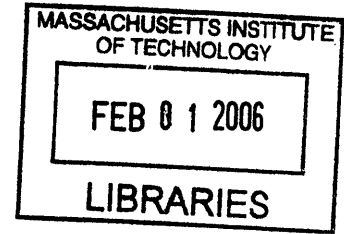# Genetic approaches to studying coronary heart disease in SR-BI/apoE double knockout mice

by

Sharon Liz Karackattu

B.S. Interdisciplinary Studies, Biochemistry and Molecular Biology
University of Florida, 2000

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy
at the
Massachusetts Institute of Technology

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Genetic approaches to studying coronary heart disease in SR-BI/apoE double knockout mice

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Abstract

Coronary heart disease (CHD) is a major healthcare concern in both developed and developing nations. The principal cause of CHD is atherosclerosis, the buildup of fatty deposits in vessel walls. SR-BI/apoE double knockout (dKO) mice are a novel mouse model of human CHD, exhibiting spontaneous coronary occlusive atherosclerosis, myocardial infarction, cardiac dysfunction and premature death. This model represents a unique system in which to study the two principal underlying mechanisms of atherosclerosis and CHD, inflammation and lipoprotein lipid metabolism. The first part of this thesis demonstrates that B- and T-lymphocytes do not play a significant role in development or progression of atherosclerosis or CHD in SR-BI/apoE dKO mice. More importantly, this study eliminates B- and T-lymphocyte-driven immunoglobulin-mediated inflammation as a significant mechanism instigating or exacerbating myocardial injury in dKO mice. Strikingly, the mice generated for this study also demonstrate that genetic background influences both average lifespan and lifespan variability of dKO mice. The second study investigates alterations in lipoprotein metabolism on CHD and demonstrates that hepatic lipase deficiency, despite raising plasma cholesterol levels, retards development of both aortic and coronary occlusive atherosclerosis in dKO mice. This reduction in atherosclerosis is associated with delayed onset and/or progression of hypertrophy, myocardial infarction and cardiac dysfunction as well as a 37% extension in lifespan. Both of these studies, along with others, suggest SR-BI/apoE dKO mice experience atherosclerotic coronary-occlusion-induced ischemic heart disease and that they may be a valuable tool in which to investigate the etiology of as well as influences of genetic, environmental and pharmacologic manipulations on CHD.

Thesis Supervisor: Monty Krieger

Title: Professor of Biology

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A. Yesilaltay, M. Gabriela Morales, L. Amigo, S. Zanlungo, A. Rigotti, S. L. Karackattu, M. H. Donahee, K. F. Kozarsky, and M. Krieger. Effects of hepatic expression of the HDL-receptor SR-BI on lipoprotein metabolism and female fertility. *Endocrinology* 2006. In Press

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Marcus JS, Karackattu SL, Fleegal MA, Sumners C. Cytokine-stimulated inducible nitric oxide synthase expression in astroglia: role of Erk mitogen-activated protein kinase and NF-kappaB. Glia. 2003 Jan 15;41(2):152-60.

Acknowledgements

At first I thought I would save the writing of Acknowledgements until the end of the dissertation writing process, a sort of fun denouement to the culmination of five years of work. However, experiencing bouts of writer's block while trying to put together my Introduction, I decided that getting started on the Acknowledgments would at least be progress.

Going back and identifying all those who made an impact in your life is no easy feat. My life as I know it comes as a series of very serendipitous events. The fortunate turns of fate and the people that were around each corner were instrumental in getting me to this point.

First, I would like to thank Jackie Lees and David Housman for their guidance and taking the time to be members of my thesis committee for the past five years. I would also like to thank Richard Hynes and Denisa Wagner for serving on my thesis defense committee.

I was lucky in that I got a high school education that very few are privileged to receive. I only realized this via my experiences as a teaching assistant, how difficult it is to thoroughly impart knowledge, how much patience, preparation and understanding it takes to be a good teacher. I got a lot of conceptual understanding in high school that most people don't even get in university or graduate school. To this day I carry the knowledge imparted to me by a slew of fantastic educators. To Mr. Youngman, Mr. Dalsass, Mr. Williams, Mr. Davis, Mr. Perry, Ms. Halcomb, Ms. Tate, Mrs. Schillit and Mrs. Timberlake: Thank you for TEACHING me the sciences and math and to read, write and think critically.

In college, I very fortunately fell under the mentorship of Dr. Colin Sumners who took me on as a transient undergraduate researcher despite the very small contribution that I would likely make to his lab. He personally taught me to pipet and without his support and influence, I doubt that I would be at MIT today.

I am also blessed with a group of friends that I will carry through life. They have stuck by me through college and graduate school, have been there for all of the ups and downs and important times in my life and will continue to be there throughout. They are the ones whom I meet up with at least once a year, the ones my children will call Uncle and Auntie and grow up playing with their kids. So here I will thank Krithi and Dennis, Aparna and Sapna.

Auspiciously enough, I ended up in a lab that was a family away from home with labmates that were close friends in addition to colleagues. So I want to thank Songwen, Ayce,

Sara F. and Sara V., Shangzhe, Yu, Sotiri, Marsha, Kathy, Thomas, Kaarin, Hussein and Eliza for all of the tea room chats, the scientific advice and teaching me the protocols I needed help with and then some. They made the experience of graduate school about more than just the daily grind at the bench.

My PI, Monty Krieger, deserves special thanks. I have never met anyone like him and I find his energy, dedication and caring truly astounding. At one point, I came to the conclusion that he must not sleep more than two hours a day. In addition to being a brilliant scientist, a great teacher and an amazing mentor, he also knows how to have a good time: cutting up the dance floor at my wedding, playing ping pong with us at the holiday party and just having lunch in the tea room with us when his busy schedule allowed. Monty was much more than just a PI, as even my other friends noted, and I am lucky to have spent my graduate years as a member of his laboratory.

What can I say about my husband Matt that would do him justice? He is my best friend and my confidante. He pushes me to work when I am feeling indolent, and makes me take a break when I've been working too hard. He puts up with all the highs and lows, the crazy moods, the outbursts and the funks. He shares my humors and my goals. He exposes me to new experiences. We supported each other through quals, we survived a year of engagement and wedding planning and by the time we both exit, we will have seen each other through the writing of three theses in total. Of the many important things I leave MIT with, he is number one.

The ultimate thanks go to the people that shaped me before I could walk and talk. My parents and my brother who knew me before the possibility of graduate school at MIT ever existed. They who quizzed me on endless spelling and vocabulary lists, taught me multiplication tables, hauled my piles of library books home, helped with all those dioramas, collages and science projects. So Mom, Dad and Jerry, this thesis and this Sharon would not exist if it weren't for all your love and support.

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List of Abbreviations

ABCA1: ATP-binding cassette A1

ApoA-I: apolipoprotein A-I

ApoE: apolipoprotein E

CE: cholesteryl ester

CETP: cholesteryl ester transfer protein

CHD: coronary heart disease

CPT-1: carnitine palmitoyltransferase 1

CR: chylomicron remnant

CSPG: cell surface proteoglycan

CVD: cardiovascular disease

dKO: double knockout

ECG or EKG: electrocardiogram

ECM: extracellular matrix

Epo: erythropoietin

FH: familial hypercholesterolemia

FPLC: fast protein liquid chromatography

HDL: high-density lipoprotein

HDL-C: HDL cholesterol

HDL-CE: HDL cholesteryl ester

HL: hepatic lipase

HMG-CoA reductase: hydroxymethylglutaryl coenzyme A reductase

HSPG: heparan sulfate proteoglycan

IDL: intermediate-density lipoprotein

IFN-γ: interferon gamma

IGF-1: Insulin-like growth factor-1

IP-10: interferon-inducible protein-10

I-TAC: interferon-inducible T-cell α chemoattractant

KO: knockout

LCAT: lecithin:cholesterol acyltransferase

LDL: low-density lipoprotein

LDL-C: LDL-cholesterol

LDLR: low-density lipoprotein receptor

LPL: lipoprotein lipase

LRP: LDL-receptor related protein

LV: left ventricle or left ventricular

LVEDV: left ventricular end diastolic volume

LVESV: left ventricular end systolic volume

NFAT: nuclear factor of activated T-cells

NHLBI: National Heart, Lung and Blood Institute

NK: natural killer (cell)

NPC1L1: Niemann-Pick C1 Like 1

MCP-1: macrophage chemoattractant protein-1

MI: myocardial infarction

MIG: monokine induced by interferon gamma

Ox-LDL: oxidized LDL

PDGF: platelet-derived growth factor

PLTP: phospholipid transfer protein

PPAR: peroxisome proliferator-activated receptor

RAG1 and RAG2: recombinase activating genes 1 and 2

RBC: red blood cell

RCT: reverse cholesterol transport

RV: right ventricle or right ventricular

SCAP: SREBP cleavage activating protein

SMC: smooth muscle cell

SRA: scavenger receptor A

SR-BI: scavenger receptor class B type I

SR-BIatt: Strain of mice expressing attenuated levels of SR-BI as result of a promoter mutation
(Arai, Rinninger *et al.* 1999)

SREBP: steroid regulatory element binding protein

TC: total cholesterol

TG: triglycerides

Tg: transgenic

TGF-β: transforming growth factor beta

tKO: triple knockout

TNF-α: tumor necrosis factor alpha

UC: unesterified cholesterol

VCAM-1: vascular cell adhesion molecule-1

VLA-4: very late antigen-4

VLDL: very low-density lipoprotein

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Chapter One

Introduction

Cardiovascular Disease: Economic and Societal Consequences

Cardiovascular disease (CVD) has been the number one cause of death in the United States every year since 1900, with the exception of 1918. The economic impacts of CVD are staggering, reaching an estimated 393.5 billion dollars in 2005 for expenses in the US alone. Coronary heart disease (CHD) and stroke are the two most common forms of CVD. CHD is the single most common killer of males and females in both Europe and the US, taking 1 out of every 5 lives. Over 50% of people who died suddenly from CHD experienced no previous symptoms of this disease. Current NHLBI estimates place the average number of years of life lost due to a heart attack at 11.5 (Association 2005).

With soaring financial consequences and global mortality rates on the rise, many studies have focused on identifying, understanding and treating the behavioral and physiological risk factors associated with CHD and stroke. The primary cause of both CHD and stroke is atherosclerosis, the buildup of fatty deposits in artery walls that can result in ischemia (obstructed blood flow) and subsequent hypoxia (oxygen-starvation) of tissues. The resultant oxygen-deprivation induced damage to brain tissue is referred to as a stroke whereas such injury to heart muscle is experienced as a heart attack or myocardial infarction (MI).

Development of atherosclerosis is closely linked to cholesterol and lipoprotein metabolism, lipoproteins being the lipid and protein complexes that transport nonpolar lipids through the circulation. The risk for atherosclerosis is directly proportional to levels of plasma total cholesterol (TC) and low-density lipoprotein (LDL; the "bad" cholesterol) and inversely related to high-density lipoprotein (HDL; the "good" cholesterol). As of 2002, over 50% of the American population over the age of 20 had total cholesterol levels exceeding 200 mg/dl, placing them at risk for atherosclerosis, CHD and stroke (Association 2005). Furthermore, over 45% of

Americans have LDL levels higher than 130 mg/dl and 26% exhibit HDL levels lower than 40 mg/dl, both of which are conditions defined as risk factors for CHD. Strikingly, the trend of high cholesterol is now appearing increasingly in children with approximately 10% of adolescents aged 12-19 exhibiting plasma total cholesterol levels above 200 mg/dl (Association 2005).

My thesis projects investigated the roles of lymphocyte-mediated inflammation and hepatic lipase regulated lipoprotein metabolism in SR-BI/apoE double knockout (dKO) mice, a recently characterized mouse model of CHD. This introductory chapter discusses a variety of topics pertinent to lipoprotein metabolism, atherosclerosis, CHD and the experimental models used to study them. To facilitate reading, the introduction has been subdivided into five major sections. The first section of this chapter will focus on cholesterol and lipoproteins with special attention on HDL and LDL, the two most studied lipoproteins with respect to atherosclerosis, their metabolism and receptors. Section two will cover development of atherosclerotic plaques and mouse models used to study mechanisms of atherogenesis. The third section introduces pathophysiological processes underlying CHD as well as animal models of CHD including detailed coverage of the SR-BI/apoE double knockout (dKO) mouse. Sections four and five give background on RAG2-dependent lymphocyte deficiency and hepatic lipase (HL) respectively with regards to atherosclerosis and heart disease, in preparation to discuss their influence on the phenotype of SR-BI/apoE dKO mice.

Section One: Cholesterol and Lipoprotein Metabolism

In the early 1900s, scientists had speculated that lipids in the circulation were in an emulsion or associated with proteins. By mid-century, they had separated lipoproteins (lipid-protein complexes) into two primary fractions with α_1 and β mobilities and noted associations between higher levels of α-lipoprotein and lower incidence of heart disease. John Gofman and

collaborators developed methods to further separate lipoproteins into subclasses and reliably assay their concentrations leading to observations that certain classes of lipoproteins were particularly atherogenic; thus not only was risk for atherosclerosis dependent on total cholesterol levels but also on the relative distribution of various cholesterol-carrying lipoproteins.

Given the close associations between plasma cholesterol and lipoprotein levels to atherosclerosis and CHD, elucidating the physiological, cellular and genetic mechanisms underlying lipid metabolism is critical to understanding the etiology of this disease.

I.   Cholesterol

Cholesterol has the chemical formula $C_{27}H_{45}OH$ and the structural formula shown in Figure 1. Cholesterol is a critical component of cell membranes and regulates membrane fluidity. It is also a precursor to many physiologically relevant molecules including steroid hormones, vitamin D, and bile salts (Figure 1). Cells can obtain cholesterol either through endogenous biosynthetic pathways from mevalonate and isoprenoid precursors or take up exogenous cholesterol from circulatory lipoproteins via cell surface receptors (Rigotti, Miettinen et al. 2003). The rate limiting enzyme in cholesterol biosynthesis is hydroxymethylglutaryl coenzyme A reductase (HMG CoA reductase), the target for statin drugs (Rigotti, Miettinen et al. 2003).

Cholesterol is packaged into lipoproteins in two forms: cholesteryl ester and unesterified cholesterol. Cholesteryl esters are esterified to fatty acids such as oleate and linoleate and found in the nonpolar lipid core. Unesterified cholesterol is found in the lipoprotein outer shell. The chemical form of cholesterol within atherosclerotic plaques has implications for its relative biomechanical strength. Plaques containing mainly cholesteryl ester, which is liquid at body temperature, are more susceptible to rupture whereas lesions containing large amounts of cholesterol monohydrate, which crystallizes, are less prone to fracture and consequent

thrombosis. Lipid-lowering therapies can decrease the incidence of atherosclerotic complication by strengthening plaques via increasing collagen content, lowering tissue factor expression and causing more of the cholesterol in the core of the plaque to accumulate as free cholesterol monohydrate (Libby, Aikawa et al. 2000) (see Section Two).

Cholesterol (animal)

Cholesteryl ester

Bile acid (deoxycholic acid)

Steroid hormone (testosterone)

Vitamin D₃
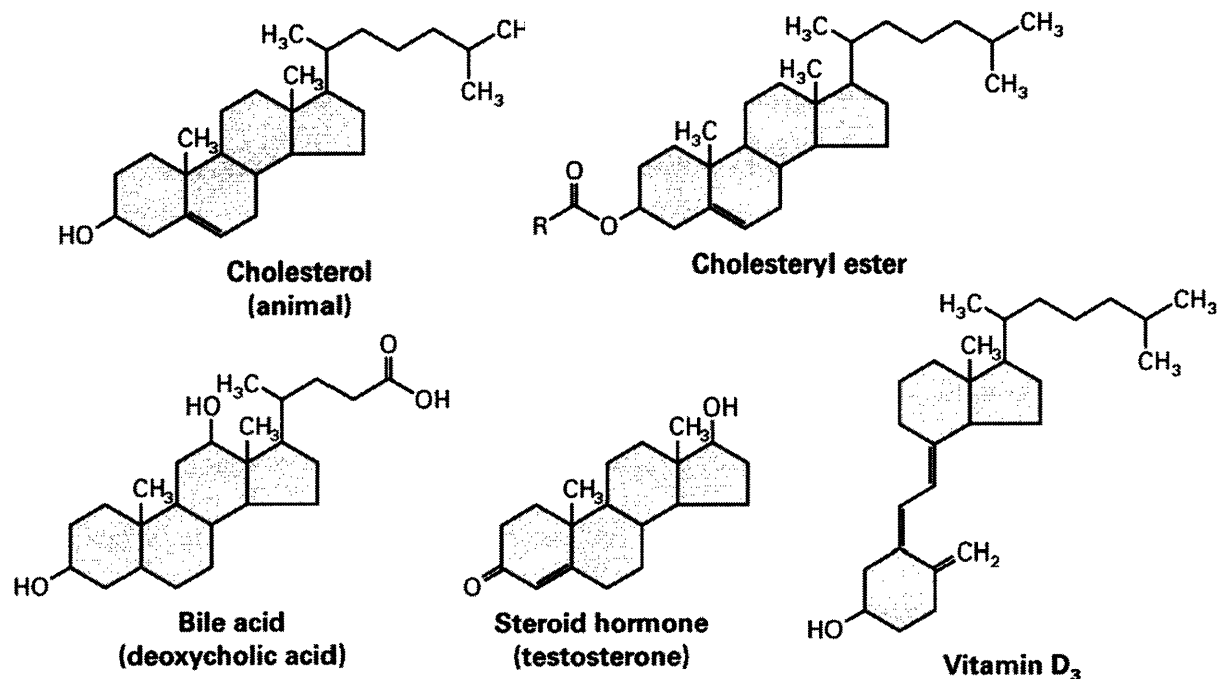
Figure 1: Cholesterol and some physiologically relevant derivatives. From Molecular Cell Biology, 5th Edition, W.H. Freeman & Company (Lodish, Berk et al. 2004).

II. Lipoproteins

Lipoproteins are spherical particles with nonpolar lipids (triglycerides and cholesteryl-esters) sequestered in the core and an outer shell consisting of proteins, phospholipids and unesterified cholesterol. The major classes of lipoproteins are characterized by their unique densities, as determined by their lipid-to-protein ratio, and the specific apolipoprotein molecules on their outer surface (Table 1). Gofman's early data suggested that different classes of lipoproteins were differentially correlated to risk for atherosclerosis and CHD. It was later

confirmed that VLDL and LDL levels were directly associated with risk for atherosclerosis, with

LDL being the most atherogenic of the lipoproteins (Steinberg 2004).

Particle	Diameter (nm)	Density (g/cm³)	Core TG:CE	Major apolipoproteins	Source
Chylo-micron	75-1200	<0.95	97:3	A,B-48,C,E	Intestine
VLDL	30-80	0.95-1.006	75:25	B-100,C,E	Liver
IDL	25-35	1.006-1.019	45:55	B-100,E	VLDL
LDL	18-25	1.019-1.063	12:88	B-100	VLDL
HDL	5-12	1.063-1.21	11:89	A,C	Chylomicrons and VLDL

Table I: Comparison of major lipoproteins

A. LDL

1. LDL structure

LDL is derived from the action of the enzymes hepatic lipase (HL) and cholesteryl ester

transfer protein (CETP) on IDL (Rigotti, Miettinen et al. 2003), and is the major carrier of

cholesterol, as cholesteryl ester, in humans. LDL has a lipid core containing triglycerides and

cholesteryl ester whereas the outer shell is comprised of phospholipids, unesterified cholesterol

and a single molecule of apolipoprotein B-100 (Krieger 1999) (Figure 2).
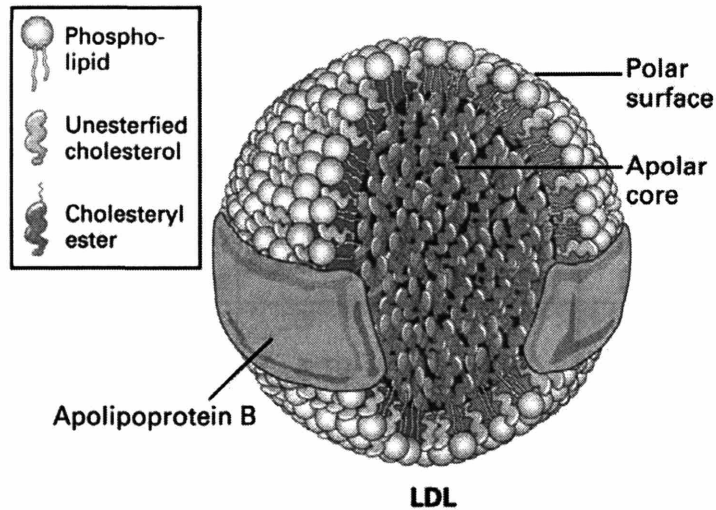
Figure 2: Low-density lipoprotein. From Molecular Cell Biology, 5th Edition, W.H. Freeman & Company (Lodish, Berk *et al.* 2004)

2. Receptor-mediated endocytosis of LDL by LDLR

The receptor-mediated pathway regulating plasma LDL levels has been well-characterized by the work of Brown and Goldstein and has led to statin therapy for hypercholesterolemia. As outlined in Figure 3, whole LDL particles are taken up by LDL receptors (LDLR) on the surface of cells via clathrin-coated pit receptor-mediated endocytosis. LDLR can then be recycled to the cell surface. To release cholesterol, the LDL particles are degraded inside the cell within lysosomal compartments. Expression of LDLR is controlled by a feedback mechanism through the membrane bound transcription factor, sterol regulatory element binding protein (SREBP), which regulates transcription of HMG-CoA reductase, LDLR and other genes involved in cholesterol and fatty acid biosynthesis (Vance and Van den Bosch 2000). SREBP is activated by a protease that is in turn activated by SREBP cleavage activating protein (SCAP). Insufficient cellular cholesterol levels result in activation of SREBP which migrates into the nucleus and promotes transcription of HMG-CoA reductase and LDLR, allowing the cell to synthesize as well as take up more cholesterol.

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HMG CoA reductase

clathrin

LDL receptors

Cholesterol ester

Protein

LDL

Cholesterol

ACAT

Cholesteryl oleate

Amino acids

LDL binding→Endocytosis → Lysosomal hydrolysis→Enzyme regulation
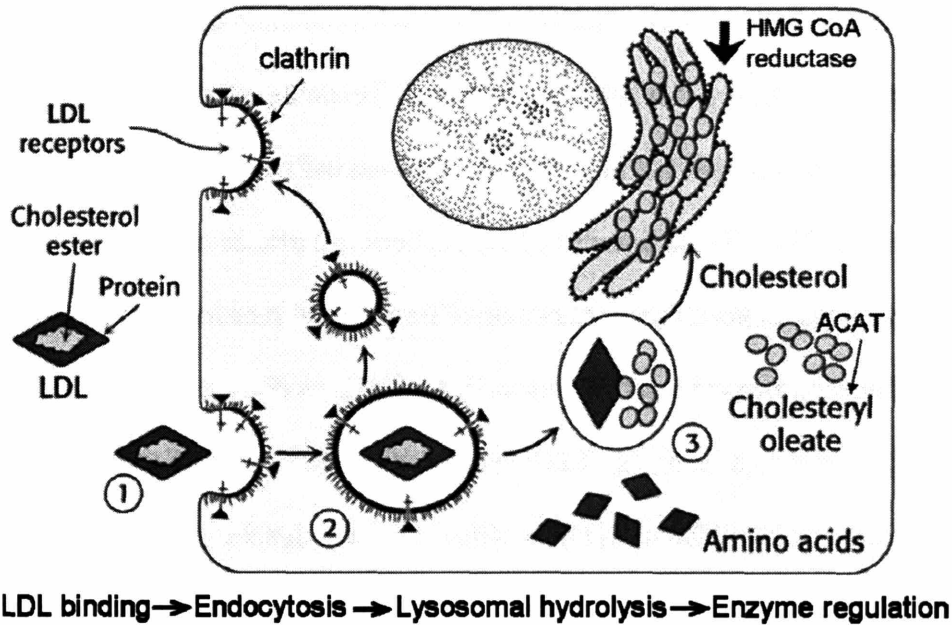
Figure 3: Clathrin-coated pit receptor mediated endocytosis of LDL particles.

Brown and Goldstein discovered the LDL receptor by studying familial hypercholesterolemia (FH), a monogenic inherited disease. Patients with FH lack functional LDL receptors, resulting in the accumulation of high plasma levels of LDL, severe atherosclerosis and death during teenage years (Vance and Van den Bosch 2000). The deduction that cells in the atheromas of FH patients must have alternate mechanisms of uptaking LDL, since they lacked functional LDLR, led to the discovery of scavenger-receptors as described below (Krieger 1999; Steinberg 2005).

B. HDL

1. Structural Classification of HDL

Rather than being a single macromolecular entity, HDLs are a group of lipoprotein particles containing nearly equal amounts of lipid and protein, that have been subclassified based on their apolipoprotein compositions and physical properties (Rigotti, Miettinen et al. 2003). ApoA-I and apoA-II are the two major apolipoproteins in HDL, with smaller amounts of apoA-

IV, apoE, apoC, apoD and others (Krieger 1999). There are three main classes of HDL 1) discoidal particles comprised of phospholipids and apoA-1, existing only transiently in the circulation 2) small, lipid-poor apoA-1/phospholipid particles called pre-β HDL present at low levels in the plasma and 3) α-HDLs, abundant, large, spherical particles with nonpolar lipid cores and two or more apolipoproteins on their outer shells. Based on ultracentrifugation density, the α-HDLs are classified into two main subfractions, HDL_3 ($1.125 < d < 1.21$ g/cm^3) and HDL_2 ($1.063 < d < 1.125$) (Barter, Kastelein et al. 2003) and further divided into five subgroups based on size: HDL_{2b} (mean diameter 10.6 nm), HDL_{2a} (9.2nm), HDL_{3a} (8.4 nm), HDL_{3b} (8.0 nm) and HDL_{3c} (7.6 nm). HDL is also divided into two subpopulations based on their relative contents of apoA-1 and apo-AII (Rigotti, Miettinen et al. 2003). An HDL particle's relative contents of these two apolipoproteins is thought to regulate its affinity for cell surface receptors and lipid transfer properties.

2. Formation of HDL

ApoA-I and apoA-II are secreted by the liver, with a small fraction of apoA-I also being synthesized by the intestine. Once in the plasma, these apolipoproteins accumulate phospholipids and small amounts of unesterified cholesterol from cells and lipoproteins becoming discoidal HDLs. Discoidal HDLs can also be formed from surface components shed by chylomicrons as their triglycerides are hydrolyzed by lipoprotein lipase (LPL).

The transport protein ATP-binding cassette A1 (ABCA1) is essential for the transfer of cellular lipids to nascent HDL particles, contributing to their transformation into spherical particles (Rigotti, Miettinen et al. 2003). ApoA-1-containing HDLs serve as substrates for the enzyme lecithin:cholesterol acyltransferase (LCAT) which esterifies their cholesterol, providing the particle with a core of cholesteryl ester and ultimately converting the discoid into a spherical

HDL (Barter, Kastelein et al. 2003). The esterification of cholesterol also creates a gradient of unesterified cholesterol between the HDL particles and cells, favoring, and possibly critical for, the efficient transfer of unesterified cholesterol from cells to HDL(Rigotti, Miettinen et al. 2003).

3. HDL Metabolism and Removal

Cholesteryl ester transfer protein (CETP) transfers HDL cholesteryl ester (HDL-CE) to other plasma lipoproteins, such as VLDL, IDL and LDL, for additional transport, with most destined for the liver. In species that express CETP, a large portion of HDL-CE is indirectly taken up by hepatic IDL and LDL endocytic receptors (Rigotti, Miettinen et al. 2003). HDL triglycerides and phospholipids are hydrolyzed by HL, LPL, endothelial lipase and secretory phospholipase A_2 whereas dissociated apoA-I can be recycled into mature or nascent HDLs or metabolized and excreted by the kidneys (Barter, Kastelein et al. 2003; Rigotti, Miettinen et al. 2003).

By the late 1990's, the pathways regulating cellular metabolism of LDL were well understood, however relatively little was know about HDL metabolism. In rodents and most likely humans as well, HDL can directly deliver cholesteryl ester to cells in addition to indirect delivery via CETP and other lipoproteins. Endocytic pathways for HDL cholesterol (HDL-C) delivery to cells have been reported (Fidge 1999), however a major mechanism for direct transfer of HDL cholesterol via a scavenger receptor differs fundamentally from the receptor-mediated endocytic pathways described for LDLR, and is discussed in detail below.

III. Scavenger-Receptors

Scavenger receptors are multiligand cell-surface molecules that bind to chemically modified lipoproteins such as oxidized or acetylated LDL. Scavenger receptor activity was first identified in macrophages by the Brown and Goldstein group as they investigated the

mechanisms underlying LDL accumulation in macrophage foam cells of fatty streak atherosclerotic lesions. The characterization of macrophage scavenger receptors, expression of which are not regulated by intracellular cholesterol pools as is LDLR, suggested a mechanism by which LDL modified in the subendothelial space could contribute to the conversion of macrophages into lipid-rich foam cells via unchecked engorgement of lipoproteins (Krieger 1999). Studies demonstrating that disruption of scavenger receptor A (SRA) function in hypercholesterolemic mice reduces atherosclerosis, as well as other in vivo experiments with SRA types I and II and the class B scavenger receptor CD36, have revealed a role for these receptors in atherogenesis (Libby 2000; Rigotti, Miettinen et al. 2003).

There are multiple classes of scavenger receptors for modified LDL as defined by their ligand-binding properties. SR-AI and SR-AII were the first macrophage scavenger receptors identified. Scavenger receptors are grouped into classes based on structural similarity, usually defined by sequence homology, and within a class individual proteins or "types" are named by roman numerals. The different types of scavenger receptors in a class can arise due to alternative RNA splice forms from a single gene as in the case of SR-BI and SR-BII or from distinct genes as in the case of SR-CI and SR-CII (Krieger 1999).

One member of the class B scavenger receptors, CD36, is expressed in monocytes/ macrophages, endothelial and epithelial cells, adipose tissue and various cultured cell lines. CD36 binds a variety of ligands including modified LDL, anionic phospholipids, long chain fatty acids and collagen (Krieger 1999). The physiological roles of CD36 are not clear but may include mediating cell adhesion, fatty acid transport, signal transduction and phagocytosis of damaged neutrophils.

After characterization of CD36, the cDNA for the scavenger receptor SR-BI was isolated

via expression cloning from a Chinese hamster ovary (CHO) cell variant. Subsequently the

murine, rat, human and bovine homologs of SR-BI were cloned. Human SR-BI maps to

chromosome 12 whereas mouse SR-BI maps to chromosome 5. Tissue-specific expression and in

vitro activities of human and mouse SR-BI are similar.

IV. Scavenger Receptor, Class B, Type I (SR-BI)

A. Structure and Function

Originally cloned for its ability to bind modified LDL, scavenger receptor class B type I

(SR-BI) was later characterized as the first physiologically relevant receptor for HDL. SR-BI is a

509 amino acid, 82 kDa fatty-acylated N-glycosylated transmembrane protein, with short

cytoplasmic amino- and carboxy-terminal tails each adjacent to a hydrophobic, putative

membrane spanning domain which together flank a large extracellular loop (Figure 4) (Krieger

1999). SR-BI can bind native LDL with high affinity but does not efficiently mediate

endocytosis of LDL compared to LDLR. Though SR-BI binds modified LDL tightly, it is

inefficient at mediating internalization and degradation of these particles. SR-BI can also bind

VLDL and anionic phospholipids, the latter suggesting a role for SR-BI in recognizing and

clearing apoptotic cells during development, tissue remodeling and disease processes (Rigotti,

Miettinen et al. 2003).

Figure 4: Topology of murine SR-BI. From Krieger 1999, Annual Review of Biochemistry (Krieger 1999).

The mechanism by which SR-BI regulates the cellular metabolism of HDL is not yet fully understood. SR-BI exhibits nonreciprocal cross competition between HDL and LDL, in which HDL can block all LDL binding but LDL poorly inhibits HDL binding. Because of this phenomenon, LDL is not thought to effectively interfere with HDL binding to SR-BI in vivo. Mutational analyses aimed at dissecting sites relevant for the various activities of SR-BI have demonstrated that alteration of specific residues can inhibit HDL association without disrupting LDL binding (Gu, Lawrence et al. 2000).

Large, spherical, low-density HDLs with high lipid content bind well to SR-BI whereas pre-β HDLs are poor substrates (Rigotti, Miettinen et al. 2003). Rather than mediate the internalization and lysosomal degradation of whole lipoprotein particles like LDLR, SR-BI mediates lipid transfer from HDL via *selective uptake* as outlined in Figure 5. Selective lipid uptake involves the binding of HDL (or other native lipoprotein ligand) to SR-BI and the temperature-dependent reversible transfer of lipoprotein core cholesteryl esters, without

endocytosis of outer shell phospholipids or proteins, to the cell membrane succeeded by irreversible internalization and nonlysosomal hydrolysis of the cholesteryl ester molecules. The lipid depleted HDL particle subsequently loses affinity for SR-BI and dissociates into the plasma where it can act as an acceptor for lipids from cells or be catabolyzed by the kidneys (Krieger 1999; Rigotti, Miettinen et al. 2003). From HDL, SR-BI can also mediate the transfer of phospholipids, unesterified cholesterol, triglycerides, α-tocopherol as well as the artificial marker lipids DiI, BODIPY-cholesteryl ester, pyrene-labeled phospholipids and cholesteryl ethers, with more hydrophobic lipids i.e. cholesteryl ester, triglycerides and unesterified cholesterol being transferred more easily (Rigotti, Miettinen et al. 2003). SR-BI can selectively uptake lipids from LDL, however the fraction of transferred lipids is lower than that from HDL (Rigotti, Miettinen et al. 2003).
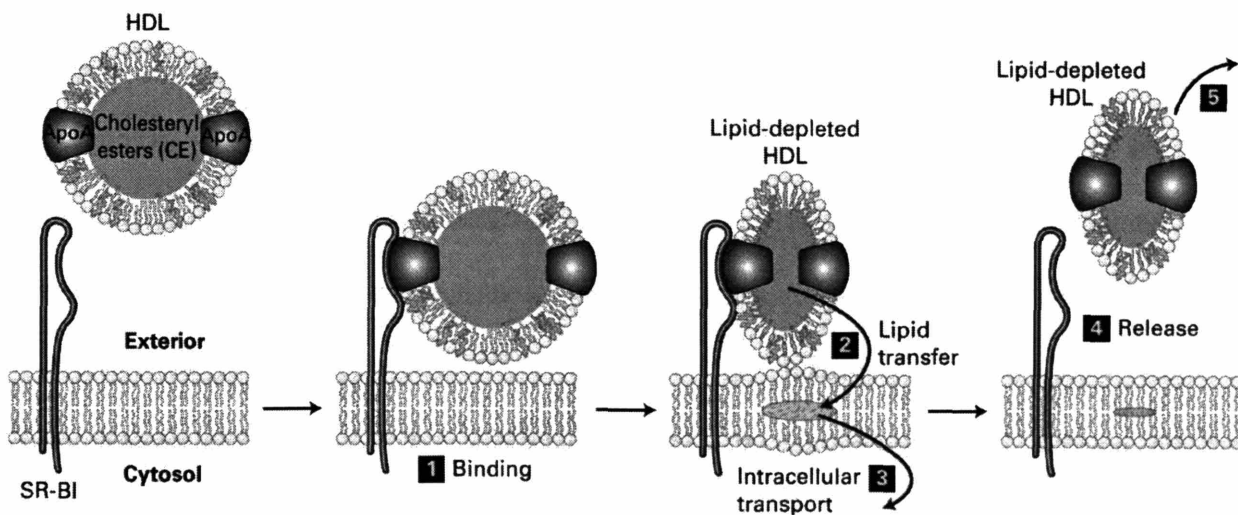
Figure 5: Cycle of selective uptake of core lipids from HDL by SR-BI. From Molecular Cell Biology, 5th Edition, W.H. Freeman & Company. (Lodish, Berk et al. 2004)

In addition to mediating transfer of lipids from lipoproteins to cells, studies demonstrate that SR-BI can also participate in the efflux of excess cellular cholesterol from cells to lipoprotein acceptors. SR-BI mediates bidirectional transfer of unesterified cholesterol between cells and HDL. SR-BI-mediated movement of lipids seems to be ATP-independent and net flux of lipids appears driven by the concentration gradient between cells and the extracellular donor/acceptor particles (Ji, Jian *et al.* 1997; Jian, de la Llera-Moya *et al.* 1998; Rigotti, Miettinen *et al.* 2003).

B. SR-BI In Vivo

1. Tissue SR-BI Expression

In adult mammals, SR-BI is most highly expressed in the liver and steroidogenic tissues i.e. adrenal glands, ovaries and testes, the predominant sites of selective lipid uptake (Acton, Rigotti et al. 1996; Landschulz, Pathak et al. 1996; Rigotti, Miettinen et al. 2003). The liver expresses the highest levels of total tissue SR-BI consistent with its role in 90% of selective HDL-CE uptake as well as 50% of HDL-CE clearance in CETP-deficient rodents (mice and rats) and 20% of HDL-CE clearance in CETP-expressing animals (Rigotti, Miettinen et al. 2003). Most hepatic SR-BI expression in vivo occurs on the sinusoidal (basolateral) surfaces of parenchymal cells, though SR-BI expression has also been shown in Kupffer and liver endothelial cells (Rigotti, Miettinen et al. 2003). Various studies suggest, that at least in some species, hepatic SR-BI expression is not sensitively feedback-regulated by hepatic cholesterol content and/or plasma HDL-cholesterol levels (Rigotti, Miettinen et al. 2003).

In the adrenal glands, SR-BI is mainly expressed on the surfaces of steroidogenic parenchymal cells, and adrenal levels of SR-BI are upregulated in apoA-I-deficient, HL-deficient and LCAT-deficient mice indicating that these lipoprotein-related genes may modulate SR-BI

expression in the adrenals (Rigotti, Miettinen et al. 2003). Ovarian SR-BI is predominantly expressed in the theca interna cells, which synthesize androgens from cholesterol, but not in granulosa cells that convert the androgens to estrogens. In rodents, granulosa cells express SR-BI during corpus luteum formation which correlates to increased serum progesterone levels in vivo (Rigotti, Miettinen et al. 2003). In the testes, SR-BI is primarily expressed in steroidogenic Leydig cells but is also found in lesser amounts in Sertoli cells where it may play a role in phagocytosis of apoptotic spermatogenic cells (Rigotti, Miettinen et al. 2003).

Though SR-BI is not requisite for intestinal cholesterol absorption, it is expressed on the luminal surfaces of intestinal epithelial cells. SR-BI can uptake unesterified cholesterol that is not packaged into lipoproteins in vitro, suggesting that it may play a role in intestinal cholesterol absorption. Furthermore, treatments that impair bile secretion into the intestinal lumen suppress SR-BI expression and the cholesterol absorption inhibitor ezetimibe, targeted to the putative intestinal sterol transporter NPC1L1, also binds and blocks SR-BI activity (Rigotti, Miettinen et al. 2003).

SR-BI levels are low in monocytes but expression increases as they differentiate into macrophages. SR-BI has been detected in macrophage-derived, lipid-laden foam cells in the atherosclerotic plaques of apoE(-/-) mice and humans, suggesting that SR-BI may function in lipid-deposition in these diseases (de Villiers and Smart 1999; Hirano, Yamashita *et al.* 1999; Zhang, Yancey *et al.* 2003).

2. SR-BI, HDL and Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) refers to the HDL-mediated transfer of cholesterol from peripheral tissues to the liver where it may be secreted into bile or alternatively processed (Figure 6). HDL efficiently removes excess cellular cholesterol in an ATP-dependent manner

from peripheral tissues via ABCA1. SR-BI can mediate ATP-independent transfer of cellular cholesterol to HDL, but the physiological relevance of SR-BI in this early stage of RCT is still unclear. Following esterification of cholesterol by LCAT, HDL can then either directly, or indirectly, through transfer of cholesteryl ester by CETP (in those species that express it) to other lipoproteins, transport cholesterol to the liver for biliary excretion either in the form of cholesterol or bile acids. HDL can also transport cholesterol to steroidogenic tissues for storage in lipid droplets and hormone synthesis. HDL and reverse cholesterol transport are reviewed extensively (Fielding and Fielding 1995).
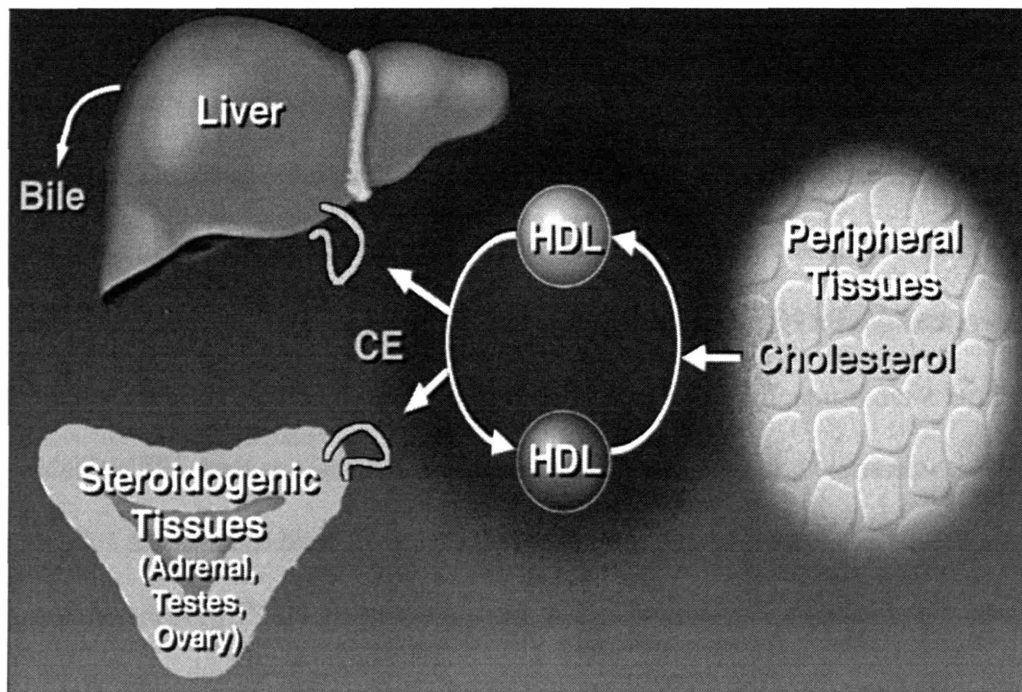
Figure 6: Role of SR-BI and HDL in reverse cholesterol transport. From M. Krieger. Derived from New England Journal of Medicine.

The inverse correlation between HDL cholesterol levels and risk for atherosclerosis lends itself nicely to the mechanism whereby HDL functions to transport excess cholesterol from cells, including those in vessel walls, to the liver for excretion. However, the atheroprotective

functions of HDL may alternatively extend to HDL's ability to inhibit LDL oxidation and protect

endothelial cells from oxidized LDL and stimulate endothelial nitric oxide production, as well as

HDL's beneficial effects on inflammation and coagulation. Moreover, the association between

HDL levels and atherosclerosis may simply be a marker for other anti-atherogenic physiology

(Yuhanna, Zhu et al. 2001; Rigotti, Miettinen et al. 2003).

3. In Vivo SR-BI Studies: Transgenic and Knockout Mice

Elucidation of the physiological roles of SR-BI in vivo came from studies in which

hepatic SR-BI overexpression was achieved via adenovirus-mediated gene transfer or generation

of transgenic mice as well as gene targeting approaches to create SR-BI null or SR-BI attenuated

expressing mice.

a. Lipoprotein and Biliary Phenotypes of SR-BI Deficiency

Initial studies using recombinant adenoviruses to transiently overexpress SR-BI in the

liver resulted in drastic reductions in plasma HDL and apoA-I levels and a corresponding

increase in hepatic biliary cholesterol levels, suggesting that the increase in biliary cholesterol

was attributable to increased uptake of plasma HDL cholesterol (Kozarsky, Donahee et al. 1997).

These results were corroborated by transgenic mice with hepato-specific overexpression of SR-

BI (Rigotti, Miettinen et al. 2003).

Analysis of SR-BI(-/-) mice demonstrated that SR-BI deficiency results in a 2.2-fold

increase in plasma total cholesterol levels compared to wild-type controls, with the increase

concentrated in HDL-sized particles as determined by molecular size fractionation of plasma

lipoproteins (Rigotti, Trigatti et al. 1997). Furthermore, the HDL particles of SR-BI(-/-) mice

were abnormally large, more heterogeneous and enriched in apoE compared to those of wild-

type mice (Rigotti, Trigatti et al. 1997). However, wild-type and SR-BI(-/-) mice have similar

levels of apoA-I, suggesting that the increase in HDL particle size and cholesterol was attributable to a defect in hepatic cholesterol uptake from HDL (Rigotti, Trigatti et al. 1997).

Another mutant mouse strain with attenuated levels of SR-BI expression (SR-BIatt) mice demonstrated reduced hepatic SR-BI expression associated with moderate increases in plasma total cholesterol levels and HDL size, reduced hepatic-selective HDL cholesterol uptake and decreased clearance of HDL-CE(Arai, Rinninger et al. 1999). These studies reveal that SR-BI is required for maintenance of normal plasma HDL cholesterol levels and structure and that the increased HDL cholesterol levels and particle size is a result of impaired hepatic selective uptake. SR-BI-deficient mice also exhibit specific reduction in biliary cholesterol secretion without decreases in other bile components (bile acids, phospholipids). Together, the increase in plasma HDL cholesterol levels and concomitant decrease in biliary cholesterol concentration in SR-BI-deficient mice support the notion that SR-BI plays an important role in the latter stages of reverse cholesterol transport (Rigotti, Miettinen et al. 2003).

b. Other Phenotypes of SR-BI Deficiency

Deficiency of SR-BI also produces adrenal, fertility and hematopoetic dysfunctions. SR-BI(-/-) mice have depleted adrenal and ovarian cholesteryl ester stores (Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999). Female SR-BI(-/-) mice are infertile whereas males do not demonstrate overt reproductive abnormalities (Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999). SR-BI(-/-) females exhibit normal gross ovarian morphology, hormone levels, estrus cycles and numbers of ovulated oocytes as well as retain the ability to produce normal levels of plasma progesterone upon induction of pseudopregnancy (Rigotti, Trigatti et al. 1997; Rigotti, Miettinen et al. 2003). However, SR-BI(-/-) mice ovulate dysfunctional oocytes; pre-implantation embryos from SR-BI(-/-) mice isolated the morning after mating exhibit aberrant

nonrefractile morphology, fail to develop into multicell morulas and blastocyts by day 3 as do

embryos from wild-type controls, and usually die at the one or two-cell stage (Trigatti, Rayburn

et al. 1999). Furthermore, the embryos appear similar to oocytes mechanically damaged during

pro-nuclear injections and this abnormal morphology can be reproduced in embryos from wild-

type females by treating them with cholesterol-binding compounds like nystatin or filipin that

can disturb cell membrane structure (Trigatti, Rayburn et al. 1999). SR-BI is not highly

expressed in ovulated oocytes, however it is expressed in the expanded cumulus cells

surrounding ovulated oocytes, cells derived from follicular granulosa cells thought to be

important in oocyte development (Trigatti, Rayburn et al. 1999). These studies suggest that

absence of SR-BI either directly (via perturbed oocyte cholesterol flux or altered membrane

structure) or indirectly (abnormal plasma lipoproteins surrounding the developing follicle)

modulates oocyte development and maturation (Rigotti, Miettinen et al. 2003). However studies

demonstrate that proper function of SR-BI(-/-) ovaries can be restored in a normal-lipidemic

environment i.e. one created by transgenic hepatic expression of SR-BI in SR-BI(-/-) mice

(Yesilaltay Ayce 2006) or when SR-BI(-/-) ovaries are transplanted into SR-BI(+/+)/RAG2(-/-)

(lymphocyte-deficient) hosts (Miettinen, Rayburn et al. 2001). Partial fertility (~40%) is

observed in SR-BI(-/-)/apoA-I(-/-) females; the apoA-I null mutation lowers the elevated total

cholesterol and HDL levels caused by SR-BI deficiency (Miettinen, Rayburn et al. 2001). In

addition, a reduced (non-Mendelian) yield of SR-BI(-/-) offspring from heterozygous mothers

(Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999) and SR-BI expression patterns later in

pregnancy (Hatzopoulos, Rigotti et al. 1998; Wyne and Woollett 1998) indicate a role for SR-BI

in steroid hormone synthesis and nutrient transport during fetal development and functioning of

extraembryonic tissues including the placenta, yolk sac and trophoblast (Trigatti, Rayburn et al. 1999).

SR-BI(-/-) mice also demonstrate mild reticulocytosis or defective maturation of red blood cells, with spiculated erythrocytes that often contain small membrane-bounded inclusions. The erythrocyte dysfunction is worsened by diet- (1% cholesterol-containing diet) or apoE-deficiency-induced hypercholesterolemia (Holm, Braun et al. 2002) and is discussed in greater detail below. Both the infertility and reticulocytosis are thought to be linked to the abnormally high lipoprotein unesterified cholesterol to total cholesterol (UC:TC) ratio in SR-BI(-/-) mice (see below) and these phenotypes are corrected when the mice are treated with the hypolipidimic, anti-oxidant drug probucol (Miettinen, Rayburn et al. 2001; Braun, Zhang et al. 2003).

Section Two: Atherosclerosis and relevant mouse models

I. Atherosclerosis

A. Origins of the 'Lipid Hypothesis' of atherogenesis

Though it is now generally accepted that plasma cholesterol and lipoprotein levels are strong indicators of risk for CHD, as late as the 1980's, the causal relationship between cholesterol levels and atherosclerosis, the 'lipid hypothesis', was still controversial. At the turn of the century, atherosclerosis was still considered a degenerative condition accompanying aging.

The idea that atherosclerosis could be modulated through dietary lipid intake was pioneered in 1913 by the Russian experimental pathologist Nikolai Anitschkow, who induced atherosclerotic lesions, similar to those observed in humans, in rabbits by feeding them purified cholesterol dissolved in sunflower oil. Through his experiments over the next few years,

Anitschkow and colleagues determined the following points that would later be recognized as instrumental to the development of atheromatous lesions (Steinberg 2004):

1. In the earliest lesions, also called 'fatty streaks', most lipid was concentrated in lipid-filled cellular vacuoles, thus identifying 'foam cells'

2. Postulated that foam cells were white blood cells that had infiltrated the artery wall.

3. Severity of the lesion was directly proportional to the level of blood cholesterol increase and time of exposure to elevated plasma cholesterol.

4. Longer periods of cholesterol feeding resulted in the deposition of connective tissue and the formation of a fibrous cap, converting the fatty streak into a complex fibrous plaque.

5. Removal of the cholesterol-rich diet led to reversion of fatty streaks but only partial regression of fibrous plaques.

6. Noted the connection between localization of plaques and hemodynamics, with lesions tending to form earlier at sites of turbulent blood flow.

Despite Anitschkow's landmark contributions to understanding the etiology of atherosclerosis, his animal model studies were dismissed at the time as not being applicable to the human condition. The cholesterol-feeding results in herbivorous rabbits were not reproducible in rats or dogs with omnivorous diets similar to humans. Later studies demonstrated that atherosclerotic lesions could be induced in almost every animal species including non-human primates. In the latter half of the century, multiple studies examining the effects of dietary cholesterol and saturated fat intake, identification of classes of lipoproteins with varying correlations to atherogenic risk, as well as the acceptance of statin-drug-based cholesterol-lowering therapy affirmed the 'lipid hypothesis' of atherosclerosis and established plasma

cholesterol levels as an important factor underlying the etiology of atherogenesis. These data

finally convinced the majority of the scientific and medical community that lowering plasma

cholesterol would be beneficial in reducing risk for atherosclerosis and CHD (Steinberg 2004).

B. Alternate theories of atherogenesis

Though lipid metabolism is now widely recognized as a key mechanism in atherogenesis,

the complex nature of atheromatous lesions gave way to alternate explanations for their

formation. Beginning with Anitschkow, various researchers had noted that atheromas contained

lipid, monocyte/macrophages, lymphocytes, smooth muscles cells (SMCs), extracellular matrix

(ECM) and a fibrous cap. Early fatty streak lesions are comprised mainly of lipid-rich

macrophage-derived foam cells whereas the bulk of mature plaques that are responsible for

significant coronary artery stenosis (narrowing of the vessel lumen) is composed of SMCs and

the ECM they secrete. Many groups focused on the importance of SMC proliferation resulting in

the 'response to injury hypothesis' of atherogenesis (Steinberg 2004).

The premise of the 'response to injury hypothesis' proffered by Ross and Glomset said

that mechanical injury could lead to desquamation of the vascular endothelium, exposure of

underlying matrix proteins, subsequent platelet adherence and release of factors, such as platelet-

derived growth factor (PDGF) which could in turn induce SMC proliferation. Chronic or

repeated injury would result in development of complex plaques. This theory suggested that

elevated plasma cholesterol levels could be a potential cause of endothelial injury and the source

of lipids in the plaque, but hypercholesterolemia was not a required condition for atherogenesis.

Though later experiments revealed that the endothelial layer overlying plaques is intact, this

hypothesis did emphasize the important role of communication between leukocytes and cells of

the artery wall (Steinberg 2004).

Subsequently, the inflammatory component of atherosclerosis and the interactions of various cells types, as initially emphasized by Ross, became a major focal point for atherogenesis research. The presence of monocytes/macrophages and T-lymphocytes in plaques had long been noted, and the secretion of chemoattractant cytokines (chemokines), like macrophage chemoattractant protein-1 (MCP-1), by endothelial cells at athero-prone sites to recruit immune cells were discovered to be among the earliest events preceding lesion formation.

C. Current views of atherogenesis

Current theories on atherogenesis emphasize the coaction of hypercholesterolemia, inflammation and cell-cell interactions in the development of atherosclerotic plaques. The formation of an atheroma can be divided into three distinct stages: initiation, progression and complication.

LDL was identified as the lipoprotein with the highest atherogenic potential. Initiation of atherosclerotic lesions begins when LDL infiltrates and becomes entrapped within the subendothelial space or intima. LDL, especially when in excess, is prone to entering artery walls where it accumulates, possibly via binding of apoB to cell surface proteoglycans (CSPGs) in the arterial intima (Libby, Aikawa et al. 2000; Skalen, Gustafsson et al. 2002). Several studies suggest that smaller, dense, lipid-poor LDL particles are more atherogenic than larger ones, possibly because they can more easily cross between endothelial cells, are more sensitive to pro-atherogenic modifications, or because they have increased exposure of positively-charged side chains that bind to CSPGs and increase their residence time in the arterial intima (Hurt-Camejo, Olsson et al. 1997; Veniant, Withycombe et al. 2001). It is hypothesized that once caught beneath the endothelium, the LDL phospholipids become oxidized. When associated with ECM macromolecules, LDL is susceptible to modification (Hurt-Camejo, Olsson et al. 1997).

Modified LDL containing phospholipids such as oxidized lysophosphophotidylcholine, as well as aldehyde-conjugated and glycated forms of LDL are found in atherosclerotic plaques (Libby, Aikawa et al. 2000). Modified LDLs induce release of cytokines, expression of cell adhesion molecules recruiting leukocytes, and scavenger receptors promoting foam cell formation, as well as other inflammatory processes that contribute to the developing atheroma (Libby, Aikawa et al. 2000). As well as stimulating the innate immune system, oxidized LDL can also activate the cellular immune response by serving as an antigen recognized by T-lymphocytes recruited to the lesion (Libby, Aikawa et al. 2000).

In response to the modified lipoproteins and possibly local hydrodynamics of blood flow, endothelial cells may express adhesion molecules that allow leukocytes to stick to the vessel walls of the affected areas. The adhesion molecules expressed by the vascular endothelium fall into one of two main classes: selectins, that mediate transient or saltatory interaction of leukocytes with the endothelium, and immunoglobulin-like molecules that maintain more permanent contacts between leukocytes and endothelium. Of the selectins, P-selectin is preferentially expressed by endothelial cells overlying human lesions (Libby 2000). Among the earliest changes in the endothelium at sites of plaque formation in cholesterol-fed rabbits and apoE-deficient mice prone to atherosclerosis is the expression of immunoglobulin-like vascular cell adhesion molecule-1 (VCAM-1), which binds to the VLA-4 receptor on the surface of leukocytes recruited to the plaque (Libby 2000).

In addition to expressing adhesion molecules that bind leukocytes to the region, vascular endothelial cells release chemokines like macrophage chemoattractant protein-1 (MCP-1) recruiting monocytes to the site of the lesion. Other lymphocyte chemoattractants such as IP-10, MIG and I-TAC, have been identified in human plaques (Libby 2000). Once bound, chemokines

direct extravasation of leukocytes through the artery wall into the subendothelial space. Mice deficient for MCP-1 or its receptor demonstrate reduced atherosclerosis and foam cell deposition in artery walls (see below) (Libby 2000).

Once in the intima, monocytes morph into macrophages that then engulf the oxidized lipoproteins, presumably through cell surface molecules like scavenger receptors that preferentially bind modified pro-atherogenic lipoproteins, ultimately becoming lipid-engorged foam cells. Hypercholesterolemic mice lacking functional scavenger receptor A (SRA) exhibit attenuated atherosclerosis, demonstrating a potential role for these receptors in atherogenesis (Libby 2000). The lipid-laden macrophages form "fatty streaks", smooth raised plaques beneath the epithelium that mark the initial stages of atherosclerosis. Foam-cell-based fatty streak lesions may be reversible and do not manifest clinical consequences (Libby 2000).

Further progression of the lesion to the second phase of atherosclerosis involves migration of SMCs into the fatty streak and formation of a fibrous connective tissue cap. The "response to injury" hypothesis of atherosclerosis suggested that denudation of the endothelium causes platelet aggregation, degranulation and the release of molecules like PDGF which promotes SMC proliferation and ECM accumulation. It is now known that lesions can form in the absence of endothelial injury and cells in the vascular wall or infiltrating leukocytes may themselves secrete PDGF. Various cytokines such as interferon gamma (IFN-γ), transforming growth factor beta (TGF-β) and interleukins contribute to lesion progression by differentially modulating SMC proliferation and ECM synthesis via a balance of growth-stimulatory and growth-inhibitory signals (Libby 2000).

Complication of the atheroma includes calcification, thrombus formation, rupture and hemorrhage which can result in ischemia, stroke, myocardial infarction and death. The majority

of MIs are generally thought to be caused by plaque rupture and resulting thrombosis as opposed to arterial stenosis and luminal obstruction by the atheroma itself (Libby 2000). Most coronary thromboses are the consequence of disruption of the plaque's fibrous cap, permitting contact between blood and thrombogenic substances like tissue factor in the lipid-rich core of the lesion. The amount of collagen in the plaque's fibrous cap, dependent on the rate of collagen biosynthesis by SMCs in the lesion, determines its tensile strength. While cytokines like PDGF and TGF-β promote collagen synthesis, IFN-γ inhibits protein expression in SMCs. Interestingly, IFN-γ is secreted by activated T-lymphocytes and T-cells have been shown to accumulate at the sites of fatal plaque rupture and thrombosis (Libby 2000). A decrease in plaque collagen content can also be the result of degradation by matrix metalloproteinases and collagenases secreted by leukocytes and SMCs in the lesion upon stimulation by inflammatory cytokines (Libby 2000). The delicate cytokine-based cross-talk between cells in the plaque may ultimately regulate the balance of collagen biosynthesis and degradation that establishes the lesion's stability and its susceptibility to rupture.
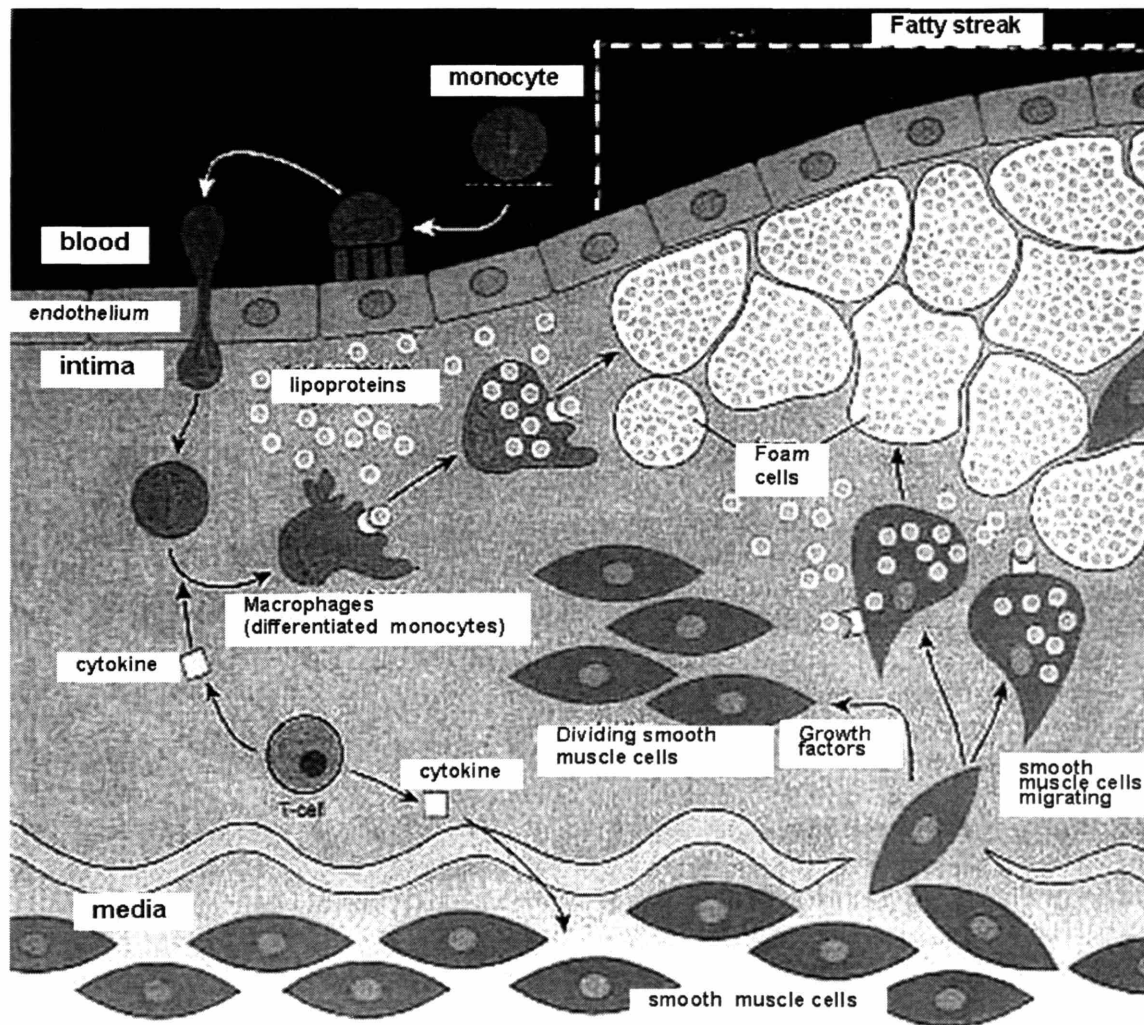
Figure 7: Formation of atherosclerotic plaques depends on the complex interactions between monocyte/macrophages, T-lymphocytes and smooth muscle cells via a variety of cell surface receptors and cytokines. Macrophage engulfment of lipoproteins converts them into foam cells, ultimately trapping them beneath the endothelium and forming a fatty streak. Adapted from http://www.activeweb.pl/images/atherosclerosis_damage.jpg.

II. Mouse Models of Atherosclerosis

The promise of animal models in the study of human atherosclerotic disease etiology was recognized at the turn of the 20th century. Mice, with their large litter sizes and short reproductive cycles, represent a relatively small, inexpensive, easily maintained system in which

to conduct a variety of studies. However, most mouse strains are naturally resistant to even diet-induced atherosclerosis and only the simplest type of lesions can be induced in C57BL/6 mice with prolonged fat-feeding. The development of techniques to create gene knockout and transgenic mice provided extensive opportunities to study mechanisms by which specific genes modulate atherogenesis.

Though fatty streak lesions could be induced in C57BL/6 mice using a high-fat/high-cholesterol diet, the generation of mice with targeted mutations in the apoE (*Apoe*) gene in 1992 (Plump, Smith et al. 1992; Zhang, Reddick et al. 1992) and the LDL receptor gene in 1993 (Ishibashi, Brown et al. 1993) provided the first mouse models of atherosclerosis that demonstrated human-like lesions progressing from fatty streaks to complex lesions with fibrous caps. ApoE(-/-) mice and LDLR(-/-) mice are now the two most widely used mouse models of atherosclerosis. Mice overexpressing human apoB are susceptible to atherosclerosis on a high-fat diet (Purcell-Huynh, Farese et al. 1995). ApoB, apoB-100-only and apoB-48-only transgenic mice are used to study the atherogenicity of apoB-48 versus apoB-100 containing lipoproteins, usually by crossing apoB transgenic mice to either apoE(-/-) or LDLR (-/-) mice. The apoB transgenic mouse lines will not be discussed here but are described in detail (Purcell-Huynh, Farese et al. 1995; Veniant, Pierotti et al. 1997; Powell-Braxton, Veniant et al. 1998; Veniant, Withycombe et al. 2001).

Though the kinetics of atherosclerosis has been characterized more extensively in apoE(-/-) mice, the lesions of apoE(-/-) and LDLR(-/-) mice are essentially equivalent. Plaques form in a time-dependent manner, begin in the proximal aorta and spread to more distal regions, and are prevalent at sites of turbulent blood flow such as arterial branch points (Knowles and Maeda 2000). Results from the two different models are comparable independent of the measurement

used to characterize or quantify their lesions (i.e. lipid content of aorta, cross-sectional plaque size in proximal aorta, cellular composition of plaque materials) (Knowles and Maeda 2000).

A. LDLR Knockout Mice

Though LDLR(-/-) mice have moderately elevated cholesterol levels of 200-300 mg/dl (two times the wild-type level of 100-150 mg/dl) when maintained on a chow diet, they generally do not develop spontaneous atherosclerosis (Ishibashi, Brown et al. 1993; Ishibashi, Goldstein et al. 1994). However, by 7 months of age, these mice exhibit cholesterol levels of 400-2000 mg/dl, massive cutaneous xanthomas (fatty deposits in the skin), and develop complex aortic atherosclerotic plaques when fed a high-fat, high cholesterol diet (Ishibashi, Goldstein et al. 1994; Knowles and Maeda 2000). The increased cholesterol in LDLR(-/-) mice is predominantly concentrated in apoB-100 containing LDL-like particles (Ishibashi, Brown et al. 1993; Veniant, Withycombe et al. 2001; Rigotti, Miettinen et al. 2003).

B. ApoE Knockout Mice

1. Apolipoprotein E

Apolipoprotein E is a ubiquitously expressed 34 kDa glycoprotein found on the surfaces of chylomicrons, VLDL and certain classes of HDL, that has been implicated in the clearance and recycling of lipoproteins and their remnants as well as many other physiological and cellular processes (Davignon, Cohn et al. 1999; Knowles and Maeda 2000; Mahley and Rall 2000; Swertfeger and Hui 2001). ApoE regulates metabolism of VLDL, LDL and chylomicrons and their remnants by serving as a ligand for both LDLR and LDLR-related protein (LRP) (Mahley and Rall 2000). As a component on a subpopulation of HDLs, apoE can modulate cellular cholesterol influx and efflux and may play a role in the late stages of RCT by acting as a ligand in the delivery of cholesterol to the liver for biliary excretion, especially in species deficient in

CETP. In addition, apoE binds cell surface heparin sulfate proteoglycans (HSPGs) and may facilitate SR-BI-mediated selective uptake of cholesteryl esters from apoE containing HDLs (Mahley and Rall 2000)

2. ApoE(-/-) Mice

Though very young animals show no obvious phenotypes, apoE(-/-) mice are hypercholesterolemic with plasma total cholesterol levels of 400-600 mg/dl that are elevated to 1800-4000 mg/dl when fed a high-fat diet (Plump, Smith et al. 1992; Zhang, Reddick et al. 1992; Zhang, Reddick et al. 1994). Unlike wild-type mice that carry the majority of their cholesterol in the HDL fraction as determined by fast protein liquid chromatography (FPLC) using molecular size exclusion separation columns, the plasma cholesterol of apoE(-/-) mice is mainly concentrated as apoB-48-containing remnant VLDL sized particles with a small peak in the HDL size fraction (Veniant, Withycombe et al. 2001). In addition to being hypercholesterolemic, apoE(-/-) mice develop spontaneous advanced aortic atherosclerotic plaques on a low-fat diet after 3-4 months of age (Plump, Smith et al. 1992; Zhang, Reddick et al. 1992; Knowles and Maeda 2000). The rate of atherogenesis in these mice can be accelerated by feeding them a lipid-enriched diet (Zhang, Reddick et al. 1994; Knowles and Maeda 2000). However, low levels of apoE expression, even below that required to lower plasma cholesterol, in the liver, macrophages derived from wild-type bone marrow, or adrenal glands reduce atherosclerosis in apoE(-/-) mice suggesting that apoE influences atherogenesis independent of its role in regulating lipid metabolism (Boisvert, Spangenberg et al. 1995; Linton, Atkinson et al. 1995; Van Eck, Herijgers et al. 1997; Thorngate, Rudel et al. 2000).

The characterization of these well-defined inbred and mutant mouse strains, the predictability of spontaneous atheroma development as well as the general advantages of mice

being small and relatively easily maintained make LDLR(-/-) and apoE(-/-) mice invaluable tools in which to study environmental, pharmacological and genetic modifiers of atherosclerosis. A genetic analysis approach in which inbred strains of mice carrying various targeted mutations are crossed into an apoE(-/-) or LDLR(-/-) background has identified several key genes that modulate atherogenesis. A selected list is given in Table II.

Gene of Interest	Effect of gene mutation	Atherosclerosis Model	Diet	Fold change in atherosclerosis
MCSF -/-	Monocyte/macrophage deficiency	ApoE KO	Chow	5x decrease
MCP-1 -/-	Deficiency of monocyte/macrophage/ And T-lymphocyte chemoattractant	LDLR KO	High fat	5x decrease
RAG1 -/-	Complete B- and T-lymphocyte deficiency	ApoE KO	Chow	1.7x decrease
RAG1-/-	Complete B- and T-lymphocyte deficiency	ApoE KO	High fat	No change
RAG2-/-	Complete B- and T-lymphocyte deficiency	ApoE KO	High fat	No change
ICAM -/-	Disruptions in cell-cell adhesion	ApoE KO	Chow	1.4x decrease
P-selectin-/-	Disruptions in cell-cell adhesion	ApoE KO	Chow	2x decrease
P-selectin -/-	Disruptions in cell-cell adhesion	LDLR KO	High fat	1.25x decrease
E-selectin -/-	Disruptions in cell-cell adhesion	ApoE KO	Chow	1.4x decrease
P, E-selectin -/-	Disruptions in cell-cell adhesion	LDLR KO	High fat	2x decrease
Hepatic Lipase -/-	Increased plasma TC, HDL	ApoE	Chow	4x decrease

Table II: Influence of selected genes on atherogenesis in LDLR KO or apoE KO mice. Adapted from Knowles and Maeda 2000.

Section Three: Mechanisms and Models of CHD

I. Myocyte hypertrophy

Cardiac hypertrophy is the enlargement of individual myofibers in response to increased biomechanical stress. Physiological hypertrophy occurs during post-natal heart development or in response to exercise. Pathologic hypertrophy, which is the focus of this discussion, is cardiomyocyte enlargement as a result of intrinsic stimuli like that in familial hypertrophic cardiomyopathy or extrinsic as in valvular heart disease or arterial hypertension (Frey and Olson 2003). Unlike physiologic hypertrophy, pathologic hypertrophy can result in myocardial interstitial fibrosis (scarring and collagen deposition). Pathologic myocardial hypertrophy can normalize ventricular wall tension and signals that control cardiomyocyte growth may enhance survival in response to pro-apoptotic stimuli (van Empel and De Windt 2004). Hypertrophy has traditionally been considered an adaptive response requisite to maintain cardiac output under stress, but regardless of the underlying cause, prolonged hypertrophy increases risk for sudden death or progression to heart failure. Cardiomyocyte hypertrophy is characterized by increased myocyte size, enhanced protein synthesis and higher sarcomere organization (Frey and Olson 2003). Though cardiomyocytes are terminally differentiated cells, these changes are preceded and accompanied by re-induction of the "fetal gene program"(Frey and Olson 2003).

A. Models of cardiac hypertrophy

Several cell signaling pathways, some of which may participate in both physiologic and pathologic forms, have been implicated in cardiomyocyte hypertrophy and will be briefly discussed below. Though some in vitro studies have been conducted on cultured neonatal cardiomyocytes, much research elucidating cellular mechanisms underlying hypertrophy in vivo has been done using animal models. Transgenic mouse models of hypertrophy include the CNA-

Tg, G_q-Tg and calsequestrin-Tg mouse. Banding or constriction (tying off with suture) of the aorta usually in rats, mice and rabbits is a surgical intervention method aimed at created pressure-overload hypertrophy that can ultimately lead to myocardial interstitial fibrosis (Frey and Olson 2003). Spontaneous hypertensive and Dahl-salt-sensitive rats are alternative widely used models of pressure-overload hypertrophy. In addition, cardiac hypertrophy can be induced in rodent models through administration of β-adrenergic agonists such as isoproterenol or norepinephrine (Frey and Olson 2003).

B. Molecular pathways involved in cardiomyocyte hypertrophy

A variety of cellular proteins, receptors and signaling circuits have been reported to play a role in cardiomyocyte hypertrophy. These include GPCRs, calcineurin-calmodulin-NFAT and PIK3 signaling, MAPK kinase circuits, small GTPases of the Ras and Rho families, NF-κB, the multi-ligand receptor Gp130 which binds interleukins 6 and 11(IL-6/11), cardiotrophin-1 (CT-1), and leukemia inhibitory factor (LIF) and its downstream JAK/STAT pathway (Frey and Olson 2003).

1. Cardiac hypertrophy and lipid metabolism

The adult myocardium relies on mitochondrial oxidation of long-chain fatty acids as its primary energy source. Cardiac hypertrophy is associated with a decrease in fatty-acid oxidation and metabolic reversion of the heart to utilizing glucose as its main source of energy, a characteristic of fetal hearts. The metabolic shift towards increased glucose usage may be an adaptive response to hypoxia because it reduces myocardial oxygen consumption per mole of ATP generated. However, chronically impaired fatty acid oxidation may lead to increased myocardial lipid deposition. Genes involved in fatty acid metabolism are regulated by the peroxisome proliferator-activated receptors (PPARs) which may also be negative regulators of

cardiac hypertrophic signals. PPARα is downregulated in pressure-overload hypertrophy as are

several lipid metabolism enzymes such as carnitine palmitoyltransferase 1 (CPT-1) responsible

for regulating mitochondrial fatty acid uptake (Frey and Olson 2003).

The role of PPAR in modulating cardiac hypertrophy suggests two possible mechanisms:

1) that altered cardiac lipid and energy metabolism are secondary to other causes of

cardiomyocyte enlargement or 2) that abnormal lipid metabolism in the heart precedes and

promotes hypertrophy (Frey and Olson 2003). Supporting the latter possibility is the observation

that several inherited fatty acid oxidation disorders are associated with LV hypertrophy.

In addition, statin-mediated inhibition of HMG CoA-reductase, the rate-limiting enzyme

in cholesterol biosynthesis, attenuates hypertrophy in a variety of rat, mouse and rabbit models.

The inhibition of HMG-CoA reductase may limit the buildup of isoprenoid precursors that would

otherwise isoprenylate and activate multiple proteins implicated in hypertrophy including the

small G proteins Ras, Rac1 and RhoA.

2. Calcineurin-Calmodulin-NFAT signaling

Elevations in cellular Ca^{2+} levels cause association of calmodulin with the serine-

threonine phosphatase calcineurin and its subsequent activation. Calcineurin then

dephosphorylates transcription factors of the NFAT family (nuclear factor of activated T-cells),

unmasking nuclear localization signals. NFAT translocates to the nucleus where it activates gene

transcription (Frey and Olson 2003).

The Calcineurin-NFAT pathway is active in cardiomyocytes. Constitutive cardiac

activation of calcineurin in transgenic mice induces massive cardiomegaly that leads to heart

failure (Frey and Olson 2003). Several studies inhibiting calcineurin signaling in the heart either

by overexpression of endogenous inhibitors or a catalytically inactive form of calcineurin that

acts as a dominant-negative protein prevents hypertrophy in various models including aortic

banding and isoproterenol drug-induced (Frey and Olson 2003). Combined, these data indicate a

role for calcineurin-NFAT signaling pathways in cardiac hypertrophy.

3. PIK3 signaling pathways

Phosphoinositide-3-kinases (PI3K) are a group of enzymes with both lipid and protein

kinase activities that can be activated by receptor tyrosine kinases (RTKs) such as the insulin-

like growth factor-1 (IGF-1) receptors and G-protein coupled receptors (GPCRs) including α and

β adrenergic receptors (Frey and Olson 2003). PI3K has been associated with several signaling

functions including cell growth, proliferation and survival. PI3K is activated in pressure-

overload hypertrophy and overexpression of constitutively active PI3K results in cardiac

hypertrophy in transgenic mice (Frey and Olson 2003). Furthermore, dominant-negative forms of

PI3K results in reduced heart-to-body weight ratios in Tg mice (Frey and Olson 2003). The

PI3K-dependent alterations in heart size are attributable to growth of individual myofibers as

opposed to increased myocyte proliferation (Frey and Olson 2003).

A primary downstream target of PI3K is the serine-threonine kinase Akt, transgenic

overexpression of which is sufficient to induce hypertrophy in mice (Frey and Olson 2003). Akt

can inhibit GSK-3β, a negative regulator of hypertrophy. GSK-3β inhibits NFAT-mediated gene

activation by masking its nuclear localization signal and causing it to translocate back into the

cytoplasm. Several hypertrophic stimuli including isoproterenol, endothelin-1 and pheneprine

result in PI3K-mediated phosphorylation and inactivation of GSK-3β suggesting that GSK-3β

inactivation is required for cardiomyocyte hypertrophy (Frey and Olson 2003). Murine

expression of mutant GSK-3 that cannot be inactivated inhibits ET-1, isoproterenol and pressure-

overload induced hypertrophy (Frey and Olson 2003). The interaction between GSK and NFAT

also emphasizes the crosstalk between the PIK3 and the calcineurin pathways in regulating hypertrophy.

4. G-protein coupled receptors in hypertrophy

The receptors for angiotensin II (angII), endothelin-1 (ET-1) and α-adrenergic receptors are coupled to $G_{q/11}$, and have been shown to be sufficient to mediate cardiomyocyte hypertrophy when stimulated by agonists. Transgenic overexpression of these receptors or their downstream mediator G_q produces cardiac hypertrophy eventuating in cardiomyopathy with reduced contractility. Furthermore, overexpression of dominant-negative forms of G_q attenuated aortic-banding induced hypertrophy (Frey and Olson 2003). Overexpression of G_s, β1-adrenergic receptors coupled to G_s, or the downstream effector protein kinase A (PKA) all produce cardiomyocyte hypertrophy that progresses to cardiac dysfunction and fibrosis (Frey and Olson 2003).

II. Mechanisms of Cardiomyocyte Injury and Death

Both of the two principal mechanisms of cell death, apoptosis and necrosis, have been implicated in ischemia-induced cardiomyocyte injury and death. Ischemia lasting 20 minutes or longer causes irreversible cardiac damage with extensive cell loss by necrosis and apoptosis (Scarabelli and Gottlieb 2004). Studies on the mechanisms involved in cardiomyocyte death employ in vitro cultured cardiomyocytes as well as canine and rodent models of cardiac ischemia with or without subsequent reperfusion and demonstrate roles for both apoptosis and necrosis. However the relative contribution of each mechanism to myocardial infarction is still controversial and may depend on the series of events that pre-empt the ultimate damage to the heart. Some evidence suggests that apoptosis is primarily responsible for early cardiomyocyte

death in the first 6-8 hours following MI whereas necrosis is a secondary event occuring 12

hours to 4 days post-MI (Blankesteijn, Creemers et al. 2001).

A. Apoptosis

Apoptosis is an ATP-dependent, cell-autonomous method, initiated by healthy or

sublethally injured cells, to eliminate damaged or unwanted cells without inciting an

inflammatory response that could damage surrounding tissue (Scarabelli and Gottlieb 2004).

Apoptosis includes profound morphological alterations including cell shrinkage and membrane

blebbing associated with externalization of phosphatidylserine from the inner to the outer leaflet

of the cell membrane (Gottlieb and Engler 1999). The changes in membrane structure are

accompanied by alterations in membrane potential, ion flux and water balance that lead to

cytoplasmic acidification (Gottlieb and Engler 1999). However, in apoptosis, membrane

integrity is preserved by tissue transglutaminase, an enzyme that crosslinks various cytoskeletal

proteins forming an outer envelope, until neighboring cells or professional phagocytic cells can

ingest the dying cell. Maintenance of an intact plasma membrane serves to limit inflammation by

sequestering cellular contents (Gottlieb and Engler 1999). In addition, activation of cysteine

proteases called caspases is a hallmark of apoptosis. These enzymes cleave cytoskeletal proteins

as well as activate other proteins such as endonucleases responsible for fragmentation of DNA, a

late stage feature of apoptosis. DNA fragmentation is not required for apoptosis and can be

completed by endonucleases expressed by phagogytic cells that ingest the cell corpse (Gottlieb

and Engler 1999).

Apoptosis of cardiomyocytes was first characterized in 1994 using nick-end labeling to

detect DNA fragmentation through studies in rabbits on ischemia-reperfusion injury (Gottlieb,

Burleson et al. 1994), which refers to tissue damage in situations where blood-flow is blocked

for some period of time and then re-established. TNF-α-mediated generation of ceramide, an intracellular signaling molecule derived from sphingomyelin, has been implicated in cardiomyocyte apoptosis. In addition, p38/MAPK pathways have been shown to play a role in both cardiomyocyte hypertrophy and apoptosis.

Since then, activated caspases have been identified in apoptotic cardiomyocytes and caspase inhibition protects against cell death in cardiomyocytes subjected to metabolic inhibition and recovery, a method used to simulate ischemia-reperfusion (Gottlieb and Engler 1999). Furthermore, recent studies demonstrate a role for caspase-3 in cleaving the cardiac myofibrillar proteins troponin T, α-actin and α-actinin (Communal, Sumandea et al. 2002). It has been suggested that transcription factors induced by hypertrophic stimuli prepare the cardiomyocytes for re-entry into the cell cycle, however, these terminally differentiated cells, being unable to divide, default to an apoptotic program (Communal, Sumandea et al. 2002). Moreover, nuclear condensation and fragmentation is only rarely completed in these cells. If continued cleavage of cytoplasmic proteins continues without nuclear degradation, the myocyte loses contractile function but can persist in a "zombie" state (Communal, Sumandea et al. 2002). However, degradation of the nucleus proceeds to cell death via apoptosis (Communal, Sumandea et al. 2002).

The activation of caspases occurs through two distinct, but interlinked, pathways. One pathway exists through activation of cell surface receptors including Fas and tumor necrosis factor receptor (TNFR), that aggregate upon ligand binding, leading to the intracellular association of proteins that interact through a conserved region called the death domain, an example of which is fas-associated death domain (FADD). FADD then recruits and assists in the activation of caspase-8 through its death effector domain. Hypoxia has been shown to upregulate

fas in myocytes and oxidative stress can induce aggregation of these receptors (Gottlieb and

Engler 1999).

An alternative mechanism of caspase activation involves mitochondria, specifically the

release of cytochrome c from the mitochondrial intermembrane space into the cytoplasmic

compartment where it participates in caspase activation. Various proteins regulating

mitochondrial integrity, such as Bcl-2, which inhibits cytochrome c release, and Bax that

promotes its release, play a role in apoptosis. Another protein, cytochrome c interacting factor of

apoptosis (CIFA) has been shown to penetrate the mitochondrial outer membrane and release

cytochrome c from the electron transport chain. Cytochrome c release has been demonstrated in

ischemia and reperfusion (Gottlieb and Engler 1999).

Apoptotic disruption of the mitochondrial electron transport chain also has consequences

for the energy metabolism in cells undertaking the cell death program. Loss of mitochrondrial

function forces the apoptic cell to resort to less efficient glycolytic means of generating ATP,

with the concomitant production of lactic acid possibly contributing to cytoplasmic acidification.

Moreover, depletion of energy stores before the cell has undergone the requisite apoptotic

transformation may cause it to divert to a necrotic, pro-inflammatory death pathway.

That death receptor aggregation, cytochrome c release and caspase activation all may

play a role in cardiomyocyte apoptosis presents a series of potential therapeutic targets aimed at

limiting cardiomyocyte death. Even though apoptosis is now thought to be the principal method

of cell death in ischemic and reperfused tissues, its significance in chronically ischemic and

hypoxic myocardium is not as well characterized. For example, pre-conditioning, a method in

which a brief period of cellular stress induces a protective response that reduces tissue death

when cells are exposed to ischemic stress that would ordinarily cause more substantial damage,

ameliorates apoptosis in ischemic and reperfused tissues. However, pre-conditioning does not seem to reduce tissue damage resultant from permanent ischemia. Moreover, blocking events in cardiomyocyte apoptosis may backfire if the damaged cell defaults to death by necrosis as has been observed in cultured fibroblasts (Gottlieb and Engler 1999).

Apoptosis was originally disregarded as a mode of death for terminally differentiated such as cardiomyocytes because it was thought that autonomous cell death was limited to developing tissues and actively dividing cells in continuously renewing populations (Scarabelli and Gottlieb 2004). Though myocyte and endothelial cell apoptosis was detected in rabbit hearts exposed to 30 minutes of ischemia succeeded by 4 hours of reperfusion, it was not detected in normal or *continuously ischemic* myocardium (Gottlieb, Burleson et al. 1994; Scarabelli and Gottlieb 2004). Cardiomyocyte apoptosis detected in ischemic/reperfused hearts predominantly occurs during the reperfusion phase (Scarabelli and Gottlieb 2004). Reperfusion of ischemic tissues accelerates but restricts apoptosis of damaged cells, whereas apoptosis develops more slowly and is more extensive during continuous ischemia (Scarabelli and Gottlieb 2004). In addition, the severity of ischemia-induced ATP shortage in the dying cell determines whether it will execute an apoptotic or necrotic program. If energy stores are nearly depleted, the cell dies by necrosis whereas if there exists a limited ATP supply, the cell may enter into apoptosis that culminates when the tissue is reperfused.

B. Necrosis and Inflammation

Necrosis involves the killing of damaged cells by immune cells such as cytotoxic lymphocytes, neutrophils and natural killer cells resulting in the leakage of cytoplasmic components and a subsequent inflammatory response. Necrosis is recognized as the typical form of ischemic cell death because release of cytosolic cardiomyocyte proteins such troponins and

creatine kinase MB have been documented in patients presenting with acute myocardial infarction (Scarabelli and Gottlieb 2004).

One feature of apoptotic cells is the transition of phosphatidylserine to the outer plasma membrane leaflet, signaling phagocytic cells to engulf the remains of the dying cell before spillage of cytoplasmic contents occurs. However, delayed or impaired phagocytosis results in secondary necrosis of the apoptotic remnants (Scarabelli and Gottlieb 2004), a shift that occurs most often in cardiomyocytes exposed to severe continuous ischemia. In chronically ischemic tissue monitored over several days, there is a switch from apoptosis to necrosis during the latter stages, with a transition period in which cells demonstrate characteristics of both apoptic and necrotic cell death (Scarabelli and Gottlieb 2004). Cardiomyocytes are incapable of ingesting neighboring apoptotic cells, and the energy requirements for apoptosis indicate that dying cardiomyocytes may not be able to fully sustain the ordered metabolic processes to complete DNA fragmentation and degradation into apoptotic bodies (Tomei and Umansky 2001). Both of these observations suggest that while cardiomyocytes may initiate apoptosis, infiltration of the damaged tissue by professional phagocytes is required to finish the job. This secondary cardiomyocyte necrosis instigates an inflammatory response which can contribute both to exacerbating myocardial injury as well as subsequent wound healing and cardiac remodeling processes (Blankesteijn, Creemers et al. 2001).

Whether ischemic cardiomyocytes predominantly die through an apoptic or necrotic program is still debated and ultimately dependent on both the intracellular and extracellular environment in which the damaged cell resides. Regardless of the mechanism of cardiomyocyte death, inflammation remains a key component in myocardial infarction and subsequent tissue remodeling. Ischemia significant enough to cause infarction produces an inflammatory response

which is accelerated if the tissue is reperfused (Frangogiannis, Smith et al. 2002). Inflammation can extend myocardial damage and corticosteroids administered to check inflammation have been shown to decrease infarct size in canine models of experimental MI (Frangogiannis, Smith et al. 2002). Necrosis of cardiomyocytes releases mitochondrial fragments which can trigger early-acting cytokines and components of the complement system within 12-16 hours of onset of ischemia (Blankesteijn, Creemers et al. 2001) and recruit neutrophils and monocytes to the damaged myocardium (Frangogiannis, Smith et al. 2002). Complement depletion reduces myocardial necrosis and infarct size in various animal models (Frangogiannis, Smith et al. 2002).

Data from a canine model of ischemia/reperfusion demonstrated that post-ischemia cardiac lymph contains pro-inflammatory cytokines including TNF-α and histamines, and degranulated mast cells, the sources of these cytokines have been detected in ischemic heart sections. Other studies have implicated cytotoxic $CD8^+$ T-lymphocytes (CTLs) in cardiomyocyte injury during myocardial infarction. Moreover, these sensitized CTLs can also kill healthy cardiomyocytes in vitro, suggesting that they may be involved in damage of healthy viable tissue beyond the ischemic region (Varda-Bloom, Leor et al. 2000).

III. Myocardial fibrotic scar formation and cardiac wound healing

Cardiomyocytes lost to ischemia-induced damage, being terminally differentiated, cannot be replaced by similar cells derived from the surrounding viable tissue. The result is the deposition of granulation tissue and the formation of a fibrotic scar in the infarcted region in a process similar to wound healing (Blankesteijn, Creemers et al. 2001).

The first step in cardiac wound healing is clearance of dead and injured cardiomyocytes and their debris. This is accomplished by polymorphonuclear (PMN) granulocytic neutrophils that infiltrate the ischemic tissue within the first day of the infarction and are subsequently

followed by arrival of mast cells, lymphocytes and macrophages (Blankesteijn, Creemers et al. 2001).

Once debris is cleared 2-3 days post-MI, granulation tissue rich in inflammatory cells, fibroblasts and blood vessels forms at the border zone of the infarcted tissue. Cardiac granulation tissue is composed of specialized fibroblasts, called myofibroblasts, that have acquired contractile properties and phenotypic characteristics of smooth muscle cells including expression of α-smooth muscle cell actin (α-SMAc) regulated by TGF-β (Frangogiannis, Smith et al. 2002). Myofibroblasts are responsible for ordered deposition of the ECM proteins fibrin, tenascin, fibronectin and interstitial collagen types III and I, the last of which contributes tensile strength to the healing infarct (Blankesteijn, Creemers et al. 2001). Whereas fibroblasts in skin wounds are abundant in granulation tissue and lost during transition into the scar, some myofibroblasts can persist in scar tissue several years after the MI and subsequent fibrosis (Blankesteijn, Creemers et al. 2001).

Necrotic cardiomyocytes in the center of the infarcted region are eventually replaced by myofibroblasts and macrophages that migrate inwards from the border zone. The myofibroblasts form highly organized arrays parallel to the epi- and endocardium, their contractile properties increase tensile strength of granulation tissue allowing it to withstand the rhythmic stretching of the ventricular wall (Blankesteijn, Creemers et al. 2001). Misalignment of myofibroblasts in the infarct can cause excessive dilation and heart failure (Blankesteijn, Creemers et al. 2001).

Another key process in scar formation is the degradation of ECM proteins soon after initiation of MI to allow movement of wound-healing cells into the damaged myocardium. ECM degradation occurs through various proteases including matrix metalloproteinases (MMPs), plasmin, gelatinases and collagenases and is generally restricted to the infarcted area

(Blankesteijn, Creemers et al. 2001). However, an imbalance between ECM degradation and synthesis can result in potentially fatal complications such as ventricular wall thinning or rupture of the infarcted region (Blankesteijn, Creemers et al. 2001). Mice deficient in these proteinases, their precursors or activators are protected against cardiac rupture and ventricular enlargement but demonstrate impaired migration of cells into the infarct and scar formation when undergoing surgically induced MI (Frangogiannis, Smith et al. 2002). Maturation of granulation into a scar is characterized by a decrease in cell numbers via apoptosis as well as complete crosslinking of collagen fibers deposited within the infarcted region.

IV. Heart Failure, Conduction Defects and Cardiac Dysfunction

A terminal complication of myocardial infarction is the progression to heart failure and eventual death. With each stroke, the left ventricle is responsible for systemic circulation of oxygenated blood through arteries while the right ventricle pumps oxygen-poor blood through the pulmonary artery to the lungs. Heart failure can be defined as any syndrome in which a structural or functional cardiac defect impairs the ability of the ventricle to fill with or eject blood (Hunt, Baker et al. 2001) to sufficiently meet the metabolic requirements of the body.

Maintenance of proper cardiac electrophysiology is essential to preserving heart function. The final event in fatal myocardial infarction is usually associated with electrical conduction irregularities (James 1997) and most arrythmias occur in the context of heart failure (Kleber and Rudy 2004). Cardiac excitation involves generation of action potentials by individual cells and propagation of the electrical signal cell-to-cell via specialized intracellular gap junctions in the intercalated disks that couple cardiomyocytes (Kleber and Rudy 2004). The action potentials that synchronize cardiac contraction originate in the pacemaker cells of the sinoatrial (SA) node. Proper ventricular contraction depends on the transmission of electrical impulses from the SA

node through the atrioventricular (AV) node, bundle of His, right and left bundle branches and the Purkinje fibers that comprise the specialized conduction network of the heart. The Purkinje fibers are large, diffusely located conducting cells that rapidly distribute the impulse throughout the ventricles, causing simultaneous depolarization and contraction of all ventricular cardiomyocytes (Dubin 2000).

The resting membrane potential of cardiomyocytes is -90 mV. Depolarization of cardiomyocytes causes influx of Ca^{2+} through voltage-gated Ca^{2+} channels. The small influx of extracellular Ca^{2+} stimulates contraction of cardiomyocytes by triggering release of intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR) into the cytosolic compartment through Ca^{2+} release channels called ryanodine receptors in a process termed calcium-induced calcium release (CICR). The Ca^{2+} is then free to interact with troponin C and initiate contraction of cardiac sarcomeres. Cardiac relaxation occurs via the re-uptake of Ca^{2+} into the SR by the Ca^{2+}-ATPase, which stores Ca^{2+} for the next contraction (Chien 2000). This process is generally referred to as excitation-contraction coupling and has been reviewed (Ebashi 1991; Ferrier and Howlett 2001).

Electrocardiography is a technique used to evaluate the transmission of electrical signals through the cardiac conduction system. The ECG signal has three main features. The P-wave demonstrates atrial depolarization. The QRS complex occurs approximately 0.15 seconds later in humans signaling ventricular depolarization followed by the T-wave caused by ventricular repolarization. Depolarization occurs simultaneously through the ventricles outward from the endocardium to the epicardium. The deflections in the ECG trace are a function of the position of the electrodes and the vectors of depolarization and repolarization they register. For example, depolarization moving away from a positive electrode records a negative wave in the ECG signal. Deviations in the ECG trace, especially in the ST segment which should be flush with the

baseline, indicate compromised blood flow to the heart muscle and abnormal ventricular

electrical activity. Specifically, episodes of depression of the ST segment denotes ischemia and

occurrence of subendocardial MI whereas ST elevation indicates acute transmural myocardial

infarction (Dubin 2000). In humans, such electrophysiological conduction abnormalities

indicating ischemia and myocardial injury are usually clinically exposed when the heart is taxed

by exercise stress tests.

Normal ECG Signal   **ST Depression**   **ST Elevation**
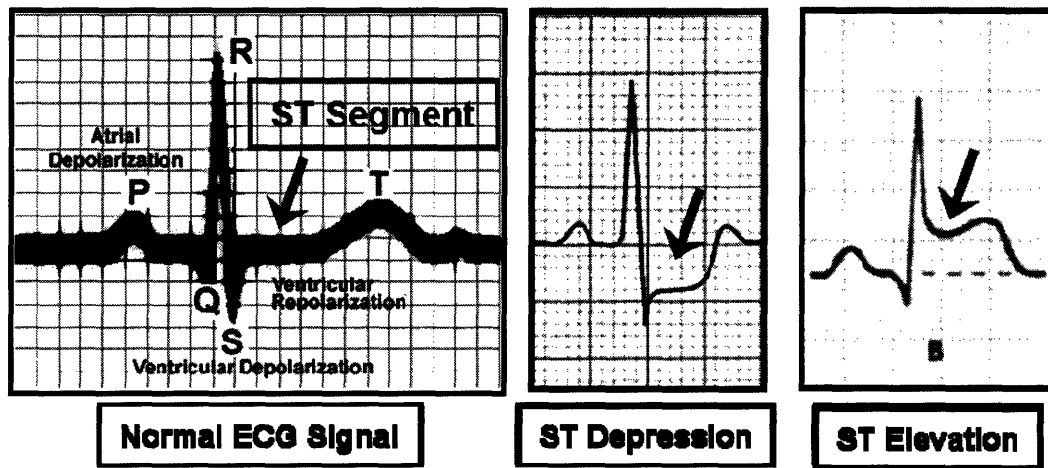
Figure 8: Examples of a normal ECG signal and the conduction abnormalities ST depression

(indicative of ischemia) and ST-Elevation (indicative of myocardial infarction).

Compiled from http://students.med.nyu.edu/erclub/ekgs/ekgseg.gif

http://www.postgradmed.com/issues/2004/06_04/tak1.gif and

http://www.unm.edu/~lkravitz/Media/stelevation.jpg

Myocardial infarction can result in both systolic and diastolic dysfunction. Systolic

dysfunction is characterized by reduced contractility and ejection fraction (percentage of the left

ventricular diastolic volume ejected with each stroke) as well as LV dilation. Systolic

dysfunction involves reductions in both speed, as manifest by decreased pressure development

observed by hemodynamic measurements ($\pm$dP/dt), and magnitude, as determined by ejection

fraction, of ventricular contractions. Diastolic dysfunction is impaired relaxation of the heart and can occur in the context of normal cardiac ejection fraction.

The failure of cell survival signals, such as those mediated by cardiotrophin-1, to prevent cardiomyocyte apoptosis is critical to the initiation of heart failure (Chien 2000). The cellular and molecular mechanisms underlying abnormal myocyte contraction and heart failure include alterations in cytosolic and intracellular Ca^{2+} concentrations, signal transduction, asynchronous release of Ca^{2+} upon myocyte stimulation, myofibrillar ATPase activity, and function of SR and sarcomere associated proteins (Chien 2000; Sjaastad, Wasserstrom et al. 2003). These pathways have been extensively reviewed (Hunter and Chien 1999; Chien 2000; Hoshijima and Chien 2002; Sjaastad, Wasserstrom et al. 2003).

Several methods have been described for invasive and non-invasive measurements of cardiac dysfunction and adapted to accurately gauge heart function in small rodent models. Among the invasive techniques are measurements of hemodynamics and blood pressure. Non-invasive procedures include magnetic-resonance imaging and echocardiography to measure left ventricular chamber dimensions, wall thickness and mass as evaluations of ejection fraction, contractile function and hypertrophy. The techniques used to evaluate cardiac function and their practical use in rodent models of heart failure are reviewed in depth elsewhere (Christensen, Wang et al. 1997; Doevendans, Daemen et al. 1998; Lorenz 2002; Roth, Swaney et al. 2002).

V. Models used to study ischemia, cardiomyocyte injury and myocardial infarction

Although the deleterious effects of *ischemia* on cardiomyocytes are often attributed to *hypoxia*, it is important to consider the definitions of these two terms. Myocardium requires an adequate oxygen supply to maintain oxidative phosphorylation, the only metabolic process that can provide sufficient high energy phosphates to sustain proper myocardial contraction

(Verdouw, van den Doel et al. 1998). Ischemia is an obstruction of blood flow to tissues which subsequently results in both decreased oxygenation and impaired removal of metabolic wastes (Verdouw, van den Doel et al. 1998). Myocardial ischemia is a condition in which there exists an imbalance in oxygen-demand and oxygen-supply resulting in anaerobic respiration and reduced contractile function (Verdouw, van den Doel et al. 1998). Hypoxia refers to oxygen starvation and can occur even in the presence of normal blood flow should there be a reduction in oxygen content or carrying capacity of the blood. Studies on the effects of ischemia must be interpreted differently from those on hypoxia because in the latter condition there is adequate removal of metabolic wastes (Verdouw, van den Doel et al. 1998).

A. In vitro models of global ischemia

Cultures of embryonic, adult or freshly isolated adult cardiomyocytes have been used to study biochemical and electrophysiological responses of cells to anoxia and hypoxia. Because these cardiomyocytes are isolated from surrounding matrix proteins, cytokines and cells, all changes detected can be attributed to cell-autonomous processes (Verdouw, van den Doel et al. 1998). In addition, co-cultures of cardiomyocytes with other cells such as lymphocytes present in myocardial infarction can help elucidate the relevance of cell-cell interactions during different phases of ischemia, reperfusion and myocardial infarction (Varda-Bloom, Leor et al. 2000). However major caveats in the use of cultured cardiomyocytes include ascertaining stability and viability of the cells as well as alterations in cellular phenotype or function resulting from procedures used to isolate the cells (Verdouw, van den Doel et al. 1998).

Another in vitro model used to investigate mechanisms of acute myocardial ischemia and hypoxia is the isolated Langendorff perfused heart. In this model, intact hearts are isolated from animals and blood or other oxygenated solution is forced into the coronary arteries via a cannula

placed in the aorta (Verdouw, van den Doel et al. 1998). The isolated hearts, after re-establishing coronary perfusion and allowing equilibration, can be used to study ischemia by restricting or ceasing perfusion as well as hypoxia by partially or completely eliminating oxygen from the perfusate (Verdouw, van den Doel et al. 1998). This system allows differentiation between the effects of hypoxia versus accumulation of metabolites from anaerobic respiration (Verdouw, van den Doel et al. 1998). However drugs tested in this system are often used at toxicity levels incompatible with living animals. Moreover, the Langendorff preparation does not allow left ventricular ejection of perfusate to measure cardiac output and is a non-working model (Verdouw, van den Doel et al. 1998). However, another model developed by Neely in which the cannula is inserted into the left atrium is a working isolated heart model that permits measurement of left ventricular outflow (Verdouw, van den Doel et al. 1998). Studies conducted in isolated perfused hearts are most useful in examining metabolites in the effluent perfusate. Isolated heart studies are most often performed in rat and rabbit hearts, but larger porcine hearts are also used.

B. In vivo surgical models of ischemia and myocardial infarction

Most studies of ischemia, MI and reperfusion occur in vivo though evidence for myocardial ischemia-reperfusion injury was first observed in isolated Langendorff perfused rat hearts (Black and Rodger 1996). Animal models used to study ischemia-reperfusion and MI in vivo include rats, dogs, pigs, rabbits and cats. Mouse models are becoming of greater interest recently because of the potential to study the effects of specific genes on these processes using knockout and transgenic mice (Black and Rodger 1996). The extent of the collateral circulatory network as well as area at risk for infarct are important determinants as to whether a particular

animal model will develop significant infarcts in response to ischemia. (Verdouw, van den Doel et al. 1998).

Chronically-instrumented conscious animals are the most accepted model of human myocardial ischemia (Verdouw, van den Doel et al. 1998). Severe ischemia is produced by inflating a balloon tied around a coronary artery, most often the left anterior descending or left circumflex coronary arteries, a method often referred to as coronary ligation (Verdouw, van den Doel et al. 1998). The ligation can be removed after a period of ischemia in order to study the effects of reperfusion or left permanently ultimately causing extensive MI, heart failure and death.

C. In vivo spontaneous models of ischemia and myocardial infarction

Animal models of spontaneous ischemia and myocardial infarction are exceedingly rare. Most atherosclerosis-prone hyperlipidemic animal models do not result in significant coronary artery occlusion, plaque rupture or thrombosis such as to progress to extensive MI and fibrosis much less cardiac dysfunction, heart failure and death.

Coronary atherosclerosis-prone Watanabe heritable hyperlipidemic (WHHL) rabbits selectively bred for high risk of MI and thus designated WHHLMI rabbits (Shiomi, Ito et al. 2003) are a recently characterized model of spontaneous ischemia and MI. Originally identified as a model of familial hypercholesterolemia and atherosclerosis due to an LDL receptor deficiency, the rabbits were selectively bred for high predisposition to coronary atherosclerosis and then again for susceptibility to MI (Shiomi, Ito et al. 2004). WHHLMI rabbits develop spontaneous severely occlusive (>90% of the luminal area) coronary atherosclerotic lesions associated with high incidence of fatal MI between the ages of 11 and 35 months (Shiomi, Ito et al. 2003). WHHLMI rabbits develop extensive transmural myocardial lesions with inflammatory

infiltration, functional and conduction defects recorded by ECG measurement, and death whereas their progenitor WHHL rabbits prone to coronary lesions develop only patchy focal infarctions. The incidence of MI increased from 23% to 97% after selective breeding (Shiomi, Ito et al. 2003), suggesting the presence of genetic modifiers that influence the penetrance and severity of MI in this model. However, WHHLMI rabbits still show no evidence of coronary thromboses or plaque rupture (Shiomi, Ito et al. 2004), the complications associated with CHD fatality in humans. Moreover, the length and variability of time over which these rabbits experience MI and eventually die from the cardiac insult may make it difficult to test the value of therapeutic interventions on lifespan.

Surprisingly, apoE(-/-)/LDLR(-/-) mice exhibit plasma total cholesterol levels similar to apoE(-/-) mice (Ishibashi, Herz et al. 1994), though they have a relative increase in IDL/LDL size particles. However apoE(-/-)/LDLR(-/-) mice have been reported to demonstrate increased atherosclerosis and older mice are more susceptible to hypoxic stress-induced MI and fibrosis relative to apoE(-/-) mice (Bonthu, Heistad et al. 1997; Caligiuri, Levy et al. 1999), again suggesting the increased atherogenicity of smaller (IDL/LDL) versus larger (VLDL) apoB-containing lipoproteins. Despite the occurrence of aortic lesions in 3-6 month-old, fat-fed LDLR(-/-) and apoE(-/-) mice, they usually do not develop spontaneous coronary arterial atherosclerosis or MIs by this age. Coronary occlusive lesions and associated myocardial fibrosis have been reported with variable penetrance in older (9-20 months of age) chow-fed apoE(-/-)mice (Nakashima, Plump et al. 1994; Calara, Silvestre et al. 2001) and 7-month-old, fat-fed apoE (-/-)/LDLR(-/-) mice(Caligiuri, Levy et al. 1999).

VI. Role of SR-BI in atherogenesis and coronary heart disease

A. SR-BI protects against atherosclerosis in LDLR(-/-) mice

SR-BI could influence atherogenesis via its participation in the early stages of RCT where it modulates efflux of excess cholesterol from peripheral cells to HDL (Rigotti, Miettinen et al. 2003). In addition, SR-BI may influence atherogenesis by its role in the late stages of RCT where it mediates delivery of HDL cholesterol to the liver for biliary excretion (Rigotti, Miettinen et al. 2003). Murine SR-BI deficiency produces a phenotype in which plasma HDL-cholesterol levels are increased, suggesting a potential pro-atherogenic role for SR-BI. However, biliary cholesterol secretion is concurrently reduced, indicating a possible anti-atherogenic function for SR-BI whereby it participates in the body's excretion of excess cholesterol. Thus, the influence of SR-BI on atherogenesis was unclear.

Several studies suggest that SR-BI activity is atheroprotective. When fed a lipid-enriched diet, SR-BI(-/-)/LDLR(-/-) and SR-BIatt/LDLR(-/-) mice exhibit elevated plasma total cholesterol levels and increased aortic root atherosclerosis compared to SR-BI-positive LDLR(-/-) mice (Huszar, Varban et al. 2000; Covey, Krieger et al. 2003; Rigotti, Miettinen et al. 2003). Other studies demonstrate that adenovirus-mediated transient or stable transgenic hepatic overexpression of SR-BI can reduce atherosclerosis in LDLR(-/-) mice fed atherogenic diets (Arai, Wang et al. 1999; Kozarsky, Donahee et al. 2000; Rigotti, Miettinen et al. 2003). However, experiments with human apoB Tg mice, demonstrate that moderate levels of hepatic SR-BI expression protect against diet-induced atherosclerosis whereas very high hepatic levels of SR-BI expression were not athero-protective, indicating that effects of SR-BI overexpression depend on both the model of atherosclerosis and level of expression and extremely high unphysiological levels of SR-BI expression may not be beneficial.

The generation SR-BI/apoE double KO (dKO) mice demonstrated unequivocally that SR-BI is protective against atherosclerosis, at least on the hypercholesterolemic apoE(-/-) mouse background. The dKO mouse presented a novel, unprecedented system in which to study atherosclerosis and coronary heart disease as discussed in detail below.

B. The SR-BI/apoE dKO Mouse: Model of accelerated atherosclerosis and CHD

1. Atherosclerosis

Atherosclerosis is dramatically increased in dKO mice with massive aortic root and sinus atherosclerotic lesions appearing at 4-7 weeks of age on a chow diet whereas neither SR-BI(-/-) or apoE(-/-) mice exhibited appreciable plaques at this young age (Trigatti, Rayburn et al. 1999). The lesions were cellular and often had cellular caps that stained positive for smooth muscle actin (Trigatti, Rayburn et al. 1999), attesting to the complexity of these plaques (Trigatti, Rayburn et al. 1999). Furthermore, dKO mice also developed advanced lipid-rich coronary occlusions resulting in arterial stenoses by 5-6 weeks of age, making them an unprecedented model of spontaneous coronary atherosclerosis. These coronary plaques also often contained acellular lipid-rich, possibly necrotic cores, fibrin deposition suggestive of hemorrhage and clotting, and cholesterol clefts (cholesterol crystals) characteristic of human lesions (Braun, Trigatti et al. 2002).

Mechanisms underlying the dramatic increase in atherosclerosis engendered by the deficiency of SR-BI on the apoE(-/-) background may include: 1) alterations in relative concentrations of pro-atherogenic (increased VLDL and abnormal HDL) and anti-atherogenic (loss of normal HDL) lipoproteins, 2) perturbations in flux of cholesterol in vessel walls, possibly attributable to direct SR-BI mediated efflux 3) inhibition of RCT pathways for excess cholesterol excretion, as demonstrated by decreased biliary cholesterol and abnormally large

HDL consequent to absence of hepatic SR-BI 4) influence of SR-BI deficiency in other organ and metabolic systems that affect the cardiovascular system (Trigatti, Rigotti et al. 2000; Rigotti, Miettinen et al. 2003).

2. Cardiac Phenotype

dKOs are visibly small (20% reduction in body weight) compared to healthy littermates. These mice appear sickly, ruffled and lethargic and die between 5 and 8 weeks of age with a mean lifespan of 6 weeks (Braun, Trigatti et al. 2002). By 5-6 weeks of age, the dKO mice exhibited grossly visible multiple MIs in the atrioventricular groove of the left ventricle in addition to other regions (Braun, Trigatti et al. 2002). Histochemical staining demonstrated the invariable presence of scar tissue in the upper outflow tracts as well as in various other locations such as the right ventricular wall, interventricular septum and apex. These lesions contained fibrotic connective tissue, dilated mononuclear inflammatory cells, indicating lymphocyte infiltration, and very few remaining myocytes. Staining with Oil Red O, a dye coloring neutral lipids, as well as F4/80, a monocyte/macrophage marker demonstated that the lesions contained extensive lipid deposits and macrophages; the colocalization of lipids and macrophages suggests the formation of foam cells (Braun, Trigatti et al. 2002).

By 5-6 weeks of age SR-BI/apoE dKO mice exhibit cardiac dysfunction and hypertrophic hearts compared to controls (1.6-1.8 fold increase in heart-to-body-weight ratio). MRI analysis corroborated that this enlargement was a result of increased tissue mass and not dilation. Hemodynamic measurements reveal that dKO mice had low blood pressure, low heart rate as well as 3-fold reduction in LV systolic contractility and diastolic relaxation (70% reduced ±dP/dt). LV end systolic volume was increased and ejection fraction ([LVEDV-LVESV]/LVEDV = volume of blood ejected with each stroke) was 50% reduced in dKO mice.

Electrocardiography demonstrates that dKO mice also have conduction defects (bradycardic episodes, widened QRS complexes, ST elevation and depression) that can be exacerbated by anesthesia.

3. Hematopoetic Phenotype

SR-BI dKO mice fed a chow diet are anemic and have both low hematocrit (about 34% for dKOs vs. 45-50% in controls) and hemoglobin counts.

As erythrocytes develop from hematopoetic progenitor cells in the bone marrow, they first expel their nuclei, becoming reticulocytes that have irregular morphology, still retain cytoplasmic organelles and are rarely found in the circulation. Reticulocytes then degrade their organelles, ribosomes and RNA, terminate protein synthesis and cell surface transferrin receptor expression, and undergo cytoskeleton rearrangements that allow them to develop into mature, biconcave discoid erythrocytes (Holm, Braun et al. 2002).

SR-BI/apoE dKOs exhibit severe reticulocytosis, with virtually 100% of their red blood cells being reticulocytes. The reticulocyte-like red blood cells of dKO mice are macrocytic, irregularly shaped, express transferrin receptors and RNA, and contain large intracellular membrane-bounded inclusions containing mitochrondria and ribosomes that stain positive with dyes that accumulate in acidic compartments, suggesting that these inclusions are autophago-lysosomes. The red blood cells of dKOs also contain increased unesterified cholesterol compared to controls (Holm, Braun et al. 2002; Braun, Zhang et al. 2003). In vivo, bone-marrow transplantation and transfusion studies as well as in vitro studies incubating the blood cells of dKO mice with normolipidemic serum and cholesterol sequestering drugs like methyl-cyclodextrin, result in the expulsion of these autophagolysome-like inclusions and restore a more normal erythrocyte phenotype, indicating that abnormally high cholesterol levels may be the

primary cause of the erythrocyte maturation defect (Holm, Braun et al. 2002). The circulating

reticulocytes apparently represent a defect in the normal pathway for red blood cell maturation,

because severely anemic patients often demonstrate erythrocytes containing

autophagolysosomes, and the reticulocytes of dKO mice retain the ability to expel these

inclusions and eliminate their RNA and transferrin receptor expression when placed in a

normolipidemic environment (Holm, Braun et al. 2002).

Hypoxia attributable to low hematocrit and reduced oxygen-carrying capacity of

erythrocytes may contribute to oxygen-starvation of cardiac muscle and subsequent MIs in dKO

mice. In addition, the large, aberrantly shaped red blood cells of dKO mice may also contribute

to obstructing vessels already stenosed by atherosclerotic plaques.

4. Lipoprotein Phenotype

Compared to apoE(-/-), dKO mice have 2-fold increased plasma total cholesterol levels

concentrated in the VLDL size fraction (Trigatti, Rayburn et al. 1999), suggesting that SR-BI,

which can bind apoB-containing lipoproteins may contribute either directly or indirectly to

clearance of VLDL-sized particles in apoE(-/-) mice. In addition, dKO mice have no normal-

sized HDL, but instead exhibit very large apoA-I-containing HDL-like lipoproteins, with even

greater heterogeneity than SR-BI(-/-) mice, in the VLDL and IDL/LDL size fractions (Trigatti,

Rayburn et al. 1999). Despite having similar or greater levels of IDL/LDL-size cholesterol, dKO

mice demonstrate a reduction in apoB in these fractions compared to apoE(-/-) mice (Trigatti,

Rayburn et al. 1999). Furthermore, dKO mice have extremely high unesterified cholesterol (UC)

to total cholesterol (TC) ratio (0.8 vs. 0.3 in apoE(-/-) and wild-type mice and 0.5 in SR-BI(-/-)

mice) and high lipoprotein surface-to-core lipid ratios (Braun, Zhang et al. 2003; Zhang, Picard

et al. 2005), resulting in the presence of aberrantly-shaped multilammelar, stacked and discoidal

lipoproteins in their plasma as well as the spherical particles seen in control mice (Braun, Zhang et al. 2003; Zhang, Picard et al. 2005). The abnormal ratios of lipoprotein UC/TC and surface-to-core lipids as well as malformation of lipoprotein particles is considered a key factor underlying both atherogenesis and erythrocyte maturation defects in these mice.

dKO mice exhibit reduced gallbladder bile cholesterol levels (47% compared to wild type) compared with SR-BI(-/-) mice (30% reduction compared to wild type) (Trigatti, Rayburn et al. 1999). These data suggest that dKO mice have impaired hepatic HDL cholesterol uptake, as indicated by the presence of abnormally large HDL particles, and concurrently have reduced biliary cholesterol levels, suggesting a block in the later stages of RCT (Trigatti, Rayburn et al. 1999).

5. Probucol

Treatment of SR-BI/apoE dKO mice with the lipid-lowering, anti-oxidant drug probucol essentially reversed all of the pathological phenotypes of these mice, restoring normal unesterified to total cholesterol ratios, lowering total cholesterol levels, restoring lipoprotein size and structure to that of apoE(-/-) mice, and restoring normal hematocrit and near normal erythrocyte morphology (Braun, Zhang et al. 2003). In addition, probucol treatment reduced aortic and coronary atherosclerosis as well as MI and cardiac hypertrophy, improved cardiac function and increased lifespan from a mean age of 6 weeks to that of 36 weeks (with some probucol-treated animals living out to 60 weeks of age) (Braun, Zhang et al. 2003). The pleiotropic effects of probucol as well as its multiple physiological activities make it difficult to discern the mechanisms (i.e. altering lipoprotein cholesterol metabolism and structure, restoring normal-sized HDL, correcting reticulocytosis and consequent hypoxia, or other anti-inflammatory and/or anti-oxidant effects) by which this drug corrects the dKO phenotype

(Braun, Zhang et al. 2003). However, these studies suggest that specifically altering lipoprotein metabolism may be one way in which to induce changes in, and elucidate mechanisms underlying, the pathophysiology of dKO mice.

6. SR-BI KO/*ApoeR61*^h/h^ Mice and diet-induced coronary heart disease

In 2005, Zhang et al. characterized another mouse model of CHD related to the SR-BI/apoE dKO mouse. Hypomorphic apolipoprotein E mice express a mutant form of apoE with a threonine to arginine mutation at position 61 (Thr61 →Arg61) at 2-5% of wild-type levels of apoE expression. When crossed to SR-BI(-/-) mice, the resultant SR-BI KO/*ApoeR61*^h/h^ mice were healthy, active and had cholesterol levels and lipoprotein profiles similar (but not identical) to SR-BI(-/-) mice as long the mice were maintained on a low-fat chow diet. However, upon feeding SR-BI KO/*ApoeR61*^h/h^ with a lipid-enriched diet, these mice demonstrated high plasma cholesterol levels, lipoprotein lipid ratios, cholesterol profiles and aberrant lipoprotein morphology resembling those of SR-BI/apoE dKO mice. Moreover, these mice exhibited dramatically increased aortic and coronary occlusive atherosclerosis, MI, conduction defects and cardiac dysfunction similar to dKO mice and died approximately 33 days after initiation of fat-feeding, regardless of age (25, 60 or 172 days of age) at which fat-feeding commenced.

The characterization of a diet-induced model of CHD resembling the SR-BI/apoE dKO phenotype presents a novel system in which to study the effects of therapies and procedures that could not otherwise be conducted on the tiny, juvenile dKO mice. Furthermore, these experiments suggest that small changes affecting lipoprotein metabolism can strikingly alter the incidence and rate of disease progression in dKO mice.

The SR-BI/apoE dKO and SR-BI KO/*ApoeR61*^h/h^ models recapitulate many of the hallmarks of human coronary heart disease including coronary occlusions, myocardial

infarctions, cardiac dysfunction, electrophysiological conduction defects and premature death.
These mice potentially represent an extremely unique, powerful small animal model in which to
study the etiology of CHD and evaluate the effects of various genetic, environmental and
physiological influences on the course of this disease. However there are two likely mechanisms
underlying the development of cardiac dysfunction and premature death in dKO mice: 1)
inflammatory heart disease and 2) occlusive coronary atherosclerotic ischemic heart disease. The
first set of studies in this thesis investigates the role of immunoglobulin-mediated, lymphocyte-
driven inflammation on the progression of heart disease in dKO mice. The next section of the
Introduction discusses the role of B- and T-lymphocytes on development of various forms of
cardiac pathophysiology.

Section Four: Investigating role of lymphocytes in cardiac pathophysiology of SR-BI/dKOs

I. Mouse models of Lymphocyte Deficiency

Several genetically immunodeficient mice have been crossed to apoE(-/-) and LDLR(-/-)
mice in order to elucidate the roles of various inflammatory cells in atherosclerosis. Severe
combined immunodeficient (SCID) mice have a naturally occurring autosomal-recessive
mutation in double-strand DNA break repair enzymes rendering them unable to convert
lymphocyte precursors into functional B- and T-lymphocytes, though the mutation is leaky and
the mice sometimes produce IgG and exhibit other lymphocyte functions (i.e graft rejection).
Mice with targeted mutations in either the RAG1 (Mombaerts, Iacomini et al. 1992) or RAG2
(Shinkai, Rathbun et al. 1992) demonstrate complete B- and T-lymphocyte deficiency
attributable to absence of recombination activating enzymes that induce double-strand DNA
breaks required for immunoglobulin and T-cell receptor gene rearrangements (VDJ
recombination). Targeted inactivation of an exon of the mu immunoglobulin gene produces mice

specifically deficient for functional B-cells (Kitamura, Roes et al. 1991). These lymphocyte-deficient mice still have functional null lymphocytes, or natural killer (NK) cells, that do not express rearranged surface receptors or immunoglobulins.

II. Role of lymphocytes in atherosclerosis

As mentioned above, a variety of inflammatory cells, including macrophages as well as lymphocytes, and their cytokines are thought to play an integral role in the development and progression of atheromas though the relative influences of these cells and their inflammatory cytokines on plaque development is not fully understood. The presence of CD4+ and CD8+ T-lymphocytes has been well documented in both early and more advanced fibrous human plaques. These cells recognize oxidized LDL (Stemme, Faber et al. 1995), and may induce expression of class II MHC antigens on the surface of smooth muscle cells in the lesion (Jonasson, Holm et al. 1986; Hansson, Jonasson et al. 1988; Stemme, Holm et al. 1992). The fibrous cap of these human plaques is comprised mainly of SMCs but can contain up to 20% T-cells, many of which express the surface marker HLA-DR characteristic of activation, whereas the lipid core contains predominantly macrophages (Jonasson, Holm et al. 1986; Stemme, Holm et al. 1992). Studies on the role of B-lymphocytes in atherosclerotic plaques have been even less conclusive. Patients with atherosclerosis exhibit serum autoantibodies to oxidized lipoproteins, and IgG that recognizes epitopes of Ox-LDL is found in plaques (Salonen, Yla-Herttuala et al. 1992; Yla-Herttuala, Palinski et al. 1994).

The presence of lymphocytes in human plaques is recapitulated in mouse models of atherosclerosis (Roselaar, Kakkanathu et al. 1996; Zhou, Stemme et al. 1996), thus providing models in which the roles of these cells can be studied via genetic manipulation. Despite the presence of circulating autoantibodies to oxidized lipoprotein found in mice (Palinski, Ord et al.

1994; Palinski, Tangirala et al. 1995; Daugherty, Pure et al. 1997), most groups failed to detect B-cells within atherosclerotic lesions (Roselaar, Kakkanathu et al. 1996; Major, Fazio et al. 2002), though one group claims to have found these lymphocytes in the plaques of atherosclerosis-prone apoE(-/-) mice (Zhou and Hansson 1999). Though, specific B-cell deficiency has been reported to reduce atherosclerosis in LDLR(-/-) mice (Major, Fazio et al. 2002) and immunization with antibodies against modified LDL has been shown to be beneficial, the precise role of B-cells and antibodies in atherogenesis is unclear.

Chow-fed apoE(-/-)RAG1(-/-) that are B- and T-lymphocyte-deficient have 50% reduction in the size of aortic atherosclerotic lesions compared to immunocompetent apoE(-/-) mice, however the plaques are limited to simple fatty streak lesions (Dansky, Charlton et al. 1997). This difference no longer exists when the mice are fed a Western diet that accelerates atherogenesis and produces more complex lesions (Dansky, Charlton et al. 1997; Daugherty, Pure et al. 1997). Furthermore, immunodeficient apoE(-/-)/SCID mice have a 73% reduction in aortic plaque size compared to immunocompetent apoE(-/-) controls. Transfer of $CD4^+$ T-lymphocytes into apoE(-/-)/SCID mice exacerbates lesion development (Zhou, Nicoletti et al. 2000), possibly as a result of localized IFN-γ secretion by T-cells in the artery wall (Zhou, Nicoletti et al. 2000).

These and other studies in immunodeficient apoE(-/-) mice suggest that lymphocytes play a role in the development of early lesions but are not necessary for the formation of complex plaques and that the influence of lymphocytes on atherogenesis is negated under the exacerbated hypercholesterolemia caused by fat-feeding (Song, Leung et al. 2001).

III. Role of lymphocytes in cardiomyocyte injury

In addition to participating in atherosclerosis in occlusive CHD, lymphocytes may directly contribute to ischemia-reperfusion injury, myocardial damage, cardiomyocyte apoptosis and death (Zwacka, Zhang et al. 1997; Maisel, Cesario et al. 1998; Frangogiannis, Mendoza et al. 2000; Rabb, Daniels et al. 2000; Varda-Bloom, Leor et al. 2000). Autoimmune myocarditis can be induced in susceptible mouse strains by cardiotropic viruses like coxsackie virus B3 (CVB3) that exhibit molecular mimicry of cardiac peptides or by inoculation with cardiac myosin (Neu, Rose et al. 1987). Lymphocytes have been shown to play a role in cardiomyocyte injury during autoimmune myocarditis (Guthrie, Lodge et al. 1984; Neu, Rose et al. 1987; Smith and Allen 1993; Pummerer, Luze et al. 1996; Gauntt and Huber 2003). T-cells have been implicated in the pathogenesis of both CVB3 and cardiac-myosin-induced myocarditis (Guthrie, Lodge et al. 1984; Smith and Allen 1991). T-cell depleted or deficient mice are protected against coxsackie virus induced autoimmune myocarditis, suggesting that these lymphocytes exacerbate the condition. Myocarditis can be elicited in mice simply by inoculating them with sensitized T-cells from virus-infected mice (Fairweather, Kaya et al. 2001). However, cardiac-myosin-immunized B-cell-deficient mice, though they have low or undetectable titers of plasma IgM, exhibit similar incidence and severity of myocarditis as immunocompetent littermates, indicating that B-cell-mediated priming of T-lymphocytes is not critical to development of myocarditis (Malkiel, Factor et al. 1999).

There is evidence that T-cells participate in the remodeling of cardiac tissues subsequent to MI. Anti-cardiac antibodies and sensitized T-lymphocytes have been detected in patients with acute MI (Agrawal, Gupta et al. 1978). In addition to mediating virus and cardiac-myosin-induced autoimmune inflammation, T-cells may contribute to ischemia-reperfusion injury and

cardiomyocyte damage. T-lymphocytes harvested from rats with coronary-ligation-induced MIs can specifically kill non-ischemic neonatal cardiac myocytes in culture (Varda-Bloom, Leor et al. 2000), indicating that these cells are sensitized to recognize cardiac antigens. In addition, normal rats inoculated with splenocytes from rats with coronary-ligation- and reperfusion-induced MIs and congestive heart failure, themselves go on to develop lymphocyte-mediated myocardial injuries (Maisel, Cesario et al. 1998). Lymphocyte-perpetrated cardiac insults can also result in cardiac failure in the absence of ischemia-induced MI. For example, disruption of the negative immunoregulatory receptor PD-1 in BALB/c mice produces unchecked lymphocyte-mediated immune responses resulting in dilated cardiomyopathy and death from congestive heart failure (Nishimura, Okazaki et al. 2001). Lymphocyte-deficient RAG2 mice are protected from the cardiac effects of PD-1 disruption (Nishimura, Okazaki et al. 2001).

The potential of role B- and T-lymphocytes in atherogenesis suggests that they may influence development of lesions in SR-BI/apoE dKO mice. As aforementioned, the hematoxylin and eosin (H&E) staining of dKO mouse hearts demonstrates the myocardial infiltration of dilated mononuclear inflammatory cells, however these immune cells have not yet been positively identified as lymphocytes. Of interest is the possibility that lymphocytes may be important in precipitating extensive myocardial injury in dKO mice, or that lymphocyte-driven, immunoglobulin-mediated myocarditis, as opposed to ischemic coronary-occlusive disease, is a critical underlying pathophysiological mechanism in this model.

As aforementioned, another likely cause for the cardiac dysfunction and premature death in dKO mice is occlusive coronary atherosclerotic ischemia, suggesting that altering the rate of atherogenesis might consequently change the course of heart disease in these mice. The second set of studies in this thesis investigates the effect of altering atherosclerosis on the heart disease

in dKO mice by manipulating hepatic lipase, an enzyme important in lipoprotein metabolism that has been shown to influence atherogenesis in both mice and humans. The next section of the Introduction briefly discusses hepatic lipase and its role in lipoprotein metabolism and atherosclerosis.

Section Five: Investigating alterations of lipoprotein metabolism in SR-BI/dKO mice

I. Hepatic Lipase

A. Structure, Function and Regulation

Hepatic lipase (HL), a 64 kDa lipolytic glycoprotein that hydrolyzes both triglycerides and phospholipids, is involved in processing chylomicron remnants (CRs), IDL and HDL (Thuren 2000; Jansen, Verhoeven et al. 2002; Santamarina-Fojo, Gonzalez-Navarro et al. 2004). Though primarily synthesized and secreted by the liver, HL is also present in steroidogenic tissues including the ovaries and adrenal glands (Jansen, van Berkel et al. 1978; Kuusi, Nikkla et al. 1979; Doolittle, Wong et al. 1987). Liver perfusion and cell culture studies indicate that HL and SR-BI are concurrently regulated (Wang, Weng et al. 1996) and that these two proteins work in conjunction to promote the uptake of HDL cholesterol (Marques-Vidal, Azema et al. 1994; Collet, Tall et al. 1999). In mice, HL circulates freely in the plasma whereas in humans, it is attached to endothelial cell-surface proteoglycans and released by heparin (Jansen, Verhoeven et al. 2002). HL expression has also been detected in macrophages (Gonzalez-Navarro, Nong et al. 2002)

HL participates in conversion of IDL to LDL and large lipid-rich HDL_2 to smaller HDL_3 thereby modulating the relative plasma distributions of these atherosclerosis-associated lipoprotein particle classes (Bamberger, Lund-Katz et al. 1985; Demant, Carlson et al. 1988; Clay, Newnham et al. 1992; Zambon, Austin et al. 1993; Connelly and Hegele 1998; Zambon,

Deeb et al. 1998; Nong, Gonzalez-Navarro et al. 2003). In addition to lipolytic activities, HL has

ligand-binding functions and may mediate interactions of lipoproteins with cell surface

proteoglycans and receptors such as SR-BI and LDL-receptor-related protein (LRP), thus

facilitating endocytosis and selective lipoprotein lipid uptake (Bamberger, Lund-Katz et al. 1985;

Krapp, Ahle et al. 1996; Ji, Dichek et al. 1997; Amar, Dugi et al. 1998; Dichek, Brecht et al.

1998; Dugi, Amar et al. 2000; Dichek, Qian et al. 2004; Lee, Kadambi et al. 2005).

B. Hepatic Lipase Studies In Vivo

Hepatic-lipase-deficient mice exhibit increased plasma total cholesterol, HDL cholesterol

and phospholipids but no change in triglyceride levels compared to wild-type mice. Like SR-BI(-

/-) mice, HL(-/-) mice have large, apoE-enriched HDL particles. Whereas wild-type mice

demonstrate a decrease in HDL in response to a lipid-enriched diet, HL(-/-) mice exhibit a

doubling of HDL (Homanics, de Silva et al. 1995). Despite its triglyceridase activity, HL(-/-)

mice are able to clear triglyceride-rich particles and tolerate acute fat-loading (Homanics, de

Silva et al. 1995).

Many studies have demonstrated that HL can influence atherogenic risk, though the

precise mechanisms through which HL exerts its effects are poorly understood. In humans, HL

modulates lipoprotein subclass distribution which determines atherogenic risk (Zambon, Deeb et

al. 1998). Increased HL activity is reported in familial hypercholesterolemia (Jansen, Verhoeven

et al. 2002), and has been linked to the formation of small, dense pro-atherogenic LDL particles

in humans (Zambon, Deeb et al. 1998). Conversely, reduced HL activity increases plasma HDL

cholesterol levels in both humans with congenital deficiencies and rodent models treated with

anti-HL antibodies or targeted mutations to the HL gene (Homanics, de Silva et al. 1995;

Lambert, Amar et al. 2000). HL overexpression can also lower the quantity of cholesterol carried

in pro-atherogenic apoB-containing lipoproteins and may thus exert atheroprotective effects (Dichek, Brecht et al. 1998; Dichek, Johnson et al. 2001).

Several studies in mice suggest that HL activity promotes atherogenesis. Both LCAT-Tg/HL(-/-) mice and apoE(-/-)/HL(-/-) mice exhibit significantly smaller atherosclerotic lesions in the aortic root as compared to apoE(-/-) mice, despite an increase in plasma total and VLDL cholesterol levels (Mezdour, Jones et al. 1997; Nong, Gonzalez-Navarro et al. 2003). Some of the most interesting data on the role of HL in atherosclerosis comes from apoE(-/-)/HL(-/-) mice which have cholesterol levels 1.8 times that of apoE(-/-) mice (900 vs. 500 mg/dl respectively), the increase concentrated primarily in the VLDL size fraction (Mezdour, Jones et al. 1997). Like SR-BI/apoE dKO mice, apoE(-/-)/HL(-/-) mice have elevated UC/TC ratios and cholesterol accumulates in abnormal, multilammelar lipoproteins mainly in the IDL and LDL fractions (Bergeron, Kotite et al. 1998). ApoE(-/-)/HL(-/-) mice have increased plasma HDL, and the HDL particles have enhanced ability to promote cholesterol efflux, compared to those of apoE(-/-) mice. Despite the increase in plasma total cholesterol levels, apoE(-/-)/HL(-/-) mice also demonstrate a 75% reduction in the size of aortic atherosclerotic plaques compared to apoE(-/-) mice (Mezdour, Jones et al. 1997).

As aforementioned, HL has distinct enzymatic lipolytic as well as ligand-binding functions, both of which may play a role in how HL regulates lipoprotein metabolism and atherogenic risk. Studies aimed at distinguishing the relative roles of HL's lipolytic activity from its ligand-binding functions draw on a catalytically inactive form of HL called HL-145G. Transient adenovirus-mediated and stable transgenic expression of HL-145G decreased plasma levels of HDL cholesterol as well as remnant and apoB lipoproteins independent of lipolysis (Amar, Dugi et al. 1998; Dugi, Amar et al. 2000; Dichek, Qian et al. 2004). A similar decrease in

cholesterol content of apoB-containing lipoproteins (VLDL, IDL) was reported in patients that

expressed an enzymatically defective form of HL as opposed to those who expressed no HL

protein at all (Zambon, Deeb et al. 2000). In addition, hepatic expression of HL-145G in apoE(-

/-)/HL(-/-) mice reduces plasma levels of cholesterol-rich remnants and decreases aortic

atherosclerosis; thus the presence of catalytically inactive HL protein appears more beneficial in

reducing atherosclerosis than completely eliminating HL expression altogether (Gonzalez-

Navarro, Nong et al. 2004). Furthermore, localized expression of macrophage HL in the arterial

wall increased lesion formation in apoE(-/-) and LCAT-Tg mice without altering plasma

lipoprotein metabolism (Nong, Gonzalez-Navarro et al. 2003), possibly by enhancing

macrophage uptake and retention of LDL cholesterol (Aviram, Bierman et al. 1988), suggesting

alternate mechanisms by which HL activity modulates atherogenesis.

Though evidence strongly indicates that dKO mice die prematurely from occlusive

coronary-atherosclerosis-induced ischemic heart disease and dysfunction, other processes may

contribute to the cardiac pathophysiology in these mice. Chapter 2 examines the role of B- and

T- lymphocytes on atherogenesis and B- and T-cell-driven immunoglobin-mediated

inflammatory myocarditis as a critical mechanism underlying heart disease in SR-BI/apoE dKO

mice. In chapter 3, we investigate the influence of altering lipoprotein metabolism on

atherosclerosis and heart disease by ablating hepatic lipase in SR-BI/apoE dKO mice.

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Chapter Two

Lymphocytes are not required for the rapid onset of coronary heart disease in SR-BI/apoE double knockout mice

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The author contributed all of the figures and text except as otherwise noted: Michael Picard performed echocardiography and contributed to Table I and Figure 4. Monty Krieger contributed to text and revisions. All experiments were performed in the laboratory of Monty Krieger with the exception of echocardiography performed at Massachusetts General Hospital.

Abstract

OBJECTIVE: SR-BI/apoE double knockout (dKO) mice exhibit many features of human coronary heart disease (CHD), including occlusive coronary atherosclerosis, cardiac hypertrophy, myocardial infarctions and premature death. Here we determined the influence of B- and T-lymphocytes, which can contribute to atherosclerosis, ischemia-reperfusion injury and cardiomyocyte death, on pathology in dKO mice. **METHOD and RESULTS:** The lymphocyte-deficient SR-BI/apoE/RAG2 triple knockout (tKO) mice and corresponding dKO controls generated for this study exhibited essentially identical lipid-rich coronary occlusions, myocardial infarctions, cardiac dysfunction and premature death (average lifespans:41.6±0.6 and 42.0±0.5 days, respectively). **CONCLUSIONS:** B- and T-lymphocytes and associated immunoglobulin-mediated inflammation are not essential for the development and progression of CHD in dKO mice. Strikingly, the dKO mice bred for this study (mixed C57BL/6x129-S4xBALB/c background, strain 2) compared to the previously described dKO mice (75:25 C57BL/6:129-S4 background, strain 1) had a shorter mean lifespan and steeper survival curve, characteristics especially attractive for studying the effects of environmental, pharmacologic and genetic manipulations on cardiac pathophysiology.

Introduction

Murine models of dyslipidemia, such as the apolipoprotein E (apoE) or LDL receptor (LDLR) knockout (KO) mice, have been used extensively to study atherosclerosis. However, even when subjected to high fat/high cholesterol diets, all but one of these hypercholesterolemia and atherosclerosis systems usually do not result in spontaneous development of occlusive coronary artery disease, MI, cardiac dysfunction or the premature death that are hallmarks of human coronary heart disease (CHD). The exception, mice doubly deficient for the HDL receptor scavenger receptor class B type I (SR-BI) and apoE (dKO) not only exhibit extensive aortic sinus and occlusive coronary arterial atherosclerosis (advanced plaques with fibrous caps, fibrin deposition and cholesterol clefts), but also suffer from severe CHD at a very young age (4-6 weeks)(Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003). The hearts of dKO mice are hypertrophic and exhibit LV dilation and multiple, large MIs. Severe cardiac dysfunction is demonstrated by multiple ECG abnormalities (ST elevation and depression, anesthesia-induced conductance abnormalities (e.g., bradyarrhythmias, AV blocks)), a 70% reduction in $\pm$dP/dT, and 50% reduced ejection fraction. The mice die between 5-8 weeks of age (mean 6 weeks). Thus, the many similarities in the CHD of dKO mice and humans raised the possibility that these mice may help to study the pathophysiology of CHD and genetic, pharmacologic and environmental approaches for its prevention and treatment.

B- and/or T-lymphocytes can modulate both development of atherosclerotic lesions and damage to cardiac tissue (Jonasson, Holm et al. 1986; Hansson, Jonasson et al. 1988; Boyle 1997; Dansky, Charlton et al. 1997; Laine, Kaartinen et al. 1999; Song, Leung et al. 2001; Hansson, Libby et al. 2002), and thus may contribute to pathophysiology in dKO mice. Disruption of the RAG1 or RAG2 genes (Mombaerts, Iacomini et al. 1992; Shinkai, Rathbun et

al. 1992) renders mice B- and T-cell deficient and reduces the rate of atherosclerosis development in low-fat, chow-fed apoE KO mice. Thus, lymphocytes contribute to, but are not essential for, murine atherogenesis. Lymphocytes' influence on lesion development in apoE KO mice is dramatically reduced when atherosclerosis is accelerated by a high fat/high cholesterol diet that exacerbates hypercholesterolemia (Zhou, Stemme et al. 1996; Dansky, Charlton et al. 1997; Daugherty, Pure et al. 1997). In addition to participating in atherosclerosis in occlusive CHD, lymphocytes may directly contribute to ischemia-reperfusion injury, myocardial damage and cardiomyocyte death (Zwacka, Zhang et al. 1997; Maisel, Cesario et al. 1998; Frangogiannis, Mendoza et al. 2000; Rabb, Daniels et al. 2000; Varda-Bloom, Leor et al. 2000). For example, anti-cardiac antibodies and sensitized T-lymphocytes have been detected in patients with acute myocardial infarction (Agrawal, Gupta et al. 1978). Lymphocytes have also been implicated in cardiomyocyte injury during autoimmune myocarditis(Guthrie, Lodge et al. 1984; Neu, Rose et al. 1987; Smith and Allen 1993; Pummerer, Luze et al. 1996; Gauntt and Huber 2003), and spontaneous RAG2-dependent, lymphocyte-mediated immune responses in mice lacking the negative immune regulator PD-1 cause dilated cardiomyopathy and death from congestive heart failure (Nishimura, Okazaki et al. 2001).

Here we have established the presence of T-cells in the hearts of dKO mice. We explored the roles of lymphocytes in murine CHD (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002) by generating and analyzing B- and T-cell-deficient SR-BI/apoE/RAG2 triple knockout (tKO) and corresponding dKO control mice. Complete deficiency of B- and T-lymphocytes had no discernable effects on the CHD and premature death. Thus, lymphocytes and associated antibody-driven inflammation are not essential for cardiac pathology in dKO mice.

Materials and Methods

Mice: SR-BI (-/-)/apoE (-/-)/RAG2 (-/-) triple knockout (tKO) mice and control SR-BI (-/-)/apoE (-/-)/RAG2 (+/+) double knockout (dKO, strain 2) mice were generated by crossing SR-BI(+/-)apoE(-/-) females (75:25 C57BL/6:129-S4 background, strain 1)(Trigatti, Rayburn et al. 1999) with SR-BI(-/-)/ RAG2(-/-) males (mixed C57BL/6x129-S4xBALB/c background) (Miettinen, Rayburn et al. 2001). The offspring SR-BI(+/-)/apoE(+/-)/RAG2(+/-) females were then crossed to sibling SR-BI(-/-)/apoE(+/-)/RAG2(+/-) males to generate littermate isolates of tKO and dKO (strain 2) mice as well as breeder mice that were used to maintain the colonies and generate subsequent experimental animals. DKO (strain 2) animals differed somewhat (see below) from the previously described 'strain 1' dKO (75:25 C57BL/6:129-S4 background) (Trigatti, Rayburn et al. 1999) mice. Unless otherwise noted all dKOs used were from strain 2.

Housing and Genotyping of Mice: Animals housed in micro-isolater cages in a Virus Antibody Free Facility were fed a standard chow diet ad libitum. Experiments followed MIT and NIH Animal Care guidelines. Experiments other than survival studies were conducted on mice 36 to 45 days of age. No significant differences were observed between males and females. Genotypes were determined by PCR as previously described (Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999; Miettinen, Rayburn et al. 2001).

Confirmation of the lymphocyte deficiency of RAG2(-/-) animals and qualitative assessment of the lymphoid tissues: Animals were phenotyped by flow cytometry (Becton Dickinson, FACScan, CellQuest software) of isolated splenocytes using the following antibodies (BD Pharmingen, La Jolla, CA): anti-B220-FITC (B-cells); anti-CD11b-FITC (monocyte/macrophages); and anti-TCRβ-PE, anti-CD8-FITC, and anti-CD4-PE (T-cells).

Splenocytes derived from all animals tested positive for monocyte/macrophages. However

RAG2(-/-), but not RAG2(+/+) animals, lacked mature B- and T-lymphocytes (data not shown).

Analysis of circulating leukocytes: Flow cytometric analyses of circulating leukocytes

with markers for CD4, CD8, TCR-β and B220 confirmed the presence of B- and T-cells in dKOs

and the absence of these cells in tKOs. As expected, both dKOs and tKOs had cells staining

positive for the monocyte/macrophage marker CD11b. Whole blood collected from mice was

gently pelleted (1500 rpm for 5 minutes) and erythrocytes lysed using 144 mM NH_4Cl/17 mM

Tris-HCl pH 7.4. Cells were resuspended in 500 μL PBS/0.5%BSA and blocked with rat anti-

mouse CD16/CD32 (Mouse BD Fc Block™, BD Pharmingen 553141) for 10 minutes (1 μL of

blocking antibody to 50 μL of cell suspension). Cells were then stained with complementary

combinations of the following antibodies, all from BD Pharmingen: PE-conjugated hamster-anti-

mouse TCR-β (#553172), FITC-conjugated rat-anti-mouse CD45R/B220 (#553087), FITC-

conjugated rat-anti-mouse CD11b (#557396), FITC-conjugated rat-anti-mouse CD4 (L3T4,

#553046) and PE-conjugated rat-anti-mouse CD8a (Ly-2, #553032) for 20 minutes at a final

dilution of 1:200 in PBS/0.5%BSA. All antibody incubations were carried out on ice. Cells were

washed 1x with PBS/0.25% BSA, resuspended in 300 μL PBS/0.5%BSA and analyzed on the

BD FacScan flow cytometry system (data not shown).

Lymphoid tissue: The tKO, but not the dKO, mice did not have a fully developed

thymus or normal-sized lymph nodes (as judged by visual inspection, data not shown).

Qualitative and preliminary quantitative (gravimetric) analysis indicated that there was little or

no difference in the sizes or weights of the spleens of the dKO and tKO mice.

Analysis of plasma total IgG concentrations: Plasma total IgG concentrations of dKO and tKO mice were measured using the Easy-Titer® Mouse IgG assay kit (Pierce #23300) according to kit instructions.

Plasma Lipid Composition and FPLC Lipoprotein Total Cholesterol Profiles: Plasma from nonfasting animals was obtained from blood drawn at sacrifice by centrifugation at 14,000 rpm (Spectrafuge 16M) for 10 minutes at 4°C. Lipid concentrations were determined by enzymatic assays on plasma diluted 1:4 in phosphate buffered saline (PBS) using kits (Cholesterol C-II, Free Cholesterol E and Phospholipids B) from Wako Chemical USA Inc., (Richmond, Virginia, USA). Plasma was diluted 1:4 in elution buffer (154 mM NaCl, 1 mM EDTA, pH 8) and subjected to FPLC analysis (total cholesterol determined for each fraction) either immediately following collection or after storage at 4°C as previously described(Rigotti, Trigatti et al. 1997).

Echocardiography: M-mode and two-dimensional transthoracic echocardiography were performed on lightly sedated mice (pentobarbital, 25 mg/kg IP) using a 13-MHz linear array ultrasound transducer with the instrument adjusted for maximal frame rate (Acuson Sequoia, Siemens Medical, Mountain View, CA). M-mode echocardiograms were obtained at mid-left ventricle level. Two-dimensional (2D) images consisted of long-axis views of the left ventricle (LV) and short-axis images at basal, mid and apical portions of the left ventricle. The long axis length of the LV was measured from the 2D long axis view and all other measurements were performed on the short axis at the mid LV level. The fractional shortening was calculated as a measure of LV systolic function (Weyman 1994) and the LV mass was calculated by the D^3 method.

Gravimetry: Mice were weighed and euthanized by Avertin overdose. Intact hearts were removed and rinsed clean of blood with heparin/PBS (10 units/ml) (heparin sodium salt, Sigma).Whole hearts were then blotted dry and weighed using an analytical balance.

Histology: Mice were anesthetized with 2.5% Avertin. Blood was drawn via heart puncture with a heparinized syringe or from the retro-orbital plexus with a heparinized capillary tube. Hearts were collected and rinsed in heparin/PBS, immersed in cold Kreb's buffer (120 mM NaCl/25 mM $NaHCO_3$/3.3 mM KH_2PO_4/0.8 mM K_2HPO_4/1.2 mM $MgCl_2$/1.2 mM $CaCl_2$/10 mM glucose, pH 7.4) for 30 minutes, embedded in Tissue-TeK OCT compound (Sakura Finetek) and fresh frozen using dry ice/isopentane. Serial cryosections (10μm) cut onto Fisher MicroProbe Plus slides (Fisher Scientific) were stained with Masson's Trichrome (Sigma)(Braun, Trigatti et al. 2002) or Oil red O and hematoxylin (Trigatti, Rayburn et al. 1999).

Immunohistochemistry: Cryosections (10μm) fixed in ice-cold acetone for 10 minutes were stained for macrophages (F4/80 (Serotec, diluted 1:10)), B-cells (anti-CD19, clone ID3 Pharmingen diluted 1:10)) and T cells ((anti-CD3, clone 145-2C11 Pharmingen diluted 1:10) [Anti-lymphocyte antibodies stained positive control spleen sections.]. Sections were blocked for 30 minutes at room temperature with 10% non-immune serum in PBS from the host animal of the secondary antibody and incubated with the primary antibody in PBS containing 2% serum at 4°C overnight. Negative controls omitted primary antibodies. Biotinylated secondary antibodies (Pharmingen diluted 1:50) goat-anti-rat Ig (GAR, multiple adsorption) or mouse-anti-hamster were visualized with alkaline phosphatase Vectastain ABC kit and Vector Red substrate (Vector). T-lymphocytes were also detected in 4% paraformaldehyde fixed (6 minutes) cryosections blocked (1 hour, room temperature, 10% normal goat serum (NGS) in PBS) and

immunostained with rat-anti-mouse CD4 L3T4 monoclonal antibody (Pharmingen, diluted 1:40) in PBS containing 1% NGS at 4°C overnight followed by GAR and visualization with Vectastain Elite ABC kit (diaminobenzidine substrate,Vector). Negative controls employed isotype-matched control Rat IgG_{2a}.

Statistical Analysis: $P \leq 0.05$ was considered significant (2-tailed, unpaired student's t test or ANOVA test, GraphPad Prism 4.0). Survival curves employed the Kaplan-Meier function with the logrank test (GraphPad Prism 4.0). Values are expressed as mean ± SEM.

Hematocrits: Hematocrits of tKO and dKO mice aged 34 – 37 days did not differ significantly from each other but both differed significantly from control values. The effects of the dKO genotype on hematocrit have been reported elsewhere (Holm, Braun et al. 2002).

Control (Percent)	tKO (Percent)	dKO (Percent)
44.4 ± 1.9 (n=6)	30.0 ± 0.8 (n=7)	26.5 ± 1.8 (n=6)

*P (tKO vs. dKO) = 0.0929

**Controls are pooled SR-BI+/+ApoE-/-Rag2-/- and SR-BI+/+ApoE-/-Rag2+/+ mice (hematocrit values of Rag2-/- and Rag2+/+ controls were not significantly different from each other and these values were pooled together).

Results

At 5-6 weeks of age, the hearts of dKO mice exhibit extensive fibrosis around the ventricular outflow tract and patchy MIs in the apex, right ventricular wall and interventricular septum(Braun, Trigatti et al. 2002). Figure 1A shows a representative trichrome-stained longitudinal section (41 day-old female). Healthy myocardium stained red and fibrotic tissue blue. Although hematoxylin and eosin staining suggested extensive inflammatory infiltration(Braun, Trigatti et al. 2002), the role of B- or T-lymphocytes in this CHD had not been directly examined.

Immunohistochemical analysis of lymphocytes in dKO hearts

The pan B-cell marker anti-CD19 did not stain heart sections (not shown), indicating these cells were absent, but not eliminating a potential role for immunoglobulin-mediated autoimmune inflammatory heart disease (Rose and Hill 1996; Rose and Hill 1996). However, clusters of cells that tended to coincide with regions of immune-infiltrated and damaged tissue stained with antibodies against the T-cell marker CD4 (Figure 1B and C), but not with isotype control antibodies (not shown), raising the possibility that T-lymphocytes might contribute to myocardial injury. Others have suggested that quantitation of lymphocyte infiltration can be confounded by the antibody and immunohistochemical technique employed (Roselaar, Kakkanathu et al. 1996); therefore no attempt was made to count positively staining cells and correlate them with the extent of tissue damage.

To definitively determine if lymphocytes play a role in the cardiac pathophysiology, we generated and characterized a line of SR-BI(-/-)/apoE(-/-)/RAG2(-/-) triple knockout (tKO) mice with total lymphocyte deficiency (Shinkai, Rathbun et al. 1992). The absence of B- lymphocyte function in tKOs was confirmed by analysis of plasma levels of IgG in dKO and tKO mice. DKO

mice had plasma total IgG titers similar to those of SR-BI+/+ApoE-/-Rag2+/+ control mice (0.51±0.12 vs. 0.60±0.089 mg/mL; P=0.608). Plasma total IgG titers for tKO mice and SR-BI+/+ApoE-/-Rag2-/- control mice fell below the range of detection of the assay kit used, even at sample concentrations that were 5 and 50 times higher than that used for the plasma of Rag2+/+ control mice, establishing the absence of detectable IgG in tKO mice as expected.
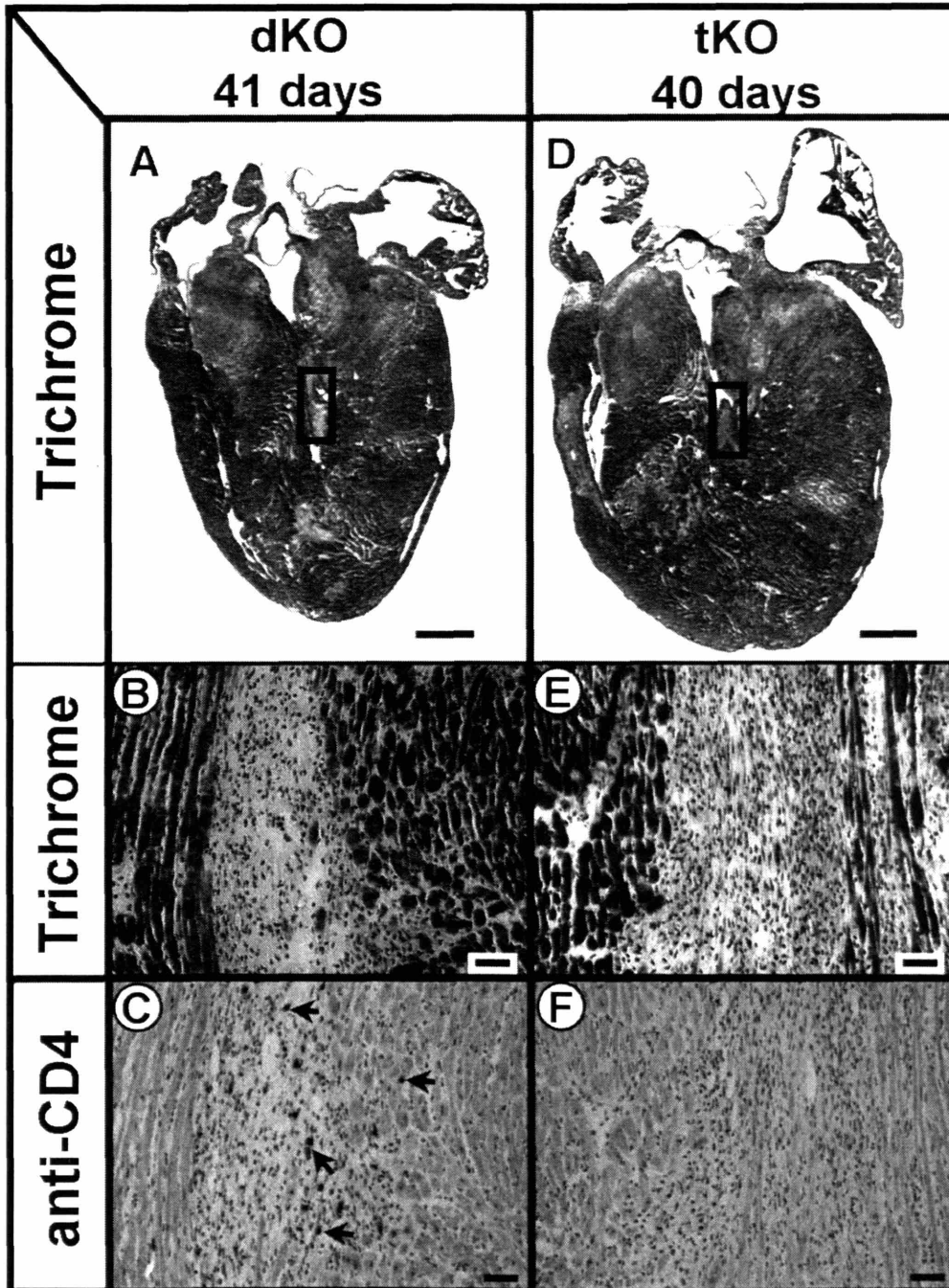
Figure 1: Histology of dKO and tKO hearts. Low (A and D) and high (B and E) magnification images of longitudinal Masson's trichrome stained heart sections (healthy myocardium, red; fibrotic tissue, blue) from 41 day-old dKO (A and B) and 40 day-old tKO (D and E) mice. Sections adjacent to those in panel B (C) and panel E (F) were stained with an anti-T-cell antibody (anti-CD4) (arrows indicate positive cells). Bars=1 mm (A and D) or 50 μm (B, C, E and F).

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Plasma lipids and lipoprotein profiles

Disruption of the RAG2 (or RAG1) gene has been shown to lower plasma cholesterol levels in apoE (-/-) or LDL receptor (-/-) mice (Dansky, Charlton et al. 1997; Reardon, Blachowicz et al. 2001; Reardon, Blachowicz et al. 2003). Thus, we compared the plasma lipid levels in dKO and tKO mice, because dyslipidemia (hypercholesterolemia, abnormally high unesterified to total cholesterol (UC/TC) ratio) is thought to be responsible for occlusive atherosclerosis and CHD in dKO mice (Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003). Consistent with previous reports (Dansky, Charlton et al. 1997; Reardon, Blachowicz et al. 2001), there was a small but significant reduction in plasma unesterified cholesterol (UC) and phospholipids in tKO animals compared to dKOs (UC (mg/dL):655±30vs.767±40 respectively; P=0.03 and phospholipids (mg/dL): 586±28vs.681±34 respectively; P=0.04), but no statistically significant differences in plasma total cholesterols (TC) (909±38vs.1001±54 mg/dL respectively; P=0.17) or UC/TC ratios. FPLC chromatographic analysis of plasma lipoproteins (e.g., Figure 2) revealed no major differences in the lipoprotein total cholesterol profiles (n=4 for each group). The slightly higher amounts of cholesterol in the VLDL-size fractions from the dKO mice relative to tKO mice is similar that reported by Reardon et al for apoE KO mice (Reardon, Blachowicz et al. 2001). The minimal alterations in plasma lipoproteins by RAG2 gene disruption appear unlikely to differentially influence atherosclerosis and CHD in dKO and tKO mice.
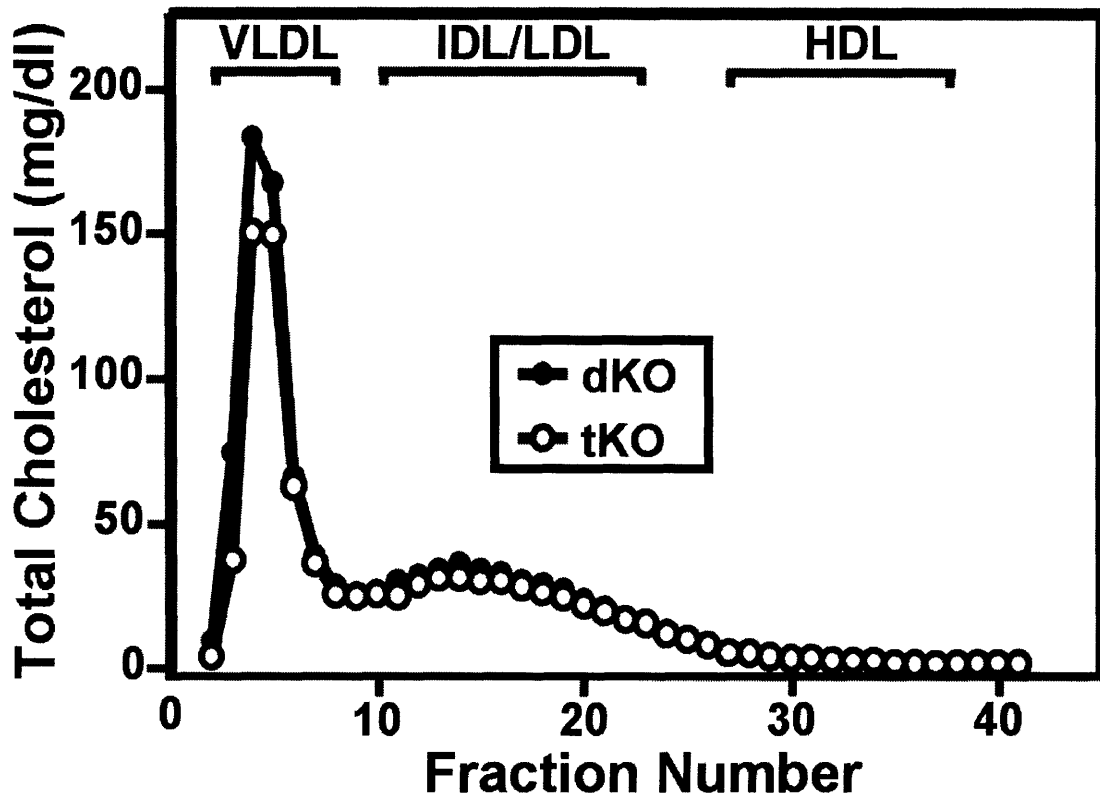
Figure 2: Lipoprotein cholesterol profiles from dKO (filled circles) and tKO (open circles) mice. Plasma lipoproteins from 39 day-old dKO and tKO mice were size fractionated (Superose 6-FPLC) and total cholesterol in each fraction (mg/dl plasma) determined. Chromatograms are representative of multiple, independent determinations.

Cardiac Histopathology

Trichrome staining of ~6 week-old tKO hearts demonstrated extensive myocardial fibrosis similar to that in dKO mice (Figure 1D, compare with 1A). As in dKO mice(Braun, Trigatti et al. 2002), neutral lipid deposits (oil red O staining) and macrophage foam cell formation (F4/80 immunohistochemical staining) coincided with regions of fibrosis (not shown). The absence of CD4^{+} cells in the hearts of tKO mice (Figure 1E and F) confirmed their B- and T-cell deficiencies (RAG2 (-/-) phenotypes). Thus, neither B- nor T-cells were required for extensive fibrosis/infarction.

Figure 3 shows that lipid-rich occlusive atherosclerotic lesions in coronary arteries of tKO mice were similar to those in dKO mice. There were no significant differences in the numbers of non-occluded, partially occluded (< 50% occluded) and completely occluded (> 50% occluded) coronary arteries (n=4 for each group). Severely occluded arteries were prevalent in areas with myocardial fibrosis, especially near the upper ventricular outflow regions. Some occlusions contained significant cellular components; others were predominantly acellular. Thus, lymphocytes did not markedly influence the nature of the occlusive coronary disease and cardiac damage.
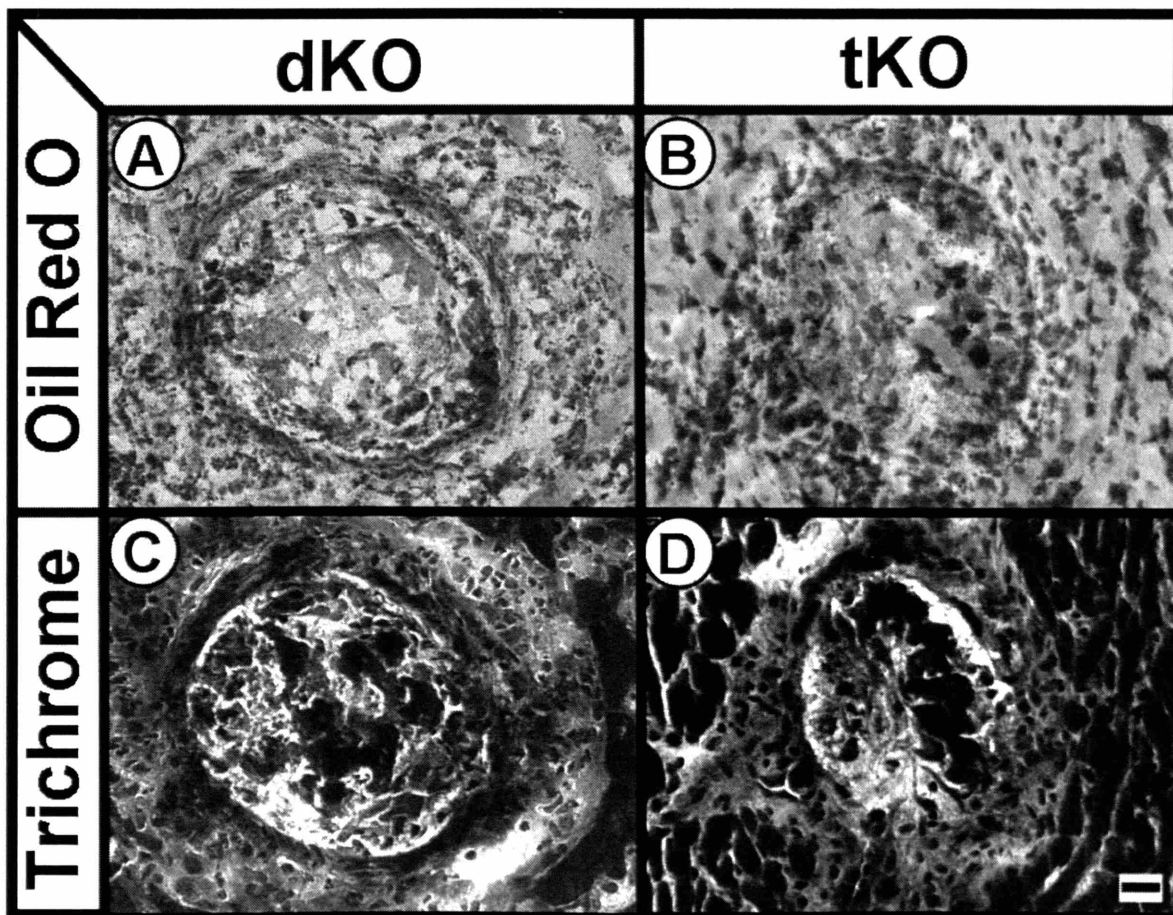
Figure 3: Coronary occlusions in dKO and tKO mice. Oil red O (A and B) and trichrome (C and D) stained coronary artery sections from 39 day-old dKO (A and C) and 43 day-old tKO (B and D) mice. Bar=20 μm.

Cardiac Structure and Function

Echocardiography was used to assess cardiac function and hypertrophy (Manning, Wei et al. 1994; Gardin, Siri et al. 1995; Tanaka, Dalton et al. 1996; Gao, Dart et al. 2000; Liao, Ishikura et al. 2002) in lightly anesthetized (pentobarbital, 25 mg/kg IP) dKO and tKO mice and their SR-BI positive littermate controls (Table I). No significant differences between RAG2(-/-) and RAG2(+/+) control mice were observed, therefore all controls were pooled.

The values of posterior wall thickness (PWT) and LV mass (absolute and normalized to body weight) for the dKO and tKO mice were not significantly different from each other, but were greater than those of controls. The quantitative effects of CHD on the PWT seen here are similar to those in other forms of cardiac dysfunction (Tanaka, Dalton et al. 1996). The echocardiographically determined increases in heart size in the dKO and tKO mice were confirmed by gravimetric analysis (Table I). The heart-to-body weight ratios of the dKO and tKO mice were similar (P=0.2405) and 1.9- and 1.8-fold larger than for age-matched controls (P<0.0001). The body weights for the dKO and tKO mice were similar (16.8±0.8 g (n=19) and 16.4±0.4 g (n=17), respectively) and smaller than that of their littermate controls (18.3±0.6 g (n=13)). The smaller size of SR-BI (-/-)/apoE (-/-) mice compared to SR-BI-positive controls was reported previously (Braun, Trigatti et al. 2002).

Left ventricular fractional shortening ([LVEDD-LVESD]/LVEDD; 'FS') was used as a measure of the heart's systolic function. Figure 4 shows representative M-mode and 2D echocardiographic images of control, dKO and tKO mice. The mean FS for control mice was 50.1% ±5.0%, corresponding to data reported by several groups for both conscious (Yang, Liu et al. 1999) and anesthetized (Gardin, Siri et al. 1995; Gao, Dart et al. 2000; Liao, Ishikura et al. 2002) mice. There were clear defects in LV wall motion in dKO and tKO mice compared to controls (Table I and Figure 4). Both dKOs and tKOs had approximately 50% lower FS than

control mice, demonstrating a substantial deficit in cardiac contractility. Importantly, there was

no significant difference in FS between the dKO and tKO mice (26.1%±6.2% vs.

24.3%±4.0%;P=0.7960). Reported FS values of approximately 10-30% for surgically-induced

models of myocardial infarction and congestive heart failure (Gao, Dart et al. 2000; Liao,

Ishikura et al. 2002), as well as genetically-induced dilated cardiomyopathy (Nishimura, Okazaki

et al. 2001), correspond well to the low values seen in the dKO and tKO mice.

During echocardiography both dKO and tKO mice exhibited significantly lower heart

rates than those of control mice, although they did not differ from each other (395±19 vs.386±24

bpm respectively). Numerous studies suggest that anesthesia administered before

echocardiography can depress heart rate (Yang, Liu et al. 1999; Takuma, Suehiro et al. 2001;

Roth, Swaney et al. 2002) that in turn can influence echocardiographically determined

parameters of cardiac function, including contractility and fractional shortening (Takuma,

Suehiro et al. 2001; Roth, Swaney et al. 2002). Moreover, these effects vary with type and

dosage of anesthetic and strain of mice studied (Yang, Liu et al. 1999; Takuma, Suehiro et al.

2001; Roth, Swaney et al. 2002). However, some studies of mice undergoing conscious

echocardiography demonstrate perturbations in heart rate, including bradycardia before training

and tachycardia after training (Yang, Liu et al. 1999; Roth, Swaney et al. 2002). The mean heart

rate for our control mice anesthetized with a low dose of pentobarbital (522±24 bpm) was similar

to that measured for conscious mice during echocardiography as reported by Takuma et al

(Takuma, Suehiro et al. 2001) and unrestrained mice undergoing telemetry (Roth, Swaney et al.

2002), suggesting the low dose of anesthetic used did not depress heart rate in the control mice.

However, electrocardiography of dKOs has shown these mice to be hypersensitive to certain

anesthetic agents (Braun, Trigatti et al. 2002). Their rapid deterioration in health and short

lifespans, prevent dKO mice from enduring the stress of multiple training sessions required for conscious, unanesthetized echocardiography. Previous electrocardiographic studies have shown that conscious unanesthetized dKO mice exhibit reduced heart rates as they approach the terminal stage of disease ((Roselaar, Kakkanathu et al. 1996) and data not shown)[_footnote: Most of the dKO and tKO mice (12/16) exhibited electrocardiographic abnormalities during echocardiographic analysis (e.g, see ECG tracings in left panels of Figure 4 and data not shown)._]. Therefore, even if echocardiographic data were obtained without anesthesia, it is likely that the dKOs and tKOs would still have exhibited heart rates significantly lower than control mice. Given these complications, it is not possible to distinguish with certainty the effects of anesthesia from those of advanced disease on heart rate and echocardiographic data obtained from dKOs and tKOs, though it seems likely that the abnormalities observed were due, at least in part, to the underlying pathology and not solely consequences of enhanced sensitivity of these mice to anesthetics.

Table I: Echocardiographic and gravimetric analyses

	FS (x100)	PWT (mm)	PWT/BW (mm/g x 100)	LV mass (Grams)	LV mass/BW (x100)	HW/BW ratio (x100)	Heart Rate (Bpm)
Control	50.1±5.0 (10)	0.65±0.03 (10)	3.04±0.19 (6)	0.062±0.006 (10)	0.266±0.015 (6)	0.509±0.010 (13)	522±24 (10)
dKO	26.1±6.2 (8)	0.80±0.04 (7)	4.96±0.52 (4)	0.114±0.007 (7)	0.761±0.134 (4)	0.979±0.028 (19)	395±19 (10)
tKO	24.3±4.0 (10)	0.76±0.03 (10)	4.64±0.15 (7)	0.106±0.013 (10)	0.650±0.094 (7)	0.933±0.026 (17)	386±24 (8)
P ANOVA	0.0014	0.0128	0.0003	0.0019	0.0036	<0.0001	0.0002
P (dKO vs. tKO)	0.7960	0.4615	0.4688	0.6505	0.5054	0.2405	0.7774

FS = fractional shortening; PWT=posterior wall thickness; BW=gravimetric body mass; HW=gravimetric heart mass. Results for RAG2(+/+) and RAG2(-/-) SR-BI-positive littermates of dKO and tKO mice were combined as controls. N in parentheses. P ANOVA all three groups; P (dKO vs. tKO) from unpaired student's t-test.
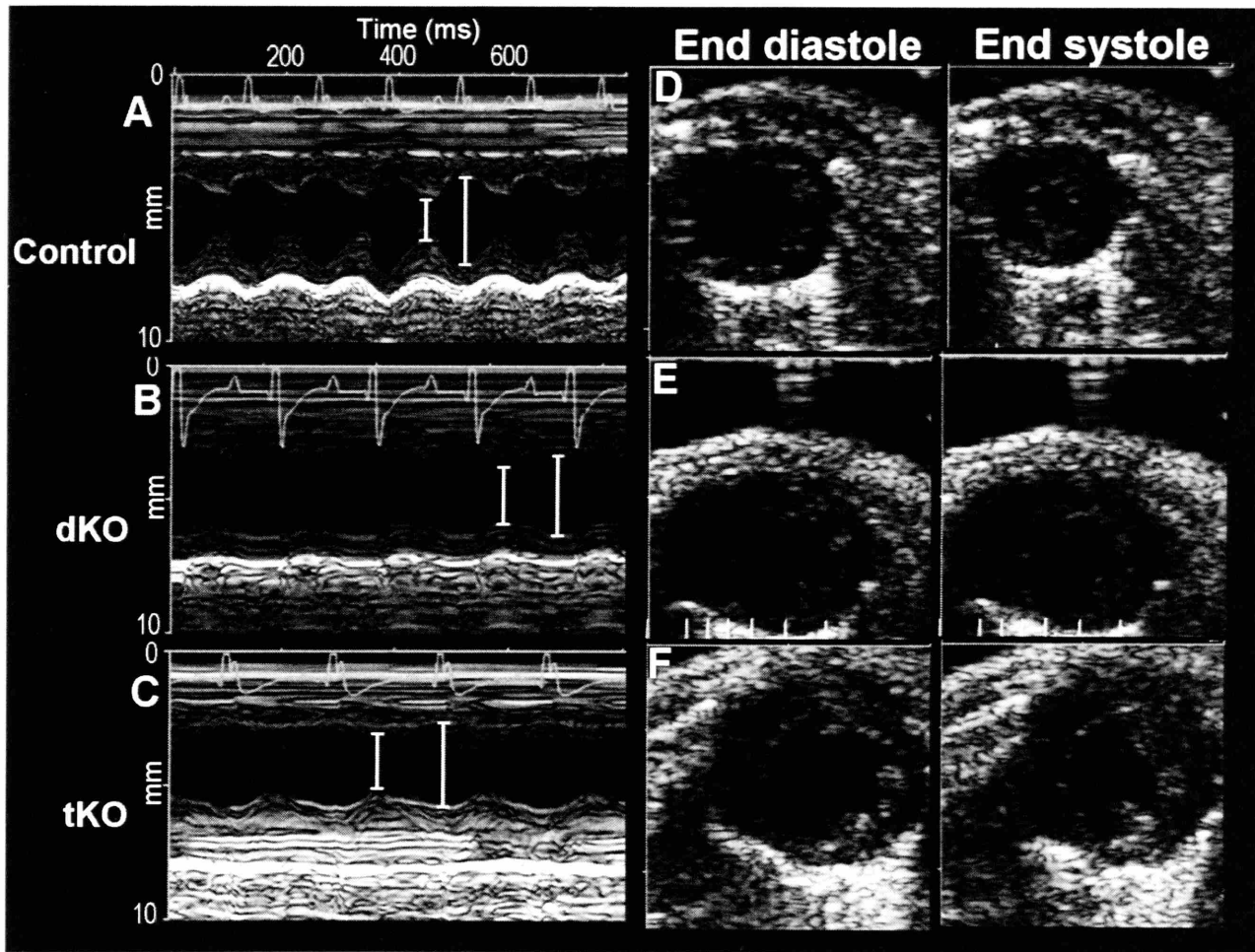
Figure 4: M-mode and 2D echocardiograms of control, dKO and tKO mice. Mice were anesthetized and subjected to transthoracic echocardiography. Representative M-mode (A-C) and 2D (D-F) images taken from 39 day-old control (SR-BI(+/-)/apoE(-/-)/RAG2(-/-)) (A and D), 43 day-old dKO (B and E) as well as 39 day-old (C) and 41 day-old (F) tKO mice. M-mode and 2D images for control and dKO mice are each from single animals, images for tKO mice are representatives from two mice (C and F, respectively).

Effects of immunodeficiency on the survival

We next determined if inactivation of the RAG2 gene altered the life expectancies of these mice. Figure 5 shows that the survival curves for dKO (strain 2,black) and tKO (red) mice were virtually identical (mean survival times (days): 42.0±0.5 (n=65) and 41.6±0.6 (n=35), respectively (P=0.3594 Logrank test)). RAG2-deficient mice have been reported to exhibit a normal lifespan when maintained in a pathogen-free facility such as that used here (Rideout, Hochedlinger et al. 2002). Thus, while it is possible that absence of an influence of RAG2 deficiency on survival of dKO mice could have arisen because of compensatory effects on the kinetics of the fatal pathologies normally exhibited by dKO mice and on independent processes due to the immunodeficiency, this seems unlikely. Therefore, B- and T-cells do not significantly contribute to the fatal pathophysiology in dKO mice.

The survival curve of the dKO mice bred for this study (strain 2, black) significantly differed from that of the previously described SR-BI(-/-)/apoE(-/-) dKO mice (strain 1, blue) (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002) revealing that genetic background variation can significantly impact lifespan. The mean survival of strain 1 (45.9±0.9 days, n=61) was significantly longer than that of strain 2 (P=0.0001). Remarkably, 82% of strain 2 dKOs died within an exceptionally narrow 9-day window (38–47 days of age), whereas the comparable range in strain 1 mice was 16-days (40–56 days). These differences are presumably due to their different genetic backgrounds (see Methods).
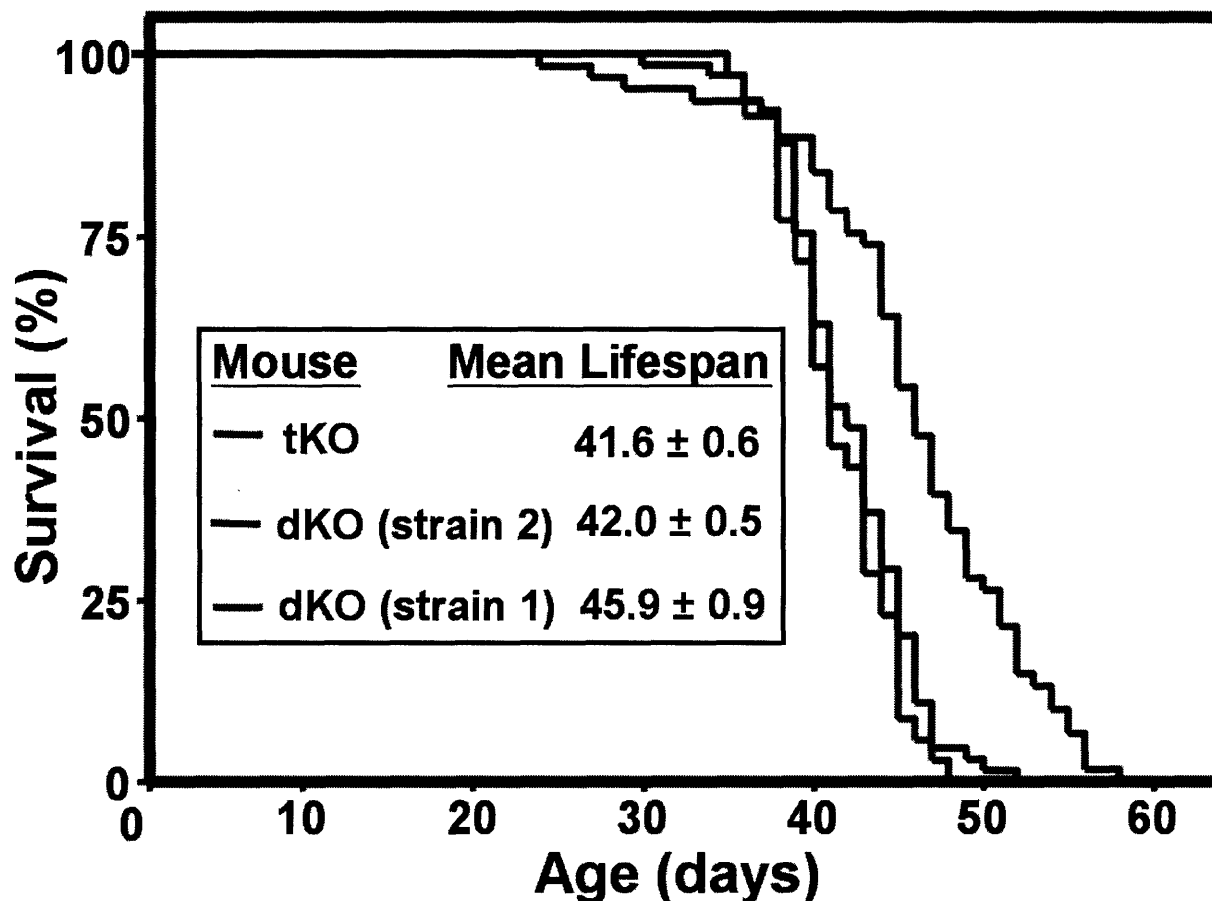
Figure 5: Effect of RAG2 deficiency and genetic background variation on survival of dKO mice. Survival curves from tKO (red line, mixed C57BL/6x129-S4xBALB/c background, n=35), dKO (strain 2) (black line, C57BL/6x129-S4xBALB/c background, n=65) and dKO (strain 1) (blue line, 75:25 C57BL/6:129-S4 background, n=61) mice.

Discussion

Low-fat-chow-fed SR-BI/apoE dKO mice rapidly develop fatal occlusive atherosclerotic CHD that closely resembles that in humans(Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003). The simultaneous absence of apoE and the HDL receptor SR-BI, both of which have been shown to protect against murine atherosclerosis(Zhang, Reddick et al. 1992; Reddick, Zhang et al. 1994), is responsible for hypercholesterolemic dyslipidemia exceeding that observed in either single KO mouse. Advanced atherosclerotic plaques can be seen in the hearts of these mice as early as 4-4.5 weeks of age (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002)and appear to be critically important for CHD pathogenesis (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003)

Here we examined the role of lymphocytes in the CHD of dKO mice by generating immunodeficient tKO mice, which are dKO mice lacking B- and T-cells due to homozygous disruption of the RAG2 recombinase gene (Shinkai, Rathbun et al. 1992). We compared the phenotypes of dKO and tKO mice, because previous studies have implicated lymphocytes in both atherosclerosis and myocardial damage after ischemic injury (see Introduction). Although occlusive coronary atherosclerosis in dKO mice appears to be the primary cause of CHD and premature death in dKO animals (Braun, Trigatti et al. 2002) other mechanisms could contribute to pathology. For example, immunoglobulin-mediated inflammatory heart disease can cause murine MI and death, even in the absence of hypercholesterolemia (Rose and Hill 1996; Rose and Hill 1996; Ayach, Fuse et al. 2003). Thus, analysis of the B- and T-cell-deficient tKO mice permitted us to determine if immunoglobulin-mediated or other B- and T-cell-associated inflammatory heart disease played a role in this CHD model.

We observed that, although the immune infiltrate in the damaged myocardium of dKO mice contains T-cells, there were apparently no differences in the occlusive coronary atherosclerosis, MI, cardiac dysfunction and survival of dKO and tKO mice. Thus, immunoglobulin-mediated inflammatory heart disease is not a critical underlying mechanism in the CHD in dKO mice, and B- and T-cells do not play a key role in the onset or progression of disease in this model. These findings are consistent with the previous observations that B- and T-cells can influence, but are not essential for, murine atherosclerosis and that their influence on atherogenesis is difficult to detect in apoE KO mice with high-fat-diet-induced, exceptionally high hypercholesterolemia (Dansky, Charlton et al. 1997; Daugherty, Pure et al. 1997). In dKO and high-fat-fed apoE KO mice, the extreme hypercholesterolemia appears to eclipse the influence of B- and T-cell deficiency on pathology. Additional studies are necessary to determine the influence on CHD in dKO mice of other immune cells, including macrophages, neutrophils and natural killer (NK) cells, which are present in RAG2-deficient mice (Williams 1996; Frangogiannis, Smith et al. 2002; Taverna, Moher et al. 2004).

Interestingly, the strain of dKO mice generated for this study by extensive inbreeding (strain 2) generated animals that exhibited a significantly shorter period over which most (82%) of the mice died (38-47 days) than that of the mice used for the first report of this model (Braun, Trigatti et al. 2002)(40-56 days, strain 1). Thus, these new strains of dKO and tKO mice appear to be especially attractive for evaluating the consequences of environmental, pharmacologic and genetic manipulations on the pathophysiology in this model of CHD. Disease progression is rapid, variation in times of death is low (relatively few animals needed to see statistically significant alterations in disease progression), and attempts to alter disease progression in the tKO mice by treatment with biological agents (e.g. antibodies, viral vectors) cannot illicit

lymphocyte-mediated immune responses that might otherwise confound analysis of the results from immunocompetent animals. Furthermore, additional analysis of the strain 1 and strain 2 mice may possibly help to identify modifier genes that influence the rates of disease progression and premature death in these animals.

Acknowledgements

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Chapter Three

Hepatic lipase deficiency delays atherosclerosis, MI and cardiac dysfunction, and extends lifespan in SR-BI/apoE double knockout mice

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Abstract

OBJECTIVE: SR-BI/apoE double knockout (dKO) mice exhibit many features of human coronary heart disease (CHD), including occlusive coronary atherosclerosis, cardiac hypertrophy, myocardial infarctions and premature death. Here we determined the effects on this pathology of hepatic lipase (HL) deficiency, which has been shown to decrease atherosclerosis in apoE knockout mice despite raising plasma cholesterol levels. **METHOD and RESULTS:** The SR-BI/apoE/HL triple knockout (tKO) mice generated for this study lived significantly longer (37%) than corresponding dKO controls (average lifespans: 63.0±0.8 vs. 46.0±0.3 days), despite their increased plasma cholesterol levels. At 6 weeks of age, compared to dKO mice, tKOs exhibited significantly less aortic root and coronary artery occlusive atherosclerosis, and improved cardiac structure and function. However, by 9 weeks of age the hearts of tKO mice exhibited lipid-rich coronary occlusions, myocardial infarctions and cardiac dysfunction essentially identical to that of 6 week-old dKO mice.

CONCLUSIONS: HL deficiency delays the onset and/or progression of atherosclerosis via a SR-BI independent mechanism. Extent of occlusive coronary arterial lesions was more closely associated with cardiac dysfunction and lifespan than the amount of aortic root atherosclerosis, suggesting that these occlusions in dKO mice are responsible for ischemia, myocardial infarctions and premature death.

Introduction

Though apolipoprotein E (apoE) or LDL receptor (LDLR) knockout (KO) murine models of dyslipidemia are often used to study lipoprotein metabolism and atherosclerosis (Reardon and Getz 2001), they usually do not exhibit spontaneous occlusive coronary artery disease, MI, cardiac dysfunction and premature death, hallmarks of human coronary heart disease (CHD). Double knockout (dKO) mice deficient in the HDL receptor (scavenger receptor class B type I, SR-BI) and apoE exhibit extensive aortic sinus atherosclerosis (advanced plaques with fibrous caps (Trigatti, Rayburn et al. 1999) that contain macrophages (unpublished data, 2005)), occlusive coronary arterial atherosclerosis (cellular and acellular plaques containing lipid (including cholesterol clefts), collagen and fibrin deposits (Braun, Trigatti et al. 2002)), and acute CHD when young (4-6 weeks old) (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003). At six weeks of age, dKO hearts exhibit multiple, large infarctions with extensive fibrosis around the ventricular outflow tract and patchy MIs in the apex, right ventricular wall and interventricular septum (Braun, Trigatti et al. 2002). In addition, they are hypertrophic with LV dilation, and exhibit severe dysfunction, including multiple ECG abnormalities (ST elevation and depression, anesthesia induced conduction abnormalities (e.g., bradyarrhythmias, AV blocks)), a 70% reduction in $\pm$dP/dT, and 50% reduced ejection fraction. They die between 5-8 weeks of age (mean 6 weeks) (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002). Similarities between dKO and human CHD raised the possibility that these mice may help to study the pathophysiology of CHD and to develop genetic, pharmacologic and environmental approaches for prevention and treatment.

Hepatic lipase (HL) hydrolyzes triglycerides and phospholipids and is involved in processing chylomicron remnants, IDL and HDL (Jansen, Verhoeven et al. 2002). HL is primarily synthesized and secreted by the liver and is found in steroidogenic tissues (Doolittle, Wong et al. 1987). HL participates in conversion of IDL to LDL and large lipid-rich HDL to smaller HDL,

thereby modulating their relative plasma distributions (Demant, Carlson et al. 1988; Clay, Newnham et al. 1992; Connelly and Hegele 1998; Zambon, Deeb et al. 1998; Nong, Gonzalez-Navarro et al. 2003). In addition to lipolytic activities, HL has ligand-binding activity and may mediate interactions of lipoproteins with cell surface proteoglycans and receptors, such as SR-BI and LDL receptor-related protein (LRP), thus facilitating endocytosis and/or selective lipoprotein lipid uptake (Krapp, Ahle et al. 1996; Ji, Dichek et al. 1997; Amar, Dugi et al. 1998; Dichek, Brecht et al. 1998; Dugi, Amar et al. 2000).

Many studies have demonstrated that hepatic lipase can influence atherosclerosis, though mechanisms through which this occurs are poorly understood. Increased HL activity has been linked to formation of small, dense pro-atherogenic LDL particles in humans (Zambon, Deeb et al. 1998). Conversely, reduced HL activity increases plasma HDL cholesterol levels in both humans (congenital deficiencies) and rodents (anti-HL antibodies or HL KO mice) (Homanics, de Silva et al. 1995; Lambert, Amar et al. 2000). ApoE/HL double KO mice exhibit significantly smaller aortic root atherosclerotic lesions than apoE single KO mice, despite an increase in plasma total and VLDL cholesterol (Mezdour, Jones et al. 1997) and altered lipoprotein structure and composition (Bergeron, Kotite et al. 1998). There are also studies showing that HL can be anti-atherogenic (Dugi, Brandauer et al. 2001; Dichek, Qian et al. 2004). Thus, while HL modulates atherosclerosis, its precise effects vary depending on the system under study.

Here, we examined the role of atherosclerosis in CHD in dKO mice by examining the effects of HL deficiency. We found that HL deficiency significantly reduced aortic root and occlusive coronary arterial atherosclerosis in dKO mice, delayed the onset and/or progression of CHD, and increased longevity (37%). Our results support the proposal that occlusive coronary atherosclerosis is the likely cause of MI, cardiac dysfunction and premature death in dKO mice. In addition, our study demonstrates that SR-BI is not essential for HL's influence on atherogenesis.

Materials and Methods

Mice

HL-/- mice were obtained from Jackson Laboratory (Bar Harbor, ME). SR-BI(-/-)/HL(-/-) mice were first generated by crossing C57BL/6 HL(-/-) mice and 50:50 C57BL/6:129-S4 SR-BI (-/-) mice and then intercrossing their offspring. SR-BI(-/-)/apoE(-/-)/HL(-/-) triple knockout (tKO) mice and control SR-BI(-/-)/apoE(-/-)/HL(+/+) double knockout (dKO) mice were generated on a 75:25 C57BL/6:129-S4 background by crossing SR-BI(+/-)/apoE(-/-) females (Trigatti, Rayburn et al. 1999) with SR-BI(-/-)/HL(-/-) males. The offspring SR-BI(+/-)/apoE(+/-)/HL(+/-) females were then crossed to sibling SR-BI(-/-)/apoE(+/-)/HL(+/-) males to generate littermate tKO and dKO mice as well as breeder mice that were used to maintain the colonies.

Animals were housed in micro-isolater cages and fed standard chow (Prolab 3000, PMI Feeds, St. Louis, MO) and housed as previously described (Karackattu, Picard et al. 2005). Experiments followed MIT and NIH Animal Care guidelines. Experiments other than survival studies were conducted on dKOs age 37 to 48 days and tKOs aged 37 to 68 days. No significant differences were observed between males and females. HL genotype was determined via PCR using the primers 5' TTC TCG GAG CAA AGT TCA CCT AAT 3' and 5' GTG ATT CTT CCA ATC TTG TTC TTC 3'. All other genotypes were determined by PCR (Rigotti, Trigatti et al. 1997; Trigatti, Rayburn et al. 1999).

Plasma Lipid Composition and FPLC Lipoprotein Total Cholesterol Profiles

Plasma from nonfasted and 4 hour fasted animals was obtained from blood drawn at sacrifice by centrifugation at 14,000 rpm (Spectrafuge 16M) for 10 minutes at 4°C. Lipid concentrations were determined by enzymatic assays on plasma diluted 1:5 in phosphate

buffered saline (PBS) using kits (Cholesterol C-II, Free Cholesterol E and Phospholipids B) from

Wako Chemical USA Inc., (Richmond, Virginia, USA) and the EZ HDL kit from Trinity Biotech

USA (Berkeley Heights, NJ) (Braun, Zhang et al. 2003). Plasma from nonfasted mice was

diluted 1:4 in elution buffer (154 mM NaCl, 1 mM EDTA, pH 8) and subjected to FPLC analysis

(total cholesterol determined for each fraction) either immediately following collection or after

storage at 4°C as previously described (Rigotti, Trigatti et al. 1997; Braun, Zhang et al. 2003).

No significant differences in lipids were observed between males and females.

Morphologic and Histologic Analyses

Mice were weighed and anesthetized/euthanized with an overdose of 2.5% Avertin.

Blood was drawn from the retro-orbital plexus with a heparinized capillary tube and used for

hematocrits.

Hematocrits: Hematocrits were measured using microcapillary tubes and a hematocrit

centrifuge. Hematocrits of dKO mice aged 41–48 days, tKO mice aged 40-46 days and tKO

mice aged 61-67 days did not differ significantly from each other (31.6 ± 0.7 [n=26], 32.6 ± 1.5

[n=10] and 31.5 ± 1.3 [n=10] respectively, P ANOVA=0.7555) but all differed significantly from

control values (45.4 [n=13], P<0.0001). The effects of the dKO genotype on hematocrit has been

reported elsewhere(Holm, Braun et al. 2002).

Gravimetry: Intact hearts were collected from euthanized mice and rinsed clean of blood

with heparin/PBS (10 units/ml) (heparin sodium salt, Sigma).Whole hearts and spleens were then

blotted dry and weighed using an analytical balance.

Histology: Hearts were then immersed in cold Krebs-Hanseleit buffer (120 mM NaCl/25

mM $NaHCO_3$/3.3 mM KH_2PO_4/0.8 mM K_2HPO_4/1.2 mM $MgCl_2$/1.2 mM $CaCl_2$/10 mM glucose,

pH 7.4) for 30 minutes, embedded in Tissue-TeK OCT compound (Sakura Finetek) and fresh frozen using dry ice/isopentane. Serial cryosections (10μm) cut onto Fisher MicroProbe Plus slides (Fisher Scientific) were stained with Masson's Trichrome (Sigma)(Braun, Trigatti et al. 2002) or Oil red O and hematoxylin (Trigatti, Rayburn et al. 1999).

Quantification of Aortic Atherosclerosis: Mice were sacrificed as described above. Hearts were perfused with cold PBS containing either 5mM EDTA or 10U/ml heparin, collected, immersed in Kreb-Hanseleit buffer and embedded in OCT(Trigatti, Rayburn et al. 1999; Braun, Zhang et al. 2003). Serial 10 μM sections were cut from the root of the aorta through aortic sinuses going up 350-400 μM (3-4 sections per slide). Sections were stained with Oil Red O and hematoxylin and eosin(Trigatti, Rayburn et al. 1999). SPOT imaging software (Diagnostic Instruments, MI) was used to quantify the lesion area per cross section in ten to twenty sections per mouse which were then averaged to provide mean lesion area per mouse.

Quantification of Occlusive Coronary Arterial Atherosclerosis: Coronary occlusive atherosclerosis was quantified by counting vessels in five or more Oil Red O-stained heart sections per mouse, and scoring vessels by visual inspection as open (essentially no atherosclerosis), partially (<50%) occluded or severely (>50%) occluded, and calculating the average percent of vessels in each category. In each group, one sample was a longitudinally sectioned heart and sections quantified were generally taken from the center of the heart. The remaining samples in each group were each transversely sectioned and sections quantified were from the upper third of the heart.

ECGS: Mice were placed under 2% isofluorane anesthesia. Subcutaneous electrodes with three leads were surgically implanted in mice between the ages of 24 and 33 days. Two incisions were made to implant the electrodes with type 2 leads: one at the back of the neck where the leads exit and one in the midline thorax where the leads are sutured onto the thoracic cavity. One lead was sutured onto the thoracic wall on the lower left side of the heart at the level of the xyphoid process and the other was sutured onto the thoracic wall at the upper right side of the heart beneath the clavicle. The third lead was placed along the spine to serve as a ground. The connector plug for the electrodes was sutured at the junction of the head and neck. Mice were allowed to recover from surgery for one day before the electrode connector plug was attached to a commuter connected to a signal processor designed and built by the Krieger and Mark laboratories (unpublished) that recorded ECG signals and allowed the mouse to move freely. Mice were housed singly and fed normal chow and water. Their lifetime ECGs were recorded continuously. MatLab 6.5 software was used to view and analyze the ECG signals.

FOOTNOTE: These ECG methods were not detailed in the original manuscript.

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Statistical Analysis: $P \leq 0.05$ was considered significant (2-tailed, unpaired student's t test or ANOVA test, GraphPad Prism 4.0). Survival curves employed the Kaplan-Meier function with the logrank test (GraphPad Prism 4.0). Values are expressed as mean $\pm$ SEM.

Results

To assess the effects of inactivation of the HL gene on CHD in SR-BI/apoE dKO mice,

we generated from common precursor animals two new strains on genetically similar mixed

75:25 C57BL/6:129-S4 backgrounds: SR-BI/apoE/HL triple knockout (tKO) and control SR-

BI/apoE dKO mice. The effect of HL gene inactivation on lifespan of dKOs is shown in Figure

1 (dKO, black curve, tKO, gray). tKOs survived 37% longer than dKOs (mean lifespans (days):

dKO, 46.0±0.3 (6.6 weeks, n=160); tKO, 63.0±0.8 (9 weeks, n=94), P<0.0001 Logrank test). We

also compared the lifespans of sibling dKOs and tKOs generated by intercrossing SR-BI(+/-

)/apoE(-/-)/HL(+/-) mice. These tKO mice lived 12-37 days (25-84%) longer than their dKO

siblings (n(tKO)=15, n(dKO)=16). In both strains, a small fraction of mice (dKOs, 4.8%; tKOs,

10.5%) died within ten days of weaning, possibly due to hypersensitivity to the anesthetic

(Braun, Trigatti et al. 2002) used during sampling for genotyping or as yet unidentified relatively

uncommon genetic or environmental factors. These animals are included in Figure 1, but not in

the calculation of mean lifespans.

At 6 weeks of age dKOs appeared hunched and sickly (e.g. lethargy) with ruffled fur,

whereas tKOs were healthier (sleeker fur, more active). Moreover, at this age tKOs weighed

significantly more than dKOs (18.5±0.4 vs. 16.4±0.3 gm). By 9 weeks the tKOs had grown

larger (20.9±0.7 gm), but otherwise resembled the ill 6 week-old dKOs. To investigate

mechanisms underlying the extended lifespan of the tKOs, we further characterized the mice at

~6 weeks (37-48 days) of age, designated dKO-6 and tKO-6 and ~9 weeks (60-68 days, tKO-9).

As previously reported, by 6 weeks of age ~50% of the dKO mice died and the surviving animals

exhibited occlusive atherosclerosis, MI and heart dysfunction (Braun, Trigatti *et al.* 2002; Braun,

Zhang *et al.* 2003; Karackattu, Picard *et al.* 2005), whereas virtually all of the tKO mice were alive. We expected that surviving tKO-9 mice (mean age of death) might resemble dKO-6 mice.
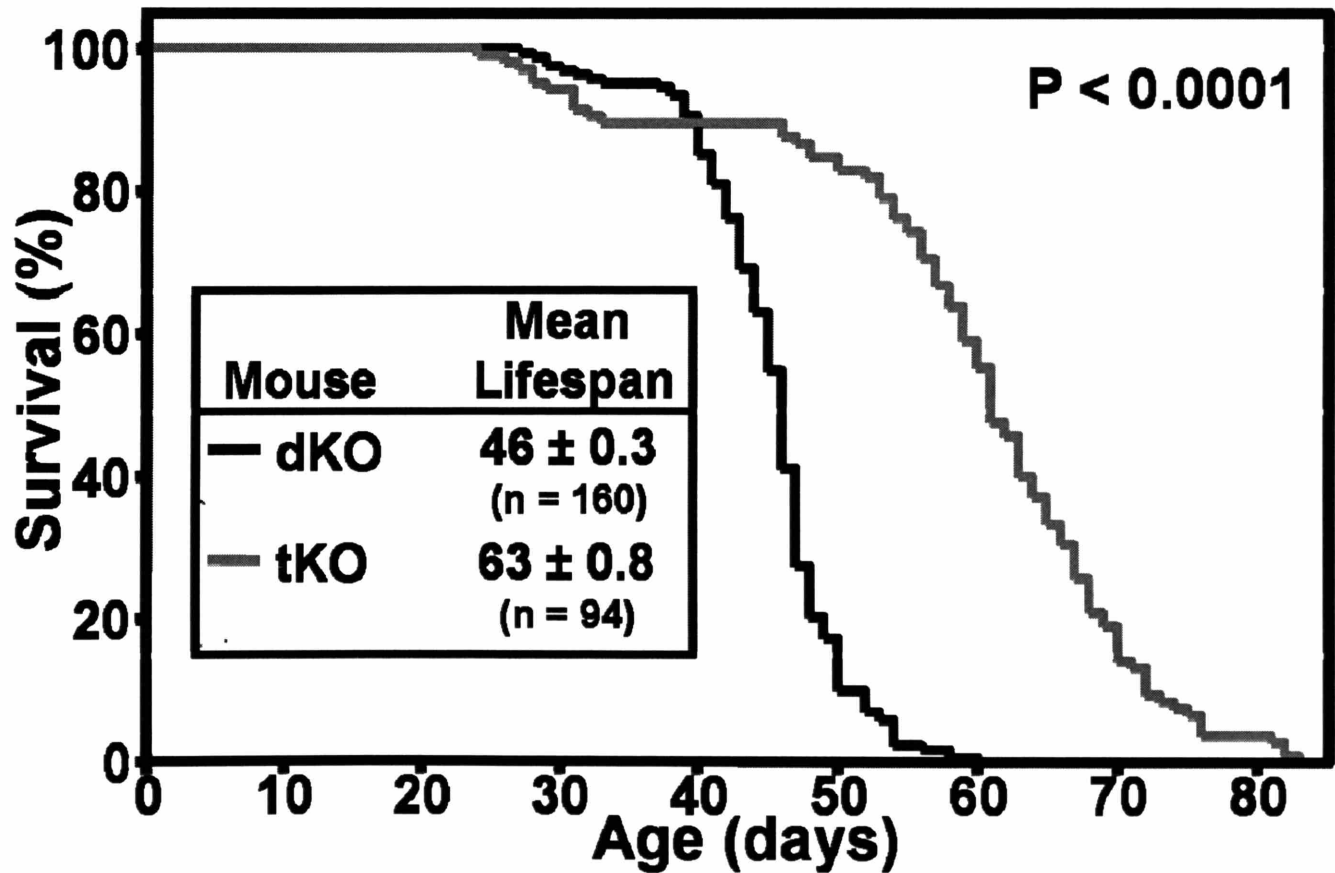
Figure 1: Effect of HL deficiency on survival of dKO mice. Survival curves for tKO (gray line) and dKO (black line) mice. Mice that died at 33 days of age or younger are included in the graph but were not included in the calculation of mean lifespan.

Cardiac Function and Structure

As previously reported (Braun, Trigatti et al. 2002), dKO-6s exhibited a variety of electrocardiographic (ECG) abnormalities associated with CHD, including ST depression and ST elevation indicative of ischemia and myocardial infarction (Braun, Trigatti et al. 2002) (Figure 2A). In contrast, the ECGs of tKO-6s were normal (Figure 2B). However, as the tKOs approached 9 weeks of age, they exhibited ECG abnormalities similar to those of dKO-6s (e.g., ST depression and elevation, Figure 2C), suggesting that, as is the case for dKOs, CHD may be the primary cause of premature death. Thus, inactivation of HL may have slowed the initiation and/or progression of CHD. The gross characteristics of the hearts support this suggestion. Hearts from tKO-6 mice were similar in surface appearance to CHD-free control hearts (Figure 3A and C), whereas those from dKO-6s and tKO-9s were markedly enlarged and exhibited surface patches characteristic of large MIs (Braun, Trigatti et al. 2002) (Figure 3B and D). Although the heart to body weight ratio for tKO-6s was significantly larger than that for controls (1.3-fold), indicating that the tKO-6 hearts were not normal; this cardiomegaly, due at least in part to hypertrophy (Zhang, Picard et al. 2005), was significantly less than that of dKO-6s (1.9-fold) or tKO-9s (1.7-fold) (Figure 3E).

Masson's trichrome (Braun, Trigatti et al. 2002) stained longitudinal heart sections (Figure 4A-C, healthy myocardium stains red, fibrotic tissue blue) showed that, unlike the massive fibrosis/MI present in virtually all dKO-6 and tKO-9 hearts (A and C) - with especially marked left ventricular dilation and fibrosis in the outflow tracts of the most ill tKO-9s, tKO-6 hearts were relatively healthy, with little fibrosis (B) or intramyocardial neutral lipid deposition (Braun, Trigatti et al. 2002) (Oil Red O staining, data not shown). Thus, tKO mice develop MIs,

cardiomegaly, left ventricular dilation and cardiac dysfunction (ECGs) similar to those of dKO

mice, but onset and/or progression of disease is slower in tKOs.
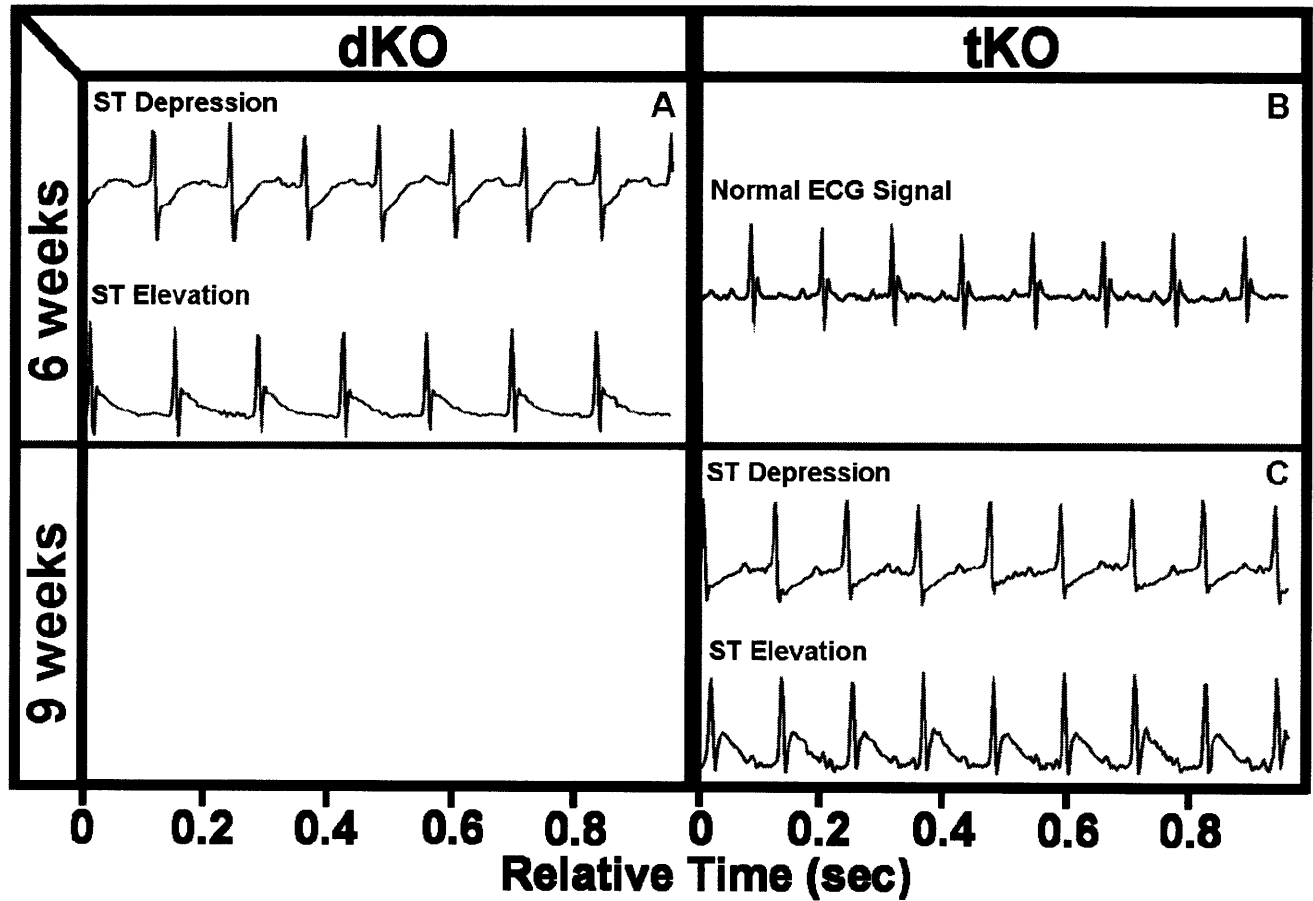
Figure 2: ECG analysis of cardiac function. Representative ECGs (n=4) from unanesthetized

dKO-6 (A; ST depression and elevation), tKO-6 (B; normal ECG) and tKO-9 (C; ST depression

and elevation) mice.
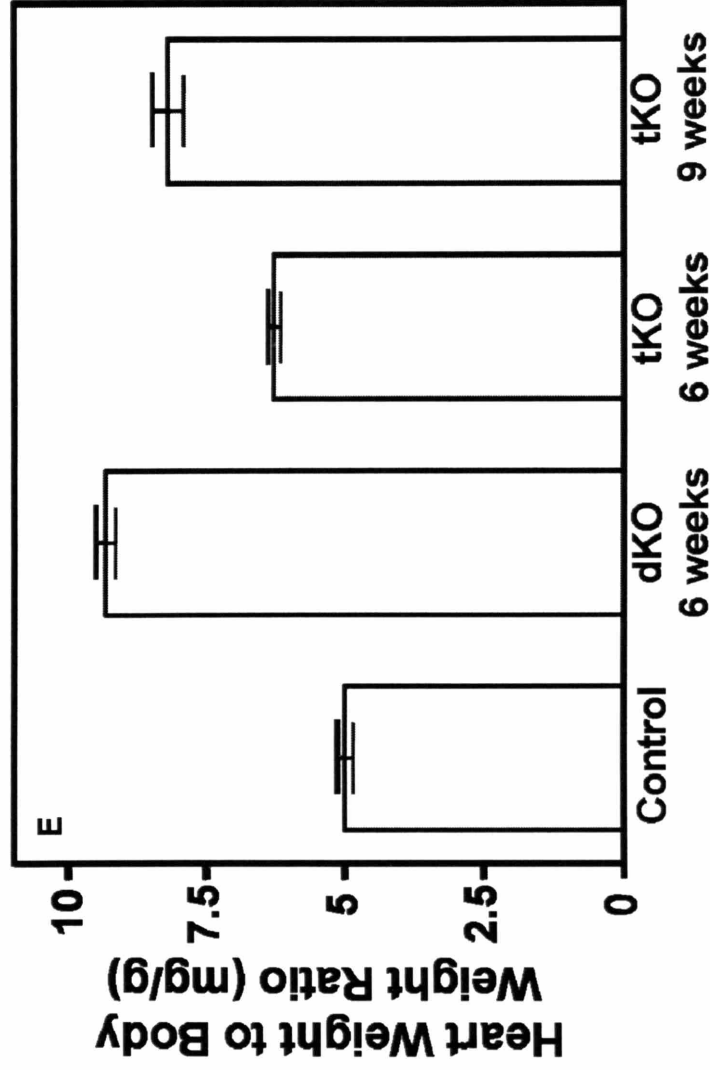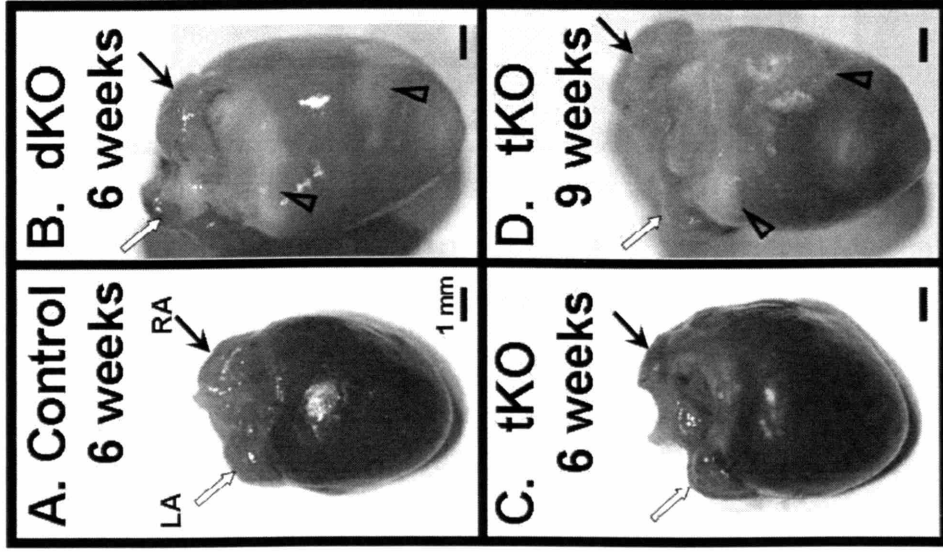
Figure 3. Anatomic and gravimetric analyses of hearts from 6 week-old SR-BI(+/-)/apoE(-/-)/HL(-/-) control, dKO and tKO and 9 week-old tKO mice. Intact hearts were photographed (A–D); and weighed (E; values represent mean±SEM of heart to body weight ratios; all pairwise comparisons *P*<0.0001). The values (mg/g) were: control, 4.85±0.12, n=20; dKO-6, 9.39±0.19, n=62; tKO-6, 6.28±0.12, n=47; and tKO-9, 8.20±0.29, n=39.
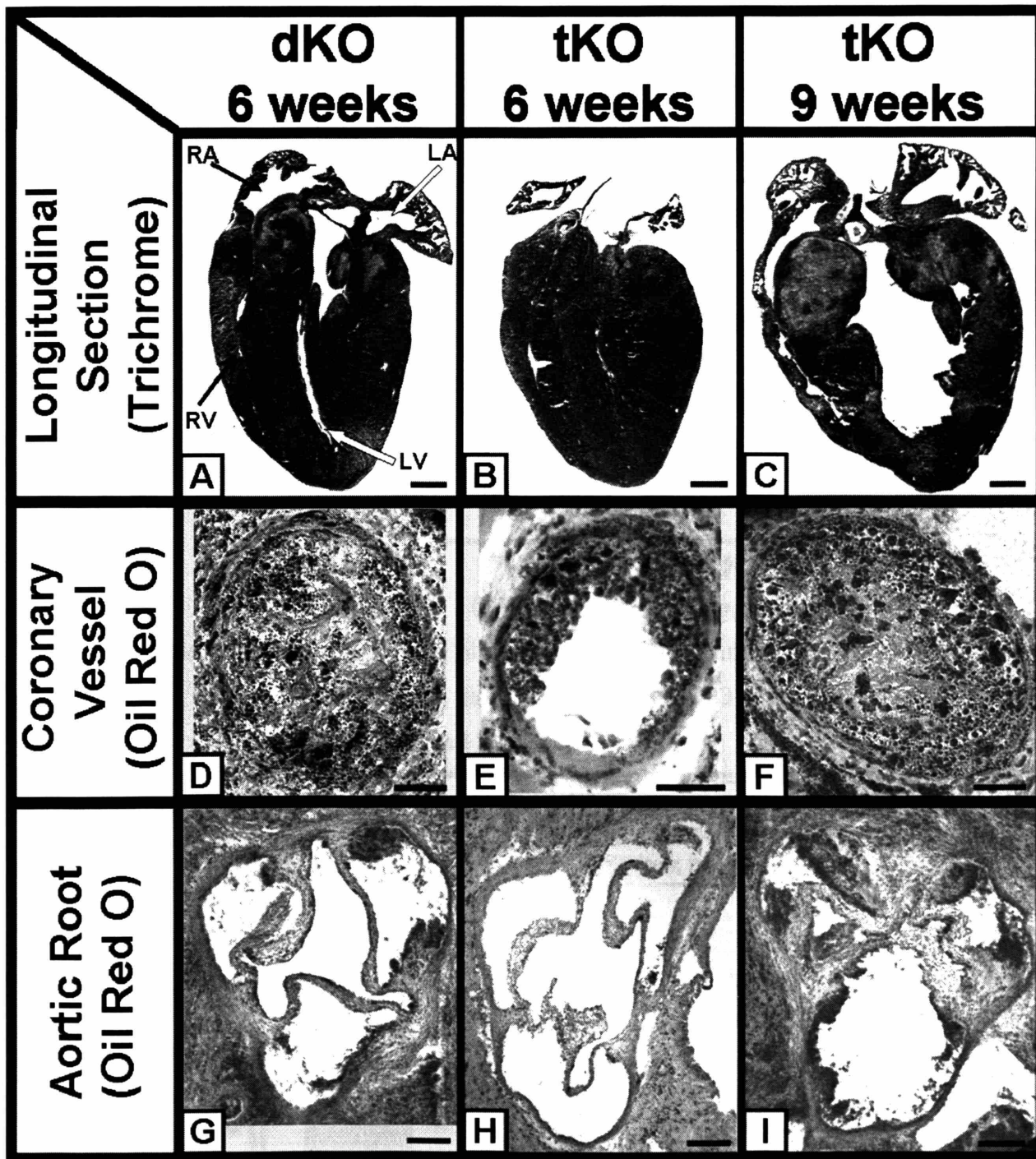
Figure 4: Histologic analysis of hearts. Panels A-C: Longitudinal Masson's trichrome stained heart sections (healthy myocardium, red; fibrotic tissue, blue) from 40 day-old dKO (A), 42 day-old tKO (B) and 60 day-old tKO (C) mice. **Panels D-F:** Oil Red O stained coronary artery sections (neutral lipid red) from 45 day-old dKO (D), 44 day-old tKO (E) and 68 day-old tKO (F) mice. **Panels G-I:** Oil Red O stained aortic root sections from 43 day-old dKO (G), 44 day-old tKO (H), and 62 day-old tKO (I) mice. Bars=1 mm (A,B,C), 40 μm (D,E,F) or 200 μm (G,H,I).
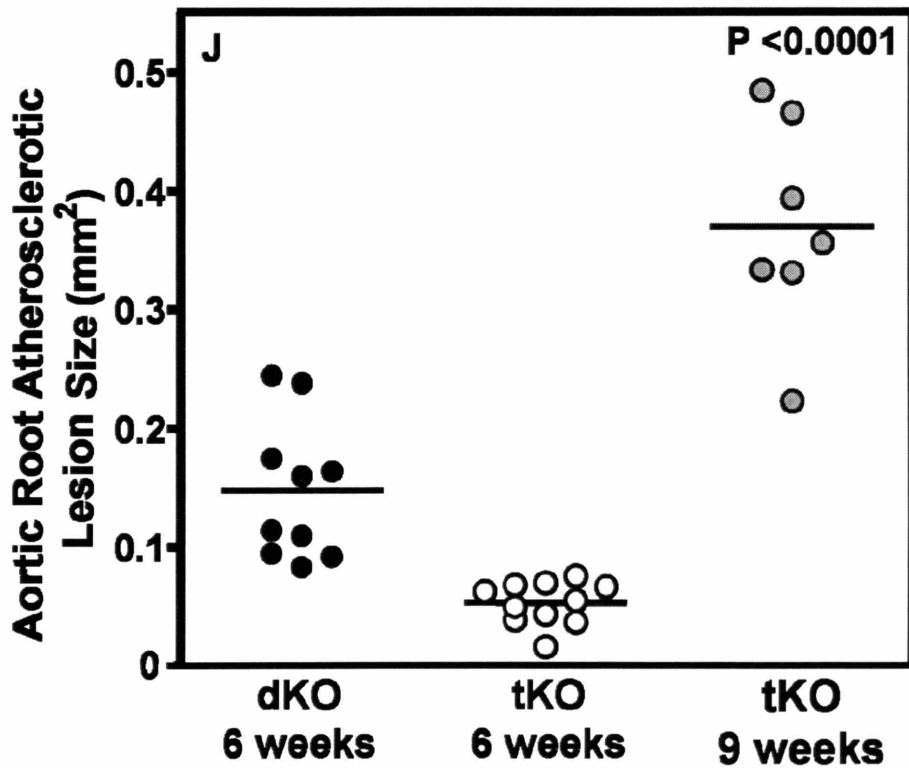
Figure 4 Panel J: Cross-sectional areas of oil red O-stained lesions in the aortic root region. Average lesion areas (mm$^2\pm$SEM, horizontal lines): dKO-6, 0.148$\pm$0.019; tKO-6, 0.053$\pm$0.005; and tKO-9, 0.370$\pm$0.034. All pair-wise comparisons had P values <0.0001.
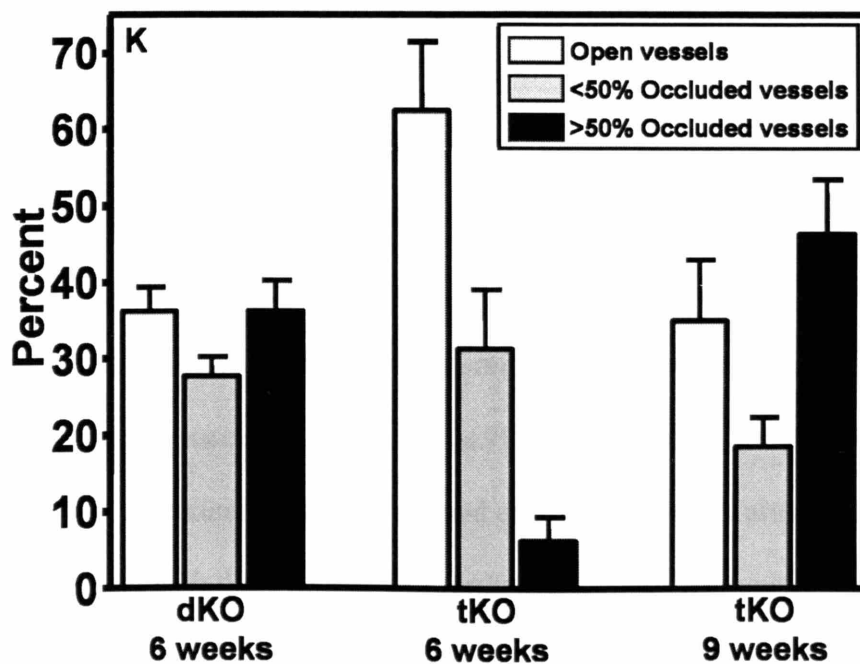
Figure 4 Panel K: Extent of coronary arterial occlusions. The percent of essentially unoccluded (white bars), partially (<50% of luminal cross-sectional area; gray bars) and severely (>50%; black bars) occluded coronary vessels in dKO-6, tKO-6 and tKO-9 mice.

Effects of hepatic lipase deficiency on aortic root and occlusive coronary arterial atherosclerosis

To determine if extended lifespan and improved cardiac pathology of HL-deficient tKO-6 mice was associated with reduced atherosclerosis, we quantified aortic root and occlusive coronary arterial atherosclerosis. Figure 4 shows representative images of Oil Red O stained aortic roots (panels G-I) and quantitative analysis of lesion sizes (J). Compared to lesion areas in dKO-6s, the plaques were 3-fold smaller in tKO-6 (H) and 2.5-fold larger in tKO-9 (I) mice.

Comparable results were observed in coronary arteries. Vessels were scored in at least five Oil Red O-stained sections per mouse and divided into three categories: severely (>50%) occluded, partially (<50%) occluded and open (no visible plaque). In dKO-6 mice (Figure 4D and K), severe and partially occluded lesions were common ((36±4%, 28±3%, respectively, n=5 mice), while 36±3% of the vessels were open. Occlusions, which were either predominantly acellular or contained significant cellular components (Braun, Trigatti et al. 2002; Karackattu, Picard et al. 2005), were prevalent in areas with myocardial fibrosis, especially near the upper ventricular outflow regions. Unlike the dKO-6s, most vessels in tKOs-6s (Figure 4E and K, n=5) were completely open (62±9%) with 31±8% partially and only 6±3% severely occluded. Thus, at 6 weeks of age, blood flow and oxygenation of the cardiac muscle in tKO-6s would have been better than in dKO-6s. However, in tKO-9s (n=5 mice), 46±8% of the arteries were severely occluded, 19±4% partially occluded and only 35±9% open (Figure 4F and K), values comparable to those in dKO-6s . The extents of aortic root and occlusive coronary arterial atherosclerosis were grossly correlated with the extent of MI, cardiomegaly, cardiac function and lifespan, suggesting atherosclerosis is most likely responsible for CHD and premature death in dKO mice and that HL deficiency may extend life by slowing the onset and/or progression of atherogenesis.

For tKO-9 and dKO-6 mice, there was a substantially larger difference in aortic root

atherosclerosis (~3-fold) than in the number of occluded coronary vessels (1.02-fold), even

though sampling occurred at their respective mean ages of death (9 or 6 weeks). Occlusive

coronary arterial atherosclerosis is expected to cause myocardial ischemia and consequently MI,

cardiac dysfunction and death, whereas previous studies have shown that extensive aortic root

atherosclerosis alone is not usually associated with MI and overt cardiac dysfunction (Zhang,

Reddick et al. 1994; Zhang, Reddick et al. 1997; Calara, Silvestre et al. 2001). Thus, the closer

correlation of occlusive arterial than aortic root atherosclerosis with the cardiac phenotypes is not

surprising.

Effects of hepatic lipase deficiency on lipoprotein abundance and composition

Figure 5 and Table I show that HL deficiency had little influence on the size distribution

of the lipoproteins in dKOs and only a small effect on relative lipid compositions. At 6 weeks of

age the fasting plasma total cholesterol level in tKOs was ~1.6-fold higher than in dKOs, a

difference due primarily to increases in the VLDL-size range (Figure 5); however, by 9 weeks of

age the plasma total cholesterol in tKOs dropped to that in dKO-6s. Nonfasting (Table II) and

fasting plasma lipid levels were similar. Based on comparisons with healthy SR-BI(+/-)/apoE(-/-

)/HL(-/-) littermates of tKO mice at 6 (n=5, TC=745±58) and 9 (n=7, TC=817±90) weeks of

age, the decrease in plasma cholesterol between 6 and 9 weeks in tKO mice was not simply

attributable to normal aging and/or sexual maturation. The mechanisms underlying this reduction

are unclear, though it is noteworthy that higher lipoprotein levels may be cardioprotective,

possibly by limiting inflammation, and reduced lipid levels may be associated with a poor

prognosis in chronic heart failure (Rauchhaus, Koloczek et al. 2000; Rauchhaus, Clark et al.

2003).

We observed no significant differences in the fasting triglyceride (TG) levels of tKO-6

and dKO-6 mice or the HDL cholesterol levels of dKO-6, tKO-6, and tKO-9 mice (Table I, see

references 27,28). Though triglyceride levels were similar in dKO-6 and tKO-6 mice, they were

higher in tKO-9 mice. The ratios of surface (phospholipids plus unesterified cholesterol) to core

(cholesterol ester plus triglyceride) lipids were similar for dKO-6s and tKO-9s and significantly

higher than in tKO-6s (Table I). As previously reported, higher lipoprotein surface to core lipid

ratios are associated with greater atherogenic potential in different SR-BI-deficient models of

CHD (Braun, Zhang et al. 2003; Zhang, Picard et al. 2005). Thus, we did not observe substantial

HL-dependent differences in the lipoproteins. Further studies are necessary to determine if subtle

differences in lipoprotein structures due to HL deficiency, or if HL-dependent changes in

lipoprotein metabolism distinct from changes in particle structure (e.g., as a consequence of the

lipoprotein binding activity of HL) (Gonzalez-Navarro, Nong et al. 2004), were responsible for

the striking effects on atherosclerosis in dKO mice.

Table I: Fasting plasma lipid data from dKO-6, tKO-6 and tKO-9 mice

	dKO-6 n=32[†]	tKO-6 n=20[†]	tKO-9 n=12[†]	P(dKO-6 vs. tKO-6)[‡]	P(dKO-6 vs. tKO-9)[‡]	P(tKO-6 vs. tKO-9)[‡]
Total Cholesterol (TC)*	895±33	1419±73	906±59	<0.0001	0.8579	<0.0001
Unesterified Cholesterol (UC)*	694±22	997±43	710±38	<0.0001	0.7187	<0.0001
Phospholipids (PL)*	545±18	838±38	602±29	<0.0001	0.1110	0.0001
Triglycerides*	55±5 (31)	47±6 (19)	82±9	0.2894	0.0055	0.0025
HDL cholesterol*	219±7 (28)	229±6 (19)	220±11 (11)	0.3016	0.9144	0.4373
Surface to Core Ratio§	5.2±0.3 (31)	4.2±0.2 (19)	5.1±0.4	0.0067	0.9013	0.0262
UC/TC Ratio	0.78±0.01	0.71±0.01	0.79±0.02	<0.0001	0.5810	<0.0001
PL/TC Ratio	0.61±0.01	0.60±0.01	0.67±0.02	0.2951	0.0048	0.0025

*mg/dl

†'n'=number of animals per group (variations in parentheses).

‡ P-values for comparisons determined using unpaired student's t-test, 2 tailed.

§Surface to core lipids = (PL+UC)/(esterified cholesterol=TC-UC(Zhang, Picard et al. 2005).

Table II: Nonfasting whole plasma lipid data for dKO-6, tKO-6 and tKO-9 mice.

	dKO (6 weeks) n=18	tKO (6 weeks) n=15	tKO (9 weeks) n=9	P (dKO-6 vs. tKO-6)	P (dKO-6 vs. tKO-9)	P (tKO-6 vs. tKO-9)
Total Cholesterol (TC)*	835±32	1395±66	963±67	<0.0001	0.0603	0.0003
Unesterified Cholesterol (UC)*	618±25	1041±47	746±58	<0.0001	0.0254	0.0008
Phospholipids (PL)*	546±26	947±37	673±53	<0.0001	0.0213	0.0003
UC/TC Ratio	0.74±0.01	0.75±0.01	0.77±0.01	0.5840	0.0840	0.2308
PL/TC Ratio	0.65±0.01	0.68±0.01	0.70±0.02	0.0785	0.0375	0.5685

*mg/dl

'n' = number of animals per group with deviations indicated in parentheses. P-values for pairwise comparisons were determined using

unpaired student's t-test, 2 tailed.

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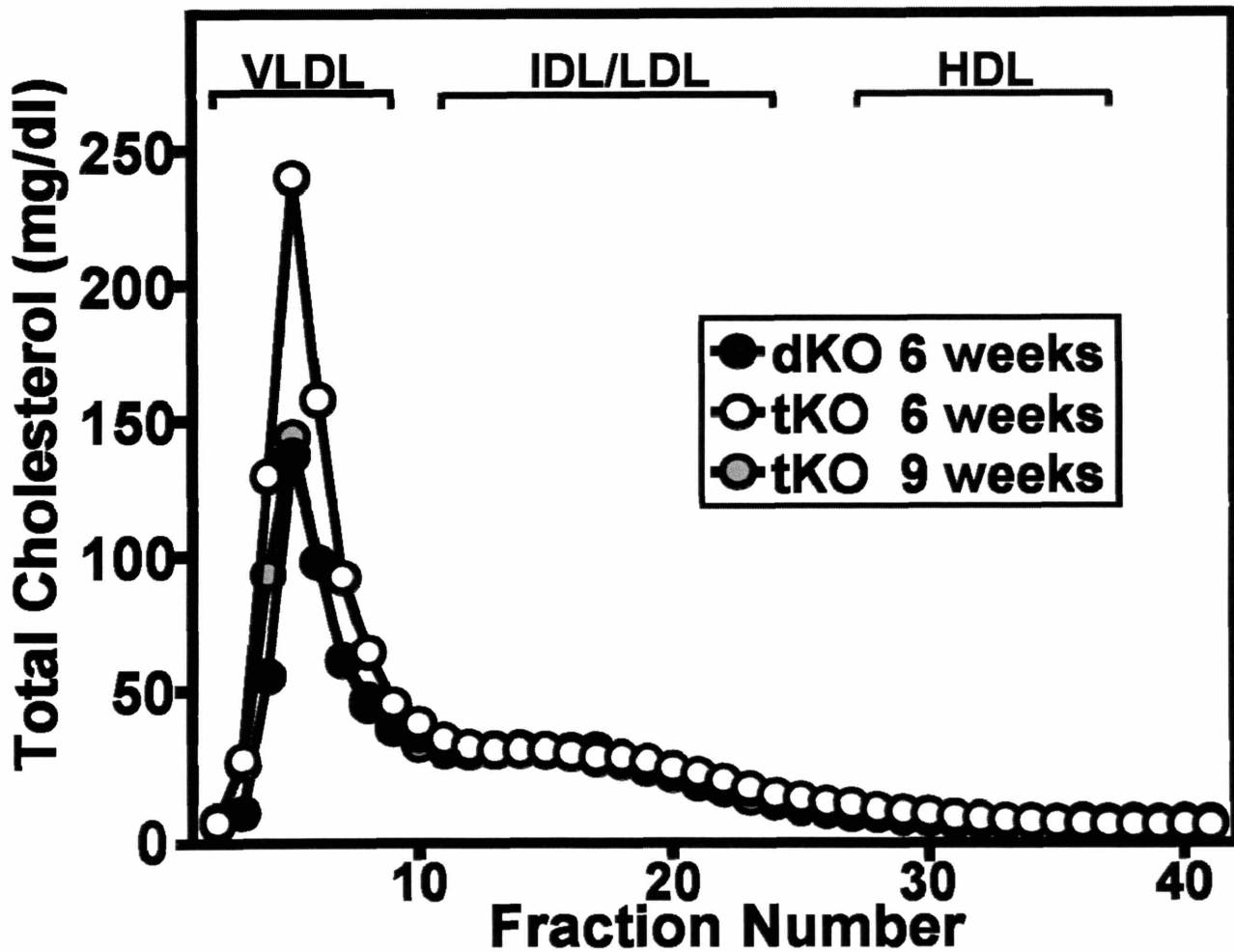
Figure 5: Lipoprotein cholesterol profiles from mice. Plasma was size fractionated (Superose 6-FPLC) and total cholesterol in each fraction (mg/dl plasma) determined. Chromatograms shown are from 42 day-old dKO (black filled circles) and tKO (open circles) mice, and 62 day-old tKO mice (gray circles) and are representative of multiple, independent determinations.

Discussion

The SR-BI/apoE dKO mouse exhibits many hallmarks of human coronary heart disease

(CHD) (see Introduction) (Braun, Trigatti et al. 2002). It and its variant, the SR-BI

KO/*ApoeR61*[h/h] mouse (Zhang, Picard et al. 2005) (see below), provide uniquely powerful tools

to investigate the mechanisms underlying CHD and to identify potential therapeutic targets and

approaches. For their promise to be realized, it is necessary to determine if the CHD etiology and

pathology in these models are similar to those in humans. Following initial characterization of

dKO mice (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002), there appeared to be two

potential causes of MIs, cardiac dysfunction and premature death: B- and T-cell mediated

inflammatory heart disease or ischemic disease due to occlusive atherosclerosis. Previous

studies, in which B- and T-lymphocyte production was blocked by deficiency in the RAG2 gene,

demonstrated that B- and T-lymphocyte mediated inflammatory heart disease is not essential for

CHD in dKO mice (Karackattu, Picard et al. 2005)

In an attempt to directly test the role of atherosclerosis, we generated SR-BI/apoE/hepatic

lipase (HL) triple KO (tKO) mice with the goal of changing the rate of onset or progression of

atherosclerosis. Numerous reports indicate that HL can have either pro-atherogenic or anti-

atherogenic activities in humans and mice, depending on the details of the system under study

(Dugi, Brandauer et al. 2001; Zambon, Hokanson et al. 1999; Zambon, Brown et al. 2001). For

example, alterations in HL expression result in strikingly different effects in apoE KO and LDLR

KO mice, despite the similarities in morphology of their aortic atherosclerotic lesions. HL

deficiency dramatically reduces aortic root atherosclerosis in apoE knockout mice (Mezdour,

Jones et al. 1997), via poorly understood mechanisms that likely include alterations in

lipoprotein metabolism, indicating a pro-atherogenic role for HL. In contrast, hepatic

overexpression of human HL in LDLR/HL double KO mice reduces aortic atherosclerosis, indicating an anti-atherogenic activity of overexpression (Dichek, Qian et al. 2004). Unlike the dKO mice examined here, these and other murine models do not exhibit significant occlusive coronary arterial atherosclerosis. Thus the influence of HL on murine atherosclerosis of this sort has not be defined previously; although examination of HL effects on coronary arterial atherosclerosis in humans has been reported (Dugi, Brandauer et al. 2001).

The diverse effects of HL on atherosclerosis in different systems is not surprising, because HL is a complex protein that exhibits distinct lipolytic (Dugi, Amar et al. 2000) and ligand-binding (Krapp, Ahle et al. 1996; Ji, Dichek et al. 1997; Dichek, Brecht et al.1998; Ji, Lauer et al. 1994; Choi, Komaromy et al.1994; Lambert, Chase et al.1999; Dichek, Johnson et al. 2001) activities that have multiple effects on lipoprotein composition and metabolism, and thus could influence atherogenesis via a variety of mechanisms (Gonzalez-Navarro, Nong et al. 2004; Dichek, Qian et al. 2004). For example, in apoE KO mice, HL deficiency may be anti-atherogenic because it raises HDL cholesterol, increases the capacity of HDL to promote cellular cholesterol efflux in vitro (Mezdour, Jones et al. 1997), prevents HL-mediated increases in the atherogenicity of VLDL particles (Stender and Zilversmit 1981; Nordestgaard, Stender et al. 1988; Mezdour, Jones et al. 1997), increases plasma apoA-I and apoA-IV levels (Mezdour, Jones et al. 1997; Bergeron, Kotite et al. 1998), or alters the phospholipid composition of lipoproteins (Bergeron, Kotite et al. 1998; Jeong, Schissel et al. 1998; Park, Panek et al. 2004). In addition, expression of catalytically inactive HL has been shown to produce a dramatic reduction of atherosclerosis in apoE/HL double KO mice (Gonzalez-Navarro, Nong et al. 2004), but not in LDLR/HL double KO mice (Dichek, Qian et al 2004). Furthermore, HL might influence atherosclerosis independently of its effects on systemic lipoprotein metabolism. For example,

bone marrow transplantation experiments demonstrate localized HL expression by macrophages can dramatically influence aortic atherosclerosis (Gonzalez-Navarro, Nong et al. 2002; Nong, Gonzalez-Navarro et al. 2003). Thus, the effects of HL deficiency on aortic root, and particularly on coronary arterial, atherosclerosis in dKO mice could not be known a priori.

Indeed, HL deficiency significantly reduced both aortic root and coronary arterial occlusive atherosclerosis in dKO mice, with 64% and 42% reductions, respectively, at 6 weeks of age. Despite the striking effects of HL deficiency on atherosclerosis, we did not observe alterations in relative cholesterol and phospholipid levels in lipoproteins or the size distribution of lipoproteins, although as expected HL deficiency was accompanied by increased plasma total cholesterol. Thus, as in other model systems, the precise mechanisms through which HL deficiency reduces atherosclerosis in dKO mice remain unclear. Future studies will be required to explore the possibility that in dKO mice HL deficiency resulted in subtle, yet functionally important, changes in lipoprotein structure, binding to tissues or metabolism.

As a consequence we could examine the effects of the reduced atherosclerosis on CHD. At 6 weeks of age, the reduced atherosclerosis in tKO mice was associated with a dramatic reduction in hypertrophy and almost complete prevention of MI, and electrocardiographically determined cardiac dysfunction (e.g., ST elevation and depression). Furthermore, in tKO mice there was an increase in the mean age of death from ~6 to 9 weeks. By 9 weeks of age, occlusive coronary arterial atherosclerosis in tKO mice was virtually identical to that in 6 week old dKO mice (65% vs. 64% of arteries with occlusions) and there was 1.5-fold greater aortic root disease. The abnormal cardiac phenotypes in 9 week old tKO mice resembled those of 6 week-old dKOs. Thus, the extent of occlusive coronary arterial atherosclerosis appeared to be

somewhat more closely correlated with cardiac pathology than the relative amounts of aortic root atherosclerosis.

The current study demonstrates that HL deficiency can delay the onset and/or reduce the rate of progression of atherosclerosis in the absence of SR-BI, and it seems likely that this reduction in occlusive coronary arterial atherosclerosis was responsible for the slower development of CHD and the extension of lifespan. Two other studies support this conclusion. First, the hypolipidemia and anti-atherosclerosis drug probucol dramatically blocks in dKO mice the onset and/or progression of atherosclerosis and cardiac pathology, and, increases their lifespans (mean age of death increases to 36 weeks) (Braun, Zhang et al. 2003). However, it is difficult to draw definitive conclusions about the role of lipoprotein metabolism and atherosclerosis in the CHD in dKO mice solely from the beneficial effects of probucol, because this drug exhibits multiple, pleiotropic activities (anti-oxidant, anti-inflammatory, cardioprotective in the absence of dyslipidemia) (Dage, Anderson et al. 1991; Pfuetze and Dujovne 2000; Nakamura, Egashira et al. 2002). Second, a lipid-rich atherogenic diet can induce in SR-BI KO/$ApoeR61^{h/h}$ mice fatal CHD that is remarkably similar to that in chow-fed dKO mice. SR-BI KO/$ApoeR61^{h/h}$ mice have low, rather than no, plasma apoE, rendering them susceptible to diet-induced hyperlipidemia, atherosclerosis and CHD (Raffai and Weisgraber 2002; Zhang, Picard et al. 2005).

Taken together with our earlier studies (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003; Karackattu, Picard et al. 2005), this work supports the suggestion that occlusive coronary atherosclerosis is directly responsible for ischemia-induced myocardial infarction, which in turn leads to cardiac dysfunction and premature death, a pathologic process closely resembling that in human CHD. Because rapid onset occlusive

coronary arterial atherosclerosis [Footnote: To date, occlusive thrombi have not been observed in these mice (unpublished data, 2005)] and associated MI does not usually accompany the aortic atherosclerosis seen in other common murine models of hyperlipidemia (Zhang, Reddick et al. 1994; Ishibashi, Brown et al.1993; Ishibashi, Herz et al.1994; Zhang, Reddick et al.1992; Ishibashi, Goldstein et al.1994; Caligiuri, Levy et al.1999), the dKO mouse, and its variant, SR-BI KO/*ApoeR61*$^{h/h}$ mice (Zhang, Picard et al. 2005), provide attractive small animal models of human occlusive atherosclerotic CHD for genetic and pharmacological studies of the mechanisms underlying the most common causes of heart disease and preclinical testing of new therapeutic strategies.

Acknowledgements

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Chapter Four

Discussion, Conclusions and

Future Investigation

This thesis investigated components of the two major mechanisms underlying atherosclerosis, inflammation and lipoprotein metabolism, in the SR-BI/apoE double knockout (dKO) mouse model of atherosclerosis and CHD. Chapter Two demonstrated that lymphocyte and immunoglobulin-mediated inflammation was not a significant mechanism underlying cardiomyocyte damage in dKO mice and strengthened the notion that dKO mice were dying of coronary occlusive atherosclerosis-induced ischemic heart disease. Chapter Three demonstrated that deficiency of hepatic lipase (HL), which has previously been shown to decrease atherosclerotic lesion size in apoE(-/-) mice despite increasing plasma cholesterol levels, can delay onset and/or progression of both aortic and coronary occlusive atherosclerosis in dKO mice. This decrease in atherogenesis was associated with deferment of myocardial infarction and cardiac dysfunction and a corresponding extension of lifespan.

Inflammation and Coronary Heart Disease in SR-BI/apoE dKO Mice

Lymphocytes and atherosclerosis in SR-BI/apoE dKO mice

T-lymphocytes are among the first cells to infiltrate a developing atheroma and are prominently found in the atherosclerotic lesions of both humans and animal models. The role of lymphocytes has been studied by multiple groups, usually via crossing lymphocyte-deficient RAG1 or RAG2 KO mice to atherosclerosis-prone apoE(-/-) mice (see Introduction for description of apoE(-/-) phenotype) and monitoring changes in the rate of plaque formation. Earlier studies have shown that lymphocyte-deficiency can slightly, but significantly, reduce the size of lesions in apoE(-/-) mice on a low-fat chow diet, however this effect is negated by the extreme hypercholesterolemia and accelerated rate of atherogenesis induced by a high-fat high-cholesterol diet(Dansky, Charlton et al. 1997; Daugherty, Pure et al. 1997). These experiments indicate that severity of hypercholesterolemia influences lesion development more than does

lymphocyte infiltration. Given that complete lymphocyte-deficiency fails to reduce aortic atherosclerosis in apoE(-/-) mice fed a lipid-enriched diet, it's not surprising that SR-BI/apoE/RAG2 triple knockout (tKO) mice and SR-BI/apoE dKOs, which both have approximately 2-fold higher cholesterol levels than chow-fed apoE(-/-) mice, exhibit similar levels of coronary atherosclerosis to each other. That is, we conclude that lymphocyte-deficiency did not affect coronary occlusive atherosclerosis in dKO mice most likely due to their increased hypercholesterolemia, relative to apoE(-/-) mice, on a chow diet.

Lymphocytes and myocardial infarction in SR-BI/apoE dKO mice

Of even greater interest to us in these studies was the potential influence of B- and T-lymphocytes as well as immunoglobulin-mediated inflammatory responses on the myocardial infarction, cardiomyocyte damage and fibrotic scarring in dKO hearts. The favored hypothesis is that dKO mice developed extensive lipid-rich coronary-occlusive atherosclerotic lesions that result in ischemia and hypoxia-induced myocardial injury, cardiac dysfunction and ultimately death. However, there existed the possibility that a critical mechanism underlying heart disease in dKO mice was the consequence of unchecked lymphocyte-driven inflammation, similar to autoimmune myocarditis, and that removal of these cells and their immune responses could potentially ameliorate cardiomyocyte injury, myocardial scarring and increase lifespan. Early reports on the cardiac pathophysiology of dKO mice report the presence of dilated, mononuclear inflammatory cells in the fibrotic myocardial lesions, however these lymphocyte-like cells were not positively identified(Braun, Trigatti et al. 2002). Using lymphocyte-specific markers, we confirmed the presence of $CD4^+$ T-lymphocytes in the myocardial lesions of dKO mice, though we were unable to detect either B-lymphocytes or $CD8^+$ T-cells (Chapter 2). Lymphocytes, especially sensitized cytotoxic T-cells, are thought to play a role in perpetrating cardiomyocyte

injury during myocardial infarction (Introduction). That the complete absence of functional B- and T-lymphocytes did not produce any detectable changes in either cardiac pathophysiology or lifespan strongly indicates that these cells are not crucial to the development of heart disease in dKO mice (barring the remote chance of there being equal beneficial and detrimental effects of lymphocyte deficiency on the dKO phenotype such that they cancelled to produce unobservable effects).

Though our studies definitively rule out a major role for B- and T-lymphocytes and immunoglobulin-mediated inflammation in the heart disease of dKO mice, other immune cells may still be involved. The hearts of both tKO and dKO mice stain positive for the monocyte/macrophage marker F4/80, which colocalizes with both intramyocardial lipid deposition as indicated by Oil red O staining, suggesting foam cell formation(Braun, Trigatti et al. 2002), and fibrotic scar tissue (Figure 1). Thus, B- and T-lymphocytes and their specific programs of cytokine release are not required for monocyte/macrophage infiltration and foam cell formation within the hearts of dKO mice.
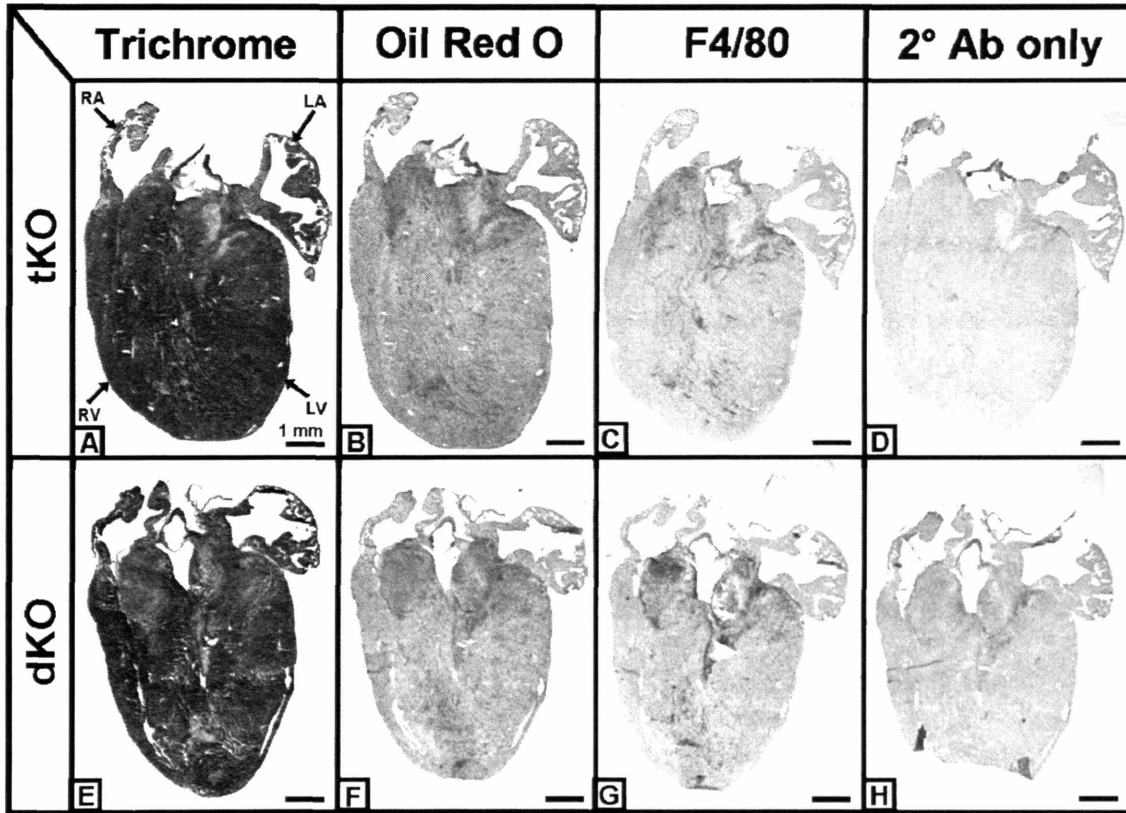
Figure 1: Macrophage staining colocalizes with myocardial fibrosis and lipid deposition in both SR-BI/apoE dKO and SR-BI/apoE/RAG2 tKO mice. Serial heart sections from a 6-week-old tKO (A-D) and dKO (E-F) mouse. In dKO mice, macrophages stained by F4/80 antibody (G) colocalize with regions of lipid deposition as stained by Oil red O (F) and fibrosis detected by trichrome staining (E). The hearts of tKO mice show a similar pattern of macrophage infiltration (C), lipid deposition (B) and fibrosis (A). Panels D and H are control sections for panels C and G respectively treated with only 2°antibody. Scale bar = 1 mm.

Method: 10 μm cryosections were air-dried for 20 minutes, fixed in -20°C acetone for 10 minutes, and blocked in 10% normal goat serum (NGS) in PBS for 30 minutes at room temperature. Sections were then incubated in 1:10 dilution of 1° antibody, mouse anti-rat F4/80, (Serotec, Raleigh, NC) in 2%NGS/PBS overnight in a humidified chamber at 4°C. Sections were then incubated for 1 hour in 2° antibody diluted 1:50 in 2%NGS/PBS. Staining was detected using the ABC-alkaline phosphatase kit and Vector Red substrate kit (Vector Laboratories, Burlingame, CA) according to instructions.

The presence of macrophages and macrophage-derived foam cells in atherosclerotic plaques has been well-documented (Introduction). Studies in which atherosclerosis-prone apoE(-/-) or LDLR(-/-) mice were crossed to macrophage colony stimulating factor (MCSF)-deficient osteopetrotic (op/op) mice demonstrate a pro-atherogenic role for monocyte-derived macrophages. Both apoE(-/-)/op/op and LDLR(-/-)/op/op mice exhibit dramatic reductions in aortic lesion size compared to MCSF-positive controls(Smith, Trogan et al. 1995; Qiao, Tripathi et al. 1997; Rajavashisth, Qiao et al. 1998). Osteopetrotic (op/op) mice carry a spontaneous frameshift mutation that results in complete deficiency of MCSF activity in tissues and serum. In addition to dysfunctional maturation of monocytes into macrophages in several tissues, osteopetrotic mice demonstrate a near complete absence of osteoclasts resulting in bone remodeling defects and skeletal and tooth deformities(Naito, Hayashi et al. 1991; Wiktor-Jedrzejczak, Urbanowska et al. 1991; Wiktor-Jedrzejczak, Dzwigala et al. 1996). Moreover, op/op mice have decreased body weight, impaired fertility and females have lactation defects, making them difficult to breed and maintain(Pollard, Hunt et al. 1991; Wiktor-Jedrzejczak, Urbanowska et al. 1991). It would be interesting to investigate the influence of MCSF deficiency on atherosclerosis and heart disease, especially infiltration and deposition of lipid-laden macrophages within the infarcted myocardium, in SR-BI/apoE dKO mice and I believe this question is currently being investigated by other groups. However, the low body weight, hemopoeitic dysfunction and frail nature of both SR-BI/apoE dKO and op/op mice as well as fertility constraints associated with both MCSF and SR-BI deficiency indicate that generating a cross between these two strains would be a challenging task.

Investigating NK cell-mediated cytotoxicity in myocardial infarction

Though mice bearing the RAG2 null mutation demonstrate a complete deficiency in mature B- and T-lymphocytes, they are not devoid of all lymphoid cells. Null lymphocytes or natural killer (NK) cells are large granular lymphoid cells that do not express antigen-specific rearranged cell surface receptors. Functional NK cells are found in lymphocyte-deficient mouse strains including RAG1- and RAG2- deficient mice, athymic nude mice and SCID mice. NK cells are generally thought to be the first line of defense against virally-infected cells in the time lapse required for proliferation and differentiation of cytotoxic T-lymphocytes (CTLs). Like CTLs, the granules of NK cells contain cytotoxic molecules including perforin and granzymes. However, unlike CTLs, which must be activated by their specific antigen before the granules appear, NK cells are constitutively cytotoxic and always contain large granules. In a mechanism similar to CTL-mediated cytotoxicity, NK cells adhere to and induce apoptosis of target cells by degranulating and releasing perforin and granzymes into the junction between the interacting cells.

A variety of NK-cell activity-deficient mutant mouse strains exist such as the Lyst (beige) mice, perforin knockout mice(Kagi, Ledermann et al. 1994) and and γ_c mutant mice(DiSanto, Muller et al. 1995). Lyst(beige) mutant mice have a severe deficiency in NK cells and also have defective CTL activity(Roder and Duwe 1979; Roder 1979). Perforin KO mice have normal numbers of CTLs and NK cells, however they are unable to lyse target cells(Kagi, Ledermann et al. 1994). Gamma-c mutant mice have a targeted deletion in the interleukin 2 receptor gamma chain, rendering them NK cell-deficient (350-fold reduction in NK cell populations), though they also have severe reductions in B- and T-cells(DiSanto, Muller et al. 1995). The significance of NK cells in atherosclerotic plaque development remains unclear. One study found that Lyst

(beige) NK-cell-deficient/LDLR(-/-) mice demonstrated an increase in atherosclerotic lesion size whereas perforin-deficient/LDLR(-/-) mice had similar lesions compared to control LDLR(-/-) mice, though both experimental groups exhibited diminished NK cell-mediated cytolytic activity(Schiller, Boisvert et al. 2002). These data seem to suggest that NK cell-mediated cytotoxicity is not important for atherogenesis and that the Lyst(beige) mutation carries additional pro-atherogenic characteristics(Schiller, Boisvert et al. 2002). However other studies including reconstitution of irradiated LDLR(-/-) mice with bone marrow derived from mice overexpressing the Ly49A receptor that inhibits activity of NK cells upon interaction with MHC molecules on the target cell, resulted in decreased atherosclerosis demonstrating that NK cell activity is pro-atherogenic(Whitman, Rateri et al. 2004). However, these data are also confounded because the activity of natural killer T-lymphocytes, $CD8^+$ cytotoxic lymphocytes and other lymphocytes expressing granzyme A, the ligand for Ly49A, would be affected as well.

The role of NK cells in ischemia-induced myocardial infarction is even more elusive. Studies on the role of NK cells in viral myocarditis indicate that these cells are among the first cells to infiltrate the infected tissue and protect the heart from exacerbated injury by only interacting with virus-infected cardiomyocytes(Kawai 1999). NK cell-deficient mice appear to have more extensive myocardial inflammation and damage in response to viral infection (Kawai 1999). However, large granular NK-cell-like lymphocytes are proposed to aggravate myocardial injury in myocarditis by perforin lysis of potentially salvageable cardiomyocytes(Kawai 1999). Diminished NK cell activity has been reported in patients with acute myocardial infarction (Klarlund, Pedersen et al. 1987; Kuroki, Miyahara et al. 1993), but more extensive studies in animal models have not been well established.

The lack of data on the importance of NK cells and perforin-mediated cytotoxicity in myocardial infarction as well as the abundance of mouse models available to study these processes suggest that this may be another interesting aspect of inflammation to investigate in the SR-BI/apoE dKO mouse model of heart disease.

Investigating the role of neutrophils in myocardial infarction

As discussed briefly in the introduction, neutrophils are thought to play an important role in myocardial necrosis and perpetrating cardiomyocyte injury. Granulocyte infiltration is a hallmark of myocardial infarction and the timeline of neutrophil migration into unreperfused human MIs was documented by Mallory et al. over 60 years ago(Baxter 2002). Neutrophil infiltration of myocardium occurs 24-48 hours after the onset of ischemia, peaking at day 4 and then nearly disappearing by day 14(Kilgore and Lucchesi 2000; Baxter 2002).

Prompt restoration of blood flow to the ischemic area, although required to preserve cardiomyocyte integrity and function, is associated with an accelerated inflammatory response and massive neutrophil infiltration. Most studies on neutrophil-mediated cardiac injury have focused on this abrupt inflammation associated with reperfusion. However, neutrophils have also been implicated in ischemic heart disease and chronic heart failure. Experimental evidence suggests that neutrophils accumulate gradually during ischemia at the periphery of the infarcted region and reperfusion simply accelerates their infiltration into the infarct(Baxter 2002). Neutrophils that enter the myocardium in response to an ischemic event may contribute to both acute and progressive tissue damage(Kilgore and Lucchesi 2000). Studies have shown that "resident" neutrophils that persist in the injured tissue after the initial ischemic insult can trigger cardiac dysfunction hours after reperfusion(Kilgore and Lucchesi 2000). There is also a

correlation between neutrophil count at the time of admission and development of chronic heart failure in survivors of acute myocardial infarction (Kilgore and Lucchesi 2000).

Neutrophil accumulation in the heart can mediate cardiomyocyte toxicity through a variety of mechanisms. The release of pro-inflammatory mediators in ischemic myocardium induces neutrophils to undergo a respiratory burst, generating oxygen free radicals including superoxide anion and hydrogen peroxide(Jordan, Zhao et al. 1999). Neutrophils also release hypochlorous acid, as well as proteases, collagenases, lipoxygenases and myeloperoxidase, all of which can damage myocardium(Jordan, Zhao et al. 1999). One phenomenon described as "no-reflow" is characterized by a lack of maintained blood flow and severe microvasculature damage in infarcted areas subjected to extended ischemia followed by reperfusion. The plugging of capillaries by rigid, activated neutrophils in the myocardial infarction was subsequently determined to be the underlying cause of no-reflow.

There are many studies suggesting that inhibition of inflammation, neutrophil depletion or interruption of neutrophil adhesion can limit infarct size in experimental models of ischemia and reperfusion(Jordan, Zhao et al. 1999; Baxter 2002). However, these reports are contradicted by numerous experiments that fail to reproduce the beneficial effects of limiting neutrophil activity or neutrophil depletion on infarct size(Baxter 2002). Neutrophil depletion can be achieved by passing the blood through neutrophil clearing filters, chemotherapy to induce neutropenia or administration of anti-neutrophil antibodies. In addition, there exist mouse models deficient for neutrophil elastase (Belaaouaj, McCarthy et al. 1998) which contributes significantly to neutrophil-mediated tissue damage through its effects on membrane charge distribution and hydrolyzes the ECM components elastin, fibronectin and collagen types III and IV(Jordan, Zhao et al. 1999). Depletion of neutrophils or inhibition of neutrophil activity in SR-BI/apoE dKO mice

may help to elucidate the role of these inflammatory cells in ischemia-induced myocardial infarction.

I have limited my discussion of potential future studies to plausible cellular mediators of inflammation in atherosclerosis and myocardial infarction. However, studies of the roles of numerous cytokines and chemical mediators of inflammation in myocardial infarction including, but not limited to, various constituents of the complement system, interleukins, IFN-γ and TNF-α are of paramount interest and could be examined in the dKO model(Mann 2002). In addition, studies on various cell adhesion and extracellular matrix molecules including E, P, L selectins, ICAM-1, VCAM-1, integrins and fibronectin that mediate leukocyte recruitment and extravasation in this model could help elucidate the roles and relative importance of these molecules in heart disease. Studies on the role of E, P, L selectins and fibronectin are already underway in collaboration with the Hynes laboratory.

In addition the development of a lymphocyte-deficient SR-BI/apoE dKO mouse strain (SR-BI/apoE/RAG2 tKO mice) with a virtually identical CHD phenotype to the original dKO mouse provides a unique immunodeficient CHD model in which to study the effects of various biologic agents and therapeutics without instigating immunoglobulin- and lymphocyte-mediated inflammatory responses that could confound results.

Effects of Strain Background and Genetic Modifiers on Lifespan

The generation of SR-BI/apoE dKO mice on a background syngeneic with that of the SR-BI/apoE/RAG2 mice produced a serendipitous discovery concerning the effects of genetic background on lifespan of dKO mice. Strikingly, 82% of the dKO mice on this background strain, an undetermined mix of C57BL/6×129-S4×BALBc which we have designated as strain 2, die within an exceptionally narrow time window of 9 days (38-47 days of age) whereas the

comparable range in the original 75:25 C57BL/6:129-S4 strain, designated strain 1, is 16 days

(40-56 days)(Karackattu, Picard et al. 2005) (Chapter 2). Furthermore, closer analysis of survival

data on the 75:25 C57BL/6:129-S4 dKOs bred as controls for SR-BI/apoE/HL tKO mice indicate

that a similar proportion of mice, 83%, die over a 13 day window of 39-52 days (Chapter 3). In

addition, the mean lifespan of strain 2 mice (42±0.5 days) is significantly shorter (P< 0.0001) than

the mean lifespans of both strain 1 dKOs (45.9±0.9 days) and the 75:25 C57BL/6:129-S4 dKOs

controls for SR-BI/apoE/HL tKO mice (reported as 46.0±0.3 days when mice ≤ 33 days old are

omitted from the calculation of mean lifespan [see chapter 3 for rationale behind this exclusion]

and falling to 45.2±0.4 days if these early dying mice are included)(Karackattu, Picard et al.

2005) (Chapters 2 and 3).

Both SR-BI/apoE/RAG2 tKO and SR-BI/apoE/HL tKO mice and their corresponding

genetically similar dKO controls were generated by the breeding scheme outlined in Appendix 1.

In addition, the lifespan of strain 1 dKOs was initially reported as 8-9 weeks, however a later,

more complete characterization of the dKO phenotype indicated that these mice die between 5

and 8 weeks of age with an average lifespan of 6 weeks(Trigatti, Rayburn et al. 1999; Braun,

Trigatti et al. 2002). These data suggest that selective breeding or random drift may result in the

association of unknown genetic modifiers responsible for modulating both average lifespan and

lifespan variability.

The observation that genetic background and associated modifier loci in animal models of

heart disease profoundly affect susceptibility to atherosclerosis, cardiac hypertrophy, cardiac

dysfunction and survival is certainly not novel. As described in the introduction, WHHLMI

rabbits were developed via selective breeding of hyperlipidemic rabbits that were increasingly

prone to development of coronary atherosclerosis and subsequently for MI, suggesting the

accumulation of innate genetic modifiers that predispose these rabbits to heart disease. More than 20 years ago, Paigen et al. demonstrated that various inbred strains of mice exhibited differences in susceptibility to diet-induced atherosclerosis and subsequently succeeded in identifying the relevant quantitative trait locus called Ath-1, which encompasses 11 known genes, to mouse chromosome 1(Paigen, Morrow et al. 1985; Paigen, Mitchell et al. 1987). Genetic background strain was also shown to drastically influence atherosclerotic lesion size, most likely by differentially modulating the temporal aspects of atherogenesis, in apoE(-/-) mice independent of variations in several cholesterol and lipoprotein parameters(Dansky, Charlton et al. 1999). Inbred strains of mice exhibit naturally occurring variations in cardiovascular traits including differences in LV end-diastolic dimension, LV mass, LV fractional shortening, heart rate and exercise endurance(Hoit, Kiatchoosakun et al. 2002). In addition, using the Calsequestrin-Tg murine model of cardiac hypertrophy and murine dilated cardiomyopathy, Suzuki et al. reported modifier loci on chromosomes 2 and 3 that influence cardiac function and survival, demonstrating the value of mouse models exhibiting robust genetic variation in disease phenotypes in identifying associated modifier genes(Suzuki, Carlson et al. 2002).

The discovery that genetic background affects mean survival and lifespan variability in SR-BI/apoE dKO mice has profound implications for future studies. First, we have realized that it is imperative to generate controls on a similar background when crossing in other knockouts or transgenes to study their effects on CHD in this model. Moreover the decreased variability in lifespan of the strain 2 dKOs has provided us with an increasingly potent model in which to study the effects of various genes, drugs and therapeutics by permitting us to see small but statistically significant differences with smaller numbers of animals. In addition, general studies of the pathophysiology and time course of CHD in the dKO model are simplified by reduction in

variability between samples, potentially allowing for a decrease in sample size required to observe reproducible and quantifiable results. Finally, the generation of dKO mice on different background strains may provide models in which to identify and map genetic loci associated with modulating the progression of CHD and survival.

Lipoprotein Metabolism and Coronary Heart Disease in SR-BI/apoE dKOs

Lipoprotein Structure and Composition in SR-BI/apoE dKOs and SR-BI/apoE/HL tKOs

By generating SR-BI/apoE/HL triple knockout (tKO) mice we demonstrated that deficiency of HL could reduce atherosclerosis in an SR-BI-independent manner and thus delay MI and cardiac dysfunction and extend the lifespan of dKO mice. Though we saw substantial decreases in both aortic and coronary occlusive lesion size in the tKO mice, we didn't observe any obvious differences in lipoprotein phenotype other than the HL-deficiency-associated increases in lipid levels(Homanics, de Silva et al. 1995; Mezdour, Jones et al. 1997; Bergeron, Kotite et al. 1998) and thus could not link the reduction in atherosclerosis with specific HL-mediated effects on lipoprotein metabolism. Though the cardiac and hematopoietic pathology of dKO mice is thought to be a consequence of their abnormally high unesterified cholesterol to total cholesterol (UC:TC) ratio (Holm, Braun et al. 2002; Braun, Zhang et al. 2003), SR-BI/apoE/HL tKO mice demonstrated a similar UC:TC ratio at both 6 and 9 weeks of age. However, tKO mice had a slightly lower (and more similar to control values) surface-to-core lipid ratio in their lipoproteins (Chapter 3) (Braun, Zhang et al. 2003; Zhang, Picard et al. 2005).

We attempted to perform electron microscopy on fractionated plasma samples of dKO and tKO mice. This work was done by Dr. Elisa Vasile, a research scientist in the Krieger Laboratory. We obtained some acceptable micrographs of lipoproteins from tKO mice (Figure 2) that suggest the presence of large multilammelar and discoidal lipoproteins similar to those

present in the plasma of apoE(-/-)/HL(-/-) mice and SR-BI/apoE dKO mice as previously reported(Bergeron, Kotite et al. 1998; Braun, Zhang et al. 2003; Zhang, Picard et al. 2005). In the case of apoE(-/-)/HL(-/-) mice, Bergeron et al. suggests that these abnormal structures are formed by SR-BI selectively removing cholesteryl ester from the core of lipoproteins. In the absence of HL to hydrolyze the excess outer shell phospholipids, the cholesteryl ester core-deficient lipoproteins fuse to form more stable multilammelar particles. That these abnormal structures form in the absence of SR-BI (in SR-BI/apoE dKO mice), and likely may also form in the absence of both SR-BI and HL suggests alternate mechanisms for the generation of multilammelar lipoproteins. Interestingly, similar lipoprotein structures have been reported in other conditions associated with elevated unesterified cholesterol and phospholipid ratios including cholestatic liver disease and familial LCAT deficiency as well as LCAT knockout mice(Miller 1990; Ng, Francone et al. 1997). Despite our efforts, we were not able to obtain acceptable images of lipoproteins from control dKOs and thus were not able to draw even qualitative assessments on alterations in lipoprotein structure attributable to HL-deficiency in dKO mice. Nevertheless, given the abnormal lipoprotein structures in the VLDL of SR-BI/apoE dKO mice(Braun, Zhang et al. 2003) and IDL/LDL of apoE(-/-)HL (-/-) mice(Bergeron, Kotite et al. 1998), it seems plausible that there might be cumulative effects of the combined deficiencies of SR-BI, apoE and HL on lipoprotein structure.
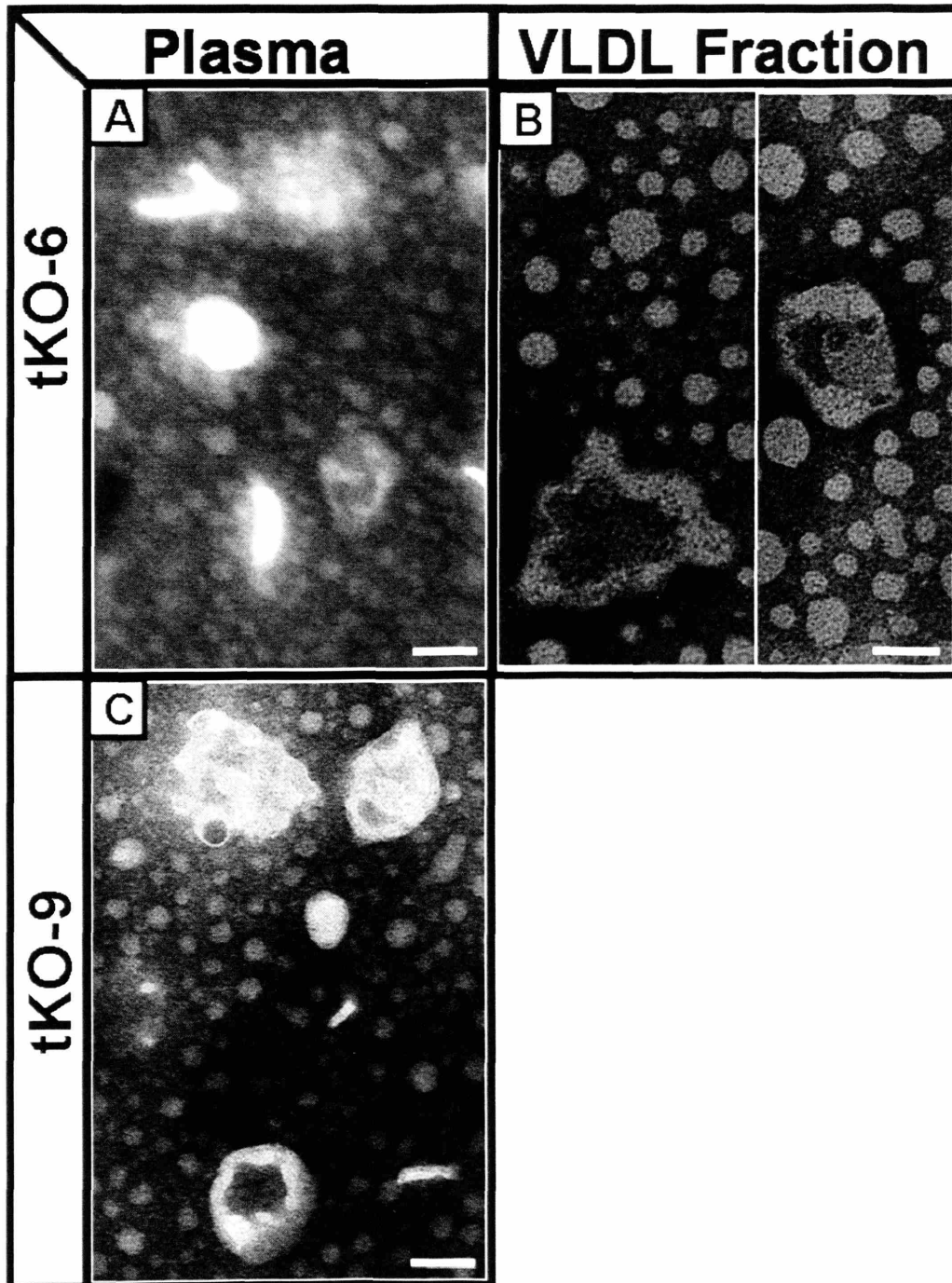
Figure 2: Electron microscopy of plasma (A) and VLDL fraction (B) from a 6-week old SR-BI/apoE/HL tKO mouse and plasma from 9-week old SR-BI/apoE/HL tKO mouse (C). Negative staining of lipoproteins with phosphotungstic acid was performed as previously described (Bergeron, Kotite et al. 1998; Braun, Zhang et al. 2003; Zhang, Picard et al. 2005). Bar = 100 nm.

The size of atherogenic lipoproteins has been correlated to their relative atherogenicity, specifically, smaller lipoproteins like LDL are more atherogenic than larger VLDL, possibly because smaller particles can more easily penetrate the endothelium and bind proteoglycans within the arterial intima (Veniant, Withycombe et al. 2001) (Introduction). HL-mediated hydrolysis is involved in the conversion of larger lipoproteins to smaller ones i.e. conversion of IDL to LDL. The absence of HL-mediated hydrolysis may skew the distribution of lipoproteins towards larger particles, thus reducing their atherogenic potential. Consistent with this notion, Mezdour et al. report that, compared to apoE KO controls, apoE(-/-)HL(-/-) mice have higher levels of cholesterol in the VLDL particle size fraction but no apparent increase in levels of apoB-48 or apoB-100 suggesting that HL-deficient mice accumulate larger apoB-containing particles and supporting a role for HL in remodeling of remnant lipoproteins (Mezdour, Jones et al. 1997). The standard molecular size exclusion FPLC technique used to fractionate dKO and tKO plasma did not demonstrate observable particle size differences in the distribution of their lipoproteins (Chapter 3). However, this technique is confounded by the abnormal, nonspherical structures of lipoproteins in these mice. Moreover, the majority of their lipoproteins fall into the earliest fraction characteristic of VLDL-sized particles, which also represents the void volume of the chromatographic column. Thus, if the lipoprotein size or morphology varies substantially between dKOs and tKOs, the difference is beyond the limits of resolution for our FPLC method and would have to be observed and quantified via other techniques such as electron microscopy, electrophoresis and ultracentrifugation.

Apolipoprotein and phospholipid composition is another parameter affecting relative lipoprotein atherogenicity that may be significantly different in tKOs and dKOs. The cholesterol esters of lamellar lipoproteins may contain fewer polyunsaturated fatty acids (Bergeron, Kotite et

al. 1998). Furthermore, increased sphingomyelin content may augment the atherogenicity of lipoproteins by making these lipoproteins more prone to aggregation upon exposure to sphingomyelinase secreted by macrophages and endothelial cells(Bergeron, Kotite et al. 1998; Schissel, Jiang et al. 1998). The elevated sphingomyelin content of lipoproteins in apoE(-/-) mice may contribute to their increased atherogenic potential and reflects a combined defect in synthesis and degradation of sphingmyelin(Jeong, Schissel et al. 1998). Moreover, recent studies demonstrate that inhibition of sphingomyelin synthesis reduces atherosclerosis in apoE(-/-) mice(Park, Panek et al. 2004). Compared to dKOs, the lipoproteins of tKO mice may be relatively poor in sphingomyelin due to the lack of hydrolysis of phosphaditylcholine, the principal phospholipid, by HL(Bergeron, Kotite et al. 1998). Thus, subtle variations in sphingomyelin content that affect the aggregation potential of lipoproteins within the arterial intima may account for the temporal differences in atherogenesis between dKO and tKO mice. The lipoproteins of apoE(-/-)HL(-/-) mice were also found to be richer in apo-AIV and apo-AI(Bergeron, Kotite et al. 1998) and apoE(-/-) mice expressing either human apo-AI or apo-AIV exhibit reduced atherosclerosis(Plump, Scott et al. 1994; Duverger, Tremp et al. 1996). Thus reduced atherosclerosis in tKO mice may be linked to subtle changes in apolipoprotein, fatty acid and phospholipid content that could only be detected through more extensive studies of lipoprotein chemistry.

Hepatic Lipase Ligand Binding Activity

As mentioned in the Introduction, hepatic lipase is thought to have two distinct activities. The enzymatic moiety remodels various classes of lipoproteins by hydrolyzing triglycerides and phospholipids. In addition, HL participates in removal of remnant lipoproteins through its "ligand-binding" or "bridging" activity, possibly in concert with other cell surface lipoprotein

receptors. In vitro studies demonstrate that cell surface-bound HL facilitates binding of remnant lipoproteins in an apoE-independent manner (Ji, Lauer et al. 1994) and in vivo, catalytically inactive HL mediates selective uptake of cholesterol from remnant lipoproteins in apoE-deficient mice (Amar, Dugi et al. 1998). HL-mediated lipoprotein-cell interactions are disrupted by heparin, suggesting that HL may act as a tether linking remnant lipoproteins to cell surface heparan-sulfate proteoglycans (Ji, Dichek et al. 1997; Mezdour, Jones et al. 1997).

Studies have demonstrated that HL can enhance cholesterol selective uptake by working in conjunction with SR-BI(Lambert, Chase et al. 1999; Lambert, Amar et al. 2000). HL can also facilitate uptake of lipoproteins (β-VLDL) by interacting with LDL-receptor-related protein (LRP), another cell surface lipoprotein receptor(Krapp, Ahle et al. 1996). Recently, the liver-specific expression of catalytically inactive HL (HL-145G) in apoE(-/-)HL(-/-) mice was found to substantially reduce cholesterol levels, particularly in the VLDL and apoB-containing fractions, without reducing levels of HDL-C or apoA-I(Gonzalez-Navarro, Nong et al. 2004). In addition, infusion of receptor-associated protein (RAP), an inhibitor of LRP, greatly reduced HL-145G mediated clearance of radiolabeled apoB-48 LDL, suggesting that the ligand-binding function of HL may facilitate clearance of apoB-containing lipoproteins via the LRP pathway. Furthermore, the ligand-binding function of transgenically expressed HL-145G was found to be particularly anti-atherogenic in apoE(-/-)HL(-/-) mice as opposed to wild-type HL(Gonzalez-Navarro, Nong et al. 2004).

Since, the ligand-binding activity of HL is also thought to facilitate SR-BI-mediated uptake of lipoproteins, it has been suggested that HL-145G may alternatively work in conjunction with SR-BI to clear lipoprotein remnants in apoE(-/-) mice (Amar, Dugi et al. 1998). If an SR-BI-dependent pathway accounts for a major mechanism by which HL-145G lowers plasma apoB-

containing lipoproteins and reduces atherosclerosis, this pathway and its beneficial effects on

plasma lipid levels and atherosclerosis would be abolished in SR-BI/apoE dKO mice, and thus

expression of HL-145G in SR-BI/apoE dKO mice would be expected to have little effect on the

cardiac phenotype. Conversely, if the LRP pathway is the major route by which HL-145G

stimulates clearance of apoB-containing, atherogenic lipoproteins, then transgenic expression of

HL-145G in SR-BI/apoE dKO mice may lower lipid levels, reduce atherosclerosis, delay MI and

cardiac dysfunction and ultimately extend lifespan. Thus the ligand-binding activity of HL and its

role in modulating atherosclerosis may prove interesting in the study of mechanisms underlying

systemic lipoprotein metabolism and CHD in SR-BI/apoE dKO mice.

Splenomegaly and Reticulocytosis in SR-BI/apoE dKO mice

One phenotype of SR-BI/apoE dKO mice that has not previously been reported is their

massive splenomegaly. A hypercholesterolemia-associated defect in erythrocyte maturation

renders them severely anemic with nearly 100% of their circulating red blood cells (RBCs)

present as reticulocytes (Holm, Braun et al. 2002). Erythropoietin (Epo) from renal peritubular

cells normally stimulates hematopoiesis by bone marrow cells. However, prolonged release or

high levels of Epo in response to acute anemia-induced hypoxia provokes erythropoiesis in the

spleen, resulting in splenomegaly or enlargement of the spleen (Binley, Askham et al. 2002;

Lenox, Perry et al. 2005). The spleens of dKO mice demonstrate evidence of extensive

erythropoiesis (unpublished histologic observations, B. Trigatti, Mark Schrenzel and Monty

Krieger). In addition, the spleen is responsible for clearing aging and abnormal blood cells from

the circulatory population. Massive destruction of RBCs, as in the case of spherocytosis, can also

contribute to splenomegaly in mouse models (Iolascon, Miraglia del Giudice et al. 1998;

Wandersee, Lee et al. 1998). SR-BI/apoE dKO mice exhibit morbidly gross splenomegaly, most

likely the result of both erythropoiesis in response to hypoxia as well as destruction of the

aberrant reticulocytic RBCs.

Despite the observation that dKO and tKO mice have similar hematocrits (Chapter 3),

splenomegaly is markedly reduced in 6-week-old tKO mice compared to dKOs (Figure 3).

However, the spleen-to-body weight ratios of 9-week-old tKO mice are similar to that of dKO

mice. The delay in progression of splenomegaly in tKO mice may arise as a result of slightly

better intrinsic oxygen-carrying capacity of the blood cells or reduced rate of destruction or

turnover of RBCs .
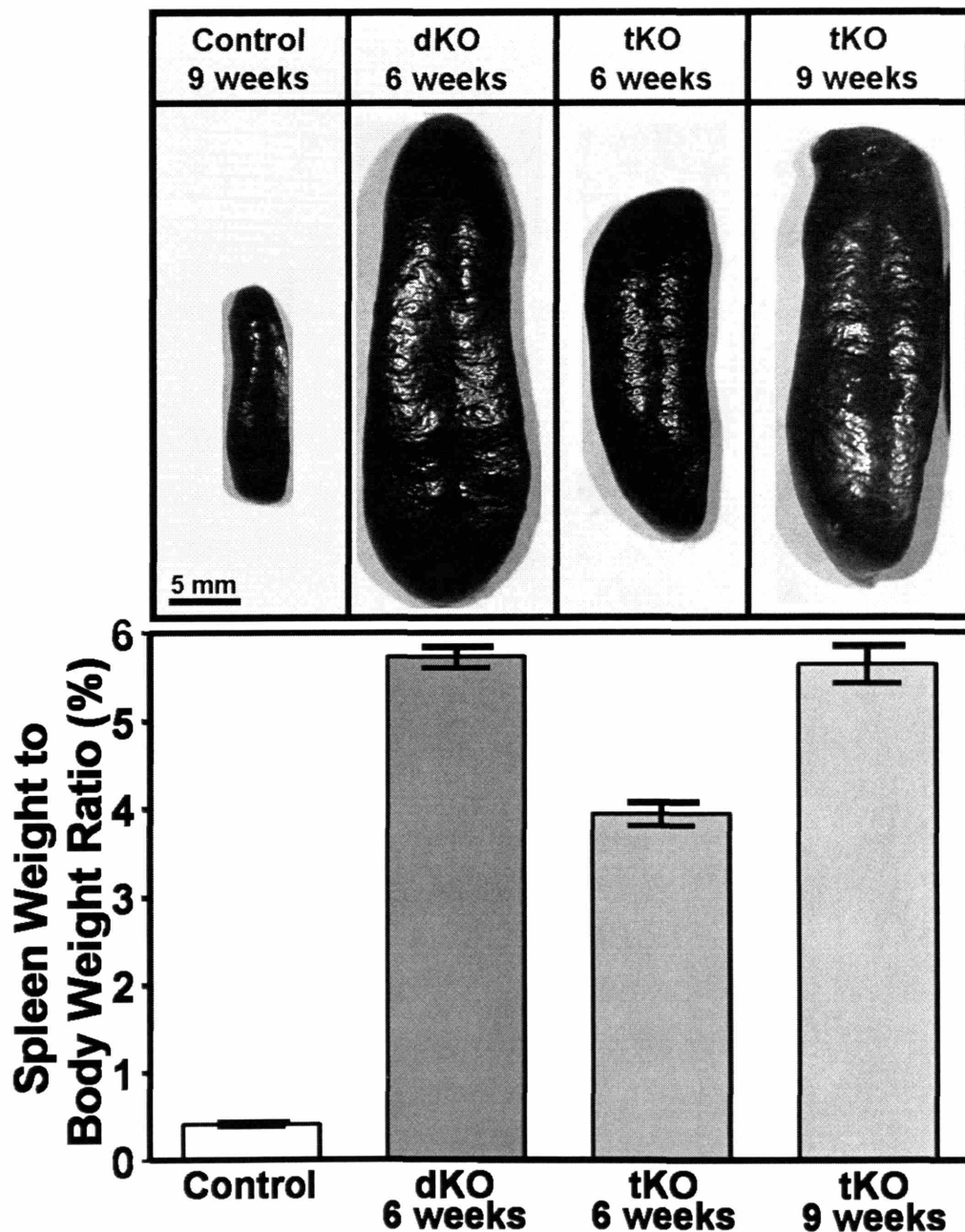
Figure 3: Massive splenomegaly in SR-BI/apoE dKO mice and SR-BI/apoE/HL tKO mice. At 6 weeks of age, tKO mice ($3.9 \pm 0.1\%$, n = 39) have significantly reduced spleen size and spleen to body weight ratio compared to dKO mice ($5.7 \pm 0.1\%$, n = 43; P<0.0001). However, by 9 weeks of age, spleen to body weight ratio in tKO mice ($5.6 \pm 0.2\%$, n = 34; P = 0.7073) is comparable to that of 6-week-old dKO mice. The spleen to body weight ratio of controls (obtained by analysis of healthy SR-BI-positive littermates of dKO and tKO mice) was significantly lower than any of the experimental groups ($0.40 \pm 0.02\%$, n = 7).

Preliminary electron microscopic studies of the morphology of red blood cells in 6-week-old SR-BI(+/-)/apoE(-/-)/HL(-/-) control, dKO and tKO mice seem to suggest that tKOs at this age may have less severe reticulocytosis; the erythrocytes of 6-week-old tKO mice did not appear to have as many autophagolysosomal inclusions (Figure 4). We did not, however, look at enough samples to make quantitative determinations on differences in the degree of reticulocytosis nor did we look at the blood of 9-week-old tKOs. Further studies including microscopy of erythrocytes, blood gas levels and plasma Epo titers may help to elucidate the underlying causes for the observed differences in spleen size of tKO and dKO mice at 6-weeks of age as well as the extended survival of tKO mice.
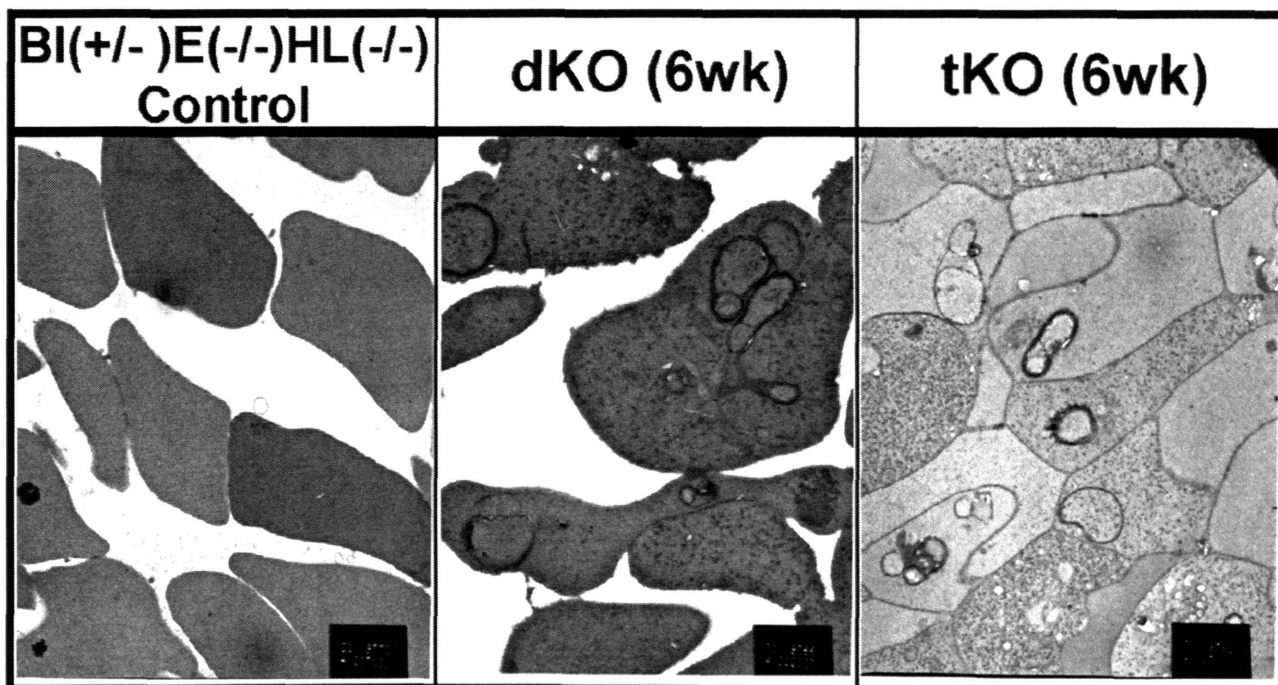
Figure 4: Preliminary electron microscopic analysis of erythrocytes from control, SR-BI/apoE dKO and SR-BI/apoE/HL tKO mice. Some of the cells in tKO mice appear to be relatively free of large autophagolysosomal inclusions and/or ribosomes compared to dKO mice. Further studies are necessary to qualitatively and quantitatively assess whether a difference between dKOs and tKOs exists. EM was performed as previously described (Holm, Braun et al. 2002).

Timecourse of CHD in SR-BI/apoE dKO Mice: Hypertrophy versus Myocardial Infarction

As presented in the Introduction, one of the key features of CHD is cardiomegaly attributable to myocyte hypertrophy. The hypertrophic response of cardiac myocytes is thought to be an adaptive response compensating for increased stress as the heart experiences ischemic damage, accrues myocardial infarctions and becomes less efficient at ejecting its ventricular volume. However, the hearts of SR-BI/apoE/HL tKO mice seem to exhibit mild hypertrophy before showing gross myocardial infarctions or significant overt histochemical evidence of fibrosis (Chapter 3). Thus, it's possible that myocyte hypertrophy in the dKO model sets in before marked observable cardiomyocyte death and fibrosis or that the hypertrophy is a response to physiological cues other than hemodynamic stress on a failing heart. For example, Epo-Tag[h] mice are rendered severely anemic by erythropoietin deficiency attributable to targeted disruption in the 5' untranslated region of the EPO gene (Binley, Askham et al. 2002). These mice exhibit anemia-induced hypertrophy to an extent similar to hypertrophy in dKO mice (Binley, Askham et al. 2002). Spherocytosis mutant mice with severe hemolytic anemia also exhibit cardiac hypertrophy (Wandersee, Lee et al. 1998). Anemia-induced hypertrophy occurs as the heart attempts to compensate for tissue oxygen-deprivation by increasing cardiac output. Since dKO mice are also severely anemic, it is conceivable that anemia and hypoxia may initiate hypertrophy before the occurrence of coronary occlusion-induced ischemic myocardial damage. The temporal dissociation between initiation of hypertrophy and extensive myocardial infarction in tKO mice may provide a model in which to study the possible independent mechanisms underlying the development of these two pathophysiological traits in the CHD of dKOs.

The Big Picture

The SR-BI/apoE dKO mouse, with its remarkable phenotype that recapitulates many of the hallmarks of human CHD, is a valuable model for the study of this prevalent condition. However, the power of this model lies in continued characterization as well as its validation as an accurate model of the pathophysiology underlying human CHD. Though multiple lines of evidence suggested that these animals were indeed dying of coronary occlusive ischemic heart disease, this had yet to be proven.

In Chapters 2 and 3 of this thesis, I manipulated two systems, inflammation and lipoprotein metabolism, that underlie atherosclerosis and heart disease and then investigated the effects of these manipulations on CHD in dKO mice. By demonstrating that lymphocytes are not critical to development of CHD we effectively ruled out the possibility that the cardiac phenotype of dKO mice was the result of lymphocyte-induced or immunoglobulin-mediated inflammatory heart disease triggered by the release of cardiomyocyte proteins, similar to the autoimmune myocarditis exhibited by mice immunized with cardiac-myosin. The generation of mice triply deficient for SR-BI, apoE and HL demonstrated that hepatic lipase deficiency still reduces atherosclerosis, both aortic and coronary occlusive, in the absence of SR-BI activity. More importantly, we were able show that the reduction in development of coronary occlusive atherosclerosis was associated with delayed onset and/or progression of hypertrophy, myocardial infarction and cardiac dysfunction as well as extended lifespan. In addition, as the incidence of severe coronary occlusions in SR-BI/apoE/HL tKO mice advanced to match that of dKOs, they also succumbed to similar pathophysiological alterations in cardiac structure and function and eventually died.

The results from these studies and those by others also have over-arching implications on the way we view the relationship between HDL, lipoprotein metabolism and atherosclerosis. Taken together, these studies demonstrate that high HDL-cholesterol levels alone do not guarantee protection against atherosclerosis. It is the ability of proteins like SR-BI and hepatic lipase to properly regulate the structure, composition and properties of HDL that determine its anti-atherogenic functions. The absence of SR-BI eliminates one pathway by which HDLs can participate in removing excess cholesterol from the body, thereby diminishing its atheroprotective activity. However the anti-atherogenic functions may also involve its anti-oxidant properties as well as its ability to stimulate nitric oxide release and migration in endothelial cells via SR-BI mediated mechanisms (Yuhanna, Zhu *et al.* 2001; Seetharam, Mineo *et al.* 2006). Further characterization of SR-BI and other proteins involved in HDL metabolism may provide insights into the discovery of potential prophylactic and therapeutic measures against atherosclerosis and coronary heart disease.

These data, in conjunction with other studies on the effect of probucol on dKO pathophysiology and the generation of the SR-BI KO/*ApoeR61*[h/h] model of diet-induced CHD, help to validate the SR-BI/apoE dKO mouse as a rapid, easily maintained and accurate model of atherosclerotic coronary occlusion-induced ischemic heart disease. We anticipate that dKO mice will be a valuable model in which to investigate underlying mechanisms of CHD, as well as evaluate various environmental, genetic, and therapeutic interventions aimed at ameliorating this disease.

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Appendices

Appendix 1: Breeding scheme used to generate SR-BI/apoE/RAG2 and SR-BI/apoE/HL tKO mice and corresponding dKO control mice.

1. Two sister females that are SR-BI +/- apoE -/- will be selected from a single breeding pair of the pre-existing 75:25 C57BL/6:129-S4 SR-BI/apoE dKO line. They will each be mated to one of two brother males of the genotype SR-BI -/- Gene X -/- (with Gene X referring to Rag2 or HL depending on the study) to create a group of mice of the genotypes SR-BI -/- apoE +/- Gene X +/- or SR-BI +/- apoE +/- Gene X +/- that are genetically equivalent.

2. The next step will be to cross females that are SR-BI +/- apoE +/- Gene X +/- to males that are SR-BI -/- apoE +/- Gene X +/- (triple heterozygous crosses). This will be done via brother-sister or cousin-cousin matings. Through these crosses we expect to get tKO mice with a frequency of about 1/32 based on Mendelian inheritance. The numbers may be slightly reduced due to a lower than expected ratio of SR-BI/apoE dKO mice observed in SR-BI +/- apoE -/- × SR-BI +/- apoE -/- matings.

3. Though animals generated through "triple het crosses" will be analyzed, the ultimate goal is to establish a line of animals that will generate tKO mice with a ¼ frequency and maintain themselves via SR-BI +/- apoE -/- Gene X -/- matings. A corresponding line of SR-BI +/- apoE -/- Gene X +/+ crosses will be established to provide control dKO animals on the same background for comparison.

Appendix 2: Comparison of lifespan of sibling SR-BI/apoE dKO and SR-BI/apoE/HL tKO mice.
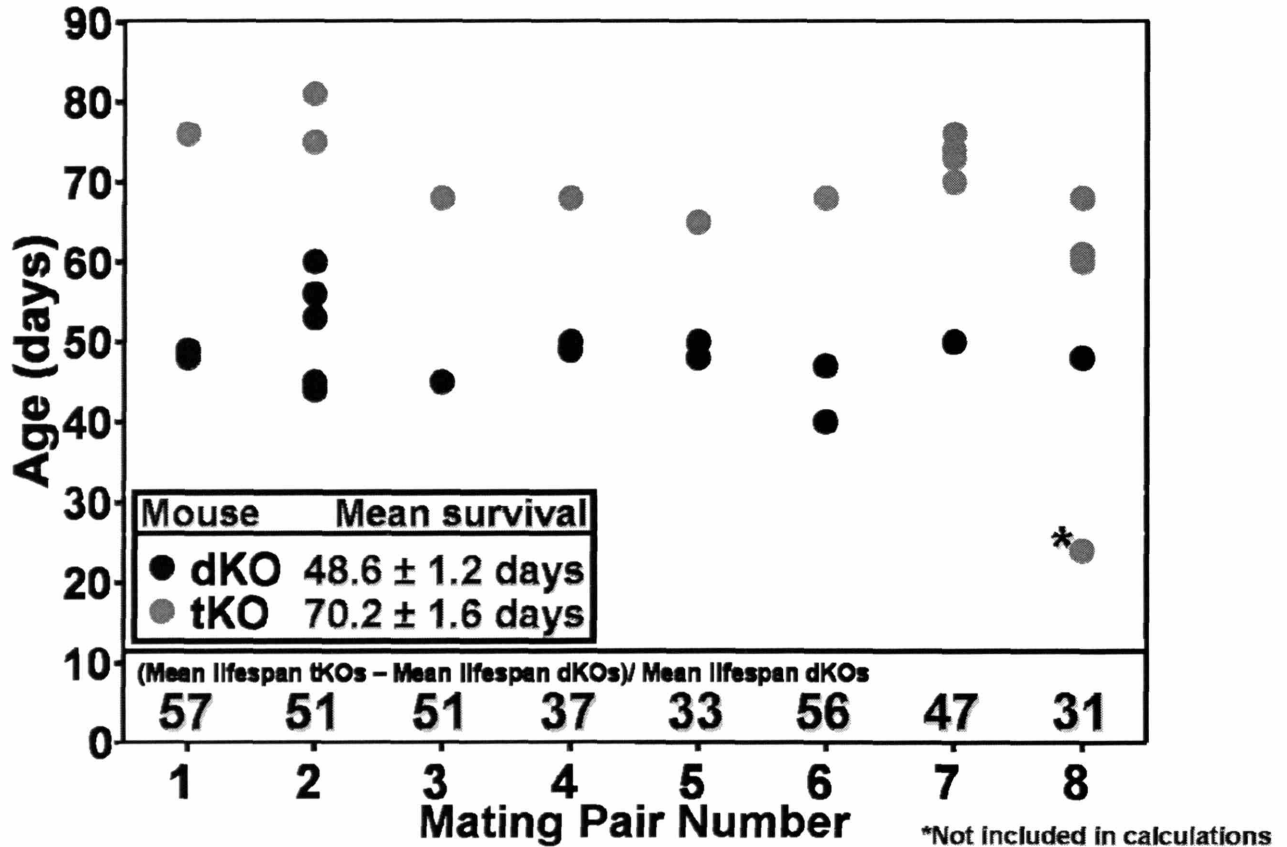
In order to verify that the difference in lifespan between dKOs and tKOs was an HL-dependent effect and not due to random divergence or segregation of unidentified modifiers of lifespan between the different sets of mating pairs used to generate each group of mice, we set up 8 mating pairs of SR-BI(+/-)/apoE(-/-)/HL(+/-) mice crossed to each other that could then produce both dKOs and tKOs. dKOs and tKOs born to a single mating pair were then compared to each other (offspring from a single mating pair are plotted along the Y-axis). In each case, with one exception that most likely died as a result of hypersensitivity to the anesthesia used for tailing), tKOs outlived their dKO siblings. The number above each mating pair represents the average increase in lifespan for the tKO compared to dKO offspring from that pair.

Appendix 3: Blood Chemistry of SR-BI/apoE dKOs and SR-BI/apoE/HL tKOs

Assay	dKO-6 (n=5)	tKO-6 (n=5)	tKO-9 (n=8)	Wild type (n=4)
Potassium	6.7±0.7	4.6±0.2	6.6±0.4	4.6±0.3
Calcium	10.2±0.2	9.8±0.1	9.7±0.5	9.25±0.1
Sodium	147.6±1.5	143.4±0.7	153.8±1.7	146.3±2.3
Chloride	109.4±0.7	107.6±0.5	109.1±1.1	109.3±1.5
Phosphorus	9.9±0.9	7.2±0.8	8.2±0.5	6.5±0.5
Total Bicarbonate	15.8±1.7	21.4±1.3	22.8±1.0	18.5±0.6
NA/K ratio	23±2	31±1	24±1	32±1
B/C Ratio	91±9	79±2	147±12	62±6
Anion gap	29±2	19±2	29±2	23±3
Albumin	2.78±0.05	2.8±0.06	2.4±0.03	3.0±0.1
Globulin	1.9±0.2	2.2±0.1	2.9±0.1	1.5±0.1
Alanine transaminase (SGPT)	29±3	25±3	37±5	26±2
Alkaline Phosphatase	304±15	427±57	109±9	221±24
Aspartate transaminase (SGOT)	116±12	106±17	263±38	66±8
Total Bilirubin	1.02±0.11	1.32±0.06	0.79±0.09	0.125±0.03
Indirect Bilirubin	0.64±0.09	0.86±0.02	0.5±0.07	0.1±0.0
Glucose	186±23	162±4	116±10	266±21
Blood Urea Nitrogen	38±3	30±2	61±5	26±2
Creatinine	0.42±0.02	0.38±0.02	0.43±0.03	0.43±0.03
Bile Acids	11.7±1.1	11.1±1.2	11.2±1.7	8.1±3.9
Cholesterol	972±62	1392±137	1017±89	65±5

Preliminary assays of plasma chemistry for wild-type, SR-BI/apoE dKO at 6 weeks of age and SR-BI/apoE/HL tKO mice at 6 and 9 weeks of age. Blood was obtained via retro-orbital puncture and spun at 4°C for 10 minutes in serum separator tubes. Serum was then sent to Division of Comparative Medicine (DCM) for analysis.

Appendix 4

EFFECTS OF HEPATIC EXPRESSION OF THE HDL-RECEPTOR SR-BI ON LIPOPROTEIN METABOLISM AND FEMALE FERTILITY

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In Press

The author participated in initial experiments with Mary Donahee and Karen Kozarsky demonstrating that transient adenovirus-mediated hepatic expression of SR-BI can restore fertility in female SR-BI deficient mice. All other studies and all text and figures were contributed by the other authors of this manuscript.

ABSTRACT

The etiology of human female infertility is often uncertain. Sterility of HDL-receptor-negative (SR-BI (-/-)) female mice suggests a link between female infertility and abnormal lipoprotein metabolism. SR-BI (-/-) mice exhibit elevated plasma total cholesterol (with normal sized and abnormally large HDL and high unesterified-to-total plasma cholesterol (UC:TC) ratio). We explored the influence of hepatic SR-BI on female fertility by inducing hepatic SR-BI expression in SR-BI (-/-) animals by adenovirus transduction or stable transgenesis. For transgenes, we used both wild-type SR-BI and a double point mutant, Q402R/Q418R, (SR-BI-RR) that is unable to bind to and mediate lipid transfer from wild-type HDL normally, but retains virtually normal lipid transport activities with LDL. Essentially wild-type levels of hepatic SR-BI expression in SR-BI (-/-) mice restored to nearly normal the HDL size distribution and plasma UC:TC ratio, while ~7-40-fold overexpression dramatically lowered plasma total cholesterol and increased biliary cholesterol secretion. On the other hand, SR-BI-RR overexpression had little effect on SR-BI (+/+) mice, but in SR-BI (-/-) mice substantially reduced levels of abnormally large HDL and normalized the UC:TC ratio. In all cases, hepatic transgenic expression restored female fertility. Overexpression in SR-BI (-/-) mice of lecithin:cholesterol acyl transferase, which esterifies plasma HDL cholesterol, did not normalize the UC:TC ratio, probably because the abnormal HDL was a poor substrate, and did not restore fertility. Thus, hepatic SR-BI-mediated lipoprotein metabolism influences murine female fertility, raising the possibility that dyslipidemia might contribute to human female infertility and that targeting lipoprotein metabolism might complement current assisted reproductive technologies.

INTRODUCTION

Plasma lipoproteins transport cholesterol (as unesterified cholesterol and cholesteryl esters) and other lipids to and from tissues where they play critical roles in maintaining cell integrity (e.g., membrane synthesis), endocrine functions (e.g., cholesterol is a precursor for steroid hormone synthesis) and fertility (Farese and Herz 1998; Rigotti, Miettinen *et al.* 2003, Miettinen, 2001 #10). Cellular unesterified cholesterol levels are tightly regulated, not only because cholesterol is an essential component of cells and a precursor of important biomolecules (Lodish, Berk *et al.* 2004), and thus deficiency would be deleterious, but also because excess unesterified cholesterol can be cytotoxic (Tabas and Krieger 1999; Simons and Ikonen 2000; Tabas 2002). To prevent this toxicity, cells esterify cholesterol for storage in cytoplasmic lipid droplets or use a variety of transporter-mediated mechanisms to export unesterified cholesterol to extracellular acceptors (Jian, de la Llera-Moya *et al.* 1998; Klucken, Buchler *et al.* 2000; Aiello, Brees *et al.* 2003; Rigotti, Miettinen *et al.* 2003; Wang, Lan *et al.* 2004). In addition, HDL appears to play an especially important role as an extracellular acceptor for cholesterol efflux (von Eckardstein, Nofer *et al.* 2001; Rigotti, Miettinen *et al.* 2003; Nicholls, Rye *et al.* 2005), a function that is commonly thought to underlie, at least in part, the well established association of elevated plasma HDL cholesterol with reduced risk for atherosclerotic disease (coronary heart disease and stroke) (Kannel, Dawber *et al.* 1961; Rhoads, Gulbrandsen *et al.* 1976; Gordon and Rifkind 1989).

HDL may also have a particularly important role in mammalian female fertility, because in many species—including humans—HDL is the main class of lipoprotein found in substantial amounts in the follicular fluid enveloping the oocytes in ovarian follicles (Shalgi, Kraicer *et al.* 1973; Simpson, Rochelle *et al.* 1980; Perret, Parinaud *et al.* 1985; Volpe, Coukos *et al.* 1991; Le

Goff 1994; Jaspard, Fournier *et al.* 1997). This is presumably due to the size limit imposed by the follicular basement membrane, which is known as the blood-follicle barrier (Shalgi, Kraicer *et al.* 1973). The follicular HDL might deliver key lipids to follicular cells or mediate cholesterol efflux from those cells (Azhar and Reaven 2002). An important mechanism by which HDL cholesterol is delivered to ovarian cells, especially steroidogenic cells, is called selective lipid uptake, which is mediated by the HDL receptor SR-BI (Glass, Pittman *et al.* 1983; Reaven, Chen *et al.* 1984; Acton, Rigotti *et al.* 1996; Rigotti, Miettinen *et al.* 2003).

SR-BI mediates cellular selective lipid uptake from HDL by binding to HDL via its apolipoprotein components and subsequently facilitating the net transfer from the core of the particle cholesteryl esters, but not the protein or most of the lipid components of the lipoprotein's outer shell (Glass, Pittman *et al.* 1983; Stein, Dabach *et al.* 1983; Acton, Rigotti *et al.* 1996; Rigotti, Miettinen *et al.* 2003). SR-BI can also mediate the bi-directional movement of unesterified cholesterol between lipoproteins and cells (Ji, Jian *et al.* 1997; Jian, de la Llera-Moya *et al.* 1998; Yancey, de la Llera-Moya *et al.* 2000; Yancey, Bortnick *et al.* 2003), and thus, in the presence of an unesterified cholesterol gradient between cells and extracellular HDL, can mediate net cellular cholesterol efflux. Therefore, SR-BI, which is most highly expressed in the liver and steroidogenic tissues (Acton, Rigotti *et al.* 1996; Landschulz, Pathak *et al.* 1996), serves as a cholesterol transporter protein on the surfaces of cells. In the ovary, SR-BI is expressed in the theca cells, luteinized granulosa cells and in the interstitial tissue (Landschulz, Pathak *et al.* 1996; Li, Peegel *et al.* 1998; Reaven, Nomoto *et al.* 1998; Svensson, Johnson *et al.* 1999). In the liver, but not steroidogenic tissues, SR-BI protein expression depends on the presence of an adaptor protein, PDZK1, that binds via one of its four PDZ domains to the C-terminal cytoplasmic domain of SR-BI (Ikemoto, Arai *et al.* 2000; Silver 2002; Kocher, Yesilaltay *et al.* 2003;

Yesilaltay, Kocher *et al.* 2005).

Mice with targeted homozygous null mutations in the SR-BI gene exhibit ~2-fold elevated plasma cholesterol carried in both normal size and abnormally large HDL particles (Rigotti, Trigatti *et al.* 1997; Trigatti, Rayburn *et al.* 1999). The ratio of unesterified cholesterol (UC)-to-total cholesterol (TC, UC:TC ratio) in the plasma is abnormally high in SR-BI (-/-) mice (~0.5 vs ~0.25 in controls) (Braun, Zhang *et al.* 2003; Van Eck, Twisk *et al.* 2003). When SR-BI (-/-) mice are crossed into an atherosclerosis-prone genetic background (Trigatti, Rayburn *et al.* 1999; Covey, Krieger *et al.* 2003) or fed an atherogenic diet (Van Eck, Twisk *et al.* 2003; Zhang, Picard *et al.* 2005) they exhibit accelerated atherogenesis. Furthermore, SR-BI (-/-) females, but not males, are infertile (Trigatti, Rayburn *et al.* 1999; Mardones, Quinones *et al.* 2001). SR-BI (-/-) females ovulate normal numbers of oocytes that are dead or defective, and thus cannot be fertilized to form multicellular embryos. Although SR-BI plays a critical role in mediating delivery of HDL cholesterol to steroidogenic cells to maintain cholesteryl ester stores and provide substrate for steroidogenesis (Temel, Trigatti *et al.* 1997; Azhar, Nomoto *et al.* 1998; Rigotti, Miettinen *et al.* 2003; Wu, Sucheta *et al.* 2003), insufficient ovarian steroid hormone synthesis does not appear to be responsible for the infertility of SR-BI (-/-) females (Trigatti, Rayburn *et al.* 1999; Mardones, Quinones *et al.* 2001). For example, these animals exhibit a normal estrus cycle and a normal post-mating increase in plasma progesterone (Trigatti, Rayburn *et al.* 1999; Mardones, Quinones *et al.* 2001).

Our previous studies showed that when ovaries from SR-BI (-/-) mice are transplanted into otherwise SR-BI-positive recipients, they appear to function normally, and that genetic or pharmacologic manipulation of lipoprotein metabolism in SR-BI (-/-) females can partially or fully restore fertility in the absence of SR-BI expression (Mardones, Quinones *et al.* 2001). These

observations suggested that infertility of SR-BI (-/-) mice appears to be a direct consequence of abnormal lipoprotein metabolism, and not due to ovarian SR-BI deficiency per se. In the current study, we explored the hypothesis that hepatic SR-BI expression can play a critical role in murine female fertility by assessing the effects of adenovirus- or stable transgenesis-mediated hepatic expression of wild-type and mutant forms of SR-BI on the lipoprotein metabolism and fertility of SR-BI (-/-) mice. We found that hepatic SR-BI expression could correct the two main defects in the structure of HDL in SR-BI (-/-) mice (abnormally large size and UC:TC ratio) and restore female fertility to virtually wild-type levels. Thus, hepatic SR-BI-mediated lipoprotein metabolism influences murine female fertility, raising the possibility that some dyslipidemic disorders might contribute to human female infertility and targeting lipoprotein metabolism in these conditions might provide a novel approach to current assisted reproductive technologies.

MATERIALS AND METHODS

Animals – All mice were fed a regular chow diet, either from Harlan Teklad (RMH 3000) or PMI

Feeds, Inc. (Prolab RMH3000) with water supplied *ad libitum*. Genotypes were determined by

PCR as previously described (63) or with modifications (Rigotti, Trigatti *et al.* 1997) by using the

primers described below. Experiments were performed with 6–10-week-old animals. All animal

studies were approved by the Massachusetts Institute of Technology and the Pontificia

Universidad Católica Committees on Animal Care. Wild-type mice and mice carrying

heterozygous or homozygous null mutations in the SR-BI gene on a mixed C57BL/6:129

background (Rigotti, Trigatti *et al.* 1997) are referred to as SR-BI (+/+), SR-BI (+/-) and SR-BI (-

/-) mice, respectively.

Generation of SR-BI Transgenic Mice – A 1.5-kilobase fragment spanning the wild-type murine

SR-BI cDNA and a cDNA encoding a mutant form of SR-BI, SR-BI$^{Q402R/Q418R}$ (SR-BI-RR), were

amplified by PCR from a plasmid (pDT188) that contains the cloned murine SR-BI cDNA

(Acton, Rigotti *et al.* 1996) and from plasmid VM54 that have been described previously (Gu,

Lawrence *et al.* 2000), respectively, by using primers MunI-SRBI-new

(TTATTCCAATTGCCGTCTCCTTCAGGTCCTGAGC) and SRBI-XhoI-rev

(CAGCACCTCGAGGGCTTATAGTGTCTTCAGGACCCTA) and a DNA polymerase with

proofreading activity, Pfu polymerase (Promega). A *Mun*I site was introduced within the primer

oligo at the 5' of the START codon of the SR-BI gene, and a *Xho*I site was introduced within the

primer oligo at the 3' of the STOP codon of the SR-BI cDNA. The 1.5-kilobase cDNA fragment

encoding the gene in its entirety was subcloned between the *Mun*I and *Xho*I sites in the pLIV-LE6

plasmid, kindly provided by Dr. John M. Taylor (Gladstone Institute of Cardiovascular Disease,

University of California, San Francisco). The SR-BI-coding region was confirmed by DNA sequencing using 4 primers spanning the gene. The pLiv-LE6 plasmid contains the promoter, first exon, first intron, and part of the second exon of the human apoE gene, and the polyadenylation sequence, and a part of the hepatic control region of the apoE/C-I gene locus (Simonet, Bucay *et al.* 1993). The new constructs harboring the wild-type and mutant copies of the SR-BI gene as well as the above-mentioned apoE/apoCI control locus elements were linearized by *Not*I/*Spe*I digestion and the resulting 6.5-kilobase fragments were used to generate transgenic mice by standard procedures (Palmiter and Brinster 1986) in C57BL/6 and 50:50 mixed C57BL/6 x 129 background mice. Founder animals were backcrossed to 50:50 mixed C57BL/6 x 129 background mice for 3-4 generations and three transgenic mouse lines, SR-BI-Tg (high), SR-BI-Tg (low) and SR-BI-RR-Tg were established. The primers AB2 (GATGGGACATGGGACACGAAGCCATTCT) and AB3 (TCTGTCTCCGTCTCCTTCAGGTCCTGA) were used to amplify solely the genomic SR-BI. The presence of the SR-BI transgene was detected by primers B-Tg1 (TGAAGCTGATGATGACCTT) and B-Tg2 (AGCAGATGCGTGAAACTTGGTGA). The transgene expression was observed mainly in the liver and some expression was observed in the kidney. The receptor in the kidney is expressed on the apical surfaces of the epithelial cells in the proximal tubules and therefore was found to face the tubular lumen rather than the plasma where it is not expected to influence substantially plasma lipoprotein metabolism. Indeed, results using liver specific adenovirus-mediated transgenesis and these stable transgenic lines were concordant, fully supporting this assumption.

The LCAT transgenic mice that carry the human LCAT gene expressed from the albumin promoter in C57BL/6 background (Francone, Haghpassand *et al.* 1997) were purchased from The Jackson Laboratory, Bar Harbor, ME. These mice were crossed to SR-BI (-/-) males. The resulting offspring were crossed to each other to obtain the animals used in the study. Unless otherwise noted, all experimental animals and controls were on the same mixed C57BL/6: 129 background.

Recombinant Adenovirus Preparation and Infection – The adenoviruses encoding wild-type SR-BI (Ad. mSR-BI) and the control virus without a transgene (Ad.ΔE1) were described previously (Kozarsky, Donahee *et al.* 1997). The adenovirus encoding SR-BI$^{Q402R/Q418R}$ (Ad.SR-BI-RR) was prepared as previously described (He, Zhou *et al.* 1998), where cDNA encoding SR-BI$^{Q402R/Q418R}$ was amplified by PCR from plasmid VM54 (Gu, Lawrence *et al.* 2000), subcloned in the *HindIII* site of the pShuttle vector, and then the recombinant adenoviral genome with the SR-BI$^{Q402R/Q418R}$ under control of the CMV promoter was generated by homologous recombination in bacterial cells (65). Large-scale production of recombinant adenoviruses were prepared from infected HEK293 cells as described previously (Kozarsky, Jooss *et al.* 1996). On day 0, SR-BI (+/+) or SR-BI (-/-) mice (2–3 months old) in 50:50 mixed C57BL/6 x 129 background were injected via the femoral vein with phosphate-buffered saline (PBS) or 1x10^{11} viral particles of Ad.ΔE1 for control groups or 1x10^{11} particles of Ad.mSR-BI or Ad.SR-BI-RR for experimental groups. Plasma, hepatic bile and liver samples were harvested at day 3 post-infection.

Hepatic Bile, Blood and Liver Sampling and Processing – Mice were anesthetized with intraperitoneal injection of pentobarbital (4.5 mg/100 g body weight) for adenovirus injections or

by 2.5% Avertin for other studies. Hepatic bile was collected for 30 min while mice were kept under anesthesia at 37 C with a heating lamp. Blood was collected by puncture of the inferior vena cava or by heart puncture with a heparinized syringe. Plasma was separated by low speed centrifugation for 10 min at 3,000xg. Hepatic bile flow, calculated by dividing the volume of bile (determined gravimetrically assuming a specific density = 1.0) by the collection time and the liver weight, was expressed as µl/min/g of liver. Bile and some plasma were stored at –20 C for lipid analysis, while liver samples were kept at –80 C prior to analysis by western blotting.

Analysis of plasma cholesterol and lipoproteins – Plasma samples from single animals, or pooled plasma where indicated, were size fractionated by fast performance liquid chromatography (FPLC) as previously described (Rigotti, Trigatti *et al.* 1997), and total cholesterol or unesterified cholesterol in each fraction or in unfractionated total plasma were determined by using commercial kits (Wako Chemical USA Inc., Richmond, Virginia, USA) or as described previously (Mardones, Quinones *et al.* 2001). Slight differences in the peak positions of the HDL from SR-BI (+/+) mice are the result of using different FPLC instruments (Fig. 2 versus Figures 5 and 7).

Biliary cholesterol analysis – After lipid extraction(Nervi, Marinovic *et al.* 1988), biliary cholesterol concentrations were measured by an enzymatic assay as previously described (Allain, Poon *et al.* 1974). Biliary cholesterol secretion rates were calculated from biliary lipid concentrations and measured hepatic bile flows, and expressed as nmol/min/g of liver.

Immunoblotting Analysis – Total liver membranes (40-50 µg of protein/sample) were size-

fractionated by 10% SDS-PAGE and immunoblotted on nitrocellulose or PVDF membranes with either polyclonal antipeptide antibodies for SR-BI (Acton, Rigotti *et al.* 1996) or NB400-104 from Novus Biologicals (Littleton, CO) or ε–COP (Guo, Penman *et al.* 1996), used as protein loading control. Antibody binding to protein samples was visualized by the enhanced chemiluminescence procedure (GE Healthcare/Amersham Biosciences) and quantified with a Macintosh Color One scanner (Apple, Cupertino, CA) and NIH imaging software version 1.6. Relative levels of hepatic SR-BI protein expression were determined after normalization for ε-COP protein levels. To quantify the relative amounts of SR-BI in different tissues, tissue lysates were serially diluted and subjected to electrophoresis/ immunoblotting, and the relative intensities of the signals were compared by visual examination of protein levels in these mice.

Immunohistochemical localization of hepatic SR-BI: – For immunoperoxidase studies, tissues were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4 C and then incubated overnight at 4 C in 30% sucrose in phosphate-buffered saline, pH 7.4. Tissues were then frozen in OCT compound (Miles Diagnostics, Elkhart, IN) and stored in liquid nitrogen. Immunoperoxidase staining was performed on 5 μm frozen sections with primary anti-SR-BI antibody (Acton, Rigotti *et al.* 1996) and secondary biotinylated anti-rabbit IgG (1/200 dilution, Vector, Burlingame, CA), and subsequently visualized with Vectastain ABC (Vector) and diaminobenzidine (Research Genetics, Inc., Huntsville, AL), according to the manufacturer's protocol.

Fertility assays – Virgin females (6-8 weeks of age) were housed continuously with wild-type males, and the numbers of litters and pups were recorded for a period of 4 months.

LCAT assays – We used two assays to measure lecithin:cholesterol acyl transferase (LCAT) activity in plasma samples.

'Endogenous' LCAT assay: Trace amounts of [^{3}H]cholesterol were equilibrated with the 'endogenous' unesterified cholesterol in the plasma lipoproteins and then LCAT activity was measured as previously described (Furbee, Francone *et al.* 2001). Briefly, 10 µl of plasma was diluted by addition of 40 µl of TBS buffer (10mM Tris pH 8.0, 140 mM NaCl, 0.01% NaN_3, 0.01% EDTA) in a glass tube containing 500,000 cpm [^{3}H]cholesterol (PerkinElmer, Inc., Wellesley, MA), which had been evaporated and dried at the bottom of the tube. The tubes were shaken vigorously overnight at 4 C. The distribution of labeled cholesterol among the plasma lipoproteins was found to be similar to that of the endogenous unlabeled cholesterol by FPLC chromatography (not shown). The samples were divided in half for simultaneous incubations with shaking for 30 min at either 37 C (experimental) or 4 C (controls). The reactions were stopped by addition of 2 ml ethanol, and lipids were extracted twice with 4 ml of hexane containing excess unlabeled cholesterol and cholesteryl oleate (50 µg each) as carriers. The amounts of labeled unesterified and esterified cholesterol were determined using thin layer chromatography and scintillation counting and the percent cholesterol esterification rate (cholesteryl ester/total cholesterol) and the plasma cholesterol esterification rate (CER, nanomoles of cholesterol converted to cholesteryl ester per hour per ml plasma) were calculated. The CER was calculated based on the specific activity of the unesterified cholesterol in the samples (determined from the mass of unesterified cholesterol in the samples measured by enzymatic methods), which varied significantly among the mouse strains used.

'Exogenous' LCAT assay: LCAT activity was determined with an 'exogenous' reconstituted

discoidal HDL substrate prepared using the sodium cholate method with an initial molar ratio of

0.8:250:12.5 of apoA-I, egg phosphatidylcholine and [^{14}C]cholesterol (Chen and Albers 1982).

Briefly, the reaction was initiated by adding 15 µl of plasma to reconstituted HDL (22 µg of

human apoA-I), 0.5% bovine serum albumin, and 5 mM β-mercaptoethanol in a total volume of

0.515 ml. After a 20 min incubation at 37 C , the lipids were extracted and analyzed as described

above. To ensure linearity, reactions did not proceed beyond 20% conversion of unesterified to

esterified cholesterol. Esterification rates were corrected by subtracting values for reactions

conducted without plasma. In addition, the specificity of the reaction was confirmed by the

complete inhibition with 1.3 mM DTNB, an inhibitor of LCAT. Plasma LCAT activity with the

exogenous substrate was determined by calculating the nmol conversion of [^{14}C]cholesterol to CE

per hour per ml plasma after correcting for the specific activity of the unesterified cholesterol due

to dilution by the endogenous unesterified cholesterol present in the plasma samples, which was

determined enzymatically as described above.

Statistical Analysis – The statistical significance of the differences was determined by one-way

ANOVA Tukey posthoc test or two-tailed unpaired Student's *t* test where appropriate.
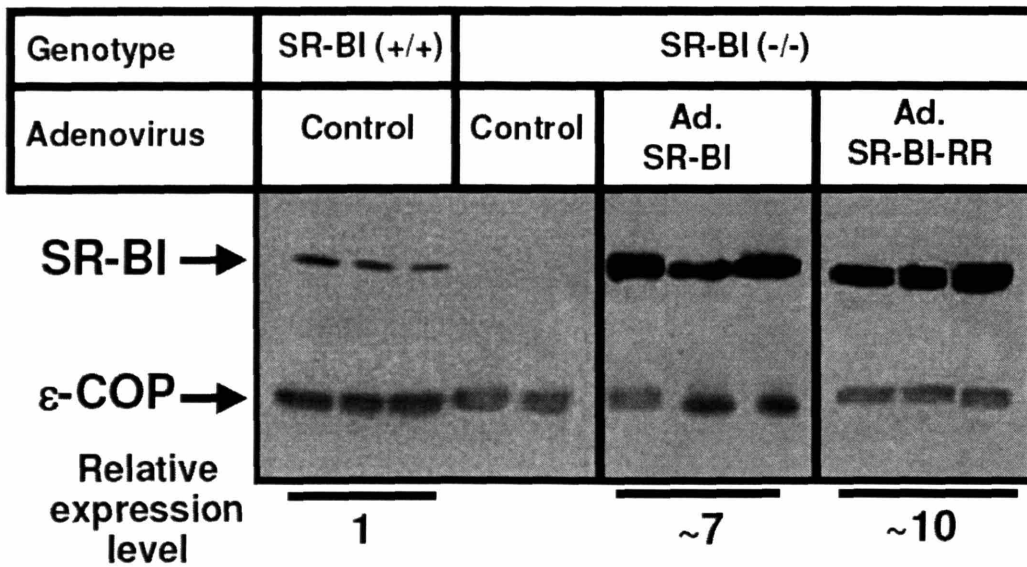
Differences are considered significant when $p < 0.05$.

RESULTS

Previous reports suggested that the influence on plasma lipoprotein metabolism of homozygous null mutation in the SR-BI gene is responsible for the infertility of SR-BI (-/-) female mice (Trigatti, Rayburn *et al.* 1999; Mardones, Quinones *et al.* 2001). The liver plays a central role in mediating the effects of SR-BI on lipoprotein metabolism (Kozarsky, Donahee *et al.* 1997; Wang, Arai *et al.* 1998; Arai, Wang *et al.* 1999; Ji, Wang *et al.* 1999; Kocher, Yesilaltay *et al.* 2003). Thus, we used adenovirus-mediated and transgenic-based approaches to increase the expression of SR-BI in the livers of SR-BI (-/-) and control mice and examined the effects on lipoprotein and lipid metabolism and on female fertility. We used as transgenes both wild-type SR-BI and a double point mutation form of the receptor (Q402R/Q418R or 'SR-BI-RR'). When expressed in cultured cells, SR-BI-RR is unable to bind to and mediate lipid transfer from normal HDL, but retains essentially wild-type binding and lipid transport activities when the larger lipoprotein LDL serves as its ligand (Gu, Kozarsky *et al.* 2000; Gu, Lawrence *et al.* 2000). We hypothesized that the mutant SR-BI-RR might catabolize or prevent the formation of the abnormal, large HDL-like particles found in SR-BI (-/-) plasma (Rigotti, Trigatti *et al.* 1997), but not normal sized HDL particles. Thus, it might help differentiate the influences of these two classes of HDL particles on the abnormal phenotypes of SR-BI (-/-) mice.

Our initial studies focused on adenovirus-mediated hepatic overexpression of SR-BI. Adenovirus treatment resulted in hepatic expression of the receptors that was ~7-10-fold greater than in control wild-type mice (see Supplemental Figure S1). We determined the effects of hepatic overexpression on plasma cholesterol levels and biliary cholesterol secretion rates (Figure 1) and the distribution of cholesterol among plasma lipoproteins as determined by FPLC size fractionation (lipoprotein profiles, Figure 2). As previously reported (Kozarsky, Donahee *et al.*

1997), Ad.SR-BI-mediated hepatic overexpression in wild-type SR-BI (+/+) mice resulted in a dramatic reduction in plasma total cholesterol (primarily in HDL, Figure 2A, filled circles) and a 2.2-fold increase in biliary cholesterol secretion (Figure 1A). Similar results were observed in SR-BI (-/-) animals (Figure 1B). Thus, hepatic overexpression of wild-type SR-BI effectively removes the cholesterol from normal sized and abnormally large HDL particles in the circulation and/or prevents the formation of these particles by rapidly clearing cholesterol from their precursors. As a consequence of accelerated hepatic HDL cholesterol clearance mediated by SR-BI, biliary secretion of cholesterol increased.

Genotype	SR-BI (+/+)	SR-BI (-/-)		
Adenovirus	Control	Control	Ad. SR-BI	Ad. SR-BI-RR
SR-BI →				
ε-COP →				
Relative expression level	1		~7	~10

Supplemental Figure S1. Immunoblot analysis of adenovirus-mediated expression of wild-type SR-BI and SR-BI-RR in the livers of SR-BI (-/-) animals.

Total liver extracts were harvested from wild-type and SR-BI (-/-) mice 3 days after injection with PBS alone or control virus without a transgene (Ad.ΔE1) (controls) or with SR-BI-expressing adenoviral vectors as indicated. The protein content was analyzed by immunoblotting using anti-SR-BI and anti-εCOP (loading control) antibodies as described under Materials and Methods. A single band of molecular weight ~82000 corresponding to SR-BI was detected in all animals except control SR-BI (-/-) mice.
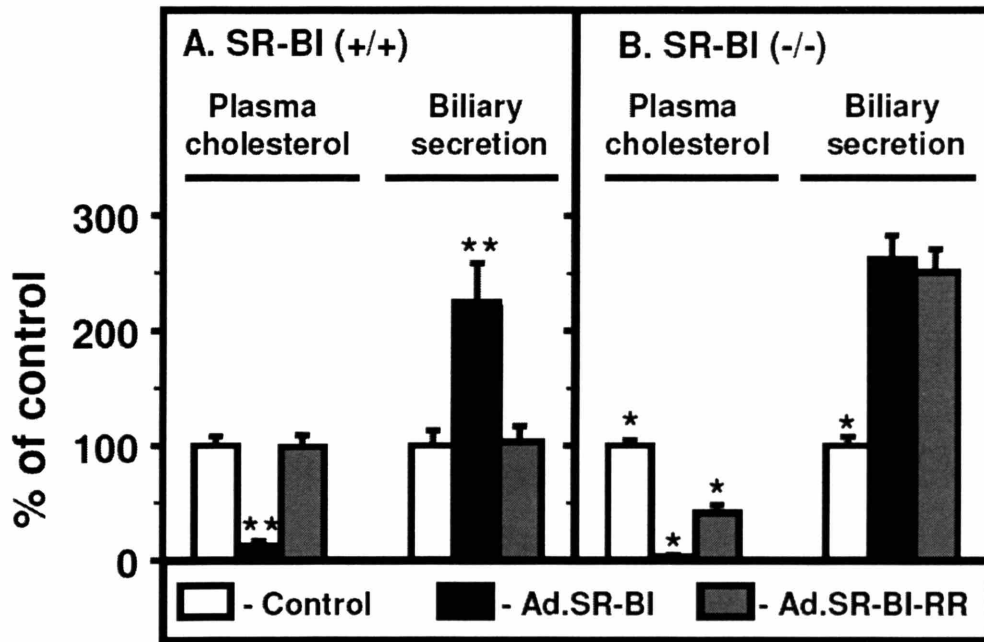
Figure 1. Effects of adenovirus-mediated hepatic overexpression of SR-BI and SR-BI-RR on plasma cholesterol levels and biliary cholesterol secretion in SR-BI (+/+) (A) and SR-BI (-/-) (B) mice. On day 0, mice were injected with PBS or control adenovirus (Control) or adenoviruses encoding SR-BI or SR-BI-RR as indicated. Some controls were injected with PBS alone. On day 3, plasma total cholesterol levels and biliary secretion rates were determined as described in Materials and Methods. The 100% of control values for plasma cholesterol and biliary secretion, respectively, were: SR-BI (+/+), 77.7 ± 6 mg/dl (n=8), 0.88 ± 0.15 nmol/min/g liver (n=8); SR-BI (-/-), 264.9 ± 13 mg/dl (n=6), 0.6 ± 0.08 nmol/min/g liver (n=5). (n=4–8 for the other values shown). The p values for one way ANOVA for total cholesterol values within each genotype are < 0.0001 and < 0.001 for biliary secretion values. Statistically significant differences by ANOVA Tukey posthoc test from the rest of the values within each genotype are indicated as: $*$, $p < 0.01$; $**$, $p < 0.001$.
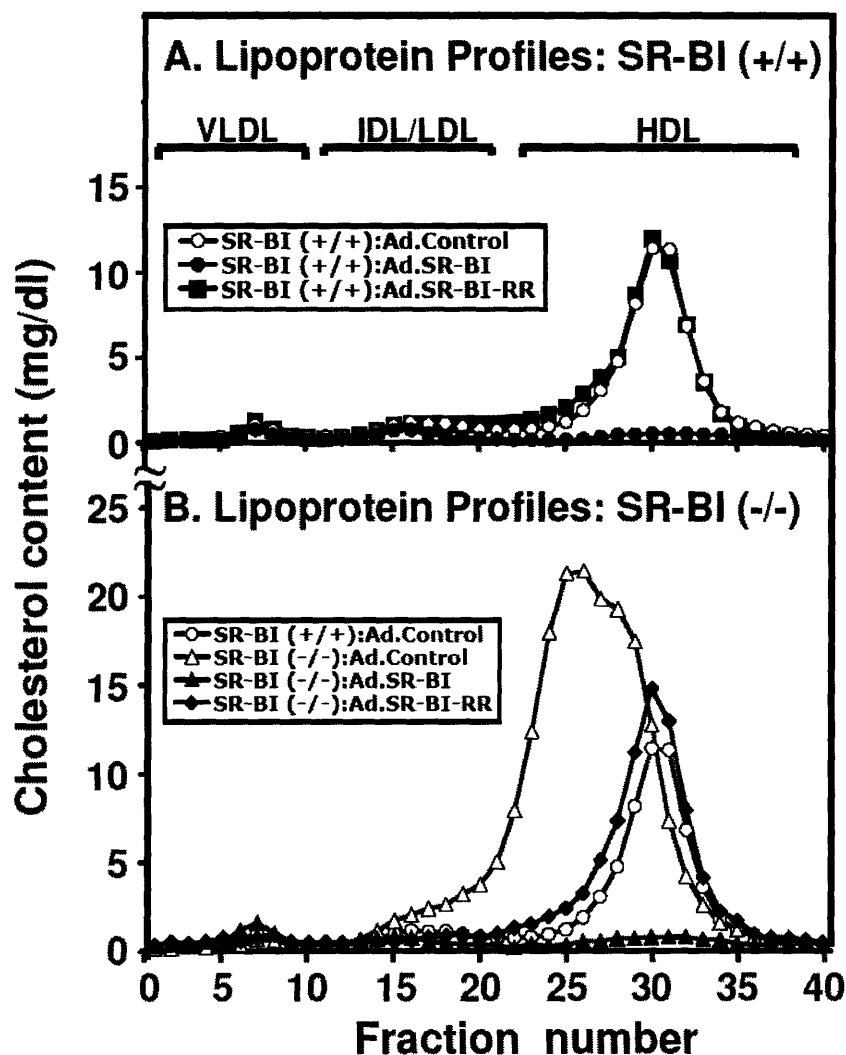
Figure 2. Lipoprotein cholesterol profiles of adenovirus-treated SR-BI (+/+) (A) and SR-BI (-/-) (B) mice. Mice were injected with the indicated adenoviruses on day 0, plasma was harvested on day 3 and then size fractionated using FPLC. The cholesterol content of the fractions was determined by enzymatic assay. Chromatograms represent average values of the total cholesterol content of FPLC fractions from independent analyses of 4–7 individual mice for each experimental group Approximate elution positions of native VLDL, IDL/LDL and HDL particles are indicated by brackets and were determined as previously described(Rigotti, Trigatti *et al.* 1997).

As expected from our in vitro studies (Gu, Kozarsky *et al.* 2000; Gu, Lawrence *et al.* 2000), transduction of SR-BI (+/+) mice with the Ad.SR-BI-RR virus had little effect on total plasma cholesterol levels, biliary cholesterol secretion (Figure 1A) or the lipoprotein profile (Figure 2A, filled squares), because the bulk of the plasma cholesterol is in normal sized HDL particles not recognized efficiently by this mutant receptor. Strikingly, in SR-BI (-/-) animals hepatic overexpression of SR-BI-RR reduced the plasma cholesterol by ~60% to the levels observed in control SR-BI (+/+) animals, apparently as a consequence of either clearing cholesterol from or preventing the formation of the larger abnormal particles without dramatically influencing normal HDL particles (Fig. 2B, filled diamonds). This Ad.SR-BI-RR-effect in SR-BI (-/-) mice was accompanied by a 2.6-fold increase in biliary cholesterol secretion (Figure 1B). It was somewhat surprising to observe comparable rates of SR-BI-mediated biliary secretion in the Ad.SR-BI and Ad.SR-BI-RR treated SR-BI (-/-) mice, because the wild-type receptor is so much more effective in reducing total plasma cholesterol. This raises the possibility that there may be an upper limit to the rate of biliary cholesterol secretion in these animals that should be explored in future studies. These results indicate that SR-BI-RR behaves in vivo as it did in vitro, in that this mutant receptor cannot efficiently process normal size HDL but may be able to mediate lipid uptake from (or prevent the formation of) larger lipoproteins, both LDL (in vitro studies) and the abnormal, large HDL particles in SR-BI (-/-) mice. Thus SR-BI-RR may be useful to differentially address the biological consequences of preventing the accumulation of these abnormal particles in the plasma.

Similar effects of hepatic transgene expression were seen in stable transgenic lines, which are more suitable for the long term studies of female fertility. We generated transgenic lines expressing wild-type SR-BI protein at either 40-fold ('SR-BI-Tg (high)'; see Supplemental Figure

S2A for immunoblotting data) or ~0.7-fold ('SR-BI-Tg (low)', data not shown), and the SR-BI-

RR mutant at 40-fold higher than normal ('SR-BI-RR-Tg', Supplemental Figure S2A). The

Q402R/Q418R mutations did not appear to interfere with the ability of the receptor to reach the

plasma membrane (see immunohistochemical analysis in Supplemental Figure S2B). Table I

shows the influence of stable transgenic expression of these receptors on plasma total cholesterol

(TC) and unesterified cholesterol (UC) levels and the UC:TC ratio in SR-BI (+/-) or SR-BI (-/-)

mice (effects on lipoprotein profiles will be described in detail below). As was the case for

adenovirus-mediated hepatic receptor overexpression, ~40-fold overexpression of wild-type SR-

BI dramatically lowered plasma cholesterol levels in both SR-BI (+/-) and SR-BI (-/-) mice. Low

level (~0.7-fold) expression of the wild-type SR-BI transgene also lowered plasma cholesterol

levels, but not as dramatically. In contrast, the ~40-fold overexpression of the SR-BI-RR

transgene resulted in only modest reductions in plasma cholesterol to values similar to those seen

in non-transgenic wild-type control animals (143 to 89 mg/dl for SR-BI (+/-) mice and 206 to 109

mg/dl for SR-BI (-/-) mice). The levels of apoA-I, the major apolipoprotein in HDL, in SR-BI (-/-

) mice are not substantially different from those in SR-BI (+/+) mice (Supplemental Figure S3

and (Trigatti, Rayburn *et al.* 1999)). This was also the case for SR-BI (-/-)[SR-BI-Tg (low)] and

SR-BI (-/-)[SR-BI-RR-Tg] mice. In contrast, plasma apoA-I levels were very low in SR-BI (-/-

)[SR-BI-Tg (high)] mice, consistent with the suggestion that small lipid poor apoA-I particles that

may be generated in these mice are rapidly cleared from the circulation (Brinton, Eisenberg *et al.*

1989; Horowitz, Goldberg *et al.* 1993; Kozarsky, Donahee *et al.* 1997).

Table I. Total plasma cholesterol levels in stable transgenic animals

SR-BI Genotype	SR-BI Transgene	Fold Protein Expression Compared to WT [z]	Plasma Total Cholesterol (mg/dl) [a]	Plasma Unesterified Cholesterol (mg/dl) [b]	Plasma Unesterified Cholesterol Ratio [c]
+/-	None (n=6)	0.5	143 ± 4 [x]	30 ± 2 [x,§]	0.21 ± 0.01 [x]
	SR-BI (low) (n=6)	~0.7	42 ± 5	11 ± 2	0.25 ± 0.04
	SR-BI (high) (n=9)	~40	6 ± 1	n/d[y]	n/d
	SR-BI-RR (n=4)	~40	89 ± 7	21 ± 4 [§]	0.23 ± 0.03
-/-	None (n=11)	0	206 ± 7	107 ± 4	0.52 ± 0.02
	SR-BI (low) (n=7)	~0.7	128 ± 11 [‡]	33 ± 4 [†]	0.25 ± 0.2 [¥]
	SR-BI (high) (n=11)	~40	7 ± 2	n/d	n/d
	SR-BI-RR (n=11)	~40	109 ± 8 [‡]	25 ± 2 [†]	0.23 ± 0.01 [¥]

[a] $p < 0.001$ by one way ANOVA and $p < 0.001$ by Tukey posthoc test for all pairwise comparisons of the plasma total cholesterol values within the same SR-BI genotype except those marked [‡] are not significantly different from each other.

[b] $p < 0.0001$ by one way ANOVA and $p < 0.05$ by Tukey posthoc test for all pairwise comparisons of the plasma unesterified cholesterol values within the same SR-BI genotype except those marked [§] and [†] are not significantly different from each other.

[c] $p < 0.0001$ by one way ANOVA and $p < 0.001$ by Tukey posthoc test for all pairwise comparisons of the plasma unesterified cholesterol ratio values within the SR-BI (-/-) genotype except those marked [¥] are not significantly different from each other. $p > 0.05$ by one way ANOVA for values within the SR-BI (+/-) genotype.

[x] Values are presented as mean ± standard error

[y] n/d not detectable due to the low total cholesterol

[z] Approximate fold protein expression for transgenic protein was determined in SR-BI (-/-) animals

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A.

Genotype	SR-BI (+/-)	SR-BI (-/-)						
Transgene	-	-	SR-BI (high)			SR-BI-RR		
Relative sample dilution	1	1	20	40	80	20	40	80
	1	2	3	4	5	6	7	8

B.
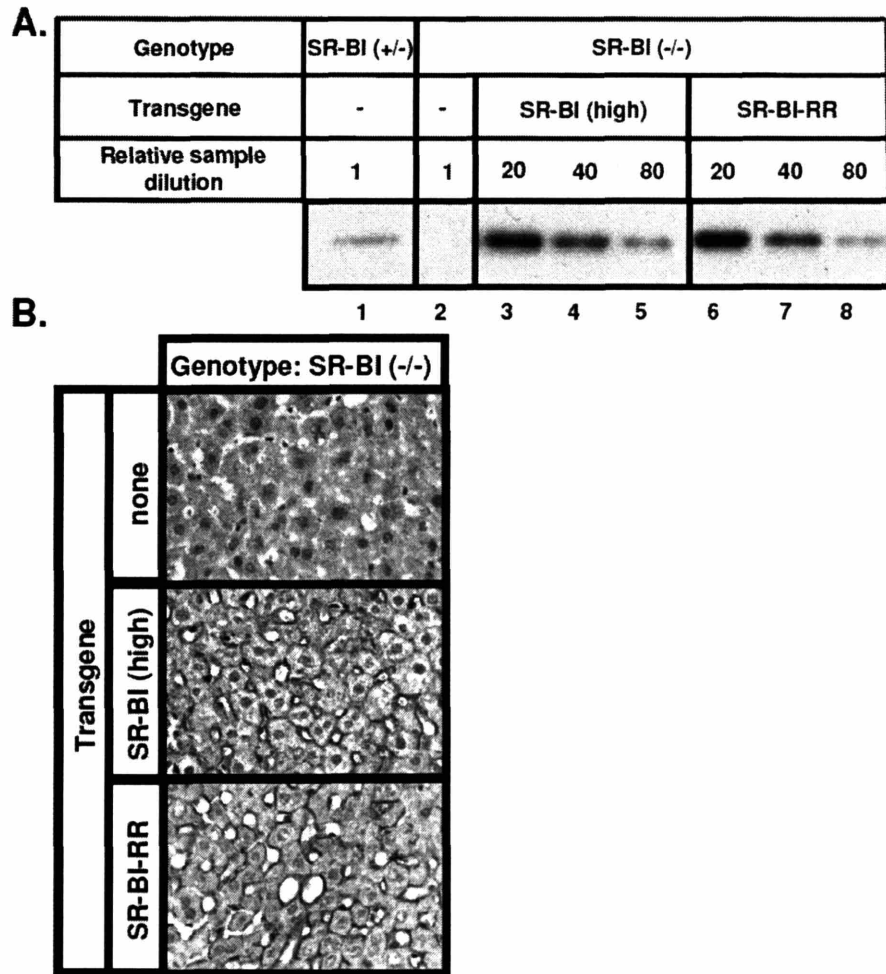
Supplemental Figure 2. Hepatic SR-BI expression in stable transgenic animals.

A. Immunoblot analysis. Lysates of livers harvested from SR-BI (+/-)or SR-BI (-/-) animals without or with the indicated transgenes with the indicated relative dilutions were subjected to immunoblotting analysis as described in Materials and Methods. A single band of of molecular weight ~82000 corresponding to SR-BI was detected in all samples except those from nontransgenic SR-BI (-/-) mice. **B.** Immunohistochemical analysis. Livers were harvested from SR-BI (-/-) animals with the indicated transgenes, fixed, frozen and sectioned, and the sections stained for SR-BI using an immunoperoxidase technique as described under Materials and Methods.

SR-BI Genotype	(+/+)	(-/-)			
SR-BI Transgene	none	SR-BI (low)	SR-BI (high)	RR-Tg	none
Apo A-I →					
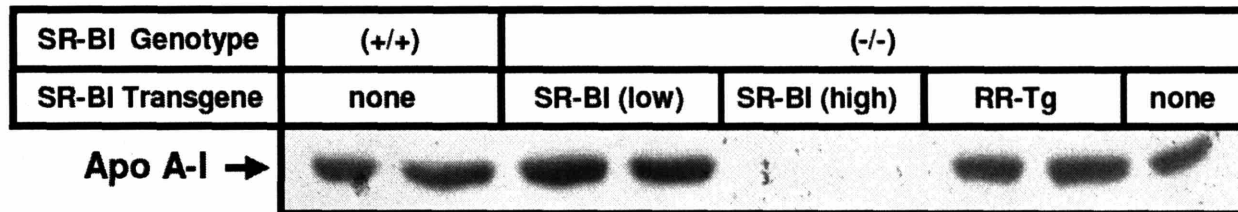
Supplemental Figure 3. Apo A-I levels in the plasma of SR-BI transgenic animals.
Plasma samples (1 µl) from mice with the indicated genotypes (marked above the corresponding lane) and stable transgene expression were analyzed by immunoblotting with anti-apoA-I antibodies. Longer exposure times (not shown) revealed the presence of very low levels of apoA-I in the SR-BI (-/-)[SR-BI (high)] lane.

The lipoprotein cholesterol profiles in Figure 3A show that both low level expression of wild-type SR-BI (SR-BI-Tg (low)) and ~40-fold overexpression of SR-BI-RR in SR-BI (-/-) mice substantially reduced the amounts of abnormally large HDL-like particles. The low level of wild-type SR-BI was more effective than the ~40-fold overexpression of SR-BI-RR. [The effects of stable high level expression of wild-type SR-BI - almost undetectable levels of cholesterol in all fractions - are not shown.] Strikingly, both low level expression of wild-type SR-BI and ~40-fold overexpression of SR-BI-RR not only reduced the total plasma cholesterol values in SR-BI (-/-) mice to nearly wild-type levels and resulted in lipoprotein profiles that were similar to that of wild-type mice, but also normalized the UC:TC ratio from its characteristically abnormally high value of 0.52 (Braun, Zhang *et al.* 2003; Van Eck, Twisk *et al.* 2003) to 0.23-0.25 (Table I). Figure 3B shows that the abnormally high UC:TC ratios present throughout the HDL size range in the control SR-BI (-/-) mice were normalized by expression of the SR-BI (low) and SR-BI-RR transgenes. [UC levels were below detection limits in the SR-BI-Tg (high) animals.]
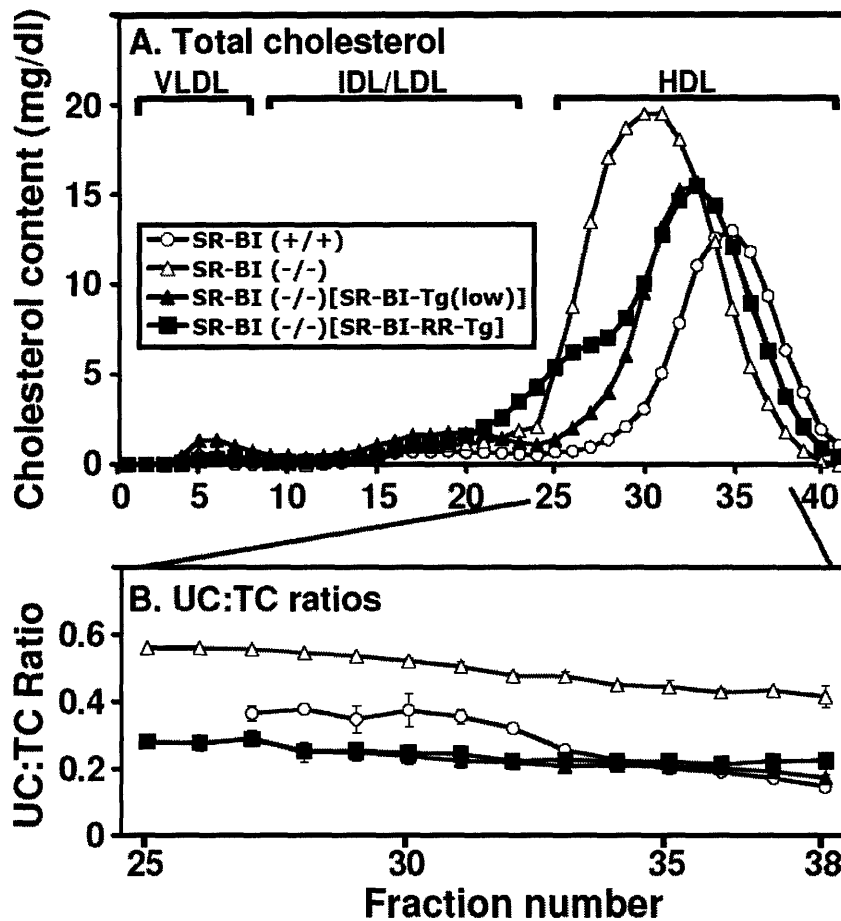
Figure 3. Effects of transgene expression on the lipoprotein cholesterol profiles of control SR-BI (+/+), SR-BI (-/-), and transgenic SR-BI (-/-) mice. Plasma was harvested from the indicated control and transgenic mice and then samples from individual animals were size fractionated using FPLC. The total cholesterol (TC, panel A) and unesterified cholesterol (UC) contents of the fractions were determined by enzymatic assays and the UC:TC ratios for the HDL-containing fractions 25-38 are shown in panel B. The chromatograms are representative of multiple, independent determinations. Approximate positions of VLDL, IDL/LDL and HDL elutions are indicated by brackets and were determined as previously described (Rigotti, Trigatti *et al.* 1997). The unesterified cholesterol values in fractions 25 and 26 from the control SR-BI (+/+) mouse were below detection limits. The error bars in panel B represent the range (n=2).

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Figure 4 shows that transgenic hepatic expression of wild-type SR-BI (both high and low levels) or SR-BI-RR restored nearly normal fertility to female SR-BI (-/-) mice, enumerated during 4 month mating periods either as % of females producing litters (panel A) or number of pups delivered per month per female (panel B). In addition, the times from the beginning of mating to delivery of the first litter were similar for the nontransgenic control SR-BI (+/-) and transgenic SR-BI (-/-) females, ranging from 21 to 23 days. These results are consistent with our previous findings that SR-BI expression in the ovary or other female reproductive organs is not required per se for female fertility, and strongly support the proposal that the presence of abnormal lipoproteins in female SR-BI (-/-) mice are responsible for their infertility (Mardones, Quinones *et al.* 2001). These findings were supported by preliminary studies using SR-BI (-/-) mice treated with adenoviruses. Fertility was restored in a large fraction of the mice injected with Ad.SR-BI encoding wild-type SR-BI and then mated 5 days later (unpublished data).
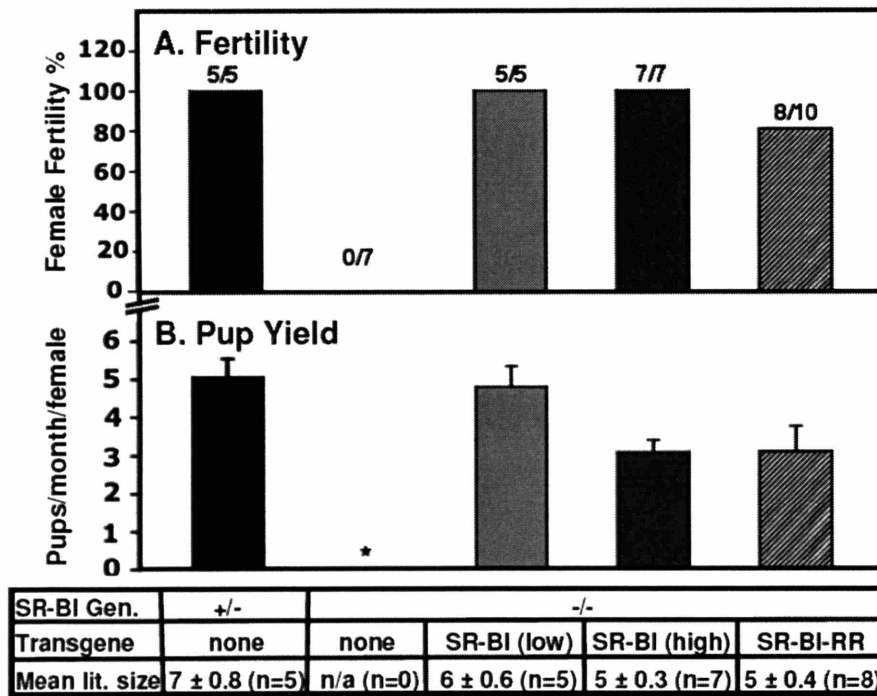
Figure 4. Effect of stable hepatic transgene expression on the fertility of SR-BI (-/-) female mice. Six-to-eight week old, individual, virgin females with the indicated SR-BI genotypes (+/-) or (-/-) and transgenes were mated continuously for 4 months to nontransgenic SR-BI (+/+) males. Females included: SR-BI (+/-) (black bars, n=5), SR-BI (-/-) (n=7), SR-BI (-/-)[SR-BI-Tg (low)] (light gray bars, n=5), SR-BI (-/-)[SR-BI-Tg (high)] (dark gray bars, n=7), SR-BI (-/-)[SR-BI-RR-Tg] (hatched bars, n=10). (A) Fertility expressed as percentage of females producing litters (the numbers of females that produced litters relative to the number of females studied are shown above the bars). (B) Average number of pups delivered per month per female mated (error bars represent SEM). The star (*) indicates statistically significant difference from the other values by ANOVA Tukey posthoc test ($p < 0.05$). The mean litter sizes, which include only those females that delivered pups, are shown below.

Given the potential causative role of the abnormally high UC:TC ratio in the plasma lipoproteins in the infertility of SR-BI (-/-) females (see Discussion), we further explored the mechanisms underlying this lipoprotein abnormality. Lecithin: cholesterol acyl transferase (LCAT) is the plasma protein responsible for the esterification of cholesterol in HDL (Glomset

1962). We used two assays to measure LCAT activity in plasma samples from SR-BI (-/-) and

control mice. The first involves the addition of an artificial, 'exogenous' [^{14}C]cholesterol-labeled

reconstituted HDL substrate to plasma samples followed by incubation and measurement of

[^{14}C]cholesteryl ester formation. This 'exogenous' substrate assay is usually considered to

provide a measure of the total active LCAT in the plasma sample (the amount of active enzyme is

rate controlling) (Chen and Albers 1982). The second LCAT assay involves the addition of trace

amounts of [^{3}H]cholesterol to the plasma samples, equilibration with the 'endogenous'

unesterified cholesterol, and measurement of the rate of [^{3}H]cholesteryl ester formation. Because

the plasma unesterified cholesterol levels varied depending on the genetic background of the mice

examined, we calculated the specific activity of the [^{3}H]labeled cholesterol in the reactions, and

thus the rate of cholesterol esterification, based on the total cholesterol present in the plasma

samples (endogenous alone for the 'endogenous' assay, or sum of endogenous and exogenous

cholesterol for the 'exogenous' assay).

Figure 5A shows that the 'exogenous' LCAT activities of control SR-BI (+/+) and SR-

BI (-/-) mice were similar (~35 and 33 nmol of CE formed/h/ml of plasma, respectively). Thus,

reduced levels of LCAT activity are not responsible for the abnormally high UC:TC ratio in SR-

BI (-/-) mice. Unlike the exogenous LCAT activity, the endogenous LCAT activity in the plasma

from SR-BI (-/-) mice was significantly less than that from SR-BI (+/+) controls (Figure 5B).

Thus, it appears that the abnormal lipoproteins in SR-BI (-/-) plasma may be poor substrates for

LCAT, and this might contribute to the abnormally high UC:TC ratio in these animals. Low

endogenous LCAT activity was also reported by Ma et al. (Ma, Forte *et al.* 2005).
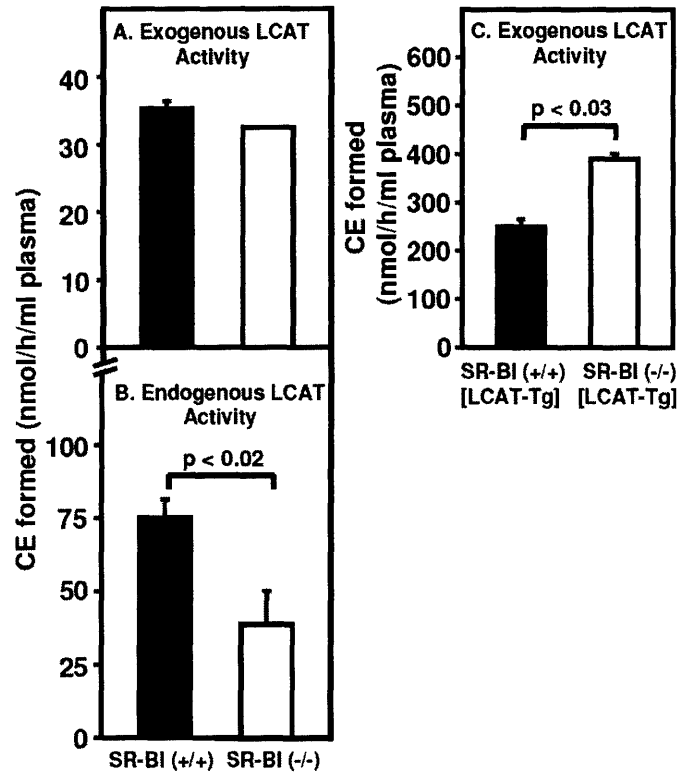
Figure 5. Exogenous (A &C) and endogenous (B) LCAT activities in plasma from nontransgenic (A & B) and LCAT transgenic (C) mice. (A & C) Exogenous LCAT activities (nanomoles cholesterol esterified per hour per ml plasma) in pooled plasma samples (n=4) from the indicated nontransgenic (A) or LCAT-Tg (C) mice were measured in duplicate during either a 20 minute (A) or 7.5 min (C) incubation at 37 C using thin layer chromatography as described in Materials and Methods. Similar results were observed when plasma samples from single animals were tested (not shown). (B) Endogenous LCAT activities in plasma samples from individual SR-BI (+/+) (n=7) and SR-BI (-/-) (n=4) mice were measured in duplicate for 30 min at 37 C after overnight equilibration with trace amounts of [^{3}H]unesterified cholesterol and analyzed by thin layer chromatography as described under Materials and Methods. Similar results were obtained from samples with pooled plasma (not shown). Values represent means and the error bars represent SEMs.

In an attempt to correct the abnormally high UC:TC ratio in SR-BI (-/-) mice and possibly restore fertility, we crossed these mice to transgenic mice (SR-BI (+/+)[LCAT-Tg]) expressing a high level of the human LCAT enzyme in addition to the endogenous murine enzyme (Francone, Haghpassand et al. 1997). The 'exogenous' LCAT activities in the LCAT transgenic mice (Figure 5C) were ~7-11-fold higher than the non-transgenic controls (compare to Figure 5A, note different scales of the y-axes), with the activity in the SR-BI (-/-)[LCAT-Tg] mice somewhat higher than in the SR-BI (+/+)[LCAT-Tg] controls. Indeed, immunoblotting experiments indicated higher LCAT protein levels in the plasma of SR-BI (-/-)[LCAT-Tg] animals compared to those in SR-BI (+/+)[LCAT-Tg] controls, possibly due to the higher LCAT binding capacity of the larger plasma lipoproteins in the receptor-deficient animals (data not shown). As expected from previous studies (Vaisman, Klein et al. 1995; Francone, Haghpassand et al. 1997), the LCAT transgene increased the total plasma cholesterol level in SR-BI (+/+) mice by 60% (Table II). Although the much smaller transgene-associated increase (18%) seen in the SR-BI (-/-)[LCAT-Tg] mice was not statistically significant (Table II), it was accompanied by an increase in the size of the already abnormally large HDL particles (FPLC analysis, shift to the left in Figure 6). Analysis of the UC:TC ratios (Table II) showed that expression of the LCAT transgene did not change this already low ratio (0.18) in the SR-BI (+/+) controls. The transgene did significantly reduce the abnormally high UC:TC ratio from 0.44 in SR-BI (-/-) mice to 0.37 in SR-BI (-/-)[LCAT-Tg] mice; however, it was still twice that of the SR-BI (+/+)[LCAT-Tg] controls. Thus, an approximately 10-fold increase in plasma LCAT activity was only able to partially overcome the apparent cholesterol esterification defect in SR-BI (-/-) mice.
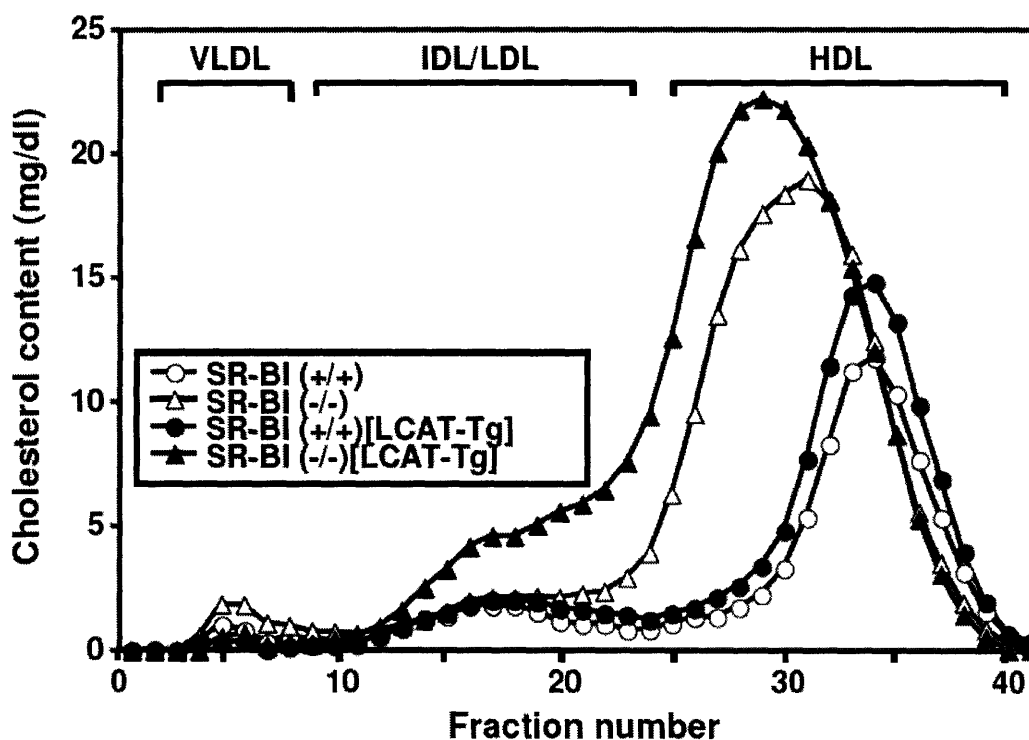
Figure 6. Effects of LCAT transgene expression on the lipoprotein cholesterol profiles of SR-BI (+/+) and SR-BI (-/-) mice. Plasma was harvested from the indicated control and LCAT transgenic mice and then pooled plasma samples (n=4) were size fractionated using FPLC. The total cholesterol contents of the fractions were determined by enzymatic assay. The chromatograms are representative of multiple, independent determinations and similar results were observed for samples from individual animals. Approximate positions of VLDL, IDL/LDL and HDL elutions are indicated by brackets and were determined as previously described (Rigotti, Trigatti *et al.* 1997).

Table II. Plasma cholesterol levels in LCAT transgenic and nontransgenic animals

SR-BI Genotype	LCAT Transgene	Plasma Total Cholesterol (mg/dl)	Plasma Unesterified Cholesterol (mg/dl)	Plasma Unesterified Cholesterol Ratio	Sample size
+/+	-	101 ± 9[a]	19 ± 2[b]	0.19 ± 0.04	n=10
	+	162 ± 12[a]	30 ± 2[b]	0.18 ± 0.02	n=21
-/-	-	249 ± 20	106 ± 7	0.44 ± 0.05[c]	n=12
	+	292 ± 14	107 ± 6	0.37 ± 0.05[c]	n=21

Values are represented as mean ± standard error

[a,b,c] $p < 0.001$

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Figure 7 shows the effects of transgenic human LCAT expression on the fertility of SR-BI (-/-) and SR-BI (+/-) females. There was virtually no effect of the LCAT transgene on the fertility of the control SR-BI (+/-) mice. The LCAT transgene did not significantly restore fertility, as measured by pups/month/female (panel B). All of the SR-BI (+/-)[LCAT-Tg] females (5/5) produced litters, whereas only 2 of 15 SR-BI (-/-)[LCAT-Tg] females each produced a single litter, and those were very small (2 vs 9 pups/litter). Thus, the very modest decrease in the abnormally high UC:TC ratio induced by the ~10-fold increase in plasma LCAT activity in the SR-BI (-/-)[LCAT-Tg] mice was not associated with a substantial increase in female fertility.
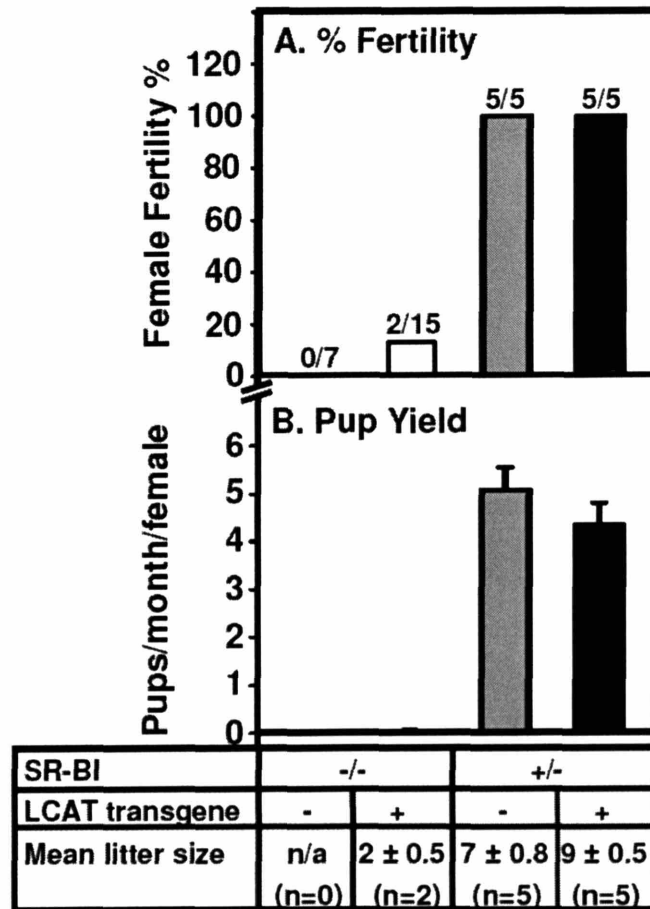
SR-BI	-/-		+/-	
LCAT transgene	-	+	-	+
Mean litter size	n/a (n=0)	2 ± 0.5 (n=2)	7 ± 0.8 (n=5)	9 ± 0.5 (n=5)

Figure 7. Effect of LCAT transgene expression on the fertility of SR-BI (+/-) and SR-BI (-/-) female mice. Individual, virgin females with the indicated SR-BI genotype [(+/-) or (-/-)] without (-) or with (+) the LCAT transgene were mated continuously for 4 months to nontransgenic SR-BI (+/+) males. (**A**) Fertility expressed as percentage of females producing litters (the numbers of females that produced litters relative to the number of females studied are shown above the bars). (**B**) Average number of pups delivered per month per female (error bars represent SEM). The mean litter sizes, which include only those females that delivered pups, are shown below. The values for the nontransgenic females, which had slightly different genetic backgrounds than the LCAT transgenic mice (C57BL/6:129 ratios 50:50 vs 75:25 in LCAT-Tg animals), were taken from Figure 6. The UC:TC values for females from the indicated genotypes are as follows: SR-BI (-/-) 0.52 ± 0.02 (n=11); SR-BI (-/-) [LCAT-Tg] 0.37 ± 0.01 (n=8) $p<0.000001$ and SR-BI (+/-) 0.21 ± 0.01 (n=6); SR-BI (+/-) [LCAT-Tg] 0.20 ± 0.01 (n=14) $p=0.73$. The p values represent statistical differences within each SR-BI genotype comparison with or without the LCAT transgene.

DISCUSSION

The abnormal lipoprotein metabolism in SR-BI (-/-) mice appears to be responsible for their female, but not male, sterility (Trigatti, Rayburn et al. 1999; Mardones, Quinones et al. 2001). The influence of hepatic SR-BI expression on female fertility has not previously been assessed directly, but would seem to be critical due to the key role hepatic SR-BI plays in plasma lipoprotein and cholesterol metabolism (Kozarsky, Donahee et al. 1997; Wang, Arai et al. 1998; Ueda, Royer et al. 1999; Kocher, Yesilaltay et al. 2003; Rigotti, Miettinen et al. 2003). Here we employed two methods to induce hepatic expression of SR-BI in SR-BI (-/-) animals: transient adenovirus-mediated or long-term stable transgenic expression. We used as transgenes both wild-type SR-BI and a double point mutation form of the receptor (Q402R/Q418R or 'SR-BI-RR'). SR-BI-RR has lost the ability to bind to and mediate lipid transfer from HDL normally, but retains essentially wild-type binding and lipid transport activities for the larger lipoprotein LDL (Gu, Kozarsky et al. 2000; Gu, Lawrence et al. 2000).

As we (Kozarsky, Donahee et al. 1997) and others (Sehayek, Ono et al. 1998; Wang, Arai et al. 1998; Ueda, Royer et al. 1999) previously showed, we found here that high level hepatic overexpression of wild-type SR-BI dramatically reduced the total plasma cholesterol in wild-type and SR-BI (-/-))- mice to nearly undetectable levels and increased biliary cholesterol levels or secretion rate. In contrast, high level hepatic expression of the SR-BI-RR mutant had virtually no effect on plasma cholesterol levels or biliary cholesterol secretion in SR-BI (+/+) mice, presumably because the normal size HDL that carries the bulk of the plasma cholesterol in these mice is not an efficient ligand for the mutant receptor. However, in SR-BI (-/-) mice hepatic overexpression of SR-BI-RR was able to reduce plasma cholesterol to nearly wild-type levels, lower to essentially wild-type values the abnormally high UC:TC ratio, correct the lipoprotein profile, and induce a substantial increase in biliary cholesterol secretion. Thus, in

SR-BI (-/-) mice high level hepatic expression of the SR-BI-RR mutant substantially normalizes the otherwise abnormal lipoprotein size and lipid composition, by either effectively removing cholesterol from abnormally large HDL particles and/or preventing the formation of these particles or their precursors. Therefore, in addition to LDL particles that are larger than normal HDL - and in contrast to normal size HDL - the abnormally large HDL particles in SR-BI (-/-) mice (or their precursors) appear to serve as effective substrates for the SR-BI-RR mutant. The SR-BI (-/-)[SR-BI-RR] transgenic mice should prove useful for future analysis of the pathophysiologic properties of the abnormal HDL in SR-BI (-/-) mice, including defective red blood cell maturation and enhanced susceptibility to atherosclerosis and coronary heart disease (Trigatti, Rayburn et al. 1999; Braun, Trigatti et al. 2002; Braun, Zhang et al. 2003).

Concurrent with the changes in the plasma lipoprotein profiles, stable transgenic hepatic expression of high or low levels of wild-type SR-BI or high levels of the SR-BI-RR mutant restored essentially normal fertility to SR-BI (-/-) females. Together with our earlier studies involving ovary transplantation, inactivation of the apoA-I gene or treatment with the hypocholesterolemia drug probucol, all of which restored production of functional oocytes by SR-BI-deficient ovaries, and thus partially (apoA-I KO) or fully (ovary transplantaion, probucol) restored fertility (Mardones, Quinones et al. 2001), these results establish that a primary mechanism by which SR-BI maintains murine female fertility is its control of plasma lipoprotein metabolism rather than its expression in reproductive organs.

The two most distinctive abnormal features of the HDL in SR-BI (-/-) mice that may influence female fertility are the substantial fraction of the HDL that is abnormally large and the high UC:TC ratio in both large and normal size particles. However, several lines of evidence indicate that large HDL particles per se are not responsible for female infertility in SR-BI (-/-)

mice. First, treatment of SR-BI (-/-) mice with the antioxidant and hypocholesterolemic drug

probucol, which lowers total plasma cholesterol by ~50%, normalizes the UC:TC ratio and fully

restores fertility, does not substantially alter the abnormally large HDL particle size distribution

(Mardones, Quinones *et al.* 2001; Braun, Zhang *et al.* 2003). Second, inactivation of the apoA-I

gene in SR-BI (-/-) mice, which lowers total plasma cholesterol by ~50% and partially restores

fertility, reduced the amounts of normal, smaller HDL particles relative to the abnormal large

particles, resulting in a mean particle size somewhat greater than that in apoA-I replete controls

(Mardones, Quinones *et al.* 2001). Third, female PDZK1 (-/-) mice are fertile (Kocher, Pal *et al.*

2003; Kocher, Yesilaltay *et al.* 2003). Hepatic SR-BI protein levels in PDZK1 (-/-) mice are ~5%

of wild-type controls, whereas SR-BI protein levels in the ovary and other steroidogenic organs

are normal (Kocher, Yesilaltay *et al.* 2003). As a consequence of the reduced hepatic SR-BI

activity, plasma cholesterol is elevated in abnormally large HDL particles somewhat reminiscent

of those in SR-BI (-/-) animals. However, the UC:TC ratio is essentially normal in PDZK1 (-/-)

mice (Kocher, Yesilaltay *et al.* 2003), perhaps as a consequence of the nearly normal

extrahepatic SR-BI expression or the normal hepatic expression of the minor, alternatively

spliced mRNA isoform called SR-BII that is not present in SR-BI (-/-) mice. Thus, large HDL

particles in PDZK1 (-/-) mice are not associated with reduced fertility.

While the presence of abnormally large HDLs per se is unlikely to cause female

infertility in SR-BI (-/-) mice, there is a striking correlation between infertility and the abnormal

UC:TC ratio. A key determinant of the UC:TC ratio is the activity LCAT, which is the plasma

enzyme responsible for esterification of unesterified cholesterol in HDL particles (Vaisman,

Klein *et al.* 1995; Francone, Haghpassand *et al.* 1997; Ng, Francone *et al.* 1997). Although the

absolute level of LCAT in SR-BI (-/-) mice appears to be the same as in control SR-BI (+/+)

animals when measured using an exogenous reconstituted HDL substrate (this study, also see (Ma, Forte *et al.* 2005)), there was significantly reduced LCAT activity in SR-BI (-/-) mice (~50%) when assessed by using the abnormal endogenous lipoproteins as the substrate. Previous studies (Rigotti, Trigatti *et al.* 1997; Trigatti, Rayburn *et al.* 1999), confirmed here, have shown that the plasma levels of apoA-I, the major activator of LCAT in plasma, are normal in SR-BI (-/-) and SR-BI (-/-)/apoE (-/-) mice. Furthermore, hepatic SR-BI or SR-BI-RR transgene expression, which restored fertility, did not increase plasma apoA-I levels. Thus, reduced apoA-I-mediated activation of LCAT due to lower plasma apoA-I levels is unlikely to be responsible for the high UC:TC ratio. We conclude that the abnormal plasma lipoproteins in SR-BI (-/-) are poor substrates for LCAT, and that this substantially contributes to the abnormally high UC:TC ratio. While this manuscript was in preparation, similar findings (essentially wild-type levels of LCAT protein, but reduced LCAT activity with endogenous substrate) were reported by Ma et al. (Ma, Forte *et al.* 2005). In SR-BI (-/-)/apoE (-/-) double knockout mice, the HDL-like particles are even larger than those in SR-BI (-/-) single knockout mice, forming lamellar/vesicular and stacked discoidal particles and the UC:TC ratio is higher (~.8 vs ~.5) (Braun, Zhang *et al.* 2003). It seems likely that the particles in the SR-BI (-/-)/apoE (-/-) double knockout mice are especially poor LCAT substrates.

In an attempt to correct the high UC:TC ratio, we crossed SR-BI (-/-) mice with transgenic mice expressing human LCAT in addition to endogenous murine LCAT. Although this resulted in an ~10-fold increase in LCAT activity (determined using exogenous substrate), the LCAT transgene only reduced the UC:TC by 16% to 0.37 and failed to restore female fertility. While species differences in the specificities of the human and murine LCAT enzymes (Liu, Bagdade *et al.* 1995) may have influenced the results, our findings suggest that the

abnormal lipoproteins in SR-BI (-/-) mice exhibit a high UC:TC ratio because they are poor LCAT substrates and that SR-BI activity normally helps maintain HDL in a state in which it can serve as an efficient LCAT substrate.

Determination of the precise mechanism by which abnormal lipoprotein metabolism in SR-BI (-/-) mice results in female infertility will require additional studies. The key lipoprotein-dependent step appears to occur prior to or immediately during ovulation, because SR-BI (-/-) females ovulate dysfunctional oocytes that are dead at ovulation or die shortly thereafter (Trigatti, Rayburn et al. 1999; Mardones, Quinones et al. 2001). There appear to be two general classes of mechanisms by which the abnormal lipoproteins in SR-BI (-/-) mice might contribute to female infertility. The first involves 'gain of function' mechanisms in which the abnormal lipoproteins are somehow ovariotoxic. In the absence of ovarian SR-BI, the abnormal lipoproteins might deliver molecules to ovarian cells that are directly toxic (this could include delivery of excessive amounts of cholesterol (Warner, Stoudt et al. 1995; Kellner-Weibel, Jerome et al. 1998; Kellner-Weibel, Geng et al. 1999; Holm, Braun et al. 2002)) or remove essential compounds, the depletion of which would be toxic, resulting in defects in ooctye development/ovulation.

Alternatively, female infertility could be a consequence of a 'loss of function' mechanism in which the abnormal lipoproteins are unable to perform a function(s) of wild-type lipoproteins. In the absence of SR-BI, the abnormal lipoproteins might be unable to deliver - or remove by efflux - key compounds to/from the ovary, for example, HDL-facilitated vitamin E uptake (Mardones and Rigotti 2004) or HDL-mediated efflux of cholesterol. Particularly, the abnormal lipoproteins in SR-BI (-/-) mice might be unable to facilitate removal of excess cellular cholesterol, because their high UC:TC ratio would prevent thermodynamically driven cellular

cholesterol efflux down a cholesterol concentration gradient (Jian, de la Llera-Moya et al. 1998; de la Llera-Moya, Rothblat et al. 1999; Yancey, de la Llera-Moya et al. 2000; Yancey, Bortnick et al. 2003). The pathogenic accumulation of intracellular cholesterol, due to either defective cholesterol efflux or abnormal enhanced delivery, appears to contribute to defective red blood cell maturation in SR-BI (-/-)/apoE (-/-) double knockout mice (Holm, Braun et al. 2002). Unesterified cholesterol efflux from the cells in the ovary may be particularly important during the preovulatory period when the granulosa cells produce progesterone following the luteinizing hormone surge. Progesterone has been shown to inhibit cholesterol esterification in vitro (Lange and Steck 1994; Mazzone, Krishna et al. 1995) at concentrations that are present in the ovary (Christenson and Devoto 2003). Thus, it has been suggested that cholesterol efflux may help cells avoid unesterified cholesterol accumulation (Balestrieri, Cigliano et al. 2001). In humans, HDL in follicular fluid is in the cholesterol poor prebeta form (Jaspard, Collet et al. 1996), a good acceptor for cellular efflux of excess unesterified cholesterol from the ABCA1 transporter (Aiello, Brees et al. 2003). In addition, LCAT is also present in follicular fluid and may contribute to efficient efflux by preventing unesterified cholesterol accumulation in the HDL (Balestrieri, Cigliano et al. 2001; Cigliano, Spagnuolo et al. 2001). Because of the limited size of murine follicles, it has been difficult to characterize their lipoprotein composition and metabolism.

The role of HDL-mediated cholesterol efflux may be especially relevant in SR-BI (-/-) mice, because SR-BI can directly mediate cholesterol efflux (Jian, de la Llera-Moya et al. 1998; de la Llera-Moya, Rothblat et al. 1999; Yancey, de la Llera-Moya et al. 2000), as well as selective lipid uptake (Acton, Rigotti et al. 1996) , and thus contribute to the bidirectional flow of lipids to maintain a local balanced environment of cholesterol. The relationship between

female infertility of SR-BI (-/-) mice and the functions of two other cell surface proteins that mediate cholesterol efflux, ABCA1 (Liu, Bortnick *et al.* 2003; Denis, Haidar *et al.* 2004) and ABCG1 (Kennedy, Barrera *et al.* 2005) is unclear. However it is noteworthy that while ABCG1 (-/-) mice exhibit normal fertility (Kennedy, Barrera *et al.* 2005), ABCA1 (-/-) females exhibit impaired fertility (placental malformation) (Christiansen-Weber, Voland *et al.* 2000) and have very low plasma HDL levels (McNeish, Aiello *et al.* 2000; Orso, Broccardo *et al.* 2000). It is possible that the normal ovarian expression of SR-BI itself, even if it were to occur in the context of the abnormal lipoproteins present in SR-BI (-/-) mice, might mitigate the deleterious influence of these lipoproteins on female fertility (e.g., by facilitating lipid transport between the lipoproteins and ovarian cells). Thus, ovarian SR-BI expression might contribute to the fertility of PDZK1 (-/-) female mice even though hepatic SR-BI expression is dramatically reduced and abnormally large HDLs accumulate in the circulation (Kocher, Yesilaltay *et al.* 2003). Our observation that transgenic hepatic expression of SR-BI or SR-BI-RR in SR-BI (-/-) females at least partially corrects their lipoprotein abnormalities and restores fertility is compatible with either class of mechanism. It is clear that not all variations in HDL structure or metabolism will substantially reduce female fertility. For example, there is no evidence of female infertility in species with naturally low HDL levels (e.g. guinea pigs) or in animals with experimentally generated low plasma HDL, such as apoA-I deficient or transgenic, hepatic SR-BI overexpressing mice (Williamson, Lee *et al.* 1992; Wang, Arai *et al.* 1998; Ueda, Royer *et al.* 1999). Thus, even small steady state levels of plasma HDL, provided these particles have the appropriate size and composition or metabolic fates, can be sufficient to prevent ovarian toxicity (e.g., accumulation of toxic cholesterol levels).

In summary, the current studies confirmed the importance of the hepatic expression of SR-BI for murine lipoprotein metabolism and further support our conclusion that the structure of HDL and its role in cholesterol transport and metabolism may be critical in determining mammalian female fertility. These findings raise the possibility that dyslipidemia might contribute to some cases of human female infertility, and in such cases targeting dyslipidemia might complement current assisted reproductive technologies.

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ABBREVIATIONS

[1]Abbreviations used are: HDL, high density lipoprotein; SR-BI, scavenger receptor, class B, type I; SR-BI-RR, SR-BI (Q402R/Q418R); LDL, low density lipoprotein; UC, unesterified cholesterol; TC, total cholesterol; PDZ, postsynaptic density protein (PSD-95)/Drosophila disc large tumor suppressor (dlg)/tight junction protein (ZO1); Tg, transgenic; Ad, adenocirus, FPLC, fast performance liquid chromatography; LCAT, lethicin:cholesterol acyl transferase; SDS-PAGE, sodiumdodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride; TBS, Tris-buffered saline; CER, cholesterol esterification rate; CE, cholesteryl ester; apoE, apolipoprotein E; apoA-I, apolipoprotein A-I, VLVL, very low density lipoprotein; IDL, intermediate density lipoprotein

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FOOTNOTE