analyzed: i) left ventricle (LV) from NF heart; ii) LV from F heart affected by DCM; iii) LV from F heart affected by ICM; and iv) right ventricle (RV) from F heart affected by DCM or ICM (94). Microarrays representing approximately 12,000 human genes were utilized. The authors identified 1,306 genes with a similar expression profile in all four cardiac situations. The upregulation of MLC1emb, calponin and SM22 represented a reversal to developmental gene expression such as occurs in cardiac remodeling and CHF. Smooth muscle myosin heavy chain and smooth muscle a-actin represented a dedifferentiation process. Several apoptosis-related genes were identified in F LV and/or RV samples, with CDKN1A showing the most marked activation. Stress-inducible metallothionein was also upregulated; this functions as an antioxidant and inhibits the production of atrial natriuretic factor (95). Novel genes included AF1a, a transmembrane protein (96), and SH3BGR. Relatively few genes were differentially expressed between F LV and F RV samples, yet the most marked difference was found in the gene profiling, an actin-binding protein being implicated in the control of actin polymerization and cytoskeletal reorganization (97). Finally, no genes were significantly differentially expressed between DCM and ICM F hearts, which indicates that all hearts were clinically in failure.

In a separate study, oligonucleotide arrays have identified specific genes expressed in patients with CHF, screening over 7,000 genes in two NF and two F human hearts with diagnoses of end-stage ICM and DCM (98). Genes were categorized into five groups: (a) cytoskeletal and myofibrillar genes [myomesin, non-sarcomeric myosin regulatory light chain-2 (MLC2) and ss-actin]; (b) genes responsible for the degradation and disassembly of myocardial proteins (alpha-1-antichymotrypsin, ubiquitin and gelsolin); (c) genes involved in metabolism [ATP synthase alpha-subunit, succinate dehydrogenase flavoprotein(SDH Fp) subunit, aldose reductase, and TIM-17 preprotein translocase]; (d) genes responsible for protein synthesis [elongation factor- 2(EF-2), eukaryotic initiation factor-4AII and transcription factor homologue-HBZ17]; and (e) genes encoding stress proteins (a- B-crystallin and m-crystallin). The study focused on the novel gene SLIM1 as being consistently downregulated in CHF. Although the precise function of SLIM1 has not been determined, it is hypothesized that it may act as a scaffold for interaction between thin filaments and the cytoskeleton (99), in a similar way to muscle LIM protein (ML). MLP knockout mice have been shown to develop DCM (100). All of the identified genes may contribute to development of the heart failure phenotype and represent compensatory mechanisms to sustain cardiac function in failing human hearts.

Similarly, Steenbergen et al. used DNA microarray profiling to investigate changes in the expression of genes involved in the apoptosis that occurs in human idiopathic DCM hearts that have progressed to CHF (101). They documented altered gene expression consistent with a pro-apoptotic shift in the TNF-a signaling pathway. Specifically, they found decreased expression of TNF- a- and NF- kB-induced anti-

apoptotic genes such as growth arrest and DNA damage-inducible (GADD) 45-b, Flice

inhibitory protein (FLIP) and TNF-induced protein 3 (A20) genes. They also observed a

significant decrease in the phosphorylation of BAD at Ser-112, which is also consistent

with a role for apoptosis in heart failure.

One option for improving left ventricular function in CHF is the use of a left

ventricular assist device (LVAD). The unloading of the F heart with an LVAD can

decrease cardiac mass and myocyte size and has the potential to improve contractile

function. Several studies have documented changes in gene expression brought about by

the presence of an LVAD. The effect of chronic ventricular unloading on myocardial

gene expression was compared before and after LVAD support in seven patients with

idiopathic DCM and end-stage CHF (102). On average, 1,374 genes were reported as

'increased' and 1,629 'decreased' after LVAD support. Upregulated genes included a

large proportion of transcription factors, genes related to cell growth/apoptosis/DNA

repair, cell structure proteins, metabolism and cell signaling/communication. LVAD

support resulted in downregulation of genes coding for cytokines. Similarly, Chen et al.

performed transcriptional profiling using a spotted cDNA microarray with 12,814 unique

clones on paired samples of LV obtained before and after placement of a left ventricular

assist device in 11 patients.103 The most significantly upregulated gene was the G-

protein-coupled receptor APJ, the specific receptor for apelin. Blaxall et al. also

examined gene expression, performing oligonucleotide arrays with LV samples from six

male patients which were harvested during LVAD placement and at the time of

explantation (104). These authors documented a significant difference in the

corresponding LVAD mediated regulation of gene expression and were able to distinguish, in a blind manner, patients with different CHF etiologies.

Finally, microarrays were utilized on paired samples of human tissue from patients with CHF with and without an LVAD (105). After several statistical analyses of the data, approximately 85 upregulated genes were identified. The focus of the study was on apelin, which was the most significantly upregulated gene, and was localized primarily in the endothelium of the coronary arteries after the mechanical offloading by the LVAD. This finding implies an important paracrine signaling pathway in the F heart. The identification of this gene led to the measurement of serum apelin levels. These were found to be increased in the serum of patients with F hearts and may serve as a biological marker in patients with CHF.

In summary, microarray analysis of CHF can reveal the underlying molecular mechanisms regulating hypertrophy and proliferation, as well as apoptosis of cardiomyocytes. These studies revealed the dysregulation of various molecular pathways involved in CHF.

2.8 Expression profiling of CHD

Congenital heart defects account for the largest number of birth defects in humans (4). The application of expression profiling in patients with CHD is still in its infancy. To date, only one microarray study has documented gene expression in CHD.

Microarray analysis was carried out in cardiac samples collected during cardiac surgery, from six normal samples and from 55 patient samples with various cardiac defects (106). Human Unigene Set-RZPD 2 cDNA arrays with 12,657 known genes

were used. The study identified specific molecular signatures for tetralogy of Fallot (TOF), ventricular septal defects (VSDs) and right ventricular hypertrophy (RVH), each of which has a distinct gene expression profile. TOF and RVH were found to be associated with genes of various functional classes, and can be clearly distinguished by different molecular signatures. The TOF signature consists of many genes involved in the cell cycle and cardiac development (e.g. upregulation of SNIP, A2BP1and KIAA1437, and downregulation of STK33, BRDG1and TEKT2) and the upregulation of ribosomal proteins S6, L37a, S3A, S14 and L13A. The RVH signature includes genes mainly involved in the stress response, cell proliferation and metabolism — for example, the upregulation of ADD2. VSDs have been associated with a specific signature consisting of downregulation of genes for ribosomal proteins S11, L18A, L36, LP0, L31 and MRPS7, and downregulation of genes involved in cell proliferation, differentiation and apoptosis (e.g. AMD1, RIPK3, EGLN1, SIAHBP1 and ARVCF). Several ion channel genes, including SLC26A8, SLC16A5, SLC4A7, KCNS2and KCNN3, were found to be differentially expressed in VSD tissues. The study also identified heart chamber-specific expression of many genes. The human heart consists of two atria and two ventricles. The atria have been found to have higher expression of genes for ECM or actin modulation, such as CST3 and PCOLCE, translation factor EEF1A and the DNA helicase REQL4, and a potassium channel subunit KCNIP2. Genes with higher expression in the ventricles encode proteins for cytoskeleton contraction, metabolism–energy turnover and cell cycle–growth, and include TMP1, FHL1, and ANKRD2.

2.9 Conclusions

The availability of cDNA and oligonucleotide microarrays with several thousand human genes makes it possible to study global changes in gene expression in CAD, CHF and CHD. These studies have identified novel genes and pathways involved in the generation of these diseases at both the organ/tissue level (coronary arteries, cardiac tissues) and cell level (endothelial cells, vascular SMCs, macrophages). It is important to note that several studies have confirmed the expression of genes previously linked with these disease processes, suggesting that expression profiling with microarrays is a valid approach for identifying gene expression alterations associated with disease. Yet, many more novel genes have also been identified, and will serve as a valuable resource for identifying novel pathways involved in the generation of disease. With the continued use of this technology, and an accelerated application to these diseases, we will eventually identify a common group of genes which will be able to serve as markers for these disease processes on a global scale. It is important, however, to note that different studies have identified different sets of genes associated with these diseases. Thus, the microarray results remain to be replicated in an independent set of samples, and caution needs to be taken when interpreting these results. In addition, a major limitation of microarray analysis is that it is usually difficult to distinguish whether the identified differential gene expression patterns are the cause or the consequence of the disease. Follow-up studies with transgenic overexpression or knockout of the gene of interest in animal models are needed to solve this issue. Furthermore, microarray analysis is biased to the genes on the arrays, and cannot be used to evaluate low-abundant transcripts. Microarray analysis also results in false-positives; it is therefore crucial that results are

confirmed using conventional technologies, such as quantitative real-time PCR, quantitative reverse-transcription PCR, Northern blot, Western blot or immunostaining analyses. Finally, the expression level of mRNA does not necessarily reflect the expression of the protein, and the pathogenic mechanism of a disease may involve protein modifications such as phosphorylation and glycosylation. Under these circumstances, proteomics will prove to be a powerful, alternative technology. The first proteomics analysis has been performed for CAD by the authors of this paper, and the study identified the ferritin light chain as a significant marker for diseased coronary arteries (3). It is important to point out that proteomics can analyze far fewer genes than can microarrays, as the resolution of the common two-dimensional gel separation of protein extracts is limited to only a few thousand protein spots. Proteomics also shares some common limitations with microarray analysis; for example, it cannot be used to evaluate low-abundant proteins/genes.

Nevertheless, microarray analysis can generate novel hypotheses relating to the pathologies of these diseases, and further studies with animal models, molecular biology, cell biology and biochemistry will validate these hypotheses and provide novel insights into the pathogenesis of disease. These studies will eventually generate novel diagnostic and therapeutic markers, and identify potential drug targets which will serve to bring about more effective management of cardiovascular disease.

CHAPTER 3

INTRODUCTION

IDENTIFICATION OF NEW GENES DIFFERENTIALLY EXPRESSION IN CORONARY ARTERY DISEASE BY EXPRESSION PROFILING

ABSTRACT

Genetic factors increase the risk to coronary artery disease (CAD). To date, a limited number of genes that potentially contribute to development of CAD have been identified. In this study, we have performed large-scale gene expression analysis of ~12,000 human genes in nine severely atherosclerotic and six nonatherosclerotic human coronary arteries using oligonucleotide microarrays. Fifty-six genes showed differential expression in atherosclerotic coronary artery tissues; expression of 55 genes was increased in atherosclerotic coronary arteries, whereas only one gene, *GST*, encoding a reducing agent, showed downregulated expression. The expression data of selected genes were validated by quantitative RT-PCR analysis as well as immunostaining. The associations of 49 genes with CAD appear to be novel, and they include genes encoding ICAM-2, PIM-2, ECGF1, fusin, B cell activator (*BL34*, *GOS8*), Rho GTPase activating protein-4, retinoic acid receptor responder, ß2-arrestin, membrane aminopeptidase, cathepsins K and H, MIR-7, TNF-α-induced protein 2 (B94), and flavocytochrome 558.

In conclusion, we have identified 56 genes whose expression is associated with CAD, and 49 of them may represent new genes linked to CAD.

Archacki SR, Angheloiu G, Tian XL, Tan FL, DiPaola N, Shen GQ, Moravec C, Ellis S, Topol EJ, Wang Q. Identification of new genes differentially expressed in coronary artery disease by expression profiling. *Physiol Genomics*. 2003;15(1):65-74.

3.1 Introduction.

Coronary artery disease (CAD) and myocardial infarction (MI) are the leading causes of death in the United States and other Western countries. An estimated 7.5 million living Americans have experienced MI, and 12.6 million have CAD (1). Approximately 1.1 million Americans developed MI in 1999, and the mortality rate was more than 45%.

CAD is a multifactorial disorder influenced by both genetic and environmental factors and their interactions. Genetic-epidemiological and twin studies strongly suggest that genetic factors contribute to the development of atherosclerosis (9). Case-control studies have been used to identify genes that are associated with increased risks to CAD and MI. These genes include the thrombospondin (TSP) genes (*TSP-1*, *TSP-2*, and *TSP-4*)[43], *LTA* gene encoding lymphotoxin-α, (29) connexin 37, (52) and the genes encoding plasminogen-activator inhibitor type 1, stromelysin-1,(52) apolipoprotein E (ApoE), Lp(a) or Apo(a), Apo AI, ApoCIII, ApoIV, fibrinogen, tissue-type plasminogen activator (TPA), plasminogen activator inhibitor (PAI-1), von Willebrand factor (VWR), platelet glycoprotein IIIa, lipoprotein lipase, cholesterol ester hydrolase, cholesterol ester transfer protein (CETP), factor V, factor VII, angiotensin converting enzyme, angiotensinogen, and endothelial nitric oxide synthase (49).

Various biochemical methods including Northern blot, Western blot, and immunostaining have also been employed to identify genes that are associated with CAD. Previous studies have implicated the upregulation of specific genes in atherosclerotic tissue including chitotriosidase (4) lumican (27), VCAM-1 (10), ICAM-1 (10), matrix metalloproteinase-9 (MMP-9) (42), and osteopontin (24) (as well as the downregulation

of endothelial nitric oxide synthase (25). Recently, subtraction suppression hybridization (SSH) was employed to identify genes that are differentially expressed in stable and ruptured atherosclerotic plaques (a major cause of thromboembolic complications) in human coronary arteries. Only a limited number of genes associated with plaque rupture were identified by SSH: the perilipin gene was found to be upregulated, and genes for ß-actin, fibronectin, and immunoglobulin-λ chain were downregulated (8).   DNA microarray technology was also performed on arteries such as common, internal, and external iliac artery, aorta, popliteal artery, posterior tibial artery, and tibiofibular trunk to define a group of 75 novel genes expressed in atherosclerotic plaques; however, the coronary arteries, the target organ of CAD, were not investigated (12).

To summarize, only a small number of genes that potentially contribute to the pathogenesis of CAD have been identified. No microarray analysis has been performed on human coronary arteries from patients with CAD. Therefore, in this study we used oligonucleotide microarrays with ~12,000 unique genes to identify genes that are differentially expressed in atherosclerotic human coronary arteries.

3.2 Material and Methods

3.2.1 Tissue Sampling of Human Coronary Arteries

Coronary arteries were obtained from explanted hearts through the Cleveland Clinic Heart Transplant Program and unmatched or rejected donor hearts from Lifebanc of Northeast Ohio (Table 1). Informed consent was obtained from all participants according to the standards established by the Cleveland Clinic Foundation Review Board on Human Subjects. Coronary artery segments measuring 5 mm in length were harvested immediately after heart extraction and cleaned of adjacent adipose and myocardial tissue.

61

The adventitial layer remained intact. The coronary arteries were harvested immediately upon explantation of the heart in the surgical suite and snap-frozen in liquid nitrogen and stored at -80°C as previously described until used (22).

Table 1. Patient characteristics.

	Atherosclerosis				No Atherosclerosis		
Sample	Age/Yrs	Race/Sex	% LAD Disease	Sample	Age/Yrs	Race/Sex	% LAD Disease
D1	49	W/M	75	N1	62	W/M	ND
D2	59	W/M	95	N2	59	W/M	ND
D3	69	W/M	75	N3	55	W/F	ND
D4	52	W/M	75	N4	75	W/M	ND
D5	60	W/M	80	N5	47	W/M	ND
D6	68	W/M	95	N6	74	W/M	ND
D7	47	W/M	95				
D8	56	W/F	75				
D9	61	W/M	75				

D1-9, patients with atherosclerosis; N1-6: patients without detectable atherosclerosis; ND, no detectable atherosclerosis; LAD, lateral anterior descending.

We selected a segment of the proximal lateral anterior descending (LAD) with obvious intraluminal plaque on inspection. The presence or absence of atherosclerosis was determined by a cardiovascular pathologist's diagnosis made upon gross examination of transverse coronary sections visually and microscopically. The atherosclerotic coronary arteries had an intraluminal stenosis of >75%, all of which had similar complexities (stage IV according to the American Heart Association classification), and non-CAD tissues had no detectable atherosclerosis.

3.2.2 RNA Isolation and Oligonucleotide Arrays

Total RNA was isolated from the coronary arteries using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was confirmed visually on a 1% denaturing agarose gel, and RNA concentration was measured using a spectrophotometer. Double-stranded complementary DNA (ds-cDNA) was synthesized from 15 μg of total RNA using the SuperScript Choice System (Invitrogen) with an HPLC-purified oligo-dT primer containing a T7 RNA polymerase promoter (Genset, La Jolla, CA) as instructed by the manufacturer. In vitro transcription was performed with 1 μg of ds-cDNA using the Enzo BioArray RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Fragmentation of biotinylated cRNA (20 μg), hybridization, washing, and staining were performed following the instructions by Affymetrix.

3.2.3 Data Extraction and Statistical Analysis.

The Human Genome U95A Array (Affymetrix) was used. Each array contains ~12,000 unique genes. One microarray was used for each human coronary artery after the quality and integrity of the RNA/cRNA sample was validated with a test array. To

make comparisons across microarrays, data sets on the arrays were normalized to a targeted total fluorescence of 300 representing total cRNA hybridized on the array.

We used GeneSpring 4.2 (Silicogenetics) for data analysis. Nine atherosclerotic coronary arteries (group D) were compared with six normal arteries (group N). All samples were treated as one group of replicates. We used the distribution of all genes in the 50th percentile to generate the median value for group comparisons, and all raw data with a score less than zero were set to zero. The algorithm to generate a list of genes that showed a statistically significant difference ($P < 0.05$) between the two groups was described previously (40). Genes were further filtered by an absolute call: present (P) or marginally present (M) in at least six of nine samples for the diseased group or four of six samples for the normal group, for upregulated genes and downregulated genes, respectively. The final list consisted of 56 genes.

3.2.4 Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (quantitative RT-PCR) was used to validate our microarray findings. Reverse transcription was performed with 5 μg of total RNA from all 15 samples used for microarray analysis using the SuperScript Choice System (Invitrogen). Quantitative RT-PCR was performed in ABI Prism 7700 Sequence Detection System using SYBR Green (Applied Biosystems) following the manufacturer's instructions with the following standard PCR conditions (94°C 10 min, and 94°C 30 s, 58°C 1 min, 72°C 45 s for 35 cycles, then 10-min extension at 72°C). Relative expression values were calculated as previously described (54). Primers were designed based on sequences from the GenBank and to span exon-intron junctions to prevent amplification of genomic DNA. The gene ß$_2$-microglobulin was used as an internal control.

3.2.5 Immunostaining

The cryosections (6 µm) of coronary arteries were fixed with paraformaldehyde (4%) and permeabilized with Triton X-100 (0.2%). The primary antibodies used include the antiserum for MMP-9 (clone 56–2A4 ICN Biomedica), ICAM-2 (Santa Cruz Biotechnology), osteopontin (Calbiochem), PIM-2 (Santa Cruz Biotechnology), CD31 (Becton-Dickinson), and α-smooth muscle actin (Sigma) (1:250). The secondary antibodies conjugated with either FITC or Cy3 were used for visualization (1:250). The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vectashield with OAD; Vector Laboratories, Burlingame, CA). The immunostained slides were visualized with a Zeiss Axiophot fluorescence microscope, and the images were captured with Photometrics SmartCature.

3.3 Results

3.3.1 Identification of 56 Genes That Are Differentially Expressed in CAD Coronary Arteries

We profiled nine coronary arteries from CAD patients and six non-CAD arteries using the Affymetrix Human Genome U95A Arrays. The mean age of the CAD group (group D) is 58 ± 7.7 yr and did not have a statistical difference from that of the normal group (group N) (62 ± 10 yr) ($P > 0.05$) (Table 1). Between the two groups (N vs. D), there were not any statistically significant differences ($P > 0.05$) in percent of genes expressed ($58 \pm 17\%$ vs. $54 \pm 15\%$), Q-score ($2.28 \pm .34$ vs. 2.45 ± 48), or background noise (43.68 ± 9.5 vs. 54.9 ± 24.6), respectively. The average mean (SD) difference of the housekeeping genes was not statistically significant (N vs. D) ($P > 0.05$): ß-actin (17,074

± 4,259 and 16,081 ± 4,674), GAPDH (8,957 ± 314 vs. 9,341 ± 1,417) and $ß_2$-microglobulin (8,541 ± 2,028 vs. 8,526 ± 2,817), respectively. These results indicate low variability and high reproducibility of our microarray analysis.

Of the ~12,000 genes analyzed by oligonucleotide arrays, we performed two independent analyses using Wilcoxon Mann-Whitney parametric test and the Student's t-test for genes that were differentially expressed (**Fig. 1**). Using a $P < 0.05$, the Wilcoxon ANOVA test generated 420 potentially differentially expressed genes between the two groups. At the same time, we used a Student's t-test ($P < 0.05$), which considers both absolute differences between the two groups, to generate a list of 372 genes. Combination of both statistical tests generated a list of 401 genes. From this gene list, 177 genes, or 44%, were upregulated by 1.5-fold, whereas 76 genes, or 19%, were downregulated. Sixty-one genes of the 177 upregulated genes met our criteria of a minimum expression level of 400, the average raw score of ~12,000 genes on each array, whereas 1 gene out of 76, or 1.3%, downregulated genes met this criteria. The final gene list generated by filtering these genes based on the call of present (P) or marginally present (M) consisted of 56 genes associated with CAD.

Statistical Group Comparison: Welch Test/Welch ANOVA
Gene List = 401 Genes

↓

Filter by 1.5-Fold Expression

↙ ↘

177 Genes with Increased Expression   76 Genes with Decreased Expression

↓   ↓

Filter by Expression Restriction with Raw data Score of 400

↓   ↓

61   1

Filter by Data File Restriction Call of "P" or "M

↓

FINAL LIST: 56 GENES
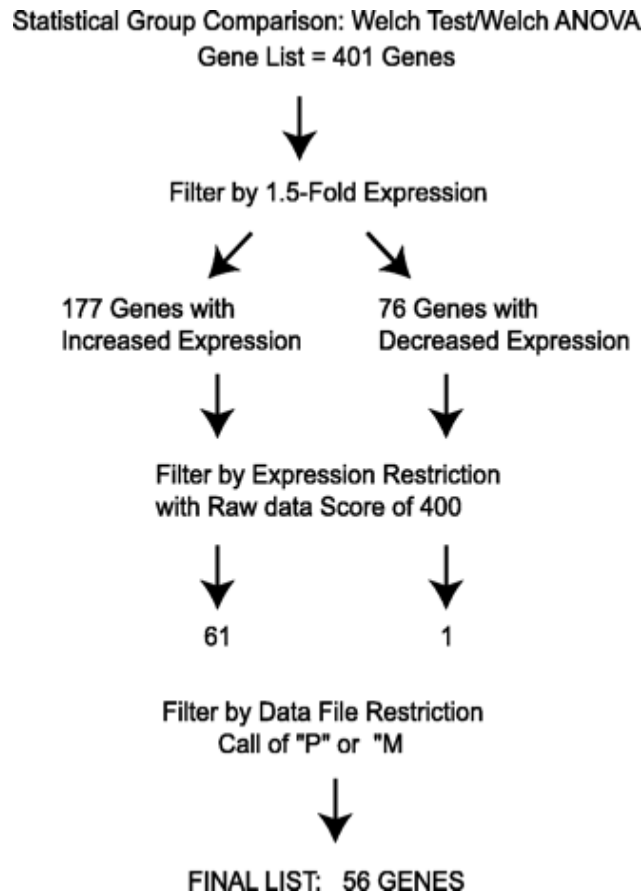
Fig. 1. Algorithm of statistical protocols used for our comprehensive cut-off points for data mining. All genes were initially filtered by a Welch test and Welch ANOVA that met the critical value of $P < 0.05$ for both tests. Genes were filtered with a mean average difference of 400 units for the diseased group for upregulated genes and normal group for downregulated genes. Genes were further filtered by an absolute call: present (P) or marginally present (M) in at least six of nine samples for the diseased group or four of six samples for the normal group for upregulated genes and downregulated genes, respectively. The final list consisted of 56 genes.

Table 2. Classification of differentially expressed genes in five groups based on their molecular activity

GenBank ID	Gene Description	Molecular Activity Relevant for CAD	Fold Change
Inflammation			
M12886[*]	T-cell receptor ß-chain (*TCRB*)	Marker of T cells presence (23)	↑
M13755[*]	Interferon-stimulated protein, 17 kDa/15 kDa	Released from lymphocytes and monocytes (7)	↑
X14046[*]	CD37 antigen	Expressed on leukocytes(36)	↑↑
M33552[*]	Lymphocyte-specific protein 1 (*LSP1*)	Modulates leukocytes migration (15)	↑
L09708[*]	Complement component 2 (*C2*)	Recruitment of monocytes to intima (10)	↑↑
U24578[*]	Complement C4b precursor (*C4B*)	Associated with immune complex disease (46)	↑
X00457[*]	MHC, SB class II, SB-α	Membrane protein, signal peptide (16)	↑
M83664[*]	MHC, class II, DP, ß-1	Cell surface glycoprotein (34)	↑↑
D32129[*]	MHC, class I, A		↑
U15085[*]	MHC, class II, DM-ß	Regulates antigen presentation (37)	↑
J00194[*]	MHC, class II, DR-α	Cell surface protein induced by INF-γ (41)	↑
AL022723[*]	MHC, class I, F		↑↑
M13560[*]	Human Ia invariant γ-chain	Antigen processing (20)	↑
AF029750[*]	TAP binding protein (tapasin) (*NGS-17*)	Ig superfamily; interacts with MHC class I(28)	↑
X57809[*]	Ig λ, light chain		↑↑↑↑
M18645[*]	Ig, C-region, V-region λ-4		↑↑↑↑
Y14737[*]	Ig heavy chain		↑↑↑
X72475[*]	Ig κ, light chain, VJC region		↑↑↑↑
U80114[*]	Ig heavy chain, variable region V4-31		↑↑↑↑
X92997[*]	Ig λ, light chain, VJC		↑↑↑↑

	region		
U88964[*]	HEM45	Cellular proliferation and differentiation (32)	↑
AF004230[*]	MIR-7 (leukocyte Ig-like receptor) (*MIR cl-7*)	Activation of MHC I-recognizing killer cells (48)	↑↑
AF060228[*]	Retinoic acid receptor responder (*RARRES3*)	Upregulates expression of scavenger receptor CD36 (51)	↑
AB006782[*]	Galectin-9	Chemoattractant for eosinophils (14)	↑
M92357[*]	TNFα-induced protein-2 (B94 protein)	Expression in endothelial cells (2), angiogenesis	↑↑↑↑

Cell necrosis/apoptosis/proliferation

M87503[*]	Interferon stimulatory transcription factor-3γ (*ISGF3-gamma*)	Serves as the DNA recognition subunit of ISGF-3 (47)	↑
Z93930[*]	X-box binding protein 1 (*TREB5*) (*XBP1*)	Regulation of MHC class II expression (39)	↑
AF000424[*]	B144 NK cell triggering receptor (*LST1*)	Inhibits lymphocytic proliferation (6)	↑
M97935[*]	STAT-91	Regulates transcription of proteins induced by INF-α (35)	↑
U53446[*]	DOC-2 (mitogen-responsive phosphoprotein)	Inhibits epithelial cell growth in ovarian cancer (21)	↑
AL049946[*]	Hypothetical protein (similar to perlecan)	Regulates smooth muscle cell quiescence (19)	↑
AF106941[*]	Arrestin-ß2	Turns off G-protein coupled receptor activation (18)	↑
M63193[*]	ECGF1	Promoter of angiogenesis (13)	↑↑
L06797[*]	Chemokine (CXC motif) receptor (fusin)	G protein-coupled receptor	↑↑
S59049[*]	B-cell activator gene (*BL34, GOS8*)	Role in leukomogenesis (50)	↑↑
X78817[*]	Rho GTPase activating protein-4 (*CI*)	Regulation of GTP binding proteins (44)	↑↑

U77735*	PIM-2 oncogene	Present in mitogenically stimulated hematopoietic cells (3)	↑↑↑
Cell migration/adhesion and matrix degradation			
U21128	Lumican	Role in cell migration, proliferation, tissue repair (27)	↑
M73255	VCAM-1	Modulates attachment of macrophage (10)	↑↑↑
M30257	VCAM-1 precursor		↑↑↑
X15606	ICAM-2 (CD102)	Mediates adhesion of lymphocytes (10)	↑
J04765	Osteopontin	Promotes migration of macrophages, cell adhesion (24)	↑↑
M22324*	Membrane alanine aminopeptidase N/CD13 (*IGFIR*)	Cell surface glycoprotein, role in motility and angiogenesis (11)	↑↑
J05070	Matrix metalloprotease-9	Vascular remodeling (42)	↑↑↑↑
X16832*	Cathepsin H	Macrophage hydrolase, possible role in MHC assembly (45)	↑
X82153*	Cathepsin K	Macrophage collagenase, role in matrix degradation (53)	↑
Lipid transfer/oxidation/metabolism			
D38255*	Steroidogenic acute regulatory protein	Transmembrane transfer of cholesterol (38)	↑
U90546*	Butyrophilin (*BTF4*)	Transmembrane transfer of lipids (26)	↑
X79389*	Glutathione-*S*-transferase 1 (*GST*)	Reducing agent (17)	↓
U29615	Chitotriosidase	Marker for the presence of macrophages (4)	↑↑
M21186*	Flavocytochrome 558	Membrane-bound oxidase, generates superoxide (30)	↑↑

Unspecified functions			
AB00087*	Small inducible cytokine, subfamily A-19	Unspecified	↑↑↑↑
AF055001*	KIAA0025	Homocysteine-inducible protein	↑
AI743134*	EST	Glia derived nexin precursor	↑
AI932613*	EST	Ig related protein precursor	↑↑↑↑
AI147237*	EST	Ig heavy chain V-III region	↑↑↑↑

EST, expressed sequence tag; ECGF, endothelial cell growth factor; GTP, guanosine triphosphate; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; IL, interleukin; ISGF, interferon stimulatory gene factor; MHC, major histocompatibility complex; MIR, macrophage Ig-like receptor; STAT, signal transducer and activator of transcription; TNF tumor necrosis factor; VCAM vascular cell adhesion molecule.

* Novel gene associated with CAD. Fold changes, upregulated: 1.5–2 (↑), 2.1–2.5 (↑↑), 2.6–3 (↑↑↑), 3.1 + (↑↑↑↑). Fold changes, downregulated: 1.5–2 (↓). Numbers in parentheses are reference citations

3.3 2 Altered expression of inflammation genes in CAD tissues.

This group includes six genes encoding the major histocompatibility complexes (MHCs), six genes encoding immunoglobulins, complement component 2 and 4b, retinoic acid receptor responder, MIR-7, HEM45, CD37 antigen, T-cell receptor ß-chain, lymphocyte-specific protein 1, human Ia invariant γ-chain, tapasin, 17 kDa/15 kDa interferon-stimulated protein, galectin-9, and tumor necrosis factor-α-induced protein-2 (B94). In the diseased coronaries, all the genes encoding immunoglobulins showed the highest fold changes in expression; consistent with the hypotheses that infection may play a role in the pathogenesis of CAD or an immune response to athero-antigens is driving this response (10).

3.2.3 Altered expression of cell necrosis/apoptosis/proliferation genes in CAD tissues. This group include genes encoding interferon stimulatory factor-3γ, *XBP1* (*TREB5*), B144 NK cell triggering receptor (*LST1*), STAT-91, DOC-2 (mitogen-responsive phosphoprotein), a hypothetical gene with homology to perlecan, arrestin-ß$_2$, platelet-derived endothelial cell growth factor (ECGF1), chemokine G-coupled receptor (fusin), B-cell activator gene (*BL34*, *GOS8*), fusin, Rho GTPase activating protein-4, and PIM-2.

3.3.4 Altered expression of cell migration/adhesion and matrix degradation genes in CAD tissues.

Genes in this group include the lumican, VCAM-1 and its precursor, ICAM-2, osteopontin precursor, membrane alanine aminopeptidase (*IGF1R*), MMP-9, and cathepsin H and K genes.

3.3.4 Altered expression of lipid transfer/oxidation/metabolism genes in CAD tissues. This group includes genes for steroidogenic acute regulatory protein, butyrophilin (*BTF4*), glutathione-*S*-transferase (*GST*), flavocytochrome 588, and chitotriosidase.

3.3.5 Altered expression of genes with unknown functions in CAD tissues.

This group includes three expressed sequence tags (EST) and two genes predicted to encode a small inducible cytokine and a homocysteine-inducible protein.

To summarize, our microarray analysis has defined 56 genes that are differentially expressed in CAD tissues. These genes can be classified into five functional groups. It is interesting that among the 56 genes associated with CAD, only one gene, *GST*, was downregulated, whereas the rest of genes were upregulated.

3.3.6 Clustering of Differentially Expressed Genes

The 56 differentially expressed genes in CAD were grouped using a hierarchical clustering method referred to as a gene tree. The resultant gene tree recapitulates the two distinct study populations, i.e., those with CAD compared with nondiseased arteries. The gene tree program clusters them in subgroups with similar genetic profiles (Fig. 2).
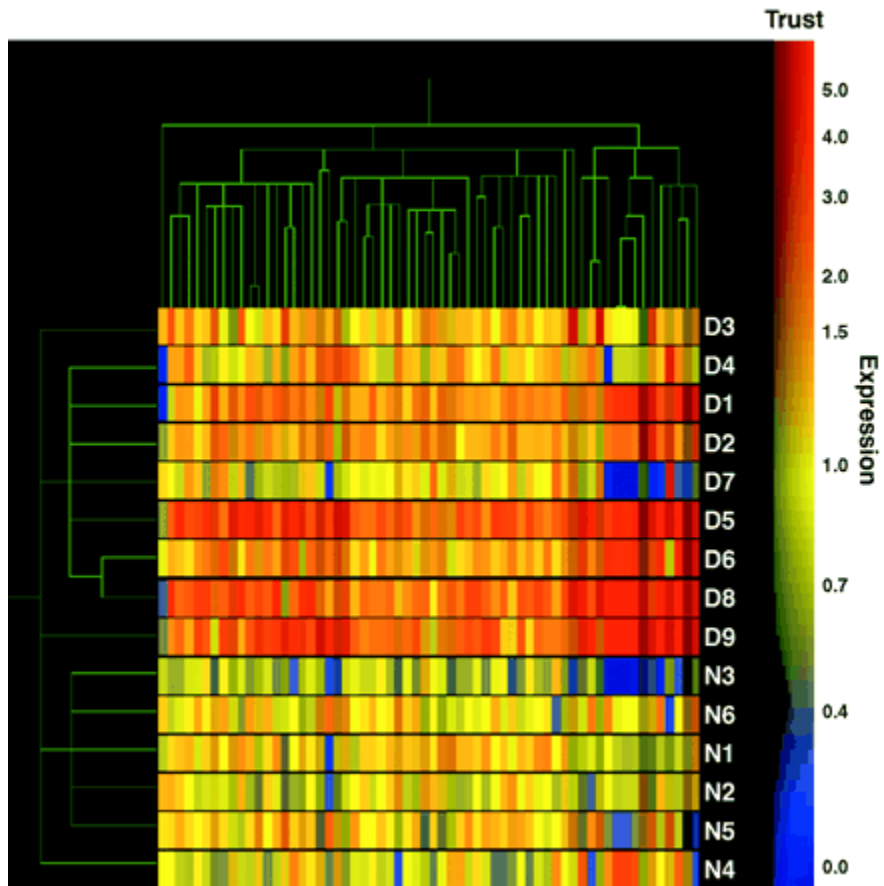
Fig. 2. Hierarchical clustering of 15 coronary arteries and 56 genes. The gene tree of nine diseased coronary arteries (*D*) and six normal coronary arteries (*N*) recapitulates the distinction between the two groups based on their similarity in gene expression. The trust shows fold changes with upregulation [up to 5x (in red)] and downregulation [starting at 0 (in blue)].

3.3.7 Validation of Changes in Gene Expression by Quantitative RT-PCR

To validate our microarray data, quantitative RT-PCR was performed using SYBR Green for 13 genes (Table 3). Consistent changes were observed between quantitative RT-PCR and microarrays for all 13 genes examined. The only gene downregulated on our microarray, *GST*, also showed decreased expression by quantitative RT-PCR. The other 12 genes showed increased expression by microarray analysis were also found to be upregulated by quantitative RT-PCR. These data provide validation of our gene expression patterns identified by microarrays.

Table 3. Confirmation of microarray analysis with quantitative real-time PCR

Gene Name	Accession No.	GeneChip	SYBR Green
Chititriosidase	U29615	2.1	4.0
Retinoic acid receptor responder	AF060228	1.7	3.4
Galectin-9	AB006782	1.6	1.4
PIM-2	U77735	2.7	2.3
STAT-91	M97935	1.6	1.2
ICAM-2	X15606	1.5	1.5
Cathepsin K	X16832	1.5	2.2
Cathepsin H	X82153	1.6	1.8
ß2-arrestin	AF106941	1.8	2.6
EST-1	AI743134	1.7	1.4
EST-2	AI932613	4.0	1.4
Lumican	U21128	1.5	4.2
Matrix metalloprotease-9	J05070	4.7	3.4
GST	X79389	-0.65	-0.07
Osteopontin precursor	J04765	2.3	2.9

Values represent fold changes in gene expression.

3.3.7 Validation of Changes in Gene Expression by Immunostaining.

To further validate the data from the microarray analysis, immunostaining was performed for four proteins encoded by the genes differentially expressed in CAD, including osteopontin (OPN), MMP-9, ICAM-2, and PIM-2 oncogene. The four coronary arteries from CAD patients are different from those used for the microarray analysis. Representative immunostaining images are shown in Figs. 3 and 4. OPN (green signal) was expressed diffusely in the media and fibrous tissue of the coronary artery and colocalized with a smooth muscle cell-specific marker, the monoclonal anti-α smooth muscle actin (red signal) (Fig. 3A). OPN showed stronger staining in the CAD coronary arteries than normal tissues (Fig. 3A), consistent with our microarray results.
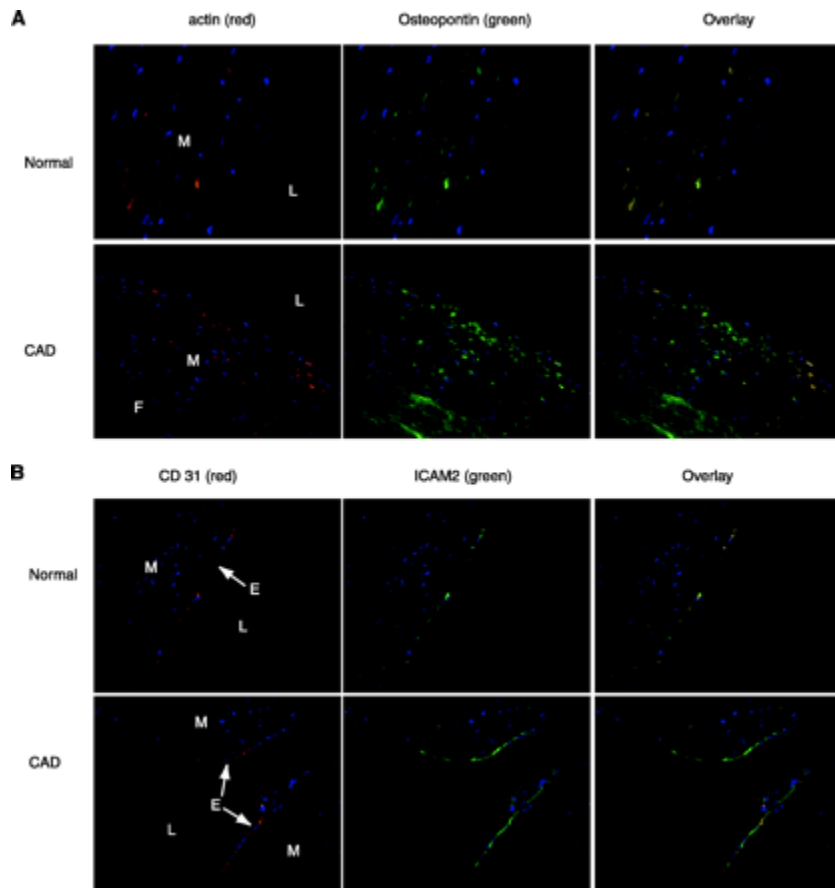
Fig. 3. Protein expression of osteopontin (*A*) and ICAM-2 (*B*) in normal and CAD coronary arteries. *A*: immunostaining images showing colocalization of osteopontin (green) and smooth muscle α-actin (red) and increased expression of osteopontin in CAD coronary arteries. *B*: immunostaining images showing colocalization of ICAM-2 (green) and endothelial cell-specific marker CD31 (red) and increased expression of ICAM-2 in CAD coronary arteries. L, lumen; M, medial layer; E, endothelial cell layer; F, fibrous tissue; CAD, coronary artery disease.
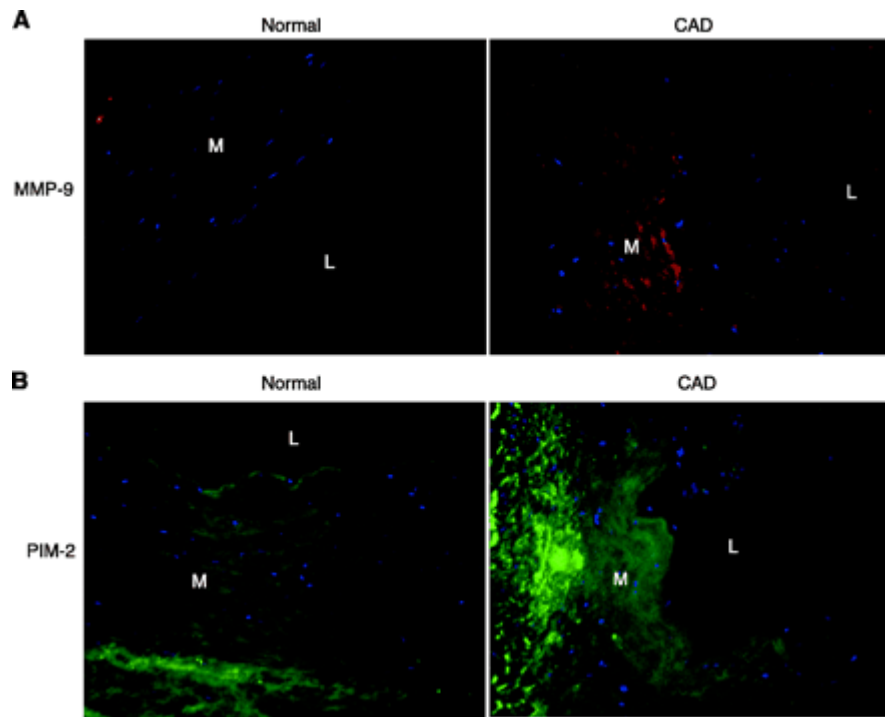
Fig. 4. Protein expression of matrix metalloproteinase-9 (MMP-9; *A*) and PIM-2 (*B*) in normal and CAD coronary arteries. *A*: immunostaining images showing increased expression of MMP-9 (red) in CAD coronary arteries. *B*: immunostaining images showing increased expression of PIM-2 (green) in CAD coronary arteries. L, lumen; M, medial layer. Note that coimmunostaining with CD31 and smooth muscle α-actin was not performed because the available antibodies are monoclonal (MMP-9 and PIM-2 antibodies) (e.g., the same anti-mouse secondary antibody will not be able to distinguish the primary antibodies for PIM-2 or CD31).

Immunostaining signal for ICAM-2 (green) colocalized with the signal for CD31 (red), an endothelial cell-specific marker (Fig. 3*B*). ICAM-2 expression is also increased in CAD tissues. MMP-9 has been shown to be expressed in macrophages (42). As shown in Fig. 4A, the immunostaining signal for MMP-9 (red) was very strong and diffusively distributed throughout the media in coronary arteries with atherosclerosis, whereas very little staining was detected in normal tissues. PIM-2 appeared to be expressed widely in the media layer of coronary arteries (green) (Fig. 4B). PIM-2 expression was increased in the CAD coronary arteries compared with arteries without the disease. Taken together, the immunostaining data illustrate the distribution and expression patterns of osteopontin, ICAM-2, MMP-9, and PIM-2 in the normal and diseased coronary arteries. Increased expression of these four proteins was observed in the diseased tissues compared with the normal ones. These data further validate results from our microarray array analysis and correlate the increased expression of the mRNA of these genes with increased protein expression.

3.4 Discussion

This is a large-scale study in which microarray analysis has been used to profile gene expression patterns using intact, human atherosclerotic and nonatherosclerotic human coronary arteries. We have identified 56 genes that are differentially expressed in patients with CAD, and 49 genes were not previously linked to CAD. Interestingly, 55 genes are upregulated and one gene, *GST,* is downregulated in CAD tissues (Table 2). Previous studies have implicated increased expression of chitotriosidase (4), lumican (27), VCAM-1 (10), ICAM-1 (10), MMP-9 (42), and OPN (24) in atherosclerosis. These results are confirmed by our microarray analysis (Table 3). This suggests that the

microarray technology is a powerful tool for identification of gene expression patterns associated with CAD.

In the clustering of the nine diseased and six nondiseased human coronary arteries, the gene tree identifies two distinct gene expression patterns: the diseased coronary arteries are clustered as a group, and the nondiseased arteries segregate as an independent group (Fig. 2). Of interest to note is among the patients with CAD, samples D8 and D9 clustered together. These two samples had the most significant occlusion (90% in D8 and 100% in D9) of the left main artery adjacent to the proximal LAD revealed by left heart catheterization.

The majority of genes associated with CAD identified in this study represent new links to CAD processes. Our microarray analysis identified 49 new genes that were not previously shown to be associated with CAD (Table 3). Some of these genes include the following: retinoic acid responder binding (RAR) protein, butyrophilin, steroidogenic acute regulatory protein (STAR), PIM-2, STAT-91, and cathepsins K and H. The *RAR* gene is regulated by vitamin A signaling pathways and has been shown to upregulate the expression of the scavenger receptor CD36 through RAR in the human monocytic cell line THP-1 (52). Butyrophilin is a structural component of the human milk fat globule and have receptor functions that mediate the transfer of lipid (26). Both cathepsins K and H are lysosomal proteases that are involved in protein degradation. Cathepsin K is a collagenase that cleaves both type I and type II collagens at their helical domains (53). Cathepsin H has been shown to be upregulated 30-fold in aortic abdominal aneurysms compared with normal aortas (45). STAR is another novel gene identified. It has been shown that in humans and genetically manipulated mice, STAR is required and is the

rate-limiting step in steroidogenesis for the transfer of cholesterol into the mitochondria (38). Two more novel genes include *PIM-2*, which is a "proviral integration site of murine" leukemia virus, is present in mitogenically stimulated hematopoietic cells (3), and *STAT-91*, which is a signal transducer and activator of transcription in hematopoietic cells (35). *GST* is the only gene whose expression is reduced in CAD tissues, and it is a reducing enzyme (17).

Many lines of evidence support the notion that inflammation plays a critical role in atherosclerotic lesion pathology. Histological analysis of unstable or ruptured atherosclerotic plaques from patients with acute coronary syndromes identified inflammatory cells including T cells, monocytes, and macrophages. Later studies showed that atherosclerosis was associated with the presence of inflammatory proteins (CRP, cytokines, chemokines) and increased expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin) as inflammatory responses from vascular cells (31, 33). Our study emphasizes the importance of inflammation in CAD. The largest group of CAD-associated genes identified in this study consists of genes involved in inflammation (Table 2). It is, however, important to note that the association of many genes in this group (e.g., genes encoding T-cell receptor ß-chain, 17 kDa/15 kDa interferon-stimulated protein, ICAM-2, CD37 antigen, tapasin, MIR-7, retinoic acid receptor responder, B94, etc.) is novel.

The immunostaining results support our microarray analysis showing not only the upregulation of the mRNA expression, but also increased protein expression. Four proteins demonstrated upregulation in atherosclerotic arteries include osteopontin, MMP-9, ICAM-2, and PIM-2. One component of atherosclerosis is smooth muscle proliferation

83

followed by connective tissue matrix deposition. Osteopontin, a protein secreted from smooth muscle cells, showed an upregulation in the atherosclerotic arteries and reflects the dynamic process of smooth muscle recruitment and proliferation. MMP-9 is secreted from macrophages, and participates in the structural remodeling of the artery as occurs with inflammation. Both proteins were found to be upregulated in the medial layer of the artery which is a site of structural remodeling, lipid deposition, and recruitment of inflammatory cells. *PIM-2*, an oncogene expressed by hematopoietic cells, also showed upregulation in the diseased arteries, although its definitive function has not been established. ICAM-2 is a protein expressed in endothelial cells as shown in **Fig.** 3*B* and showed an increased expression in CAD; however, its precise function in endothelial cells and in atherogenesis remains to be established.

We compared our gene list with another list of 75 genes connected to atherogenesis and identified by a separate DNA microarray analysis and found that the expression of the gene for immunoglobulin-λ (VJC) was upregulated in both studies. Surprisingly, other genes in our list do not match any genes identified by Hiltunen et al. (12). This discrepancy may be explained by different tissue samples used in the two studies, coronary arteries in our study compared with common, internal and external iliac artery, aorta, popliteal artery, posterior tibial artery, and tibiofibular trunk used in the other study.

It is important to note that microarray technology does have its limitations as previously described (5). Furthermore, our study is a genetic profile of the CAD coronary artery tissues at a single time point (a more advanced atherosclerotic stage), and may provide only limited information for the continuous process of atherogenesis. Another

limitation of the present study is the small sample size due to difficulties in obtaining human coronary artery tissues. In addition, the majority of tissues used were male tissues as 8/9 CAD and 5/6 normal tissues are male-derived samples. Future studies with an increased sample size, female samples in particular, will further validate our results.

In conclusion, this study represents a large-scale microarray analysis to study gene expression patterns of atherosclerosis using human coronary arteries. We identified 56 genes that are potentially involved in the pathogenesis of CAD or can be used as biomarkers for diagnosis of CAD. Our study also identified 49 genes that are not previously linked to atherosclerotic human coronary arteries. Further analysis is required to determine the exact role of each gene, if any, in development of CAD.

CHAPTER 4

INTRODUCTION

GLOBAL GENE EXPRESSION STUDIES OF ATHEROSCLEROSIS-RESISTANT
INTERNAL MAMMARY ARTERIES AND ATHEROSCLEROSIS-SENSITIVE
CORONARY ARTERIES IDENTIFIES AN ANTI-ATHEROSCLEROTIC ROLE FOR
TES IN ATHEROSCLEROSIS

ABSTRACT

Objective - Internal mammary arteries (IMA) are resistant to the development of atherosclerosis, whereas the coronary arteries are athero-prone. We hypothesize that genes differentially expressed in IMA vs. coronary arteries are involved in resisting atherosclerosis.

Methods and Results - Microarray analysis identified 29 genes differentially expressed in IMA compared to coronary arteries and identified a novel gene, TES (encoding Testin), not previously associated with atherosclerosis. Western blot analysis showed higher TES expression in IMA than LAD. RT-PCR and Western blot analyses showed that TES was consistently down-regulated at both mRNA and protein levels in patients with coronary artery disease compared with patients without disease. This indicates that reduced TES expression may be involved in the development of atherosclerosis. Consistent with this hypothesis, knockdown of TES expression by siRNA promoted oxLDL-mediated monocyte adhesion and transendothelial migration of monocytes, while overexpression of

TES in endothelial cells (ECs) blunted these processes. Overexpression of TES reduced cell migration of ECs, whereas knockdown of TES expression promoted cell migration.

Conclusion - The results indicate that the human IMA and LAD are distinctly different in gene expression. This study identifies TES as a novel atherosclerotic-resistant gene, which has anti-atherosclerotic properties.

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4.1 Introduction

Atherosclerosis is the leading cause of death and disability for both men and women in the world (1). A common treatment for coronary artery disease (CAD) is coronary artery bypass grafting (CABG) surgery. While several arteries and veins can be used as blood conduits in this procedure, internal mammary artery (IMA) grafts have demonstrated significantly better long-term results with lower complications irrespective of age, gender, race, or left ventricular function (2, 3). For example, the patency of IMA grafts seven to ten years after CABG is 85 to 95 percent when compared to saphenous vein grafts, which readily re-occlude with atherosclerosis, hypertrophies, or become fibrotic (4). Furthermore, the IMA patency 20 years after surgery is even >90% when the graft serves the LAD territory (4). Interestingly, the only disease-prone site of an IMA graft is at the anastomotic site grafted to the coronary artery suggesting surgical trauma as the inciting factor (5). Recommendations are that its use for CABG is preferred in all situations (6, 7).

The proximal LAD is the coronary artery to which the IMA is most frequently grafted with resultant improved long-term survival (7). A high-grade proximal LAD

stenosis is a major risk factor for sudden death. An acute coronary occlusion in the LAD is more likely to cause a fatal myocardial infarction (MI) compared to all the other coronary arteries with the exception of the left main trunk (4). The IMA are comparable in size to the proximal LAD, and both arteries are arteries subject to the same pro-atherogenic environment; yet the IMA is atherosclerotic-resistant and the proximal LAD is athero-prone (7). The contrasting properties of these two arteries is an interesting subject to identify the molecular mechanisms of this differential propensity to develop and not to develop disease.

We hypothesize that gene expression differences are the key to distinguish the IMA from the proximal LAD with respect to their sensitivities to the development of atherosclerosis. To test this hypothesis, we used oligonucleotide microarrays to identify the genes that are differentially expressed in the IMA compared with the LAD. We found 29 genes whose expression levels were statistically different. The functions of these genes provide insights as to why the IMA is resistant to the progression of atherosclerosis and may aid in the development of therapeutic options to prevent such life-threatening disease in the coronary artery. One of these 29 genes, namely the TES gene, was selected for follow-up functional studies to define its role(s) in the pathogenesis of CAD.

The human TES gene (protein product is Testin) is homologous to the mouse testosterone-responsive gene, TESTIN that encodes a Sertoli cell secretory protein. The human TES gene is located on chromosome 7q31.2 and encodes a 421 amino acid protein that contains a PET domain at the N-terminus (amino acids 92-195) and three LIM

domains at the C-terminus (LIM1 amino acid, 236-296, LIM2 301-357,LIM3 361-414). The LIM domains are responsible for interactions with zyxin, paxillin, Mena-VASP, α-catenin, talin, Arp7a, actin, alpha-actinin, and Gripp (8). The interactions of TES with these proteins identify its role as a cytoskeleton associated factor and a scaffolding protein that may be involved in cell adhesion, cell spreading, cell migration, and the reorganization of the actin cytoskeleton. The PET domain is involved in protein-protein interactions between Testin and actin stress fibers and may mediate the organization of the cytoskeleton. In fact, the knockdown of TES expression by siRNA leads to a loss of actin stress fibers, supporting its role in the regulation of the cytoskeleton (9). Testin localizes in the cytoplasm and appears to be a component of focal adhesions and cell-to-cell junctions.

The functional role of TES in CAD is previously unknown. In this study, we identified TES as a novel gene which is expressed in coronary arteries and may confer a resistance to atherosclerosis. We found that the expression of TES is significantly higher in the IMA than the LAD, and its expression is lower in the LAD tissues from CAD patients than from non-diseased coronary arteries. Functional studies for cellular properties relevant to atherosclerosis, including monocyte adhesion to ECs, oxLDL-induced transendothelial migration of monocytes, and EC and VSMC cell migration, were used to define the cellular mechanisms by which TES may confer a resistance to atherosclerosis.

4.2 Material and Methods

4.2.1 Tissue Samples of Human Arteries

Arteries were obtained from explanted hearts through the Cleveland Clinic Heart Transplant Program, unmatched or rejected healthy donor hearts from LifeBanc of Northeast Ohio and the Cuyahoga County Coroner's office. Each LAD was carefully and thoroughly examined for the presence or absence of atherosclerosis by microscopic examinations of consecutively dissected serial segments of the entire artery by cardiovascular pathologists. We selected a segment of the proximal LAD without intraluminal plaque. Informed consent was obtained from the participants (or their families at post-mortem exam) according to the standards established by the Cleveland Clinic Foundation Institutional Review Board on Human Subjects and the Cuyahoga County Coroner. Arteries were cleaned of adjacent adipose and myocardial tissue in explanted hearts or from cadavers (10). The adventitial layer remained intact. The specimens were snap-frozen in liquid nitrogen and stored at -80°C as previously described until used (11).

The initial cohort of patients for expression profiling with microarrays all were not affected with CAD, and came from both explanted hearts (the coronary artery) and autopsy (the internal mammary artery) (Online Table 1). For follow-up RT-PCR and Western blot analyses, we compared the arterial gene expression between these two arteries harvested from the same patient but from a larger group (n=30).

4.2.2 RNA Isolation and Oligonucleotide Arrays

Total RNA was isolated from the arteries using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Microarray analysis was performed as previously described by us as well (12, 13).

4.2.3 Extraction of Microarray Data and Statistical Analysis

The Human Genome U95A Array (Affymetrix) was used. Each array contains ~12,000 unique genes. GeneSpring (Agilent technologies) was used for data mining as previously described (12). We generated the statistical algorithm as followed on the web site (www.agilent.com).

4.2.4 Real time RT-PCR Analysis

Results from the microarray analysis were validated for selected genes by real time RT-PCR with SYBR Green (VWR). Specific primer pairs were designed for each selected gene (Integrated Biotechnologies, Inc.) (Online Table 2). Relative expression values were calculated as previously described (12, 13). The expression level of β_2-microglobulin was used as an internal control.

Online Table 1. Characteristics of study subjects from which coronary/IMA tissue samples were utilized for microarray and/or expression analyses.

Initial Microarray Cohort Case No.	Age	Gender/ Race	Clinical description
Microarray 1 – coronary	62	WM	Dilated cardiomyopathy
Microarray 2 - coronary	59	WM	Dilated cardiomyopathy
Microarray 3 -coronary	55	WF	Motor vehicle accident (MVA)
Microarray 4 - coronary	49	WM	Cerebrovascular accident (CVA)
Microarray 5 - coronary	40	WM	Restrictive cardiomyopathy
Microarray 6 - coronary	46	WM	MVA
Microarray 1 - IMA	84	WM	asthma
Microarray 2 - IMA	55	WM	Alcoholism
Microarray 3 - IMA	47	WF	MVA
Microarray 4 - IMA	57	WM	Arrhythmia
Microarray 5 -IMA	55	WM	Arrythmia
Replication studies Case No.	Age	Gender/ Race	Postmorten examinations
1	48	WM	MVA
2	46	WM	Epilepsy, alcoholic cardiomyopathy
3	53	WM	Asthma
4	41	WM	CNS hemorrhage

5	54	WM	MVA
6	46	WF	Domestic violence
7	50	WM	Suicide via carbon monoxide poisoning
8	53	BM	Head trauma
9	22	WM	Subdural hematoma
10	61	WM	Laryngeal cancer
11	54	WM	Epilepsy
12	38	WM	Heroin overdose
13	86	WM	Suicide
14	53	WF	Trauma/fall
15	81	WM	Trauma
16	50	BM	Arrythmia
17	46	WM	Gun shot wound to chest
18	56	WF	Asthma, hypothyroid, depression
19	43	WM	Alcohol abuse
20	71	WF	Suicide
21	48	WF	Dilated cardiomyopathy
22	55	WM	Depression
23	55	WM	Recurrent arrythmias
24	50	WM	Arrythmia
25	62	WM	Cirrhosis
26	62	WF	Colon cancer
27	52	WM	Trauma, hypertension
28	70	WM	CVA

29	60	WM	Trauma
30	52	WM	Suicide

Online Table 2. Primer pairs used for RT-PCR analysis.

Gene Name	Primer Pairs
HSP70	F 5' – AGGAGATCTCGTCCATGGTG-3' R 5'-CCCAGGCAAAGATCAGGAC-3'
ATF3	F 5'-GTCGGAGAAGCTGGAAAGTG R 5'- GGCTTCAGGGTTTTGGGTAT-3'
GLUT-3	F 5'-TGCTTTCTGCTCCCAGTTTT R 5'-GCACAGCTTGCAGTGTTGAT
NR4A1	F 5'-TCCAGCCTTCTTTTCCTTCA R 5'-CAAGGTGTGGAGAAGTGGGT
TINUR	F 5'-AGTCTGAGGAGATGATGCCG R 5'- GAGACTGGCGTTTTCCTCTG
CD163 Antigen	F 5'-GATTGCTCAAAGGGAGCAG R 5'-TAACTGTCCCAGCCGTCAT
CDH7	F 5'-GCTTTCCATGGGATTCAAGA R 5'-CGTTCCCGCATTATGGTACT
CALB2	F 5'-GCTCCAGGAATACACCCAAA R 5'-CAGCTCATGCTCGTCAATGT
AQP7	F 5'-CTACACGGCCATTCTCCACT R 5'-ACCACGAGGATGCCTATCAC
F13A1	F 5'-GGAATACGTCATTGGTCGCT R 5'-TTCT GGGT6TTCGACTGGTTC
COL16A1	F 5'-GCCTGTCTCGTTTGACCTC R 5'-TTTCTGCCTTCTGGCTGATT

ASAH1	F 5'-GCCTGCAAAGATGTGTCTGA R 5'-CCTATACAAGGGTCAGGGCA
C1Q	F 5'-TCCAGATATGGAGGCCTGAC R 5'-TTCACTCAGCAGCATTCACC
CYB5A	F 5'-AATGGCTTCTTTTCTCCGT R 5'-GGTGATCCCTGCCATCTCT
RAS-A PLA2	F 5'-GCAGCCGTAGAAGCCATAAC R 5'-GAGCTAGGCCAGTCCATCTG
C7	F 5'-TGGTTTTCCTCAATGGAAC R 5'-AGATGAGATCACCCCACTGC
DUSP6	F 5'-TTTGCAGCATGCTTGACTTT R 5'-GAGCCCATGATTTGGTGTCT
SLA	F 5'-AAATGCTCAGCTCCAGGAAA R 5'-ATACTGCAAGCATGTTCCCC
GLUL	F 5'-GCTCTGAGAGATGGGACAGG R 5'-TGCTTTGGGCAGTTACACAG
FACL1	F 5'-TGCAAAGTTCATTTCCTGGG R 5'-TTAGTGCAAGCC CTGTTGTG
TESTIN-2	F 5'-ATTCTAGAGAGGAGGGGACCCATAG R 5'-ATAAGCTGATAGCTATGGCTCGATA
RGS5	F 5'-ATTGATCCTTCCCTTGGACC R 5'-TGGAAACATTTGACCCACAG
Clone DKFZ 586O031	F 5'-TCCTTTGACCCCTTCATCAG R 5'GAATAAGCCAGGGGGAA

ID-H2	F 5'-AAATGCCCTTTCTGCAGTTG R 5'-TCCTTCTGGTATCACGTCTCC
FKHL7	F 5'-TTCAGCGTGGACAACATCAT R 5'-CTTGCAGGTTGCAGTGGTAG
RAMP1	F 5'-TCCTCTACCCCTTCATCGTG R 5'-GGGGGCTTCCAGGTTAATAC
LMNA	F 5'-AGTCTGCTGAGAAGGAACAGCAA R 5'-AGCTTGATGTCCAGAAGCTC
PECAM1	F 5'-GCTTCCCTTAGCACTTTCTCCTT R 5'-CTCTGCCTCCCAGGTTCAAATGAT
HBGR2	F 5'-TGCTTTCTGCTCCCAGTTTT R 5'-GCACAGCTTGCAGTGTGGAT
β2-microglobulin	5-' CGCTACTCTCTCTTTCTGGCC R-5'CGGCAGGCATACTCATCTTT

4.2.5 Cell Culture and Transfection

Endothelial cells (ECs) were harvested from the proximal LAD coronary artery and IMA were purchased from Cell Applications, Inc. The cells were isolated from non-diseased human arteries and cryo-preserved at the second passage. Cells were grown with standard media supplied ready-for-use from Cell Applications, Inc.

ECs were transfected with the TES expression plasmid using Amaxa (Program U-001) and the HUVEC Nucleofactor Kit (Cat No. VPB-1002). The human TES gene was PCR amplified with primers containing in-frame restrictions sites EcoR 1 (GCCAAT TCC TAA GAC ATC CTC TTC TT) and BamH 1 (AAG GAT CCA GAT GGA GCT GGA AAA). The PCR fragment was digested with EcoR 1 and BAMH 1 and sub-cloned into pcDNA3.1A (5.5 kb-6X histidine tagged vector) (Invitrogen). The identity of the construct was verified by direct DNA sequence analysis of the entire insert using the BigDye Terminator Cyclic Sequencing Ready Reaction Kit (Applied Biosystems) in a 3100 Genetic Analyzer (Applied Biosystems). Cells were used for assays 24 and 48 hours after transfection.

Small interfering (si) RNA for TES was purchased from Integrated DNA technologies: Sense--rGrCrA rGrCrA rCrCrU rGrCrC rArUrG rArArC rUrCrC rUrGG T and Antisense--rArCrC rArGrG rArGrU rUrCrA rUrGrG rCrArG rGrUrG rCrUrG rCrArG. Cells were transfected with the siRNA using Polyjet (SignaGen Laboratories). The optimal dose to interfere with TES mRNA expression was 1 µM.

4.2.6 Preparation of Protein Extracts by the Delipidation Method

The IMA tissues or proximal LAD coronary arteries (0.2 g/sample) were lysed in a homogenization buffer for 15 minutes at 35°C using the delipidation method as previously described (13). Protein extracts from the IMA or LAD ECs vs. VSMCs grown *in vitro* were prepared with M-PER Mammalian Protein Extraction Reagent (Pierce) and quantified with BCA Protein Assay Kit (Pierce).

4.2.7 Western Blot Analysis

Western blotting was performed as previously described (12, 13). The primary antibodies used included the antiserum for CD31 (PharMinger), and GAPDH (Santa Cruz). The antibody against Testin was kindly provided by Yingli Zhong. Quantification of bands was made with Melanie 2D gel software analysis (SIB).

4.2.8 Immunostaining

Cryo-sections (6-μm) of the proximal LAD were fixed with paraformaldehyde (4%) and permeablized with Triton X-100 (0.2%) as previously described (12) and incubated with anti-TES. Anti-Rabbit IgG conjugated with FITC (Sigma) was used to visualize TES. The sections were counterstained with DAPI for staining nuclei (Vectashield with OAD, Vector Labs). The immunostaining signals were visualized with a Zeiss Axiophot fluorescence microscope and the images were captured with Imageplus 6.0.

4.2.9 Monocyte Adhesion Assay

Fully confluent ECs were grown in CoStar96 well plates after being seeded at a density of 5,000 cells/well and stimulated with oxidized-LDL (oxLDL) (Sigma) (0.25 mg/mL) for six hours. Human HL-60 pro-myelocytic leukemia cells were cultured in Iscove's

modified Dulbecco's media, fluorescently labeled with Calcein AM (Invitrogen) for 60 minutes at 37ºC and seeded into each well coated with ECs at a density of $3x10^5$ per well. After one hour, the media were aspirated and wells were washed gently with PBS three times. Fluorescent intensity was measured in each of the 96-wells with a standard fluorometer with the CytoFluoroII program (Applied Biosystems) (exit: 485/20, emission 530/30, gain of 65) to determine the fluorescent units in each well. The rational for using HL-60 cells is that they represent immature monocytes *in vivo* which attach to and transmigrate through ECs and then differentiate into different mature lineages.

4.2.10 Cell Migration Assays

ECs and VSMCs were transfected as mentioned above and plated at a density of $0.5x10^6$ cells per cm^2 in 35 mm six well plates. The media were changed 24 hours later. Then, the confluent monolayers were wounded with a single edge razor and cells on the right side of the wound were removed. After 48 hours, the cells were stained with Coomassie Brilliant Blue for 10 minutes followed by Glacial acetic acid/methanol destaining buffer, and photographed with Image-Pro Plus and analyzer 7.0 (Mediacybernetics). The number of cells which migrated passed the wound were counted under a 4x power field.

4.2.11 Transmigration of Monocytes Assay

HL-60 cells ($3x10^5$) were pippeted into 0.4 μm pore chambers (Corning) within a Boyden chamber, which had TES transfected ECs plated 24 hours prior and then exposed to 0.25 μg of ox-LDL. FBS was used as a chemo-attractant and diluted in serum-containing DMEM media. The HL-60 cells were collected and counted from the bottom chamber 48

hours after their administration with a Bright-Line Hemacytometer (Hausser Scientific) using an Olympus CKX 41 microscope. Each experiment was repeated in triplicate.

4.2.12 Statistical Analysis. The difference between the two groups of variables was compared by the paired Student t-test. A P value of < 0.05 was considered as significant.

4.3 Results

4.3.1 Microarray Analysis Identified 13 Genes Showing Increased Expression Levels and 16 Genes Showing Reduced Expression Levels in IMA Compared with LAD

To identify the genes differentially expressed between IMA and LAD, we carried out a microarray analysis with Human Genome U95A arrays (Affymetrix). Each array contains ~12,000 unique genes. A cohort of six proximal LAD coronary arteries and five left IMA, all from non-CAD study subjects, were studied. Before any analyses were performed, all data were screened for any genes with a negative average difference value (and negative values set to zero). The average difference after log-transformation between LAD and IMA was compared by using Student's t-test and a Welch ANOVA. This initially generated a list of 2,219 genes. Then, we filtered the gene list by accepting genes that were flagged as "present" on all 11 chips and eliminated them if flagged as "marginally present or absent". This generated a list of 53 genes. Finally, we filtered the list with a set difference of 3-fold. This resulted in our final list of 29 genes (Online Table 3).

Twelve genes including *ATF3*, *TINUR*, *LMNA*, *HSP70*, *TES*, and *RGS5* showed increased expression in IMA compared to LAD. By contrast, expression of 17 genes including *CALB2*, *RASF-A-PLA2* and *PECAM1* were lower in IMA than in LAD (Online Table 3).

To confirm these microarray results, we randomly selected 7 of the 29 genes, including *HSP70*, *HBGR2*, *TINUR*, *CD163 Antigen*, *CDH7*, *AQP7*, and *F13A1,* for real time RT-PCR analysis with the RNA samples used for the original microarray analysis.

Online Table 3. List of differentially expressed genes in the IMA vs. LAD.

Gene Name	Gene Product	GenBank Number	Fold Change on Array	Function
ATF3	Human activating transcription factor 3	L19871	↑8.3	Meets the criteria of an anti-atherosclerotic gene (1,2)
HBGR2	Human glutamate receptor 2	L20814	↑7.2	Studied in CNS (3)
TINUR	NGFI-B/nur77 beta-type transcription factor homolog	S77154	↑6.5	Protects from apoptosis and reduces human macrophage lipid loading and inflammatory responses (4)
LMNA	LAMIN A/C	M13452	↑6.5	Defects associated with premature atherosclerosis (5)
HSP70	Heat Shock Protein 70	M11717	↑6.1	Cytoprotection (6)
RGS5	regulator of	AB008109	↑5.9	Gown-regulated

	G-protein signaling 5			in atherosclerotic caps, negative modulator of AT1 receptor (7)
GLUT3	Human glucose transporter-like protein-III	M20681	↑4.4	Facilitated glucose transporter (8)
cDNA DKFZp586B2022(TES)	strong similarity to mouse TESTIN 2	AL050162	↑4.3	Maintains structural integrity (9)
NR4A1	Human TR3 orphan receptor	L13740	↑3.7	Prevents SMC proliferation (10,11)
COL16A1	Homo sapiens alpha-1 type XVI collagen	M92642	↑3.2	Inducible in CD14+ monocytes (12)
FKHL7	Homo sapiens forkhead/winged helix-like transcription factor 7	AF078096	↑3.1	Development of the anterior chamber of the eye (13)
Id-2H	Inhibitor of	D13891	↑3.1	Noninvasive

	DNA binding 2			phenotype in endothelial cells breast cancer cells; negative regulator of adipogenesis (14)
RAMP1	Receptor activity modifying protein	AJ001014	↑3.0	Expressed in coronary artery and myocardium, promotes vasodilation (15)
CALB2	Calretinin	X56667	↓6.3	Calcium binding protein
CD163 antigen	M130	Z22971	↓5.5	Enhances heme oxygenase-1 activity cytoprotective responses (16)
CDH7	Cadherin-7	AF047826	↓5.4	Preserves membrane integrity, induces smooth muscle cell migration and apoptosis (17)
cDNA DKFZp586O031 (from clone DKFZp586O031)	Hypothetical protein	AL050141	↓4.9	Hypothetical protein

DUSP6	Dual specificity phosphatase 6	AB013382	↓4.7	Phosphotyrosine-kinase enzyme
C7	Complement protein C7 precursor	J03507	↓4.5	Up-regulated in plaque (18)
CYB5A	Cytochrome b5	L39945	↓4.3	Oxidative Pathways
FACL1	Human long-chain acyl-coenzyme A synthetase	L09229	↓4.3	Metabolism of carboxylic acids
SLA	Src-like adapter protein	D89077	↓3.8	It is expressed in T-lymphocyte (19)
RASF-A PLA2	Phospholipase A2, group IIA	M22430	↓3.7	Amplifying the inflammatory component of many disease (20)

AQP7	Aquaporin	AB006190	↓3.6	Involved in water, glycerol and urea transport (21)
GLUL	Glutamine synthase	X59834	↓3.5	Detoxification peroxides (22)
F13A1	Factor XIII subunit a	M14539	↓3.3	Resistance to fibrinolysis (23)
ASAH1	Acid ceramidase	U70063	↓3.2	Protects from TNFα induced cell death (24)
C1Q	C1q B-chain of complement system	X03084	↓3.1	Shown to reduce atherosclerosis (25)

Real time RT-PCR analysis showing the median fold changes are in general agreement with that from the microarray analysis (Online Table 4).

4.3.2 Assessment of Expression of a Selected Set of 11 Genes in IMA and LAD Arterial Samples from an Independent Cohort of 30 Human Subjects

Among the 29 genes identified by the microarray analysis (Online Table 3), we selected 11 genes for further validation studies (Table 1). This set of genes was selected based on their physiological relevance to atherosclerosis because of their expression or function in atherosclerotic relevant cells (ECs, VSMCs and macrophages). Real time RT-PCR analysis was carried out for each gene using matched IMA and LAD samples simultaneously harvested from 30 independent subjects. The analysis confirmed that the genes *RGS5*, *LMNA*, *TES*, and *NR4A1* showed (median values are documented) two-fold or more expression in IMA than in LAD (Table 1). Genes *PECAM1*, *F13A*1, and *RASF-A-PLA2* showed two-fold or less expression in IMA compared to LAD (Table 1).

4.3.3 Expression of TES Is Increased in IMA in Comparison to LAD

Our major focus of this study was to identify novel genes associated with a resistance to atherosclerosis. The genes with increased expression in the IMA compared to the LAD documented in our replication studies, namely four genes (*RGS5*, *LAMNA*, *TES*, and *NR4A1*) would be strong candidates that biologically might confer resistance to atherosclerosis. TES stood out to be a novel gene that was not previously related to atherosclerosis, whereas the other three genes have been studied for their properties related to atherosclerosis and vascular biology. Thus, TES was selected for further functional characterization for its relevance to atherosclerosis. To confirm the real time RT-PCR results showing increased TES expression in

Table 4. Validation of the results from microarray analysis with real time RT-PCR analysis.

Gene Name	GenBank Number	Fold-Change on Array	Fold-Change with RT-PCR
GLUL	L20814	↑7.2	↑12.1
TINUR	S77154	↑6.5	↑3.7
HSP70	M11717	↑6.1	↑7.8
CD 163 Antigen	Z22971	↓5.5	↓5.6
CDH7	AF047826	↓5.4	↓7.4
AQP7	AB006190	↓3.6	↓9.8
F13A1	M14539	↓3.3	↓5.4

the IMA compared to the LAD, Western blot analysis was performed with proteins extracted from the IMA and proximal LAD tissues. The analysis showed significant up-regulated expression of Testin (P=0.014) in the IMA compared to the LAD (Figure 1a). Similarly, *RGS-5, HSP70, ATF3, TINUR* had increased expression in IMA via Western blot, while *PECAM-1, RASF-A-PLA2, F13A1,* and *CD163 antigen* had decreased expression in the IMA (correlating with mRNA expression, data not shown).

To localize the site of expression of Testin in these arteries, we carried out immunostaining studies of IMA and LAD tissues sections with an anti-Testin antibody. The immunostaining signal for TES co-localized with the EC marker CD31, indicating strong Testin expression in arterial endothelial cells (Figure 1b). The immunostain signal for Testin was stronger in the IMA than in LAD (Figure 1b). Moreover, some immunostain signal for Testin was also detected in the media of the IMA, most likely localizing in VSMCs (Figure 1b).

4.3.4 TES/Testin Expression in LAD Is Decreased in CAD Patients Compared to Non-CAD Subjects

Because the expression level of TES is higher in the atherosclerotic resistant IMA than in the atherosclerotic prone LAD, we hypothesized that TES is involved in contributing a resistance to atherosclerosis. To test this hypothesis, we assessed the expression levels of the TES gene in coronary arteries from 10 CAD patients in comparison to 10 non-diseased coronary arteries by real time PCR analysis. Expression of TES was markedly reduced by six-fold in CAD patients compared to non-CAD subjects (P=0.000049) (Figure 2a).
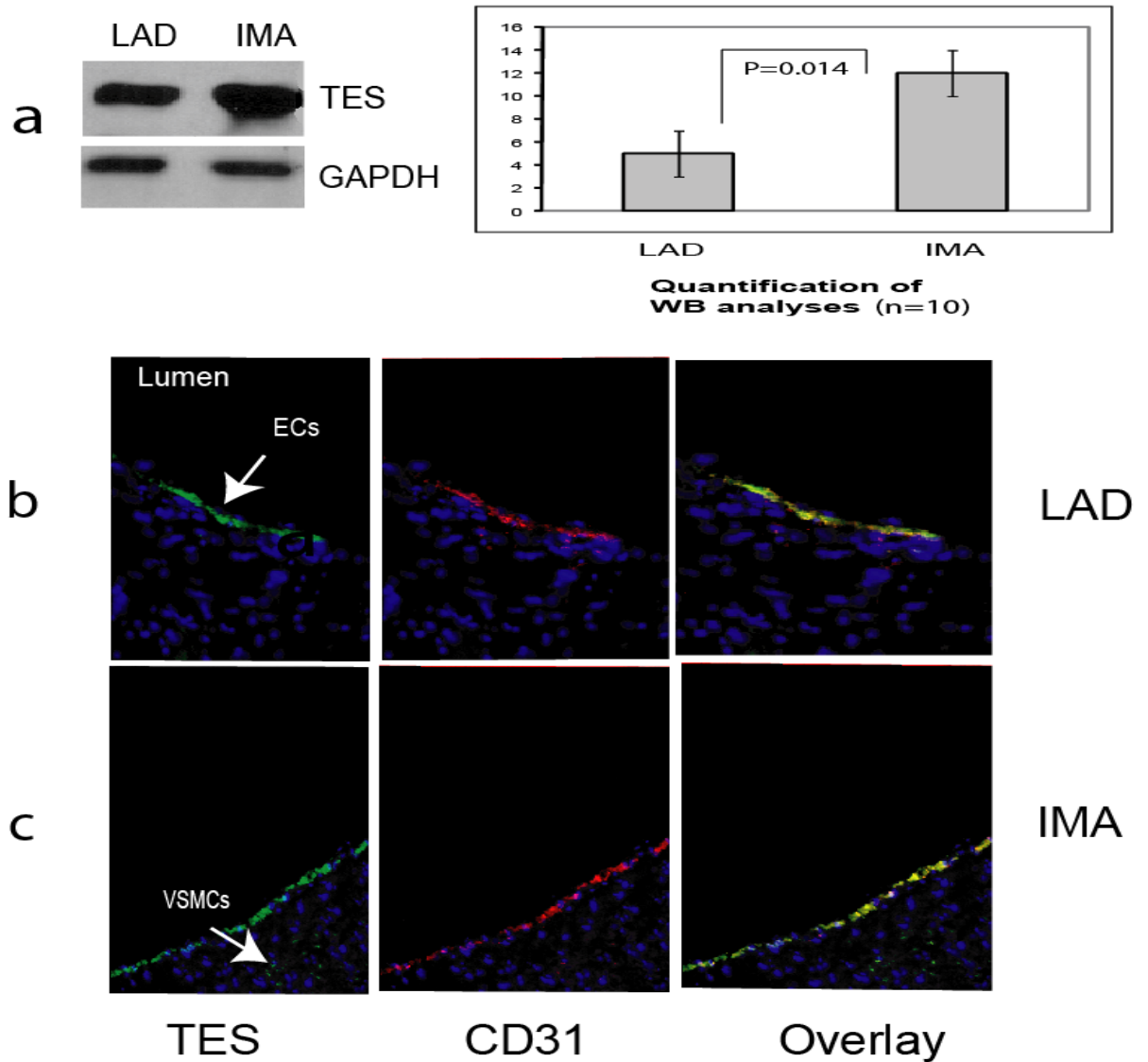
Fig. 1. Western blot analysis of IMA vs LAD for TES expression and cellular localization of TES in these arteries. Western blot analysis was performed with proteins extracted from the IMA and LAD (n=10), a representative comparison is shown (a). The expression level of Testin is higher in the atherosclerotic resistant IMA than in the atherosclerotic prone LAD (n=10/group) (P=0.014). The immunostaining signal for TES co-localized with ECs stained with CD31, a marker for ECs, in both IMA and LAD (b). In the IMA, the Testin signal was detected in ECs. Some TES signal was also detected in the artery, mostly likely VSMCs (c).
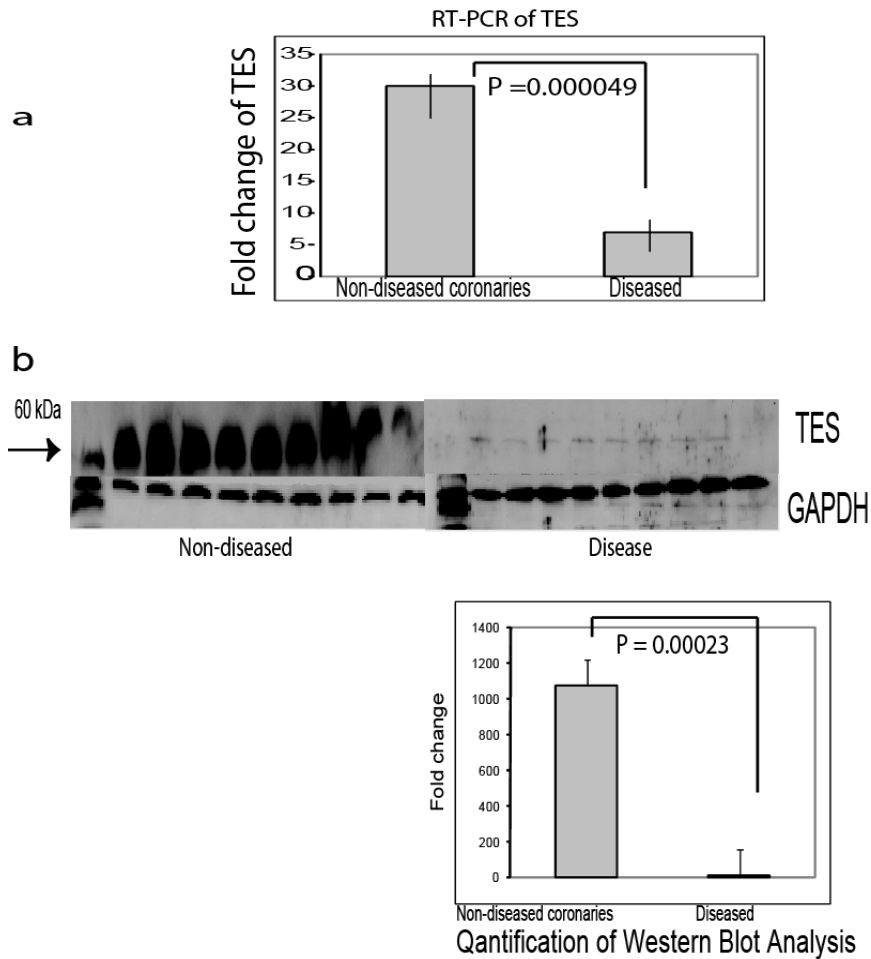
Fig 2. Decreased TES/Testin expression in coronary arteries from CAD patients. TES mRNA expression from 10 non-diseased coronaries compared to 10 diseased coronary arteries by real-time RT-PCR. The mRNA expression of TES in patients with CAD is significantly lower than in non-CAD individuals (a). Testin expression by Western blot analysis in an additional cohort of 10 CAD patients and 10 non-CAD individuals shows Testin expression is lower in patients with CAD. GAPDH was used as loading control (b).

Western blot analysis of protein extracts from LAD with a different cohort of 10 CAD patients and 10 non-CAD controls further confirmed that Testin expression was decreased in diseased coronaries in comparison with disease-free arteries (Figure 2b) (P=0.00023). The reduced TES expression is expected to correlate with decreased resistance to atherosclerosis and the development of CAD.

4.3.5 Testin Affects oxLDL-Mediated Monocyte Adhesion to LAD ECs

To identify a cellular mechanism for the association between TES/Testin expression and its sensitivity to atherosclerosis, we characterized the effects of over-expression of TES in LAD ECs and VMSCs by transient transfection with an expression construct of a 6xHis-tagged TES (empty vector as the negative control) or knock-down of TES expression by RNA interference (scrambler as control). Western blot analysis showed successful over-expression of Testin in LAD ECs and VSMCs with transient transfection of the TES expression plasmid compared to the vector, and that the TES siRNA effectively reduced the expression levels of Testin in LAD ECs and VSMCs compared to the scrambler siRNA (Figure 3a and Figure 4a).

Because adhesion of blood monocytes to the ECs of coronary arteries is a significant step in initiating atherosclerosis, we first assessed the effect of over-expression of TES and knock-down of TES expression in LAD ECs on monocyte adhesion. As shown in Figure 3b, treatment of ECs with oxLDL dramatically increased the adhesion of monocytes to the ECs, yet over-expression of TES decreased the adhesion of monocytes to ECs (compare TES to Vector; P=0.022). At the same time, knockdown of TES expression increased adhesion of monocytes to ECs, (Figure 3b; compare siRNA to scrambler; P=0.000000021).

113

4.3.6 Testin Is Involved in Cell Migration of LAD ECs and VSMCs

Because cell migration also plays an important role in the development of atherosclerosis, the effects of over-expression of Testin and knock-down of Testin expression on EC and VSMC migration were examined using a scratch assay. Over-expression of Testin significantly inhibited the migration of ECs (Figure 3c, compare TES to Vector; P=0.000000079), and similar results were obtained for VSMCs (Figure 4b compare TES to Vector; P=0.00048). Knockdown of Testin expression increased migration of both ECs and VSMCs (Figure 3c and Figure 4b, compare siRNA to scrambler). These results suggest that Testin plays an important role in cell migration of both ECs and VSMCs.
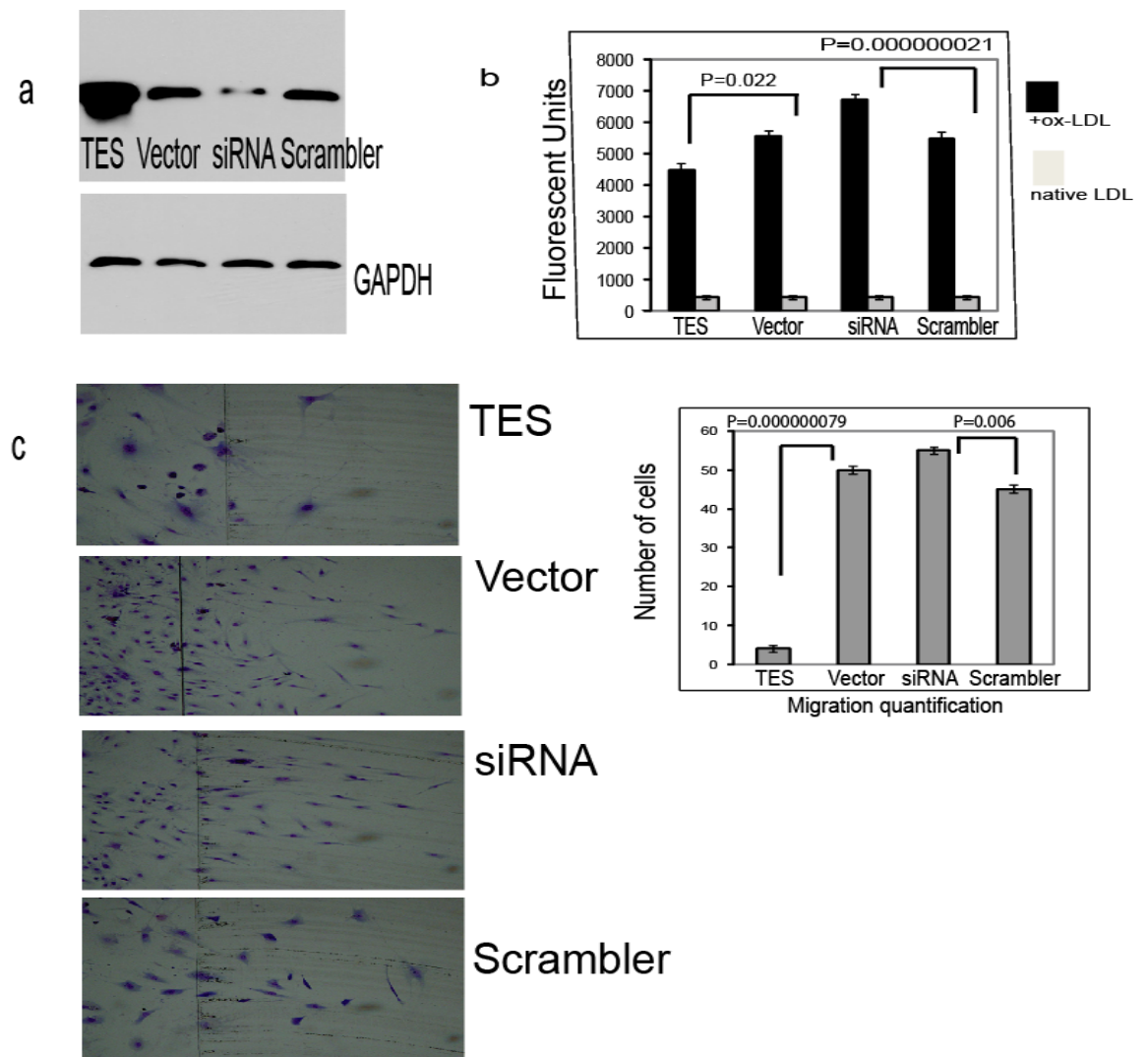
Fig. 3. Functional characterization of Testin in ECs. Western blot analysis shows that Testin was successfully overexpressed in LAD-derived ECs by transient transfection (empty vector as control) and that Testin expression was successfully knocked down by TES siRNA (scrambler as control). GAPDH served as loading control (a). Studies on monocyte adhesion to ECs. The treatment of ECs with oxLDL dramatically increased their adhesion to monocytes. Over-expression of Testin (compare TES to Vector) in ECs appeared to decrease adhesion of ECs to monocytes, knockdown of Testin expression in ECs increased adhesion of ECs to monocytes .(compare siRNA to scrambler) (b). EC Migration assays. Over-expression of Testin in ECs significantly inhibited the migration of ECs (compare TES to vector, while knockdown of Testin increased EC migration (compare siRNA to scrambler) (c). These results suggest that Testin plays an important role in EC cell migration.

115
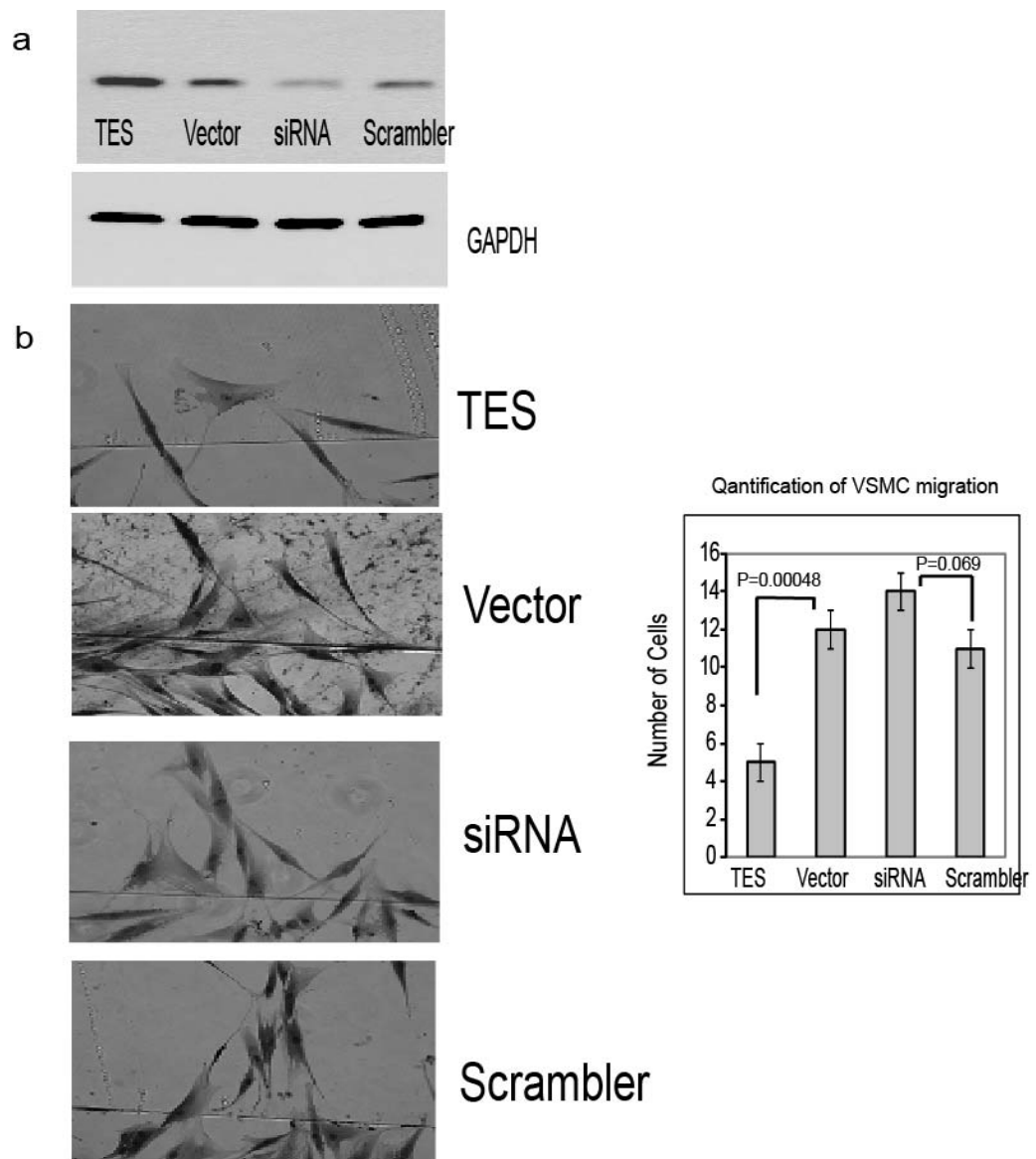
Fig. 4. Functional characterization of Testin on VSMC migration. Western blot analysis shows that Testin was successfully overexpressed in LAD-derived VSMCs by transient transfection (empty vector as control) and that Testin expression was successfully knocked down in VSMCs by TES siRNA (scrambler as control). GAPDH served as loading control (a). VSMC migration assays. Over-expression of Testin significantly inhibited the migration of VSMCs (compare TES to vector, while knockdown of Testin increased VSMCs migration (compare siRNA to scrambler) (b). These results suggest that Testin plays an important role in VSMC cell migration.

116

4.3.7 Testin Is Involved in Transmigration of Monocytes across oxLDL-treated LAD

ECs

While Testin did play a significant role in affecting the adhesion of monocytes to

ECs, we also hypothesized that Testin might also be involved in the transendothelial

migration of monocytes, which is a critical process in development of CAD.

Consequently, Testin was over-expressed in ECs derived from the LAD coronary arteries

and the cells were exposed to oxLDL (0.25 mcg/mL for 24 hours). The effect of Testin

overexpression in ECs on the transmigration of HL-60 monocytes was measured after 24

hours by plating monocytes onto confluent ECs adherent to the bottom of the Boyden

Chamber (Figure 5). Overexpression of Testin in ECs significantly reduced

transmigration of monocytes across the EC layer (Figure 5, compare TES to vector,

P=0.00000000051). Knockdown of Testin expression by TES siRNA increased

transmigration of monocytes across the EC layer (Figure 5, compare siRNA to scrambler,

P=0.028). The results indicate that Testin plays a significant role in the migration of

monocytes across the EC layer, a cellular process critical to atherosclerosis.
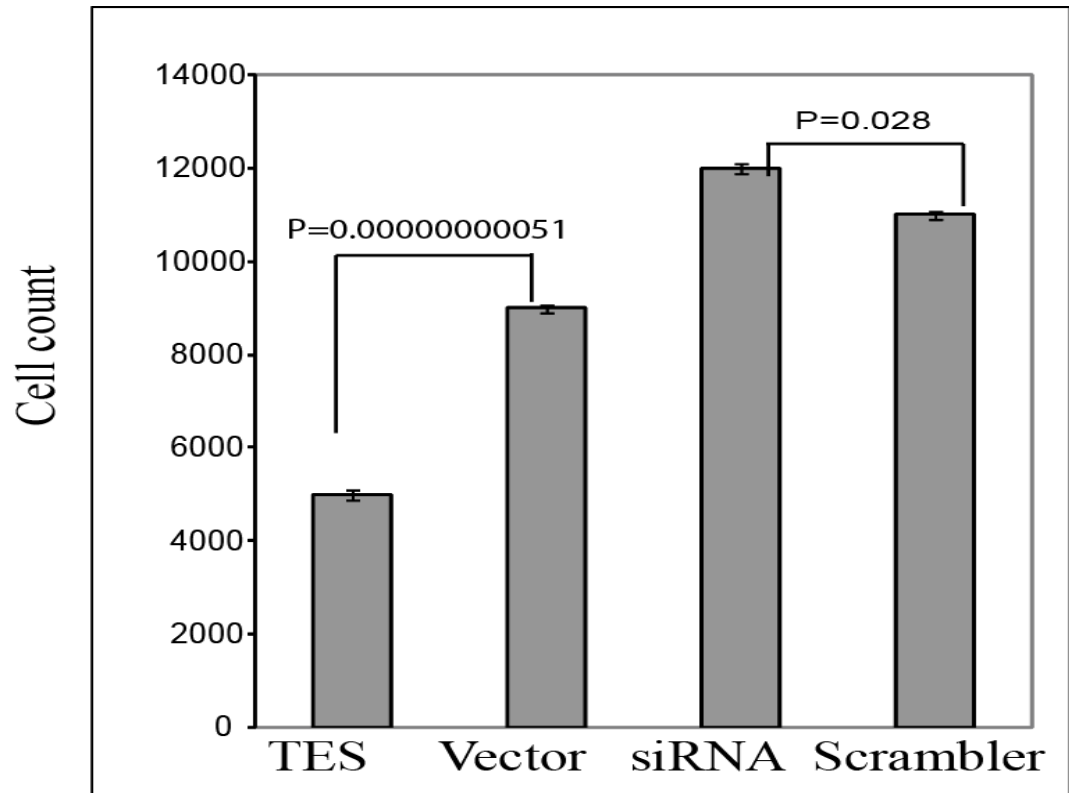
Fig. 5. Effect of overexpression or knockdown of Testin expression in ECs on transendothelial monocyte migration. Testin was over-expressed in LAD-derived ECs, which were exposed to oxLDL. The overexpression of Testin in ECs significantly reduced transmigration of monocytes across the EC layer (compare TES to vector). Knockdown of Testin by siRNA increased transmigration of monocytes across the EC layer (compare siRNA to scrambler). The results indicate that Testin plays a significant role in the migration of monocytes across the EC layer.

4.4 Discussion

This study was designed to identify the genes that confer a resistance to or protection from atherosclerosis by investigating the fundamentally different gene expression patterns between the atherosclerotic-resistant IMA and the atherosclerotic-prone proximal LAD coronary artery in human subjects. The study successfully identified a novel gene, TES (protein product Testin), whose expression level was associated with the sensitivity to the development of atherosclerosis. The expression level of TES/Testin was higher in the IMA compared to the LAD at both the mRNA and protein levels. Similarly, the expression level of TES/Testin was significantly higher in non-diseased coronary arteries than that in diseased arteries. Therefore, increased TES/Testin expression may be associated with resistance to atherosclerosis, whereas decreased TES/Testin expression is associated with a susceptibility to CAD.

This study was the first microarray analysis to use human tissue samples to compare global gene expression differences between IMA to LAD. We identified 29 genes differentially expressed between the two arteries, and follow-up replication studies with RT-PCR and Western blot analyses identified four genes with increased expression in the IMA compared to the LAD, including *RGS5, LAMNA, TES,* and *NR4A1. RGS5* encodes a regulator of G-protein signaling and was up-regulated by 5.9-fold in the IMA compared to the LAD. Consistent with our results, a previous study showed that *RGS5* expression was lost with the development of atherosclerosis and yet expressed in fibrous atherosclerotic caps (14). *LAMNA* encodes the Lamin A/C that is an important component of the nuclear lamina. Mutations in LAMNA are causes of premature aging in the Hutchinson's Gilford progeria syndrome and Werner Syndrome in which

premature atherosclerosis is one of the phenotypes (15). The *NR4A1* gene encodes the TR3 orphan receptor. Transgenic overexpression of the TR3 orphan receptor resulted in five-fold inhibition of neointimal formation after carotid artery ligation, whereas transgenic overexpression of a dominant negative variant increased neointimal formation by three-fold. This suggests that *NR4A1* confers a resistance to atherosclerosis (16).

Our study also identified genes with down-regulated expression in the IMA compared to the LAD. These included: *PECAM1, F13A1*, and *RASF-A-PLA2,* which showed two-fold or less expression in IMA compared to LAD. *PECAM-1* was down-regulated by 3-fold in the IMA. This gene mediates the attachment of monocytes to the endothelium and transendothelial migration. When *PECAM-1* expression was blocked, the consequences were reduced myocardial infarct size due to decreased neutrophil accumulation in the myocardium (17), suggesting a protective role for this gene in atherosclerosis. *Factor XIIIA* is involved in blood coagulation in which it cross-links fibrin molecules in a clot, stabilizing thrombi, and produces a "resistance to fibrinolysis" (18). The product of *RASF-A-PLA2,* namely Phospholipase A2 (Group 2A) is responsible for "amplifying" the inflammatory component of many disease processes including atherosclerosis (19). Showing multiple genes that were documented to resist or promote atherosclerosis, microarray analysis of the IMA vs. LAD is an effective approach to identify novel protective or susceptibility genes involved in atherosclerosis. Employment of this interesting strategy in the present study identified a novel gene, TES, which is associated with a resistance to atherosclerosis.

The pathogenesis of atherosclerosis starts with the adhesion of monocytes to ECs, followed by their transendothelial migration into the media of an artery. The monocytes

differentiate into macrophages which release cytokines, amplifying the inflammatory process and culminating in foam cells, the migration of VSMCs and the formation of plaque. Functional studies with the overexpression of TES or the knockdown expression of TES suggest that the cellular mechanisms by which TES mediates the development of atherosclerosis may be related to the transendothelial migration of monocytes as well as EC and VSMC migration and monocyte adhesion to the endothelium. The molecular mechanism for TES-associated atherosclerotic resistant properties is unknown. The function of TES in the coronary artery may parallel its function in the testes where it serves a role as being part of the tight junction between adjacent, germinal epithelial Sertoli cells (8). Here, TES contributes to the blood-testes barrier to protect the developing germ cells from the systemic circulation and blunt the diffusion of proteins that can damage the developing gametes. Correspondingly, TES may retard the binding to and passage of monocytes through the endothelial cells into the intima, the initial event leading to atherosclerosis.

We showed that overexpression of TES decreased monocyte adhesion to ECs and inhibited migration of ECs and VSMCs, while knockdown of TES increased the migration of these cells. This finding is supported by a report showing that the overexpression of TES in chicken embryo fibroblasts, a working model for the study of focal adhesions, decreased their motility (9). The involvement of TES in cell migration is most likely related to its interactions with multiple cytoskeletal proteins including mena, zyxin, paxillin, actin, alpha-actinin, VASP and Talin, which are involved in reorganization of actin fibers and cell motility (8), although a direct link remains to be established. The relationship between EC migration and atherosclerosis is not known.

121

However, TES inhibition of VSMCs migration may be more applicable to its contribution to a resistance of atherosclerosis.

A previous study by Qin et al used suppression subtractive hybridization (SSH) with three pooled porcine mRNA samples to identify the gene expression differences in the IMA and LAD denuded of the endothelial cells (19). Qin et al identified 24 genes, but none of the genes expressed in pigs were found in common with our final list expressed in humans. The difference may be caused by the fact that Qin et al focused only on VSMCs, or by different tissue samples used by the two different studies. Furthermore, the SSH is limited by the number of genes to be analyzed or cDNA clones selected for sequencing, whereas the microarray analysis is more a global strategy that analyze more than 12,000 genes simultaneously.

There are a few limitations of our study. Our initial cohort of LAD samples for expression profiling came from heart transplants at Cleveland Clinic. Because IMA were not available from the same heart transplantation patients, we matched the LAD samples with the IMA samples from autopsy. To minimize this complication, we carried out our replication studies in an independent population of 30 study subjects, and used matched IMA and LAD samples excised and compared from the same individuals. It is also important to point out that we focused only one aspect of the IMA, namely, its genetic profile. This information complements two other areas that may explain the molecular basis for this artery's lack of stenosis. For example, the IMA may not get atherosclerosis because: 1) based on its anatomy, it is a conduit with steady laminar blood flow which down-regulates the expression of cell adhesion molecules--processes associated with endothelial dysfunction in contrast to the proximal LAD known to have turbulent flow

(21, 22); 2) based on its histology and cell composition, it contains a unique internal elastic lamina (IEL) that is continuous, lacks perceivable gaps and serves as a barrier to the passage of inflammatory cells into the sub-intima and media of the artery, which is based on several electron microscopic studies of this artery (23). Nevertheless, our findings that a decreased expression level of TES correlates with the development of CAD and that TES plays an important role in cellular processes relevant to atherosclerosis suggest that the different genetic profiles of the IMA and LAD may be a significant factor for a resistance to atherosclerosis by the IMA.

In conclusion, we performed the first global gene expression profiling study on the human atherosclerotic resistant IMA as compared to the atherosclerotic-prone proximal LAD coronary artery. This study identified a novel gene, TES, which is associated with resistance to disease. We showed that TES expression was significantly decreased in LAD tissues from patients with CAD compared to non-CAD patients. Functional studies showed that the association between TES and CAD may be related to monocyte adhesion, transendothelial migration of monocytes and VSMC migration.

CHAPTER 5

THE FUNCTIONAL SIGNFICANCE OF THE ADENOSINE A2B RECEPTOR

IN THE INTERNAL MAMMARY ARTERY

ABSTRACT

The endothelium is the initial target that leads to cardiovascular disease. Knowing that

the internal mammary arteries (IMA) are resistant to the development of atherosclerosis,

which contrasts with coronary arteries (Cor) which are athero-prone, we hypothesize that

genes over-expressed in the endothelial cells (ECs) of these two arteries will identify

genes that resist atherosclerosis. Methods and Results—Microarray analysis showed 95

genes differentially expressed in the ECs of IMA vs Cor. The most statistically

significant different gene was the adenosine A_{2B} receptor.   This indicates the A_{2B}

receptor may be involved in a resistance to atherosclerosis. Western blot analysis showed

higher A_{2B} expression in the IMA than in coronary arteries with or without disease from

proteins harvested from these human arteries and ECs. Overexpression of A_{2B} in ECs

blunted: monocyte adhesion, cell adhesion molecule expression, migration, and the

transendothelial migration of monocytes-- processes directly associated with the

development of atherosclerosis. Knockdown of A_{2B} expression by siRNA promoted

these processes. Conclusions—ECs derived from the IMA and Cor are distinctly different

124

in gene expression, which may be responsible for their differential sensitivities for atherosclerosis. This study defined how the A_{2B} receptor may act as an atherosclerotic-resistance gene, which blunted monocyte adhesion and cell adhesion molecule expression, EC migration and retarded the transendothelial migration of monocytes.

5.1 Introduction

Endothelial cells (ECs) are the initial target affected by numerous risk factors that lead to atherosclerosis. Additionally, ECs respond to turbulent blood flow, vasomotor tone, and inflammatory cytokines--all of which play critical roles in target-organ damage. Interestingly, the human internal mammary arteries (IMA) are resistant to atherosclerosis despite being exposed to the same risk factors as the coronary artery. They have proven better long-term patency rates (1).

The IMAs resistance to disease may be the result of its superior endothelial function, because it produces more antithrombotic vasodilators such as prostacyclin and nitric oxide (NO) as well as more endothelium-derived hyperpolarizing factor than saphenous veins (2). Even in patients with CAD, atherosclerotic lesions rarely affect the IMA. This reinforces the fact that ECs harvested from different areas respond differently to the risk factors for CAD.

Since endothelial dysfunction is a key factor in atherogenesis, the current study investigated the gene expression of the ECs harvested from IMA and Cor to distinguish these arteries' differential sensitivities to the develop atherosclerosis and identify novel molecular mechanisms. Using oligonucleotide microarrays, we found 95 genes that were differential expressed, and the most statistically significant gene identified was the adenosine A_{2B} receptor (A_{2B}), an integral membrane protein. Consequently, A_{2B} was selected for follow-up functional studies to define its role(s) in ECs and how it may promote a resistance to atherosclerosis.

Four subtypes of adenosine receptors have been identified: A_1, A_{2A}, A_{2B}, and A_3. Based on pharmacologic studies, these receptors have been classified into adenylyl cyclase inhibitory (A_1 and A_3) and stimulatory (A_2) categories (3). The A_2 receptors are further subdivided into 2_A and 2_B. The A_{2B} receptor maps to chromosome 17q11.2-p12, and it consists of a single intron that interrupts the coding sequence in the region corresponding to the 2nd intracellular loop between Leu^{111} and Arg^{112}.[3]

Although the A_{2B} receptor's primary ligand is adenosine which results in termination of superventricular tachycardias and causes vasodilation (3), the biological mechanism(s) underlying the functional properties of the A_{2B} receptor are not known. However, several studies have made observations. Recent evidence obtained using A_{2B} knockout (KO) mice showed that these mice had a phenotype associated with atherosclerosis (4). The A_{2B}-KO mice displayed a significant increase in pro-inflammatory cytokines under baseline conditions, and following a challenge with lipopolysaccharide. At the same time, the A_{2B}-KO mice had up-regulated levels of the adhesion molecules E-selectin, P-selectin, and ICAM-1 in the vasculature with increased leukocyte adhesion (5). Similarly, a femoral artery injury model with A_{2b}-KO mice vs the wild type showed that the A_{2b} receptor prevented vascular lesion formation in the artery that resembles human restenosis after angioplasty (5). It was reported that BAY 60-6583, a highly selective A_{2B} receptor agonist, limited infarct size in rabbit hearts (6). In vivo studies have also shown that vascular permeability and pulmonary inflammation are significantly increased in A_{2B} KO mice subjected to hypoxia (3). These studies support the A_{2B} receptor as an atherosclerotic-resistance gene.

In this study, we identified the A_{2B} receptor as the most statistically significant gene differential expressed in the ECs of the IMA vs the Cor. We confirmed the expression of A_{2B} is higher in human IMA tissues and disease-free coronary arteries and lower in coronary arteries with atherosclerosis. Functional studies including monocyte adhesion and cell migration, oxLDL-induced transendothelial migration of EC were used to define the molecular mechanisms by which A_{2B} may confer a resistance to endothelial dysfunction which generates disease.

5.2 Material and Methods

5.2.1 Tissue Samples of Human Arteries

Arteries were obtained from explanted hearts through the Cleveland Clinic Heart Transplant Program, unmatched or rejected healthy donor hearts from LifeBanc of Northeast Ohio and the Cuyahoga County Coroner's office. The LAD was carefully examined for the presence or absence of atherosclerosis by microscopic examinations of consecutively dissected serial segments of the entire artery by cardiovascular pathologists. We selected a segment of the proximal LAD without intraluminal plaque. Informed consent was obtained from the participants (or their families at post-mortem exam) according to the standards established by the Cleveland Clinic Foundation Institutional Review Board on Human Subjects and the Cuyahoga County Coroner. Arteries were cleaned of adjacent adipose and myocardial tissue in explanted hearts or from cadavers. The adventitial layer remained intact. The specimens were snap-frozen in liquid nitrogen and stored at -80°C as previously described until used (7).

5.2.2 Immunostaining of human arteries. The cryo-sections (6-μm) of IMA and coronary arteries were fixed with paraformadehyde (4%) and permeablized with Triton X-100 (0.2%) as previously described (8).   The primary antibodies used were: CD64 (macrophages) (Santa Cruz), Adenosine A_{2B} receptor (BD Technologies), Platelet endothelial cell activating molecule (PECAM-1) (Santa Cruz), CD31 (PharMinger) and monoclonal anti-α-SMC-actin (clone 144) conjugated with cys3 (Sigma). The secondary antibody conjugated with cys3 (Sigma) or FITC (Sigma) were used for visualization (1:250). The sections were counterstained with DAPI (Vectashield with OAD, Vector Labs, Burlingame, CA).   The immunostaining signals were visualized with a Zeiss Axiophot fluorescence microscope and the images were captured with Imageplus 6.0.

5.2.3 Cell Culture and Transfection

Endothelial cells (ECs) harvested from the proximal LAD coronary artery and IMA were purchased from Cell Applications, Inc. The cells were isolated from non-diseased human arteries and cryo-preserved at the second passage. Cells were grown with standard media supplied ready-for-use from Cell Applications, Inc. All cells were used between the 3th and 6th passage and grown in a humidified incubator at $37^{o}C$, 5% CO_2 and 95% air.

The Adenosine A_{2B} receptor plasmid was a generous gift from Michelle Wong (Emory University). All cells were used for assay 48 hours after transfection.

Small interfering (si) RNA for A_{2B} was purchased from Integrated DNA technologies: Sense--rGrCrA rGrCrA rCrCrU rGrCrC rArUrG rArArC rUrCrC rUrGG T and Antisense--rArCrC rArGrG rArGrU rUrCrA rUrGrG rCrArG rGrUrG rCrUrG

129

rCrArG. Cells were transfected with the A_{2B} plasmid, siRNA, empty vector, or scrambler using Polyjet (SignaGen Laboratories). The optimal dose to interfere with A_{2B} mRNA expression was 1 μM.

5.2.4 Real time RT-PCR and Expression profiling

Total RNA was isolated from the ECs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. ECs were grown in six well plates to confluence for 48 hours and then exposed to various experimental conditions.

To identify the genes differentially expressed between the ECs cells of the IMA and Cor, we carried out microarray analysis with Human Genome U133 arrays (Affymetrix). Four microarrays were used to compare the IMA and the Cor. Each array contained ~33,000 unique genes. GeneSpring (Agilent technologies) was used for data mining as previously described (8). We generated the statistical algorithm as followed on the web site (www.agilent.com).   Results from the microarray analysis were validated for selected genes by real time RT-PCR with SYBR Green PCR (VWR) using the original cDNA for microarray analysis as well as from the total RNA extracted from another group of independent tissues. Relative expression values were calculated as previously described (8, 9). The expression level of β_2-microglobulin was used as an internal control. Specific primer pairs were designed for each selected gene of interest (Integrated Biotechnologies, Inc.) (Supplemental Table 2).

5.2.5 Western Blot Analysis.

The IMA or Cor tissues (0.2 g/sample) were lysed in a homogenization buffer for 15 minutes at $35^{\circ}C$ using the delipidation method as previously described (9).   Protein

extracts from the IMA or LAD ECs grown *in vitro* were prepared with M-PER Mammalian Protein Extraction Reagent (Pierce) and quantified with BCA Protein Assay Kit (Pierce).

Protein lysates from each experiment (25 μg/ lane) were separated by 12% SDS-PAGE Ready Gels (Bio-Rad) and transferred to nitrocellulose membranes. After incubation in blocking solution (5% nonfat milk) (Bio-Rad), membranes were incubated with the primary antibodies: Adenosine A_{2B} Receptor, VCAM-1 (Serotec), or ICAM-1, (Santa Cruz) overnight at 4ºC. Membranes were washed three times for 10 minutes (0.1% Tween 20) and then incubated with the secondary antibody, GAPDH (Santa Cruz) for one hour at room temperature. Signals in the membranes were detected with the ECL system (Amersham). Relative intensities of protein bands (and signals on immunostains) were analyzed by Melanie (Swiss Institute of Bioinformatics).

5.2.6 Monocyte Adhesion Assay

Fully confluent ECs were grown in CoStar96 well plates after being seeded at a density of 5,000 cells/well and stimulated with oxidized-LDL (oxLDL) (Sigma) (0.25 mg/mL) for six hours. Human HL-60 pro-myelocytic leukemia cells were cultured in Iscove's modified Dulbecco's media, fluorescently labeled with calcein AM (Invitrogen) for 60 minutes at 37ºC, washed three times with PBS, and then seeded into each well coated with transfected ECs (A2B, empty vector, siRNA, or Scrambler) at a density of 3×10^5 per well. After one hour, the media was aspirated and wells were washed gently with PBS three times. Fluorescent intensity was measured in each of the 96-wells with a standard

fluorometer with the CytoFluoroII program (Applied Biosystems) (exit: 485/20, emission 530/30, gain of 65) to determine the fluorescent units in each well.

Endothelial cells were incubated with ox-LDL (12.5, 25, 50, or 100 μg/mL) for 3, 12, 24, or 72 hours to determine the maximal expression of selected cell adhesion markers. Control cells were incubated for the same time periods with native LDL. Each experiment was repeated in triplicate.

5.2.7 Cell Migration Assays

ECs were transfected as mentioned above and plated at a density of 0.5×10^6 cells per cm^2 in 35 mm six well plates. The media was changed 24 hours later. Then, the confluent monolayers were wounded with a single edge razor and cells on the right side of the wound were removed. After 48 hours, the cells were stained with Coomassie Brilliant Blue for 10 minutes followed by Glacial acetic acid/methanol destaining buffer, and photographed with Image-Pro Plus and analyzer 7.0 (Mediacybernetics) under 4X power. The number of cells which migrated passed the wound was counted. Each experiment was repeated in triplicate.

5.2.8 Transmigration of Monocytes Assay

HL-60 cells (3×10^5) were pippeted into 0.4 μm pore chambers within a Boyden chamber (Corning), which had transfected ECs plated 24 hours prior and then exposed to 0.25 μg of ox-LDL. FBS was used as a chemo-attractant and diluted in serum-containing DMEM media. The HL-60 cells were collected and counted from the bottom chamber 48 hours after their administration with a Bright-Line Hemacytometer (Hausser Scientific) using an Olympus CKX 41 microscope. Each experiment was repeated in triplicate.

5.2.9 Statistical Analysis. The difference between the two groups of variables for these functional assays related to atherosclerosis was compared by the paired Student t-test. A P value of < 0.05 was considered as significant

5.3 Results

5.3.1 Endothelial cells from IMA Bind Less Monocytes *in vivo* and *in vitro*

To begin to identify the ECs role in the resistance of the IMA to atherosclerosis, we did an immunostain with CD64, a marker for macrophages. The rational for this experiment is that atherosclerosis is defined as an inflammatory process (ie the binding and infiltration of monocytes through the ECs) leading to endothelial dysfunction. Immunohistochemical stained tissue sections of IMA and Cor showed a significant (P=0.000516) difference in the presence and binding of monocytes (Figure 1A). The Cor bound significantly more monocytes that were not only attached to the EC layer, but also can be seen to have transmigrated into the media of the artery. By contrast, the signal for CD64 was limited to the intraluminal surface of the IMA.
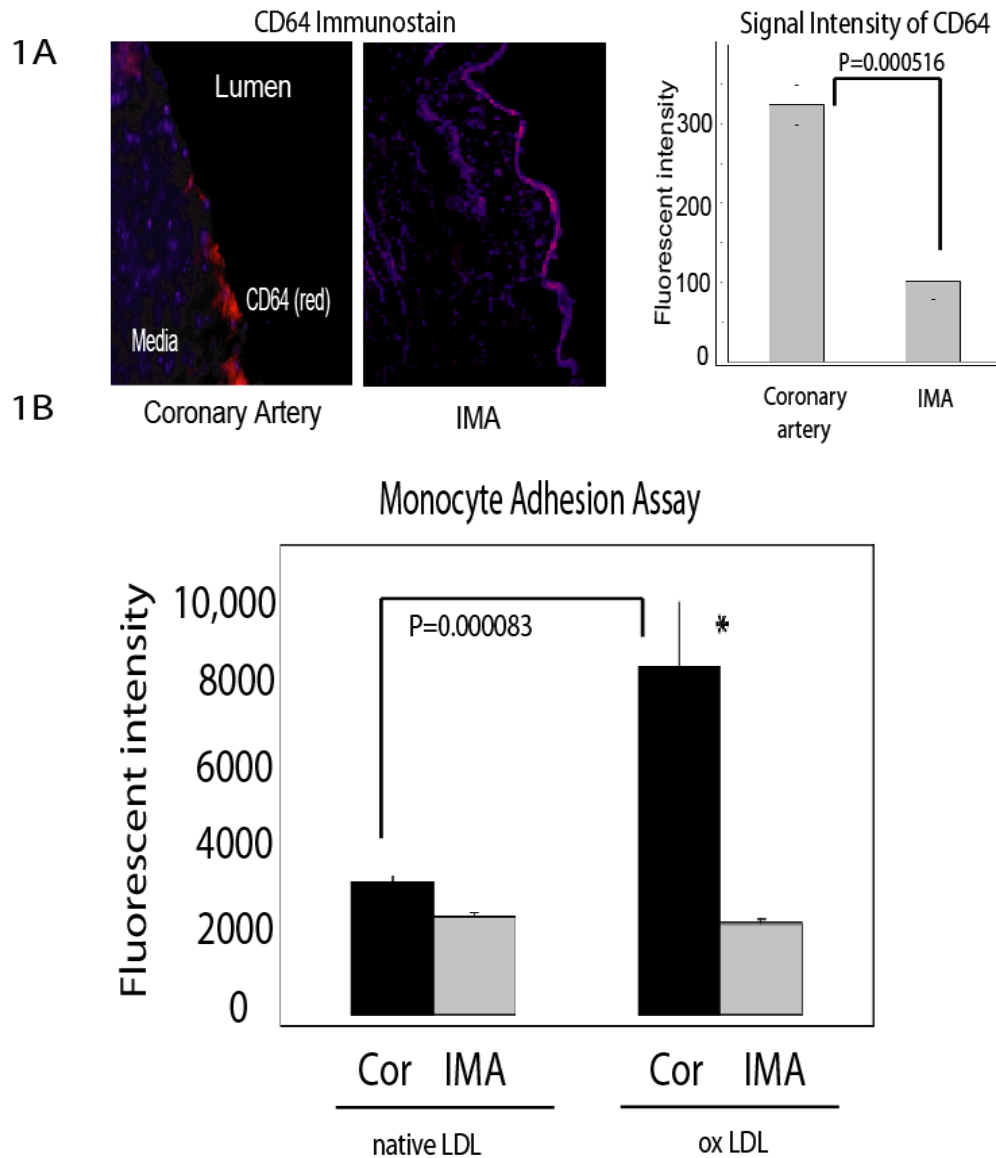
Fig. 1. The IMA contains less inflammatory cells. A, an immunostain of coronary artery vs internal mammary artery with CD64 (red) a marker on macrophages shows the IMA contains less inflammation. B. The endothelial cells derived from the IMA vs coronary artery show the IMA endothelial cells bind significantly less monocytes when exposed to ox-LDL.

In vitro, when we exposed the two groups of ECs to ox-LDL compared to native, unoxidized LDL for 24 hours (0.25mcg/mL), there was also a significant (P=0.000083) up-regulation of the binding of monocytes to the ECs derived from the Cor compared to the IMA (Figure 1B). The presence of ox-LDL did not appear to activate monocyte adhesion in the IMA derived ECs. In sum, human IMA tissue contained significantly less inflammatory cells adherent to the EC layer as well as in the media, which is a significant contrast to the Cor which had a preponderance of inflammatory cells. At the same time, the ECs harvested from Cor readily bound more monocytes in vitro when exposed to oxLDL while the ECs harvested from the IMA did not. This lack of inflammation may be attributed to unique genetic properties of the ECs of these two arteries.

5.3.2 Human IMA tissues and its ECs cells express less cell adhesion molecules.

The main molecular mechanism in which monocytes attach to the ECs cells is via cell adhesion molecules (CAMs). VCAM-1 and ICAM-1 are critical for the development of atherosclerotic lesions in which they are not expressed under baseline conditions but are rapidly induced by pro-atherosclerotic conditions. These CAMs were significantly up-regulated in the Cor vs IMA as seen by: immunostain (Figure 2A, P=0.0042 for VCAM-1; P= 0.017 for ICAM-1), by RT-PCR (Figure 2B, P=0.013 for VCAM-1; P=0.024 for ICAM-1), and by Western blot analysis from protein extracted from these tissues (Figure 2C, P=0.0000889 for VCAM-1; P=0.0023 for ICAM-1). The CAMs were also up-regulated in the ECs derived from these two arteries (Figure 2D, P=0.0043 for VCAM-1; P=0.0073 for ICAM-1). In sum, the differential expression of CAMs between the Cor vs IMA in both the arterial tissues and their ECs in vitro indicates that there is a
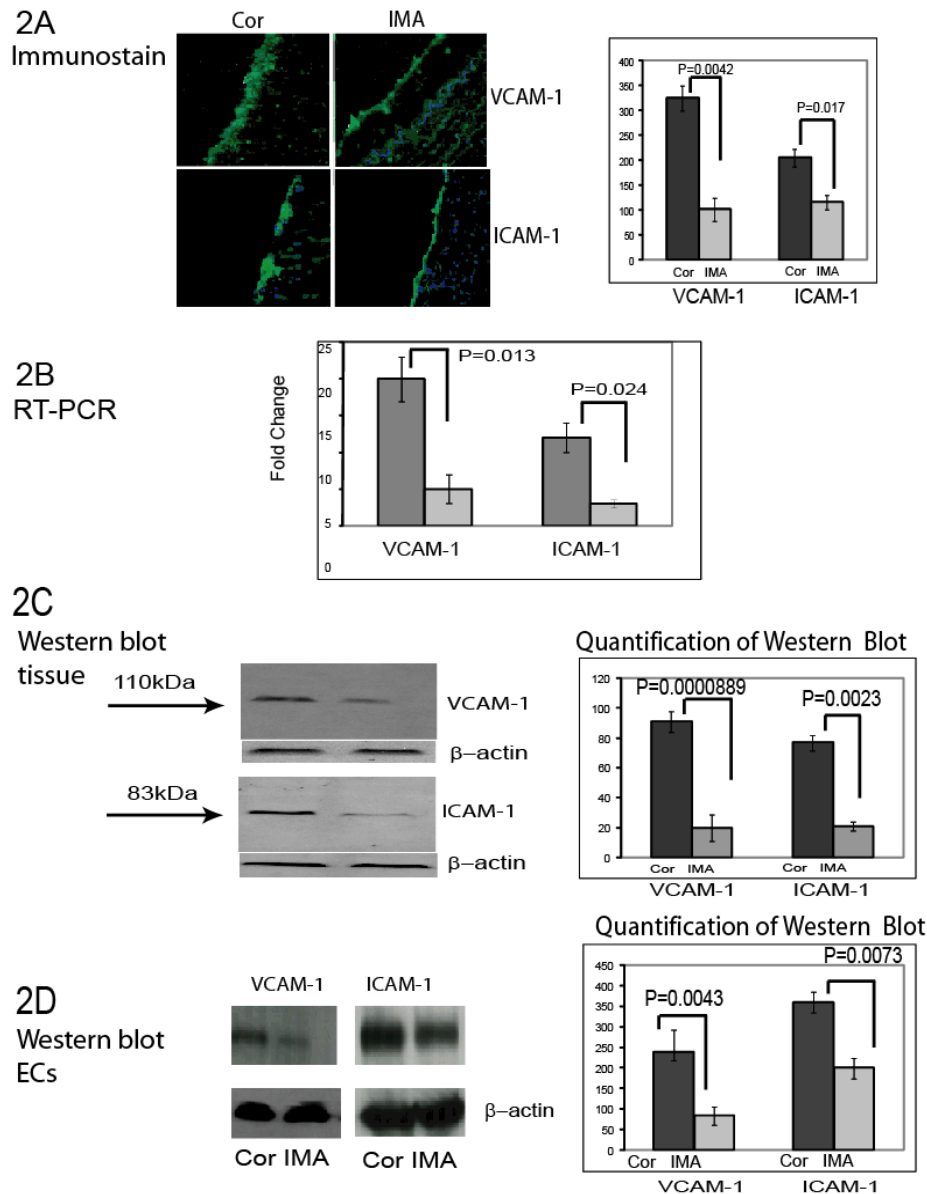
Fig. 2. The IMA expresses less cell adhesion molecules. A, an immunostain of IMA vs coronary artery for VCAM-1 and ICAM-1 show these adhesion molecules are down-regulated in the IMA. B, These cell adhesion molecules are also down-regulated at the level of transcription seen by RT-PCR, which also correlate with their translation noted in protein extracted from these two arteries. C, The IMA also expresses less cell adhesion molecules noted via Western blot analysis from protein extracted from these two tissues. D, The endothelial cells harvested from these two arteries also express these cell adhesion molecules less in vitro.

different propensity of these two arteries to develop atherosclerosis. The process is known to be initiated by monocyte binding directly to these CAMs on the ECs.

5.3.3 Microarray Analysis Identifies 95 Statistically Significant Genes in the IMA Endothelial Cells Compared to the Coronary Artery Endothelial cells.

To identify the genes differentially expressed between the ECs in the IMA and Cor and elucidate the differences that may account for the different tendencies of these arteries to develop atherosclerosis, we carried out microarray analysis with Human Genome U133 arrays (Affymetrix). Each array contains ~33,000 unique genes. A total of 95 genes were differential expressed (supplemental data). The top three genes that were most statistically significant were: Adenosine A_{2b} receptor (P=1 x 10^{-6}), Dynein (P =1.21 X 10^{-5}), and Ninjurin-1(P=1.01 X 10^{-5}) (Table 1).

At the same time, we validated a subset of these 95 genes that may be physiologically more relevant to a resistance to ECs in the IMA and their known expression in ECs (Table 2). These included 10 up-regulated genes and two down-regulated genes: Mesotrypsin (*PRSS3*), Antichymitropsin (*SERPINA3*), plasminogen activator (*PLAT*), trypsin inhibitor (*PRSS2*), Thromboxane A2 receptor (*TBXA2R*), Heme oxygenase-1 (*HMOX1*), Tissue factor pathway inhibitor 2, IL-1 receptor, Aminopeptidase (*ANPEP*), Heparin cofactor II (*SERPIND1*), and two down-regulated genes, namely, TNF-α-induced protein-1(*TNFAIP1*), and Activated leukocyte adhesion molecule (*ALCAM*). A review of these genes (Table 2) demonstrates that the ECs derived from the IMA are less atherogenic compared to the Cor. The resistance to thrombus formation is an inherent

genetic phenotype of the ECs derived from the IMA and may contribute to its lack of

disease.

5.3.4   Adenosine A$_{2B}$ Receptor Expression is Increased in the IMA compared to the coronary arteries with and without atherosclerosis.

The Adenosine A$_{2B}$ receptor was the most statistically significant gene found to

be differentially expressed in the EC of IMA and the Cor. An immunostain for this gene

Table 1. The top three statistically significant genes differentially expressed in the endothelial cells derived from the IMA vs coronary artery.

Gene Name	GenBank	Fold change on array	RT-PCR	Description
Adenosine A2b receptor	NM_000676	↑1.7	↑ 5.1	integral membrane protein stimulates adenylate cyclase activity in the presence of adenosine
Dynein	NM_006141	↓2.1	↓ 3.7	a motor protein which converts the chemical energy contained in ATP into the mechanical energy of movement.
ninjurin 1	NM_004148	↑2.5	↑ 2.9	cell surface adhesion molecule that promote growth

Table 2. Interesting genes differentially expressed in the IMA vs coronary artery endothelial cells.

Gene Name/Symbol	GenBank	Fold change on array	RT-PCR	Description
Mesotrypsin (PRSS3)	NM_002771 AW007273	↑20.9	↑18.3	plays a unique and highly specialized role in the degradation of trypsin inhibitors (22)
Antichymitropsin (SERPINA3)	NM_001085	↑10.3	↑9.4	a plasma protease inhibitor and member of the serine protease inhibitor class
plasminogen activator (PLAT)	NM_000930	↑13.6	↑10.6	involved in the breakdown of blood clots (23); a serine protease found on endothelial cells,
trypsin inhibitor (PRSS2)	NM_002770	↑15.0	↑12.3	It is an important anti-inflammatory substance (24)
Thromboxane A2 receptor (TBXA2R)	NM_001060/ D38081	↑4.2	↑3.0	a potent vasoconstrictor which regulates blood pressure (25)
Heme oxygenase-1 (HMOX1)	NM_002133	+6.6	+4.1	an inducible stress protein, confers cytoprotection against oxidative stress (26)
TNF-alpha-induced protein 1 (endothelial) (TNFAIP1)	NM_021137	↓7.0	↓6.5	Inhibition of tumor necrosis factor-α; reduces atherosclerosis in apolipoprotein E knockout mice (27)

Activated leukocyte adhesion molecule – (ALCAM)	**AA156721**	↓7.1	↓8.0	an important mediator involved in leukocyte migration into the CNS (28)
Tissue factor pathway inhibitor 2	**L27624**	↑5.7	↑5.0	exhibits antiproliferative action after vascular smooth muscle injury in addition to the ability to inhibit activation of the extrinsic coagulation cascade (29)
IL-1 receptor	**AK026803**	↑2.3	↑3.0	involved in the pathogenesis of atherosclerosis by a variety of mechanisms, including endothelial activation with expression of leukocyte adhesion molecules, increased gene expression of clotting factors and inhibitors of fibrinolysis, induction of chemokines, and increased proliferation of vascular smooth muscle cells (30)
Aminopeptidase (ANPEP)	**NM_002133**	↑6.6	↑4.0	Aminopeptidase is a zinc-dependent enzyme produced and secreted by glands of the small intestine.
Heparin cofactor II (SERPIND1)	**NM_000185**	↑9.2	↑8.0	inactivates thrombin action at the injured vascular wall (31)

in both arteries co-localized its expression with CD31, a marker for ECs (Figure 3A). The A_{2B} signal was more frequent in the IMA compared to the Cor (P=0.0031).

We further tested the expression level of this gene in three groups of ten human patients by RT-PCR and Western blot analysis. This included testing the expression of the A_{2B} gene in the IMA and the coronary artery with and without disease (average extent of disease in the coronary arteries was a 75-95% occlusion of the lumen). The Adenosine A_{2B} receptor had a similar level of expression in the IMA at both the level of transcription (Figure 3B, P=0.125) and translation (Figure 3C, P=0.231) compared to the Cor. Yet, the expression of the A_{2B} receptor was lower in the diseased coronary arteries compared to the IMA (P=0.0007) and non-diseased coronary artery (P=0.0034). In sum, microarray analysis identified a gene, namely that A_{2B} receptor that was found to be differentially expressed at the transcriptional and translation level and whose expression was down-regulated in diseased coronary arteries.

5.3.5 Adenosine A2B receptor affects ox-LDL mediated monocyte adhesion to Cor ECs.

To identify a cellular mechanism for the Adenosine A_{2B} receptor and its association with atherosclerosis, we over-expressed the Adenosine A_{2B} receptor by transient transfection. Western blot analysis showed successful over-expression of the Adenosine A_{2B} receptor in ECs (compared A2B plasmid to vector) and its knock-down expression (compare siRNA to scrambler) (Figure 4A).

Because adhesion of blood monocytes to the endothelium of coronary arteries is a significant step in initiating atherosclerosis, we first assessed the effect of over-expression of adenosine A_{2B} on monocyte adhesion. The treatment of ECs with oxLDL

dramatically increased monocyte adhesion (Figure 4B, compare siRNA to scrambler, P=0.0000000067), while the over-expression of the A_{2B} receptor significantly blunted the binding of monocytes to the ECs (Figure 4B, compare A2B plasmid
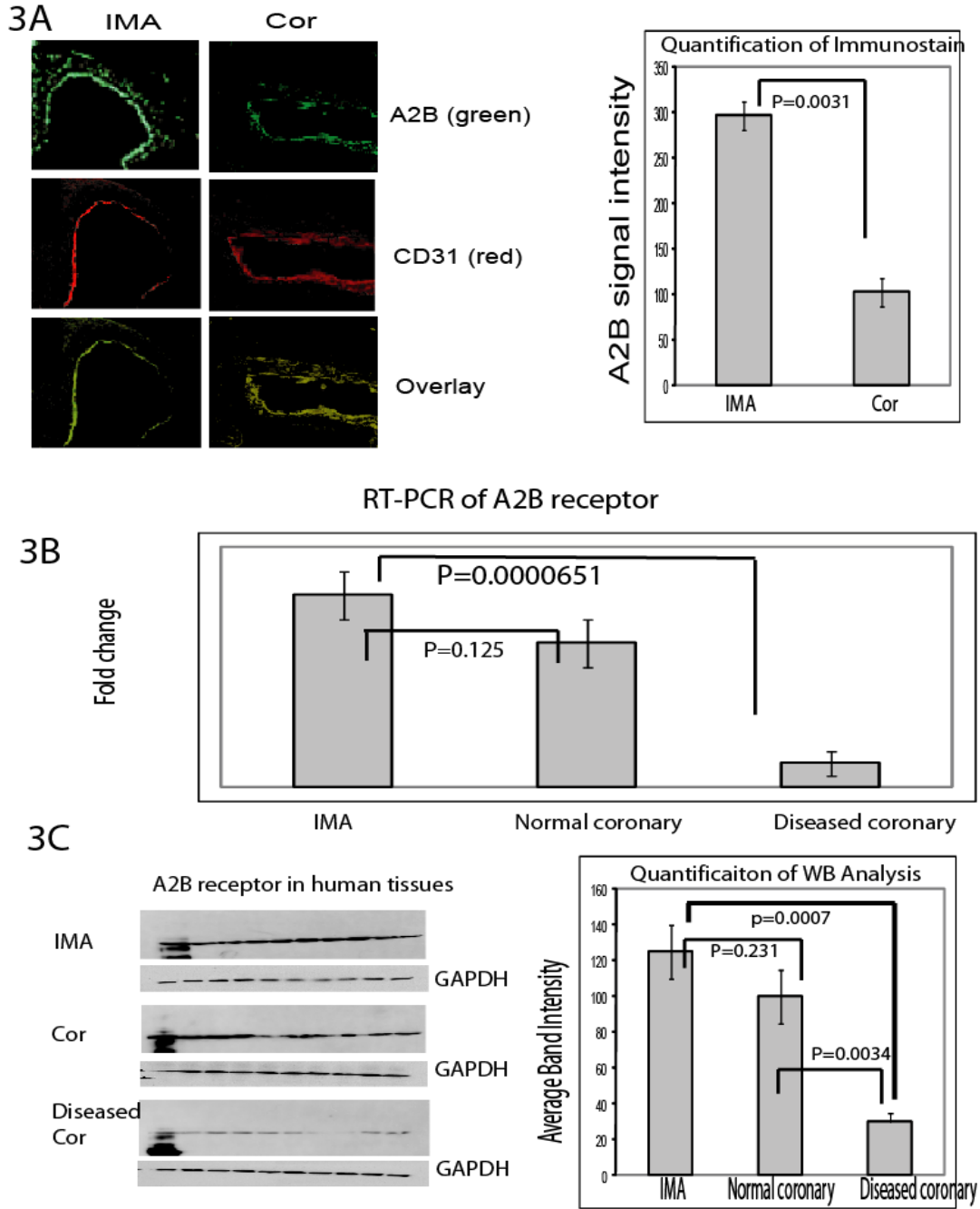
Fig. 3. Increased adenosine A2B receptor expression in the IMA vs. coronary arteries. A, an immunostain co-localized the A2B receptor (green signal) to the ECs (red signal), yellow is overlay. B, By RT-PCR from patient samples, the A2B receptor was significantly up-regulated in the IMA. C. Western blot analysis also showed the A2B receptor was up-regulated in five random patient's IMA vs coronary artery.
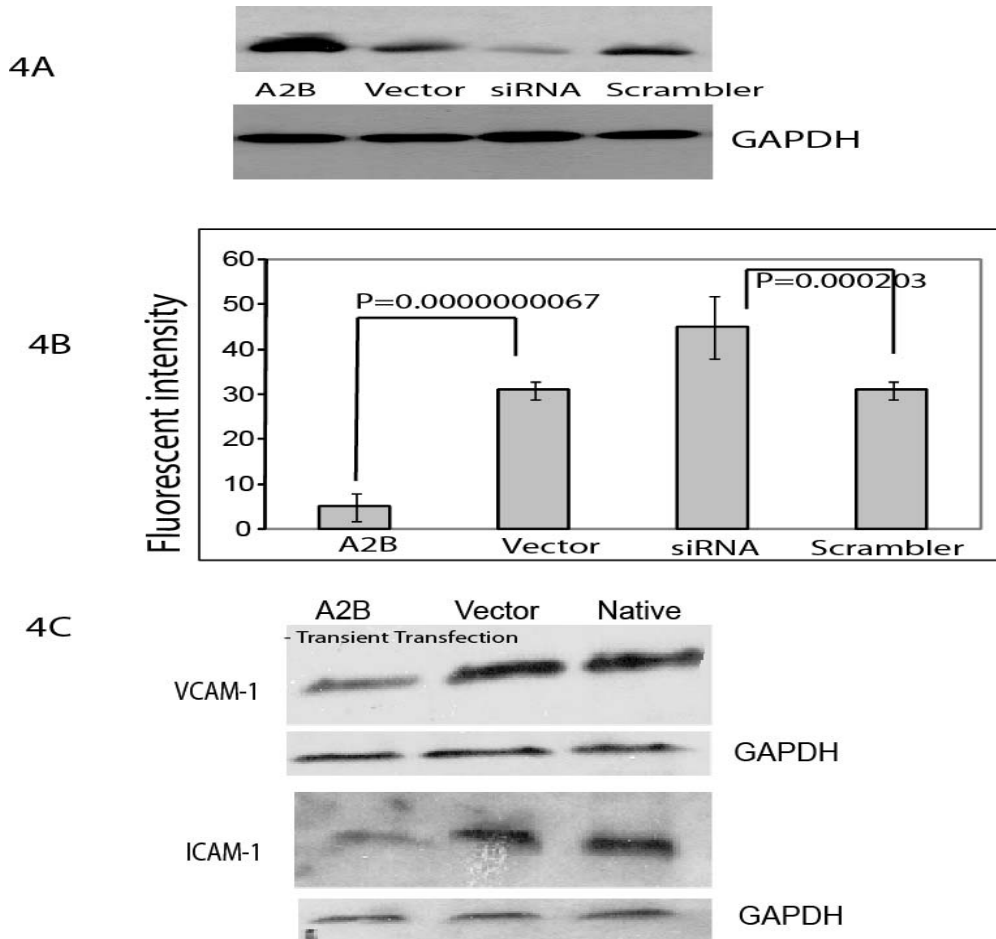
Fig. 4. Functional characterization of the A2B receptor in ECs. A. Western blot analysis shows the A2B was successfully overexpressed in EC by transient transfection (empty vector as control) and that A2B expression was successfully knocked down by A2B siRNA (scrambler as control). B, Studies on monocyte adhesion. Treatment of ECs with oxLDL dramatically increased their adhesion to monocytes. Over-expression of A2B (compared A2B to vector) in ECs appeared to decrease adhesion of ECs to monocytes, knockdown of A2B expression in ECs increased adhesion of ECs to monocytes (compare siRNA to scrambler). C, Western blot analysis for cell adhesion molecules (VCAM-1, ICAM-1, P&E selectin) were significantly down-regulated along with siRNA for A2B compared to vector (and native cells).

with vector, P=0.000203). The results show the A_{2B} receptor affects monocyte adhesion to ECs exposed to oxLDL which induces the expression of CAMs. Additionally, when we did Western blot analysis to assess the impact of A_{2B} over-expression on the expression of cell adhesion molecules (VCAM-1, ICAM-1), the A_{2B} receptor down-regulated their expression. Consequently, the atheroprotective effect of the A_{2B} receptor may affect the expression of CAMs.

5.3.6 Adenosine A2B receptor affects EC migration.

The impact of the Adenosine A_{2B} receptor on EC migration was examined using a scratch wound assay. Over-expression of the A_{2B} receptor significantly slowed the migration of ECs (Figure 5, compare A_{2B} vs vector, P=0.000076). Knock down of the Adenosine A_{2B} receptor increased migration of the EC (Figure 5, compare siRNA to scrambler, P=0.00024). These results suggest that the Adenosine A_{2B} receptor plays an important role in cell migration.

7.3.6 Adenosine A_{2B} receptor is Involved in Transmigration of Monocytes across ECs.

The Adenosine A_{2B} receptor was over-expressed in ECs derived from the coronary arteries and exposed to oxLDL (0.25 mcg/mL for 24 hours). The effect on the transmigration of HL-60 monocytes was measured after 24 hours by plating monocytes onto confluent transfected ECs adherent to the bottom of the Boyden Chamber (Figure 6). Over-expression of the Adenosine A_{2B} receptor significantly reduced transmigration of monocytes across the EC layer (Figure 6, compare A_{2B} to vector, P=0.00000000037). Knockdown of the Adenosine A_{2B} receptor increased transmigration of monocytes across the EC layer (Figure 6, compare siRNA to Scrambler, P=0.0147). The results show that

the Adenosine A_{2B} receptor plays a significant role in retarding the migration of monocytes across the EC layer, a cellular process relevant to atherosclerosis.

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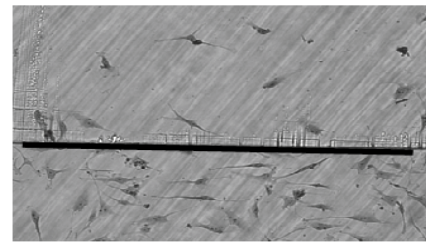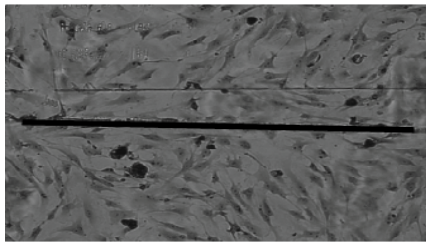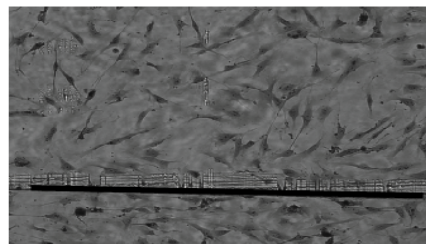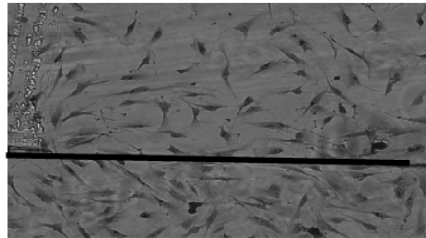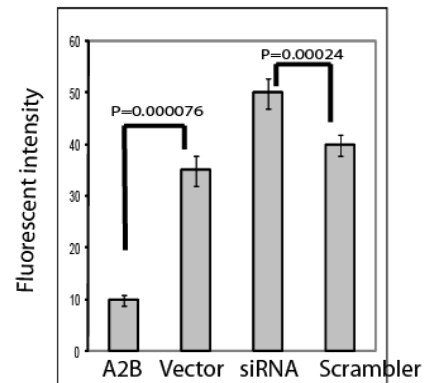
Fig. 5. EC migration assay. Over-expression of A2B in ECs significantly inhibited the migration of ECs (compare A2B to vector, while knowckdown of A2B increased EC migration (compare siRNA to scrambler). These results suggest that A2B plays an important role in EC cell migration
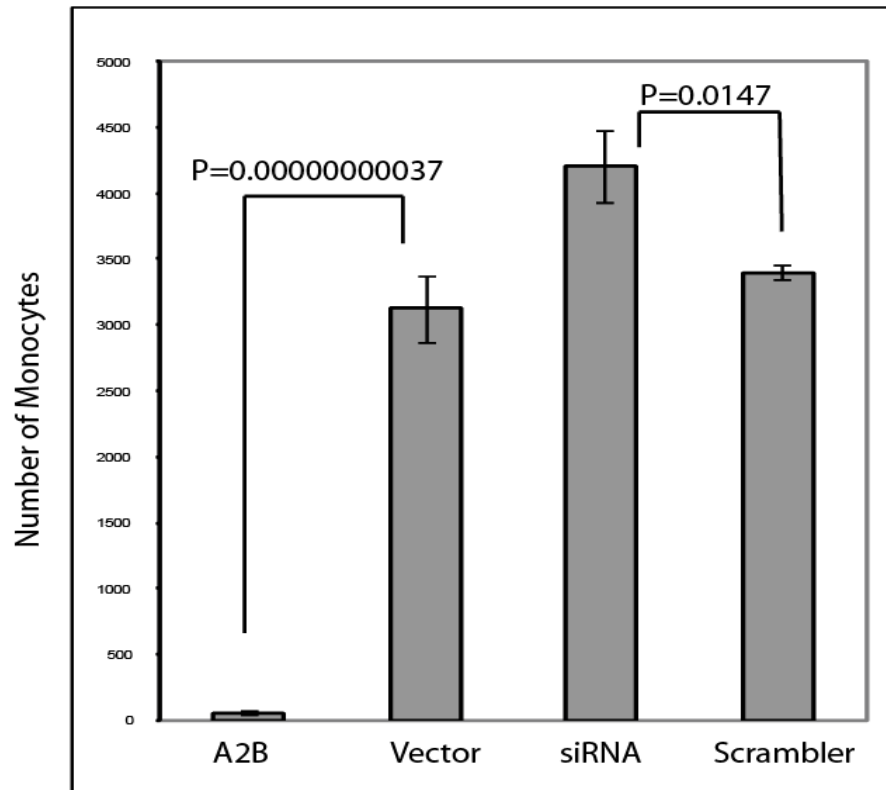
Fig. 6.   Effect of over-expression or knockdown of A2B expression in ECs on transendothelial monocyte migration. A2B was over-expressed in ECs which were exposed to oxLDL.   The over-expression of A2B in EC significantly reduced transmigration of monocytes across the EC layer (compare TES to vector). Knockdown of A2B by siRNA increased transmigration of monocytes across the EC layer (compare siRNA to scrambler). The result indicate that A2B plays a significant role in the migration of monocytes across the EC layer.

5.4 Discussion

Since the endothelial cells (ECs) are the initial site generating disease, the current study investigated the genetic profile of the ECs that were harvested from IMA compared to coronary arteries to identify genes that may account for the IMAs lack of atherosclerosis. The internal mammary artery (IMA) is the vessel of choice for coronary artery bypass grafting because of its resistance to developing atherosclerosis. This has been attributed, in part, to its superior endothelial function (10).

The rational for these studies began when we did immunostains on cross-sections of the IMAs and coronary arteries with CD64, a marker for macrophages. There was a dramatic difference in the preponderance of inflammatory cells attached to and transmigrating passed the ECs in the coronary artery compared to the IMA. Furthermore, VCAM-1 and ICAM-1, cell adhesion molecules (CAM) expressed in activated ECs which bind monocytes, were also much higher in the ECs of the coronary than IMA. Subsequently, when we exposed ECs derived from these arteries to ox-LDL, the ECs derived from the human coronary artery dramatically up-regulated the CAMs and bound more monocytes compared to the IMA. This suggested that different genetic mechanisms are active in the ECs of these two arteries. It also reinforces the fact that the coronary artery has an inherent propensity to progress toward disease.

Using microarray technology, we identified the most statistically significant gene differentially expressed in the IMA vs Cor ECs: the adenosine A_{2B} receptor. It was up-regulated in the IMA by 1.8-fold. Consequently, we showed that the over-expression of the A_{2B} receptor in the ECs derived from the coronary artery down-regulated monocyte adhesion and cell adhesion molecules, the migration of ECs, and blunted the

transendothelial migration of monocytes—processes relevant to the genesis of atherosclerosis, while knock out of this gene promoted these processes.

The signaling mechanisms that are mediated by the Adenosine A_{2B} Receptor have not been fully elucidated. The Adenosine A_{2B} Receptor is coupled to G_s proteins and when activated can up-regulate cAMP production (11). Increases in cAMP levels have been associated with the inhibition of cellular functions such as proliferation, DNA, protein and collagen synthesis (11). Similarly, Adenosine A_{2B} Receptor activation can also increase intracellular levels of calcium that can lead to activation of other signaling pathways (11). Finally, A_{2B} knockout mice was associated with increased levels of inflammatory cytokines and chemokines and it was shown to be mediated through NF-kappaB and p38 activation (12). The effects of the Adenosine A_{2B} Receptor may be mediated by the simultaneous activation of different signal transduction pathways. It appears to play an athero-protective role in the IMA and is naturally over-expressed compared to the coronary artery.

The genetic profile of a subset of genes noted by expression profiling also portray the ECs derived from the IMA as atherosclerotic-resistant. For instance, we found the ECs in the IMA have genes that are up-regulated and linked with a resistance to thrombus formation: plasminogen activator (13), trypsin inhibitor (14), activated leukocyte inhibitor (which may contribute to the significant down regulation of the CAMs in the IMA ECs) (15), tissue factor inhibitor (16), heparin cofactor II (17), as well as provide protection against oxidative stress such as heme oxygenase-1 (18), and TNF-alpha-induced protein-1 (19). It is interesting to point out that the IMA expresses many genes previously associated with a resistance to atherosclerosis has them naturally expressed.

Thus, microarray analysis of the IMA vs. LAD is an effective approach to identify novel protective or susceptibility genes involved in atherosclerosis. Employment of this novel strategy identified the A_{2B} receptor which is associated with a resistance to atherosclerosis in this study.

The limitations of this study are that the definitive mechanism of action of the A_{2B} receptor has yet to be completely elucidated. We have defined the function of the A_{2B} receptor on basic, functional processes related to atherosclerosis. A current review of the literature shows that the molecular pathways that are activated by this receptor are defined pharmacologically with several agonists and antagonists (3). And, multiple pathways are involved demonstrating the global impact this receptor may have on many biological systems. In the future, we will need to utilize the A_{2B} deficient mice to define the specific genetic alterations.

In conclusion, we documented the genetic profile of the ECs derived from the athero-resistant IMA which contrasts with those of the athero-prone coronary artery. We have shown that the gene expression differences are the key to define the IMA and coronary artery's tendencies to develop atherosclerosis, which may be mediated, in part, to the adenosine A_{2B} receptor. These results provide insights as to why the IMA is resistant to the progression of atherosclerosis and will help us understand the mechanisms, which may prevent such life-threatening disease in the coronary artery.

CHAPTER 6

DISCUSSION OF THESIS

Coronary artery disease (CAD) remains the leading cause of death not only in the United States, but world-wide (1). The statistics and unfortunate facts about CAD are often the introductory statements made by most original research articles that are investigating some aspect of atherosclerosis. Despite the identification of its risk factors, the aggressive medical management, the surgical options, and the recommendations by physicians to patients to alter specific lifestyle behaviors (i.e. quit smoking, lose weight, consume a low cholesterol/fat diet and exercise more), the incidence of CAD is remains at an all time high. Consequently, more research is needed in primary prevention.

The hope to alter the morbidity and mortality and development of CAD in this generation may reside in genetics (2). Atherosclerosis may be the "effect" directly "caused" by specific genes; and we need to target those causes. The new approach to prevent CAD in this decade is to identify genetic mutations or single nucleotide polymorphisms (SNPs) and categorize them as an "at high risk for CAD genotype". Consequently, we can identify and target these genes and/or their protein products early-on to interrupt the molecular mechanisms that lead to disease and hopefully well before

one develops it. Additionally, physicians need to continue to reinforce risk factor modification.

In our research, we started with the general hypothesis that there are novel genes yet to be identified that are not only associated with coronary artery disease/atherosclerosis, but can cause this disease. We started our genetic studies directly with the target organs: human coronary arteries. The arteries came from patients who had such advanced CAD (i.e. ischemic cardiomyopathy) that the only treatment option available was a heart transplant. There were no further options for by-pass grafting, stenting, or to even increase medical management. In fact, one can assume that not only were these patients failing medical management over their lifetime, but they had a dominant underlying genetic basis influencing their medical response to treatment. The final option: to get an entirely new heart and coronary arteries.

We performed expression profiling on the human coronary arteries with and without disease in an effort to study the differences in gene expression (3). The hearts with severe coronary artery disease came from patients with a diagnosis of ischemic cardiomyopathy, while the non-diseased hearts came from patients whose 'normal' hearts were ultimately rejected for donation at Life Banc usually because a suitable recipient was not available. This provided us with the opportunity to analyze the genetic differences between age-matched patients who had advanced disease compared to no detectable disease.

Using expression profiling with the "microarray" or "genechip" from Affymetrix, we found 56 genes were differentially expressed between patients with and without CAD

(3). Consequently, we performed a literature search on every gene to document what was not only known about it, but how its protein product may have contributed to atherosclerosis. In this setting, we also identified novel genes as future prospects to study. We categorized these genes into five categories relevant to the genesis of atherosclerosis. These included: 1) inflammation, 2) cell necrosis/apoptosis/proliferation, 3) cell migration/adhesion and matrix degradation, 4) lipid transfer/oxidation/metabolism, and genes with 5) unspecified functions.

This list of 56 genes provided the scientific community with the opportunity to see for the first time, the genes expressed not only in the human coronary artery but also to view these genes in the context of advanced coronary artery disease. In addition, we utilized an intact, coronary artery which showed gene expression not only in situ but also in the context of multiple other genes and cell types as well as in the setting of all the risk factors that impact a coronary artery. This is quite a contrast to other studies which exclusively study endothelial, smooth muscle, or inflammatory cells in vitro (4).

From our final list of 56 genes, three genes demonstrate the usefulness of studying an intact artery by expression profiling. These genes include: 1) the immunoglobulin superfamily, 2) lumican, and 3) chiotriosidase. For instance, this study demonstrated that the immune system may make a significant contribution to atherosclerosis. This was demonstrated by the fact that there was a large representative of genes that code for the immunoglobins and infiltrated the arterial tissue. These finding lend support to the observation that bacteria, such as Chlamydia pneumonia, may be an underlying factor in the genesis of CAD (5). Consequently, this finding will focus more attention as to how to prevent CAD in the setting infections or blunting an arterial immune response.

Lumican is a gene that was reported to be overexpressed during the wound-healing process in the cornea of the eye (6). The identification of this gene with our approach of using intact coronary artery re-defined the role of a gene that may not have been studied outside the field of optholomology. Subsequently since our publication, lumican has been found to be expressed by intimal and medial VSMCs in coronary atherosclerosis, and it contributes to collagen fibrillogenesis of coronary atherosclerosis (7).

Similarly, chitotriosidase had been simply identified as a marker on inflammatory cells. The chitinase enzyme family hydrolyzes chitin, a structural component found in the cell walls of many living species such as the fungi, nematodes, protozoan parasites, and insects (8). However since our publication, serum chitotriosidase activity has been considered to be a strong inflammatory marker of CAD (9). In sum, our initial study found several novel genes that might not have been related to atherosclerosis, and offered opportunities to identify novel pathways and mechanisms that may lead to disease. At the same time, we also detected genes previously associated with CAD (such as ICAM-1, VCAM-1, and osteopontin) (3). This demonstrates that microarray technology is a valid approach to study the genetics of atherosclerosis.

There are three main limitations of our initial study. These are: 1) extracting intact total mRNA from tissue (which represent gene expression), 2) the use the microarray technology, and 3) statistical analysis. To begin with, we harvested mRNA from the coronary arteries and documented global gene expression. With this approach, one often captures the genes that are significantly up-regulated and may not represent very small amounts of mRNA and/or genes with a low expression—representative of genes that may

make a contribution to regulatory pathways. An alternative method is subtractive hybridization which actually does target genes with a very low expression.

At the same time, mRNA is a very fragile molecule that can degrade within minutes after extraction from tissues or cells. The variations in handling tissues and the method of RNA extraction from samples can result in different levels of gene expression. At this point in time, there is no universal protocol to maximize the efficiency of mRNA extraction. One has to adhere to experimental technique, consistency in the timing and sampling of tissue, and even duplicate experiments using a single reference RNA. These are realistic means by which sources of error can be minimized. Fortunately, we were able to harvest our coronary arteries immediately upon explantation, place them in liquid nitrogen, and store them at -80C until they were used. This approach would minimize mRNA degradation.

At this point in time, a universal, standardized approach to microarray data analysis is problematic. For instance, microarray data is difficult to exchange between institutions because of the lack of standardization in the type of chips used (current there are oligonucleotide arrays, spotted arrays, cDNA arrays), assay protocols, and especially analysis methods. Currently, there are on-going projects to improve the exchange and analysis of data generated. For example, the "Minimum Information About a Microarray Experiment" (MIAME) checklist helps define the level of detail that should exist and is being adopted by many journals as a requirement for the submission of papers incorporating microarray results (10). The MicroArray Quality Control (MAQC) Project is being conducted by the US Food and Drug Administration (FDA) to develop standards and quality control measures which will eventually allow the use of MicroArray data in

drug discovery, clinical practice and regulatory decision-making (11). And, The MGED Society has developed standards for the representation of gene expression experiment results and relevant annotations (12).

Similarly, statistical challenges in data analysis are now taking into account effects of background noise and a standard normalization of the data. These includes: Image analysis, data processing and background subtraction (based on global or local background noise), determination of spot intensities and intensity ratios, visualisation of data and log-transformation of ratios, global or local normalization of intensity ratios. And finally, which method to detect significant changes: t-test, ANOVA, Bayesian method Mann–Whitney test methods should be tailored to microarray data sets. This takes into account multiple comparisons or cluster analyses. These methods assess statistical power based on the variation present in the data and the number of experimental replicates and can help minimize Type I and type II errors in the analyses. Unfortunately, the same gene chip raw data can still generate different final gene lists based on how one analyzes the data.

In our research to find and study novel genes associated with atherosclerosis, we also studied CAD from two different perspectives: 1) proteomics and/or protein expression and 2) examining the genetic profile of the internal mammary artery (IMA), which by contrast to the coronary artery, is resistant to disease. Although the main details of the proteomics approach are not included in this research, the summary of our findings is. Both of these approaches contributed to novel molecular mechanisms to identify genes associated with or contribute to CAD.

Using proteomics, one documents the differential expression of proteins which separates proteins based on their molecular weight and the pI (isoelectric point) (13). It provides yet another perspective on the molecular pathways involved in CAD and complements genomics. In this next step of our research, we compared the proteins extracted from diseased and non-diseased coronary arteries. The same coronary arteries that were utilized with the initial study to identify novel genes were also utilized for proteonomics. This was the natural next step to investigate after documenting the gene expression profile.

Proteomics has several advantages. First, the fold changes in the level of gene transcription may give only an estimate of its level of translation into a protein. One naturally makes the assumption that if a gene is up-regulated, then its protein product is as well. Yet, the mRNA that is transcribed may be degraded rapidly or not even translated into a functional protein product. Consequently, a gene may be transcribed into mRNA, but this does not guarantee a protein product. Second, many proteins experience post-translational modifications that profoundly affect their activities; for example some proteins are not active until they become phosphorylated. Third, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Fourth, many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules. Finally, protein degradation rate plays an important role in protein content and offers clues to molecular pathways that may be important. In sum, the main goal of our study was to identify proteins associated with CAD and generate new hypotheses and novel treatment strategies.

Like our initial study in which we utilized human coronary arteries, our second project was the first application of proteomics to study CAD. Using this technique, the proteins are extracted from the tissue, run in a first dimensional gel to separate the proteins based on their isoelectric point, then transferred to a second gel, a SDS-PAGE gel and separated based on their molecular weight. One compared the protein spots between two groups of samples to detect a differentially expressed gene, it is cut out, and sent to mass spectrography for identification. In our case, we found the ferritin light chain protein was significantly up-regulated in diseased coronary arteries. Ferritin is a major intracellular iron storage protein. It is composed of 24 subunits of the heavy and light ferritin chains, and its subunit composition affects the rate of iron uptake and release in different tissues. Additionally, ferritin stores iron in a soluble and nontoxic state. Defects in the light chains ferritin have been associated with several neurodegenerative diseases and hyperferritinemia-cataract syndrome (13).

We hypothesized that the increased expression of the ferritin light chain may contribute to the pathogenesis of atherosclerosis by modulating the oxidation of lipids within the vessel wall through the generation of reactive oxygen species (13). However, this increased expression in diseased coronary may be a consequence of rather than a cause of CAD. And it is interesting to point out that despite the mRNA of the ferritin light chain being lower in CAD patients, the expression of the corresponding translated protein was higher when compared to patients without coronary artery disease. As mentioned, this important finding shows the importance of not only looking at the genetic profile but also including the protein expression as well.

It would have been important to identify many more protein spots on the 2D gels that were differentially expressed between diseased and non-diseased coronary arteries, but the cost of Mass Spectrometry and protein sequencing is very high. Nevertheless, our study did document the global protein expression in human coronary arteries.

To continue to study the underlying genetic mechanisms that lead to CAD, we returned to expression profiling but from yet another perspective. Rather than compare coronary arteries with and without disease, we compared non-diseased coronary arteries to the internal mammary artery (IMA). The rational for this investigation was due to the fact that the IMA is resistant to atherosclerosis, the main explanation for which remains unknown. This is quite a contrast to the atherosclerotic-prone coronary artery.

We hypothesized that not only will the genetic profile of the IMA identify underlying molecular mechanisms that account for its resistance to atherosclerosis, but also reveal the processes that become dysregulated in the coronary artery. Of note, the IMA is the preferred vessel to be used in coronary artery by-pass grafting because of its low incidence of disease. Using expression profiling, we found 29 genes differentially expressed between non-diseased coronary arteries and the IMA. This was once again the first large-scale expression profile done to compare these arteries using human samples.

We found a subset of genes that reflect relevant underlying physiological processes that resist inflammation and can offer a credible hypothesis as to why the IMA resists disease as well as novel genes to study. The inflammatory process of atherosclerosis is initiated when cholesterol-containing oxidized LDL accumulate in the intima and induce the expression of cell adhesion molecules (CAM) on the surface of the

161

endothelium.   These CAM permit the attachment of monocyte and subsequently transmigration into the media of the artery. This is the first strategic location that initiates the events generating CAD. It is also known as endothelial dysfunction.

In the IMA, there was a significant difference in three genes specifically expressed in the endothelial cells: *PECAM-1, TESS-2,* and *RGS-5.* A brief discussion of the clinical, physiological relevance of these genes demonstrates why the IMA may not get disease while the coronary artery readily does. *PECAM-1* was down-regulated by 3-fold in the IMA.   This gene mediates the attachment of monocytes to the endothelium. Several groups have hypothesized that leukocytes utilize *PECAM-1* during the process of transmigration into the artery (16).When *PECAM-1* is blocked, the consequences are reduced myocardial infarct size due to decreased neutrophil accumulation in the myocardium (16). *RGS-5* was up-regulated 5.9-fold in the IMA. This gene is a negative modulator of the AT1 receptor (17). Based on experimental and clinical studies, *RGS-5* enhances   endothelium-dependent   relaxation   in   atherosclerosis   and   promotes vasodilatation (17). With the up-regulation of *RGS-5,* the IMA has an inherent mechanism to promote vasodilation and resist endothelial dysfunction. *RGS-5* is also a marker for VSMC during angiogenesis, stabilizes vessels, promotes endothelial cell survival,   and   inhibits   EC   proliferation--processes   which   are   dysfunctional   in atherosclerosis. It is also down-regulated in atherosclerotic caps (18). And finally, the novel gene *TESS-2* was up-regulated 4.3 fold in the IMA. *TESS-2* plays a role in cell-cell junctions (19,20).   Initially discovered in the testes, it forms a tight-barrier and maintains structural integrity of endothelial cells. Steady-state *TESS-2* mRNA correlates with an extensive renewal of cell-cell junctions during development (21).   With *TESS-2* up-

162

regulated in ECs, the IMA has yet another defense mechanism to resist inflammation by retarding the transmigration of monocytes.

A second strategic location that is altered in the generation of CAD is the smooth muscle cells (SMCs). It is well established that the cytokines that are released by the monocytes that have transmigrated into the media of the artery through the endothelial cells layer induce the SMCs to migrate and proliferate especially into area of plaque (22). The IMA had four up-regulated genes expressed in SMCs that have already been associated as markers for athero-resistance. Four genes encoding *ATF-3*, *TR3 orphan receptor, HSP-70* and *Nurr77* are expressed in SMCs and may contribute a resistance to atherosclerotic. These genes prevent excessive proliferation of VSMCs. The *TR3 orphan receptor* has been shown to reduce atherosclerotic vascular lesion formation (23). The proposed mechanism of action of the *TR3 orphan receptor* is that it inhibits the cell cycle by up-regulating the synthesis of the cyclin-dependent kinase inhibitor p27 (Kip1) in VSMCs (23). *ATF-3* functions as a stress-inducible transcriptional repressor and has been described to meet the criteria of an anti-atherosclerotic gene (24). Its expression is restricted to areas subjected to laminar flow (24). *TINUR* prevents apoptosis in VSMCs (25). And, while also expressed in inflammatory cells, it has been shown to reduce human macrophage lipid loading and inflammatory responses (25). Similarly, *HSP-70* was up-regulated greater than seven-fold, which reflects an inherent mechanism of action in the IMA to prevent ischemic injury (26). Even though both arteries share the same hemodynamic pressures and exposure to the risk factors for atherosclerosis (i.e. hyperlipidemia, hyperglycemia, etc), our study clearly demonstrates that at the genetic

163

level, we identified that the IMA has inherent genetic mechanism to resist the insults that lead to disease in the coronary artery.

Finally, one more strategic location plays a role in the generation of atherosclerosis are the inflammatory cells (such as monocytes). Coronary artery disease is considered an inflammatory process initiated by the infiltration of monocytes into the artery (22). We found that the IMA had three significantly down-regulated genes compared to the coronary artery that are expressed on inflammatory cells. Whether the certain subpopulations of monocytes are native to an artery (known as tissue histiocytes) or transmigrate from the blood stream has yet to identified. In any event, the lack of inflammatory cells in the IMA demonstrates a decreased inflammatory response-to-injury compared to the coronary artery.

The three genes: *CD163 antigen, Factor XIIIA*, and *PLA2,* are specifically expressed in macrophages. *CD163 antigen* is a 130 kDa transmembrane glycoprotein restricted to human monocytes and macrophages and has been found in "chronic" inflammatory diseases (27). It has been reported that anti-CD163 antibodies induce the secretion of pro-inflammatory cytokines in macrophages and promote monocyte infiltration into tissues by a nonclassical adhesion mechanism (27). *Phospholipase A2 (Group 2A)* is responsible for "amplifying" the inflammatory component of many disease processes including atherosclerosis (28). *Factor XIIIA* is involved in blood coagulation in which it cross-links fibrin molecules in a clot, stabilizing it, and producing "resistance to fibrinolysis" (29).

In spite of the complexity of the arterial tissues used for expression profiling which consists of several cell types (ECs, SCMs, or inflammatory cells), we were able to identify a subset of the genes relevant to a resistance of atherosclerosis and identify their site of expression and quantify protein translation in the IMA compared to the coronary artery. We had confirmed our microarray results with RT-PCR, immunocytochemistry, and WB analysis. Furthermore, we used cell lines of ECs and VSMCs harvested from both the IMA and LAD to study the gene expression. It is not surprising that we were able to incorporate multiple independent experiments which studied individuals genes associated with a resistance to atherosclerosis and found them to be native to the IMA.

Our approach of using intact, human coronary arteries has both its advantages and disadvantages. The advantages are that one documents gene (and protein) expression in the context of how it may be regulated physiologically in contrast to an in vitro study. At the same time, there are many factors that may influence the expression of that gene (and protein) that may not be accounted for in vitro as well. Yet, this approach also poses some limitations. One cannot dissect out one isolated gene and just study its expression. The advantage of an in vitro approach is that one can selectively alter the environmental condition (i.e. add drugs, stressors, etc) to assess its individual impact on the system such as atherosclerosis.

It is also important to point out that we documented only one aspect of the IMA, namely, its genetic profile. This information complements two other areas that may explain the molecular basis for this artery's lack of disease. For example, the IMA may not get atherosclerosis because: 1) based on its anatomy, it is a conduit with steady laminar blood flow which down-regulates the expression of cell adhesion molecules--

processes associated with endothelial dysfunction in contrast to the proximal LAD known to have turbulent flow (30), 2) based on its histology, it contains a unique internal elastic lamina (IEL) that is continuous, lacks perceivable gaps and serves as a barrier to the passage of inflammatory cells into the sub-intima and media of the artery, which is based on several electron microscopic studies of this artery (31), and ultimately 3) the expression of genes at strategic locations, namely the EC and SMCs which reflect specific underlying molecular mechanisms that resist inflammation. Our focus obviously was on the genetic profile.

With further studies on the IMA to define the nature of its tendency not to develop atherosclerosis, we began to focus on the ECs. After all, the first strategic location that is affected in an artery that triggers the cascade of events that lead to disease is the EC. It is here that all the risk factors associated with CAD bombard these cells and alter their gene expression. We hypothesized that there are unique genetic properties of the ECs of the IMA that may account for the lack of disease.

Our initial studies supported this hypothesis. When we did an immunstain on cross sections of both the coronary artery and IMA with CD64, a marker unique to monocytes, we found a preponderance of CD64-labeled monocytes not only bound to the ECs of the coronary artery but, by contrast, a near absence of this marker in the IMA. Additionally, we could see that the CD64-specific monocytes also transmigrated into the media of the coronary artery. As a result, one can conclude that there are unique differences in the ECs of these two arteries that account for the lack of inflammatory cells not only attaching to the ECs but permitting their movement into the media of the artery.

166

Similarly, when we harvested these two groups of ECs and did a monocyte adhesion assay, we found that the ECs derived from the coronary artery had a robust expression not only of cell adhesion molecules, but bound significantly more monocytes. Based on this important observation, we did expression profiling of EC cell lines harvested from these two arteries to define their genetic differences.

We found 95 genes that were differentially between the ECs harvested from both the coronary artery and the IMA. However, a subset of these genes demonstrate the unique properties of the ECs of the IMA to resist atherosclerosis. For instance, we found the ECs in the IMA have genes that are up-regulated and associated with a resistance to thrombus formation. These included: tissue plasminogen activator (32), trypsin inhibitor (33), activated leukocyte inhibitor (which may contribute to the significant down regulation of the CAMs in the IMA ECs) (34), tissue factor inhibitor (35), heparin cofactor II (36), as well as provide protection against oxidative stress such as heme oxygenase-1 (37), and TNF-alpha-induced protein 1 (38). It is interesting to point out that the IMA expresses many genes previously associated with a resistance to atherosclerosis has them naturally expressed. Thus, microarray analysis of the IMA compared to the coronary artery is an effective approach to identify novel protective or susceptibility genes involved in atherosclerosis.

At this point in time, we generated extensive gene lists expressed in the IMA and coronary arteries, and we decided to select a few novel genes to study and determine how they may play a role in atherosclerosis. We chose two novel genes: TES and the A_{2B} receptor. Both of these genes may be associated with a resistance to atherosclerosis.

The human TESTIN (TES) gene was noted to be a putative tumor suppressor gene in the fragile chromosomal region FRA7G at 7q31.1/2 (39). It has been reported to be down-regulated in cancer cell lines primarily by promoter hypermethylation (40). TES does not appear to be an enzyme, rather, it is a protein that mediates cellular functions via three LIM domains that mediate protein-protein interactions.

TES may play a role primarily in altering cell motility. Specifically, TES may interrupt the binding of proteins to the LIM3 domain of Ena/VASP proteins which act as nucleators of actin filament assembly, which generate cytoplasmic protrusions needed for cell migration (41). With TES blocking the access of the LIM3 domain (specifically the FPPP binding site), the assembly of the focal adhesion complex is disrupted and cells cannot efficiently migrate. Furthermore, the clinical implications of this alteration mean that if TES is over-expressed cancer cells, they are less likely to metastasize. However, TES is known to be down-regulated in specific malignancies. A research goal would be to prevent the down-regulation of TES to prevent cancer from metastasizing. Yet, our research has now linked TES with atherosclerosis, and we shall contribute and understanding of the molecular mechanisms of this gene in this area.

At the same time, although the A_{2B} receptor's primary ligand is adenosine which results in termination of superventricular tachycardias and causes vasodilation (42). The biological mechanism(s) underlying the functional properties of the A_{2B} receptor are not known. However, several studies have made observations. Recent evidence obtained using A_{2B} knockout (KO) mice have shown that these mice had a phenotype associated with atherosclerosis (43). The A_{2B}-KO mice displayed a significant increase in pro-inflammatory cytokines under baseline conditions, and following a challenge with

lipopolysaccharide. At the same time, the A_{2B}-KO mice had up-regulated levels of the adhesion molecules E-selectin, P-selectin, and ICAM-1 in the vasculature with increased leukocyte adhesion (43). Similarly, a femoral artery injury model with A_{2b}-KO mice vs the wild type showed that the A_{2b} receptor prevented vascular lesion formation in the artery that resembles human restenosis after angioplasty (43) It was reported that BAY 60-6583, a highly selective A_{2B} receptor agonist, limited infarct size in rabbit hearts (44). In vivo studies have also shown that vascular permeability and pulmonary inflammation are significantly increased in A_{2B} KO mice subjected to hypoxia (43). These studies support the A_{2B} receptor as an atherosclerotic-resistance gene, but its function is unknown.

Our approach has involved both over expressing these genes in both EC and SMCs as well as silencing them using small interfering RNA (siRNA) technology. We determined a function of these genes using basic, in vitro assays that are applicable to the development of atherosclerosis, namely: a monocyte adhesion assay, cell migration assay, and measured the transmigration of monocyte through an endothelial cell barrier (as done in a Boyden chamber). Because these techniques are clinically relevant, it is important to briefly re-review the rational for these experiments.

Atherosclerosis is initiated when agents such as ox-LDL come into contact with the ECs which line the inner surface of blood vessels, and it induces the expression of cell adhesion molecules and integrins. These molecules captures circulating monocytes to role, attach, and then transmigrate into the media of the artery where they differentiate into mature macrophages. Consequently, they begin to secrete cytokines which not only stimulate and recruit the smooth muscle cells to migrate and proliferate, but induce

further changes in the ECs to perpetuate the cycle. Each step of the process can be studied in vitro, and this is how we applied them to our genes of interest.

Initially, we did a monocyte adhesion assay. It was performed with ECs over-expressing or silencing either TES or the Adenosine A_{2B} receptor genes. The ECs were plated in a six-well tissue culture well, exposed to ox-LDL added to the media which induces the expression of cell adhesion molecules (CAM) to which monocytes can attach, and then adding caseinAM-labeled monocytes to quantitate the monocytes that attached.

We found that the over-expression of both TES and the Adenosine A_{2B} receptor both blunted the attachment of monocytes to the ox-LDL stimulated ECs as compared to the control group which included the empty plasmid vector. Additionally, the application of siRNA to these genes (and the scrambler) also disrupted the native expression of these genes and increased the binding of monocytes. Based on these in vitro results, it is plausible to make the hypothesis that these genes may behave similarly in situ and have this anti-atherosclerotic phenotype.

These genes also had anti-atherosclerotic properties in both ECs and VSMCs when we assayed for cell migration. Although the role of migrating ECs is not as pertinent to atherosclerosis as VSMCs, these genes blunted the migration of the cells over a 24-48 hour window. The cells which had the over-expression of these genes migrated significantly less compared to the empty vector. Interestingly, the interference of these genes with siRNA technology increased cell migration. As previously mentioned, it is quite plausible to make the hypothesis that the genes are anti-atherosclerotic.

This is further supported by the fact that when these genes were over-expressed in ECs and plated in a Boyden chamber to measure the number of monocytes that would transmigrate across the barrier into the bottom well, both TES and the Adenosine A_{2B} receptor both decreased the number of cells. This further supports a role for preventing the evolution of atherosclerosis based on the evidence of these simple, straightforward in vitro assays.

All of our current research to identify novel genes associated with CAD may culminate with a long-term investment in the gene TES. Future studies will be needed to investigate how this genes work with the continued use of genetics. We propose to: 1) utilize a knockout mouse, 2) continue basic genetic experiments, 3) perform mutational analysis, 4) identify more protein-protein interactions, and 5) not how TES may affect cell trafficking.

A Testin knock-out mouse has already been created (45). To test whether Testin was a tumor suppressor in vivo, the investigators assessed its role in a well established gastric carcinogenic model. To summarize, in mice a zinc deficient diet enhances cellular proliferation in parts of the stomach and makes them susceptible to N-nitrosomethylbenzylamine (NMBA) induced carcinogenesis. In the Testin KO group, the administration of this protocol results in significantly more gastric tumors, which supports Testin as a tumor suppressor in vivo.

Our research has now applied this gene to the generation of arterial disease, namely, atherosclerosis. We have shown that TES blunted monocyte adhesion, decreased cell motility and migration as well as decreased the transmigration of monocyte across an

EC layer. We would utilize the KO model and demonstrate/hypothesize that TES is important to maintain the vascular permeability of arteries. By administering methylene blue to the KO animals and subsequently sacrificing them, we would measure the leak of this dye into the various organs of the mouse model. A very high retrieval of the blue dye from the various organs of the KO model compared to wild type would demonstrate that TES was an important component to maintain the permeability of the artery against molecules such as monocytes. In sum, we propose that TES is necessary to maintain the integrity of the artery.

We will continue basic genetic experiments to define the molecular mechanisms of TES specifically by defining the critical domains. To review, TES consists of a cysteine rich domain (amino acid residues 1 - 90 – with no known homologies), two PET domains (90 – 200 and linker 201 – 233), and three LIM domains (234 – 300, 300 - 365 and, 366 - 421). We propose to create a series of constructs deleting three amino acid residues at a time from the C-terminal end of the gene to the N-terminal. Consequently, we would assess the impact of these constructs of the atherosclerotic assays previously described to identity which domain is critical for the function of TES.

TES has been found to be downregulated in disease states such as cancer. This is the result of the hypermethylation of its promoter (45). We propose to perform mutational analysis by creating a series of point mutation in the TES promoter and assess their impact on the expression of TES. This would demonstrate the impact and control the promoter may have on disease processes such as cancer.

Basic genetic techniques would also be important to incorporate into the clinical significant of TES. Using single stranded conformational polymorphism (SSCP) on patients with and without coronary artery disease would lead to the identification of novel SNP associated with the disease. Consequently, this will create these SNPs in plasmids and attempt to pharmacologically correct the expression of TES (if down-regulated) to improve the disease process.

Since TES has been shown to be involved with several proteins, we could demonstrate if several of these proteins (i.e. paxillin, zyxin) are affected which influence cell migration. Furthermore, more yeast-two hybrid experiments would be indicated to identify other structural proteins that TES may alter or interact with and affect cellular function.

In the future and in conclusion, I hope to continue to make progress and expand the knowledge in gene expression in the field of cardiovascular disease.   Taking a retrospective look at the extensive lists of genes that I have generated, I have many opportunities to pursue and define novel mechanisms that may lead to CAD. The field of genetics is the ultimate field of primary care prevention in internal medicine and offers one the opportunity to screen patients in advance of the development of CAD to help reduce its ever growing morbidity and mortality.

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CHAPTER 2

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APPENDIX

CHAPTER 4. SUPPLEMENTAL REFERENCES

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