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Regulation of Paraoxonase-1 and Its Role in the Development of Atherosclerosis

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Biology

by

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The dissertation of Alejandra Gutierrez is approved, and it is acceptable in quality and form for publication on microfilm:

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Dedication

This work is dedicated to my family and friends.

Especially
Maria-Isabel Gonzalez and Francisco Gutierrez
Joshua Webb Wilton
Cisco and Laura

Thank you.

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List of Abbreviations

ABCA1: ATP-binding Cassette Transporter A1

ACAT: Acetyl-Coenzyme A Acetyltransferase

Apo B: Apolipoprotein B

CAD: Cardiovascular Arterial Disease

CYP7A1: Cholesterol 7-alpha Hydroxylase

FGF15: Fibroblast Growth Factor 15

FGF19: Fibroblast Growth Factor 19

FGFR4: Fibroblast Growth Factor Receptor 4

FXR: Farnesoid X Receptor

GdCl3: Gadolinium Chloride

GFP: Green Fluorescent Protein

HDL: High Density Lipoprotein

HFH: Homozygous Familial Hypercholesterolemia

HSC: Hematopoietic Stem Cells

IL-1: Interleukin 1

IL-6: Interleukin 6

IL-8: Interleukin 8

LBP: Lipopolysaccharide Binding Protein

LCAT: Lecithin Cholesterol Acyltransferase

LDL: Low Density Lipoprotein

LDLR: Low Density Lipoprotein Receptor

LPS: Lipopolysaccharide

LRH-1: Liver Receptor Homolog 1

MCP1: Monocyte Chemotactic Protein 1

MCSF: Macrophage Colony Stimulating Factor

PBS: Phosphate Buffered Saline

PON1: Paraoxonase 1

SHP: Small Heterodimer Partner

SRA: Scavenger Receptor A

SRB: Scavenger Receptor B

TLR4: Toll-Like Receptor 4

TNFα: Tumor Necrosis Factor Alpha

VCAM-1: Vascular Cell Adhesion Molecule 1

VLDL: Very Low Density Lipoprotein

List of Figures and Tables

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ABSTRACT OF THE DISSERTATION

Regulation of Paraoxonase-1 and Its Role in the Development of Atherosclerosis

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The studies presented herein examine various aspects of paraoxonase 1 (PON1), specifically PON1's therapeutic potential and the hepatic expression of PON1. Bone marrow obtained from mice expressing a macrophage specific PON1-transgene was transplanted into atherosclerosis-susceptible mice lacking low density lipoprotein receptors (LDLR), then followed with gadolinium chloride ($GdCl_3$) treatment, an agent that induces Kupffer cell apoptosis. $GdCl_3$ treatment increased hepatic PON1 mRNA expression 9-fold in PON1-trangenic bone marrow recipient mice. After a 12 week cholesterol-enriched atherogenic diet feeding period, PON1-transgenic bone marrow recipient mice treated with $GdCl_3$ exhibited a 50% reduction in atherosclerotic lesions; this protection was not seen in non-transgenic bone marrow

recipients treated with $GdCl_3$. These findings illustrate the important role of Kupffer cells in atherogenesis and suggest that $GdCl_3$-facilitated replacement of Kupffer cells may enhance the efficacy of other HSC-based gene therapies.

In additional studies, the regulation of PON1 expression by dietary bile acids was examined. It has been previously shown that a cholic-acid containing diet reduces the hepatic expression of PON1 in athero-susceptible C57BL/6 animals, but not in athero-resistant C3H/HeJ animals. When fed an atherogenic diet containing taurocholate, C3H/HeJ mice, in contrast to C57BL/6 mice, displayed a resistance to bile acid mediated repression of hepatic PON1 mRNA expression and HDL cholesterol. Further studies demonstrated that the absence of functional toll-like receptor 4 (TLR4s) in C3H/HeJ mice could not account for this response. However, mice with a genetic deletion of either fibroblast growth factor receptor 4 (FGFR4) or farnesoid X receptor (FXR) failed to repress PON1 and CYP7A1 in response to the taurocholate-containing diet. Additional studies demonstrated that FGF-19, an FXR-inducible growth factor that binds specifically to FGFR4, decreased the expression of PON1 and CYP7A1 in HepG2 cells. The taurocholate containing diet increased the ileal expression of FGF-15, the murine homologue of FGF-19, in C57BL/6 and C3H/HeJ mice. Combined these data suggest that hepatic PON1 and CYP7A1 mRNA expression is repressed by bile acids via an FXR-mediated induction of FGF-15. The resistance of C3H/HeJ mice to bile acid-mediated repression of PON1 and CYP7A1 is, therefore, due to signaling events distal to the FGF-15/FGFR-4 association.

Chapter 1

Review of the Literature

Introduction

Inflammation and Atherosclerosis

Cardiovascular disease is the leading cause of death in technologically advanced societies (1). Atherosclerosis, the primary cause of cardiovascular disease, is characterized by the accumulation of lipids in the arterial wall (2), which subsequently induces inflammation. Studies in humans and animals demonstrate that inflammation can act by itself, or in combination with excessive LDL, to promote atherogenesis (1-6). Thus, atherosclerosis is defined as a chronic inflammatory disease.

Additional studies uncovered a link between vascular wall inflammation and the processes mediating innate and acquired immunity (7,8). The presence in atherosclerotic lesions of innate and acquired immunoglobulins that recognize bacterial antigens and epitopes, suggest that immune response may mediate the progression of atherosclerosis (9,10). The discovery that many of the reactive products that are formed when LDL undergoes oxidative modification can generate immunogenic neopeptides (11) provides further insights into the mechanisms linking immunity to atherogenesis. Autoantibodies recognizing these epitopes have been

identified in the plasma and atherosclerotic lesions of both animals and humans (12-15).

These findings provide a mechanistic understanding for the well-established relationship between elevated plasma LDL levels and atherosclerosis (16). Due to prolonged residence in the arterial wall, LDL is subject to oxidative modifications (1). These modifications induce LDL to assume inflammatory, proatherogenic properties that lead to the activation of arterial wall endothelial cells and macrophages. Several cell culture models of cells residing at the arterial wall, specifically macrophages, endothelial cells, and smooth muscle cells, are able to oxidize LDL *in vitro* (17-19). The relationship between oxLDL produced *in vitro* and the oxLDL that is involved in atherogenesis *in vivo* (2) remains to be fully established.

The arterial wall is lined by endothelial cells that resist the adhesion of leukocytes; however, pro-atherogenic inflammatory signals, such as oxLDL, IL-1, and TNFα, induce the expression of vascular cell adhesion molecules (20). Arterial wall endothelial cells that express surface selective adhesion molecules bind monocytes and other leukocytes. One such molecule, vascular cell adhesion molecule-1 (VCAM-1), binds monocytes and T lymphocytes and is expressed by endothelial cells in areas prone to lesion formation (21). VCAM-1 expression in endothelial cells has been shown to precede the appearance of macrophages in the arterial intima (22). Further, a compromised form of VCAM-1 was found to be protective in atherosclerosis-susceptible LDLR knockout and apo E knockout mice (23).

Once adhered to the arterial wall, monocytes can exhibit an induction of chemotaxis via monocyte chemotactic protein-1 (MCP-1). As a result, monocytes enter the intima where they perpetuate the inflammatory response. The release of macrophage colony stimulating factor (MCSF) at these sites causes the differentiation of blood monocytes to activated macrophages (reviewed in (2)). When monocytes/macrophages take up oxLDL they become lipid laden foam cells, which can be stimulated to secrete MCP-1 and MCSF (24) thereby perpetuating the inflammatory response. As a result, additional macrophages are recruited to the lesion site, leading to an increased lesion area. Gene expression studies in atherosclerotic lesions revealed an up-regulation of pro-atherogenic cytokines, including IL-1 and IL-6, which recruit inflammatory cells (25,26).

Atherosclerotic lesions also exhibit elevated levels of toll-like receptor 4 (TLR4) (27), a receptor that binds lipopolysaccharide (LPS) and initiates an inflammatory response (28). The TLR4 has been proposed to play an important role in atherosclerosis. C3H/HeJ mice, which lack a functional TLR4, are resistant to diet-induced atherogenesis (29). In addition, Walton *et. al.* (30) demonstrated that oxidized phospholipids, which are believed to be the proatherogenic element of oxLDL, signal through the TLR4 in endothelial cells to upregulate IL-8, a cytokine responsible for monocyte recruitment and retention at the vessel wall. Further, Miller *et. al.* (31) found that oxLDL induces macrophage spreading through the TLR4/MD2 complex. CD-14, a receptor for LPS that associates with TLR4 when bound to lipopolysaccharide-binding protein (LBP), specifically binds mmLDL and causes

morphological changes in macrophages that lead to increased oxLDL uptake (reviewed in (10)). OxLDL uptake is mediated by a number of different receptors (Figure 1.1); including class A (SRAI, SRAII) and class B (SR-B1, CD-36) scavenger receptors, which also recognize acetylated LDL (32-36).

HDL and Paraoxonase 1

HDL levels inversely correlate with atherosclerosis. One of the anti-atherogenic properties of HDL has been attributed to the presence of paraoxonase 1 (PON1). PON1 is one of the three members of the PON gene family present in humans and mice (37). PON1 has paraoxonase and arylesterase activities that are not present in PON2 or PON3 (38). In addition, PON1 displays genetic polymorphisms which are linked to susceptibility to organophosphate toxicity in varying populations (summarized by La Du (39)). The role of PON1 in lipid metabolism was elucidated after its association with HDL in the plasma was revealed (40); this association occurs through the retained N-terminal signal sequence of PON1 (41). Initially, McElveen ***et. al.*** found that PON1 activity was low in the plasma of myocardial infarction survivors (42). Interestingly, the structural similarities between organophosphates and phospholipids suggested a potential role for PON1 in lipid metabolism, particularly in the context of atherosclerosis (43). Subsequent studies demonstrated that PON1 prevents LDL lipid-peroxidation *in vitro* (44). Recent evidence suggests that PON1's anti-atherogenic properties are based on its lactonase activity. Detailed analysis of PON1 substrates indicate that PON1 is a lactonase with high activity toward statins as

well as toward lactones formed as oxidation products from polyunsaturated fatty acids (45,46). While there is no apparent link between PON1 polymorphisms and cardiovascular arterial disease (CAD), epidemiology studies show that increased plasma PON1 activities were associated with decreased CAD (47).

Liver Resident Macrophages: Kupffer Cells

Arterial wall macrophages comprise of only a small subset of the body's resident macrophages. Kupffer cells, the resident macrophages in the liver, in contrast, represent nearly 80% of the body's resident macrophages (48). Kupffer cells are primarily located in the periportal region of the liver, which allows them to phagocytose elements in the blood entering the liver via the portal blood flow (48). Kupffer cells are responsible for clearing most of the oxLDL from the plasma (49). Like arterial wall macrophages, Kupffer cells express receptors capable of oxLDL and acetyl LDL uptake. It is also known that Kupffer cells produce cytokines such as IL-1 and IL-6, which can act locally within the liver and also systemically through the blood stream (50).

Cholesterol Homeostasis

The liver indirectly affects atherosclerotic lesion formation in the arterial wall. The liver is the major tissue site controlling the plasma levels of atherogenic apo B-containing lipoproteins via VLDL production (51,52) and the removal of LDL from plasma (53,54). Apo B-containing lipoproteins are the precursors of the oxidatively

modified lipoproteins that act on vascular wall endothelial cells (3) and macrophages (15) causing inflammation and atherogenesis. In addition, Apo B-containing lipoproteins are the major source of cholesterol esters that accumulate in vascular wall macrophages (i.e. “foam cells”) (4,5,55).

Cholesterol is a necessary component in maintaining the plasma membrane in living organisms; however, its homeostasis is tightly regulated by various, sometimes redundant, mechanisms. Under most physiological conditions, cholesterol synthesis is limited by the enzyme HMG-CoA reductase (56), which reduces HMG-CoA to mevalonic acid. This enzyme is tightly regulated by cellular cholesterol levels through various mechanisms at the transcription (57), translation (58), and protein levels (59-61).

Cholesterol uptake into cells occurs via the cell-surface LDL receptor, which recognizes specific ligands, namely apo B100 or apo E lipoproteins, in cholesterol-enriched LDL particles. Lipoproteins are rapidly taken into the cell and delivered to lysosomes (62) where lipases hydrolyze the cholesterol esters to unesterified cholesterol and fatty acids. Once hydrolyzed, the LDL-derived cholesterol regulates the expression of various genes within the cell (63).

Cells are capable of esterifying free cholesterol at the 3β-hydroxyl group through the activity of ACAT1 and ACAT2. ACAT1 is mainly expressed in macrophages, whereas ACAT2 is mainly expressed in intestinal epithelial cells and liver parenchymal cells (64,65).

Cholesterol esters are hydrophobic molecules that accumulate in cells as cytoplasmic lipid droplets, as occurs in "foam cell" macrophages. Cholesterol esters formed by ACAT2 in the liver can be secreted as apo B- containing lipoproteins (66). Curiously, deficiency of ACAT1 in LDLR-knockout and apo E-knockout animals did not reduce atherosclerotic lesion formation despite decreasing plasma cholesterol levels (67). In fact, ACAT1 deficiency in macrophages increased atherosclerotic lesions in LDLR-knockout mice; however, an accumulation of free cholesterol was present within these lesions (68). These observations may be due to the extreme hyperlipidemia exhibited by these mouse models. In contrast, the deletion of ACAT2, blocked the production of lipoproteins and the formation of diet-induced gallstones in C57BL/6 mice (69).

The liver is the organ site mainly responsible for the removal of excess cholesterol from the body in the form of biliary micelles consisting of free cholesterol, phospholipids and bile acids (70). The rate limiting enzyme in the neutral pathway for synthesis of bile acids is CYP7A1, which is only expressed in liver parenchymal cells. Over-expression of a constitutively active CYP7A1 transgene protects C57BL/6 mice from diet induced hypercholesterolemia (71). Even in the absence of functional LDLRs, mice expressing the CYP7A1 transgene are protected from diet induced hypercholesterolemia (72).

Hepatic expression of CYP7A1 is highly variable and sensitive to diet (73-81). CYP7A1 is repressed by various factors including TNFα and bile acids (82,83). Bile acid-negative feedback regulation of CYP7A1 has been extensively studied (83-88).

For example, FXR-dependent repression of CYP7A1 can be mediated through SHP and LRH-1. Upon activation, FXR increases the expression of SHP, a transcriptional corepressor. SHP can interact with LRH-1, a positive regulator of CYP7A1, and impede its transcriptional activity (86). The recent findings showing that SHP knockout animals remain sensitive to bile acid repression of CYP7A1 indicates that the SHP-LRH-1 regulatory pathway is complemented by other pathways (89). Recent evidence suggests that bile acids repress CYP7A1 via JNK kinases, FGF signaling and inflammatory cytokines (84,87,89-94).

Reverse cholesterol transport provides a mechanism for the removal of cholesterol from peripheral tissues so it can be transported back to the liver for excretion from the body (95). The accumulation of excess cholesterol in peripheral cells can be prevented by increasing reverse cholesterol transport. This occurs initially via the efflux of free cholesterol from the plasma membrane onto various acceptors that are present in the plasma. These acceptors include free apo A1 protein and HDL particles (96). Free cholesterol found in the plasma HDL particle is esterified by the plasma soluble protein, LCAT (97), and is consequently taken up and processed by the liver (97-98). LCAT-mediated esterification of HDL free cholesterol drives the ability of HDL to accept cholesterol (98).

The ATP binding cassette transporter A1 (ABCA1), a member of a superfamily of trans-membrane transporters, plays a significant role in reverse cholesterol transport (99). Removal of both phospholipids and free cholesterol from the arterial wall is linked to ABCA1 (100-104). The importance of ABCA1 in

mediating the cholesterol efflux process is evident in Tangier's disease patients. Tangier's disease patients have dominant negative mutations of ABCA1 (105,106) which results in extremely low levels of plasma HDL cholesterol and apo A1 (104,107). Moreover, Tangier's disease patients accumulate vast quantities of cholesterol in macrophages, exhibit impaired reverse cholesterol transport and greater susceptibility to atherosclerosis (100,108).

The liver is the major site for plasma lipoprotein clearance (109). LDL receptor-mediated uptake of LDL is an important pathway for regulating cholesterol homeostasis (110). Nearly 70-90% of plasma LDL is removed from plasma via the LDL receptor (54,111,112). Mutations in the LDLR gene are among the most common in the human population (about 1 in 500). The rare patients (homozygous familial hypercholesterolemia (HFH)) exhibiting homozygous mutations in the LDL receptor (about $1/10^6$) have marked abnormalities in plasma lipids and develop prematurely fatal cardiovascular disease for which the only long-term effective therapy is liver transplantation (113). The liver also plays a major role in the uptake of modified forms of LDL from the plasma, such as acetyl-LDL (114) and oxidized LDL (49).

Goal of this Study

In this study we examine the role and regulation of paraoxonase 1. We hypothesize that macrophage specific expression of PON1 will ameliorate atherosclerosis in susceptible LDLR knockout animals. We use a novel gene therapy

to replace liver resident macrophages and determine the therapeutic potential of the method. Further, we describe a mechanism for the regulation of hepatic PON1 mRNA. We hypothesize that the regulation of PON1 is similar to CYP7A1, and examine a recently described pathway signaling from the intestine to the liver. Finally, we examine the proposal that HDL regulates PON1 plasma protein and activity by regulating its secretion from the plasma membrane.

Table 1.1: Macrophage receptors involved in modified cholesterol uptake.

Receptor	Ligand
CD14	MmLDL
Low Density Lipoprotein Receptor (LDLR)	MmLDL
Scavenger Receptor A I	OxLDL
Scavenger Receptor AII	OxLDL
Scavenger Receptor B1	OxLDL
CD-36	OxLDL
Macrosialin/CD68 [88]*	OxLDL

*More recently,(88) has suggested that there is no direct role for macrosialin in oxLDL metabolism.

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Chapter 2

Facilitated Replacement of Kupffer Cells Expressing a Paraoxonase-1 Transgene Is Essential for Ameliorating Atherosclerosis in Mice

Abstract

Resident macrophages (i.e., Kupffer cells) are derived from hematopoietic stem cells (HSCs) and are primarily responsible for the removal from plasma of oxidized forms of low-density lipoprotein (LDL). The therapeutic potential of Kupffer cell expression of a transgene encoding paraoxonase-1 (PON1), whose plasma activity correlates with the protection from atherosclerosis, was examined in mice rendered atherosclerosis-susceptible through genetic deletion of the LDL receptor. Mice having their bone marrow engrafted with HSCs expressing the PON1 transgene (PON1-Tg) driven by a macrophage-specific promoter were injected i.v. with saline (vehicle only) or with gadolinium chloride ($GdCl_3$), an agent that rapidly causes Kupffer cell apoptosis. One month later, $GdCl_3$-facilitated Kupffer cell apoptosis increased the hepatic expression of transgenic PON1 mRNA by 9-fold. After 12 weeks of being fed a cholesterol-enriched atherogenic diet, mice injected with $GdCl_3$ exhibited 50% reductions in both aortic sinus atherosclerotic lesions ($P < 0.0097$) and surface lesions of the abdominal aorta ($P < 0.006$). In contrast, mice receiving HSCs expressing the PON1-Tg but not treated with $GdCl_3$ showed no protection from atherosclerosis. In addition, mice engrafted with HSCs not expressing the PON1-Tg but injected with

$GdCl_3$ also showed no protection from atherosclerosis. These findings, showing that $GdCl_3$-enhanced hepatic expression of the PON1-Tg is essential for reducing atherosclerosis, indicate that Kupffer cells play an important role in atherogenesis. $GdCl_3$-facilated replacement of Kupffer cells may enhance the efficacy of other HSC-based gene therapies.

Introduction

Hematopoietic stem cells (HSCs) are attractive vehicles for the delivery of therapeutic genes to different tissues to ameliorate human disease (1-3). Because of its large size and unrestricted contact with blood (4), the liver is a key tissue target for gene therapy (5). Recent studies suggest that engraftment of hepatic parenchymal cells via HSCs occurs rarely, if at all (6). Our studies were directed to develop an alternative approach to rapidly deliver therapeutic transgenes to the liver using HSCs.

Quiescent phagocytic resident macrophages (i.e., Kupffer cells) are derived from bone marrow HSCs and make up ≈10-20% of the cells in liver (7,8). The rate of replacement of Moma-2-positive Kupffer cells from engrafted HSCs in mice is relatively slow (several months) (9). We examined whether the rate of replacement of liver resident macrophages derived from progenitor HSCs could be enhanced sufficiently to provide a more effective delivery of therapeutic transgenes.

Gadolinium chloride ($GdCl_3$) is an imaging contrast agent that is rapidly taken up mainly by liver resident macrophages (Kupffer cells) (10). The uptake of $GdCl_3$ by Kupffer cells induces apoptosis followed by a rapid replacement (within a few days) from HSCs (10). Circulating and spleen macrophages are relatively resistant to $GdCl_3$-induced apoptosis (10).

To test the hypothesis that $GdCl_3$ would enhance the rapid expression of a therapeutic transgene by Kupffer cells and ameliorate disease, we chose to quantify atherosclerotic lesion formation in a mouse model of homozygous familial

hypercholesterolemia (HFH) (11). HFH mice lack low-density lipoprotein (LDL) receptors and exhibit a severe diet-induced hypercholesterolemia that results in the formation of atherogenic oxidatively modified LDL (12), xanthomatosis, and vascular atherosclerotic lesions (11). The only effective long-term therapy for most HFH patients is liver transplantation (13). Kupffer cells are a major site for the uptake and removal from plasma of oxidatively modified forms of LDL (14,15). Oxidatively modified forms of LDL also can be metabolized and their atherogenicity inactivated by paraoxonase-1 (PON1), an enzyme produced in liver parenchymal cells and carried in plasma on high-density lipoprotein (16-22). Human and animal studies have established inverse relationships between the plasma activity of PON1 and myocardial infarction (20-22) or atherosclerotic lesion formation (16-19, 23,24). Thus, it is reasonable to propose that expression of PON1 by Kupffer cells, the cellular site of oxidized LDL uptake, might decrease atherosclerotic lesion formation.

Materials and Methods

All experiments were performed in compliance with the relevant laws and institutional guidelines of the Institutional Animal Care and Use Committees at San Diego State University, University of California, San Diego, and The Scripps Research Institute after review and approval of detailed animal experimental protocols. Based on preliminary studies showing that *Helicobacter* infection of our mouse colony affected the expression of some liver genes and atherosclerosis lesion size, all mice used in these studies were *Helicobacter*-free (using PCR).

PON1 Transgenic Mice

The coding region of mouse PON1 was inserted into the HindIII and EcoRV sites of the polylinker in the plasmid Fxba-A1, which contains the macrophage-specific human acetyl-LDL receptor promoter and the human growth hormone polyadenylation signal (25). Both strands of the resulting plasmid (pMacPON1) were sequenced.

PON1 transgene (PON1-Tg) mice were made by injecting the excised purified PON1-Tg into C57BL/6 embryos. Genomic DNA obtained from the tail of pups was screened for the presence of the PON1-Tg. Mice having the PON1-Tg and nontransgenic littermates were bred with C57BL/6 mice expressing GFP driven by the β-actin promoter (i.e., C57BL/6-TgN ACTbEGFP) 1Osb mice (26) obtained from The Jackson Laboratory.

Bone Marrow Transplantation

Six- to eight-week-old male C57BL/6 LDL receptor knockout mice (11) (obtained from The Jackson Laboratory) were given 1,000 rads of total body irradiation and then injected (tail vein) with 2×10^6 bone marrow HSCs (27). Mice were fed with a standard chow diet (No. 5015, Harlan Teklad, Madison, WI) for 4 weeks. Mice then received an injection (tail vein) of either saline (control) or saline containing $GdCl_3$ (25 mg/kg) and were fed the standard chow diet for an additional 4 weeks, after which they were fed an atherogenic diet (1.25% cholesterol, 6% fat, TD96335, Harlan Teklad).

Immunohistochemistry

Frozen liver tissue embedded in OCT compound was sectioned at a thickness of 10 μm on a Leica CM1850 cryostat. Slides with liver sections were fixed for 2 min in ice-cold acetone (-20°C) and then rehydrated for 2 min in PBS. Liver tissue was blocked for 30 min at room temperature with 10% goat serum in blocking buffer (1% BSA/0.15% Triton X-100 in PBS, pH 7.4). Fc receptors on Kupffer cells were blocked further with purified rabbit IgG (Vector Laboratories) at 0.5 mg/ml in blocking buffer for 2 h at room temperature. Tissue sections were washed with PBS and incubated at room temperature for 2 h with a rat anti-mouse Moma-2 (Serotec) antibody applied at a 1:200 dilution in blocking buffer. Sections were washed with PBS and incubated for 1 h with a 1:30 dilution of goat anti-rat IgG antibody conjugated to rhodamine (Calbiochem). Sections were washed in PBS, and 25 μl of Vectashield Mounting

Medium (Vector Laboratories) was applied. Liver sections were viewed by using a Leica Confocal Microscope to view colocalization of rhodamine (indicative of macrophages) and GFP fluorescence.

Plasma Lipids and PON1 Activity

After an overnight fast (14 h), mice were anesthetized with isofluorane. Blood was collected by retroorbital puncture. The lipids contained in individual lipoprotein fractions were quantitated by standard colorimetric assay (28). Paraoxonase activity was determined as described (29,30).

Macrophage Isolation and Characterization

Four days after mice received an i.p. injection of 1 ml of 4% thioglycolate, peritoneal macrophages were isolated, washed with 20 ml of ice-cold sterile PBS, resuspended in 10 ml of 10% FBS DMEM, and plated onto 100-mm culture dishes. After 6 h, adherent macrophages were used for the following experiments.

Total RNA was isolated from attached macrophages by using the RNeasy Minikit from Qiagen, Valencia, CA. cDNA was made from 2 μg of RNA (random primers) by using the Superscript II RT kit (Invitrogen).

Bone marrow cells were extracted from the femurs and tibias of male mice 4-6 weeks of age, both PON1-transgenic and control nontransgenic siblings. Cells were

plated overnight in RPMI medium 1640 with L-glutamine, 10% FBS, and 30% L-cell conditioned media (31). Nonadherent cells were removed at 24 h, counted, and plated at a density of 500,000 cells/ml with 10 ml per 100-mm plate. After 5 days of culture in RPMI medium 1640 with L-glutamine, 10% FBS, and 30% L-cell conditioned media, adherent cells were harvested with a cell lifter and replated in a six-well plate at a density of 500,000 cells/ml, 1.5 ml/well. L-cell media were removed 24 h later, and the cells were cultured for 24 h in RPMI medium 1640 with L-glutamine and 10% FBS. The media were removed and replaced with 1.5 ml of RPMI medium 1640 with L-glutamine and 10% FBS or 1.5 ml of RPMI medium 1640 with L-glutamine, 10% FBS, and 2 μg/ml LPS.

RT-PCR was performed on the total RNA by using random hexamers, and the cDNA was used for real-time Taqman PCR. Amplification was done in an Applied Biosystems 7700 with the conditions 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 55°C for 1 min. PCR primers for SRA, CD-36, and GAPDH are as described (32). PCR primers for IL-1β were (5′ to 3′): AGG CAG GCA GTA TCA CTC ATT GT (forward), GGA AGG TCC ACG GGA AAG A (reverse), and TGT GGA GAA GCT GTG GCA GCT ACC TGT (probe); PCR primers for IL-12 were (5′ to 3′): AAG GTG CGT TCC TCG TAG AGA A (forward), GAG CTT GCA CGC AGA CAT TC (reverse), and CAT CTA CCG AAG TCC AAT GCA AAG GCG (probe). Samples were run in triplicate and normalized against GAPDH.

Real-Time PCR Analysis of Liver cDNA

SyBr green real-time PCR analysis for the PON1-Tg and the endogenous mouse PON1 was performed on an IQ-Cycler (Bio-Rad). PON1-Tg PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 61°C for 20 sec, and 72°C for 20 sec. Mouse PON1 PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 57°C for 45 sec, and 72°C for 30 sec. GAPDH PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 55°C for 1 min. All PCR analyses were run in triplicate with a standard curve and melt curve analysis. Data were normalized to the internal GAPDH control. PCR primers for PON1-Tg were (5′-3′): ATA TCT CTA GAC CGC GGG GA (forward), GGG TGT CGG AAT AGA CTC TG (reverse); PCR primers for mouse PON1 were: GAT TGG CAC TGT GTT CCA C (forward), ATC ACT GTG GTA GGC ACC TT (reverse). PCR primers for GAPDH were: GTG TCC GTC GTG GAT CTG A (forward), CCT GCT TCA CCA CCT TCT TG (reverse).

Plasma Lipids

Plasma was fractionated into individual lipoprotein subclasses by size-exclusion FPLC (33). An aliquot of each fraction was used to measure protein (Bradford assay) and cholesterol. Plasma levels of very LDL, intermediate density lipoprotein, and LDL (together) and high-density lipoprotein cholesterol were determined by differential precipitation (33). Cholesterol quantitation was achieved by

using the commercially available Infinity Cholesterol Reagent Kits (Thermo Electron, San Jose, CA).

FACS Analysis

FACS was performed according to the protocol described by Pharmigen. Briefly, 1 μl of FcBlock (Pharmigen no. 01241D), 1 μl of anti-Gr-1(CD-11b) (Pharmigen no. 553129), and 1 μl of anti-Mac3 (Pharmigen no. 553324) were added to 100 μl of whole blood, mixed, and incubated for 30 min on ice in the dark. The mixture was then washed with 2% FBS in PBS, and the pellet was incubated with Pharmlyse Ammonium Chloride Lysing Reagent (Pharmigen no. 555899) in the dark for 30 min. The pellet was washed twice with PBS and resuspended in 1 ml of 2% FBS in PBS for FACS analysis.

Analysis of Atherosclerosis

The heart was dissected out, cleaned of fascia, and stored at -80°C. OCT compound-embedded hearts were sectioned in a cryostat until all three leaflets were visible within the aortic valve. From this point, 10-μm sections were collected for the next 300 μm of the valve region, and each section was collected on a Superfrost slide (Fisher Scientific). The lipid-rich lesions were visualized by staining the sections with oil red O followed by counterstaining with hematoxylin (27). A total of four sections taken every 40 μm were used to quantitate individual mouse lesion areas by using a

computer-assisted video imaging system. The lesion areas of the four sections were used to calculate the mean lesion area per mouse.

After excision of the heart, the aorta was stripped of its adventitial fat and dissected out from the right common carotid artery to the superior mesenteric artery. The aortas were opened longitudinally from the common iliac arteries to the aortic valve and subsequently detached and pinned flat onto the smooth black wax surface of a dissecting pan and stained with Sudan IV. The percent surface area stained red and indicative of fatty lesions was calculated by using a computer-assisted video imaging system.

Statistical Analysis

Statistical analysis of atherosclerotic lesion areas was performed by using a Mann-Whitney unpaired test. All other statistical analyses between the groups of mice were performed by using Student's *t* test (double tailed, unpaired).

Results

Development and Characterization of HSCs Expressing a Macrophage-Specific Transgene

To use HSCs as vehicles for transgenic expression by Kupffer cells, we developed mice that express a mouse PON1-Tg driven by a macrophage-specific promoter element (25). This transgene was constructed and used to produce PON1 transgenic C57BL/6 mice (i.e., PON1-Tg mice). Analysis of mRNA from PON1 transgenic C57BL/6 mice showed that the PON1-Tg was expressed only in circulating monocyte/macrophages and tissues containing resident macrophages (i.e., liver, lung, spleen, but not in heart, kidney, brain, or skeletal muscle; data not shown). Thus, expression of the PON1-Tg exhibited the expected macrophage specificity (25). PON1-Tg mice displayed no detectable abnormalities: body weight gain, size of litters, longevity, and gross appearance were indistinguishable from nontransgenic littermates.

The functional characteristics of the PON1-Tg were examined by using peritoneal macrophages obtained from PON1-Tg and nontransgenic mice 4 days after thioglycolate treatment. Peritoneal macrophages obtained from the PON1 transgenic mice expressed PON1 mRNA (Fig. 2.1A), protein (Fig. 2.1B), and enzyme activity (Fig. 2.1C), whereas macrophages from nontransgenic littermates exhibited no detectable expression of transgenic PON1 mRNA (Fig. 2.1A), no immunodetectable PON1 protein (Fig. 2.1B), and ≈60% less esterase activity (Fig. 2.1C). The level of

transgenic PON1 mRNA expressed by thioglycolate-elicited peritoneal macrophages was ≈10% of the level of endogenous PON1 mRNA expressed by liver (comparison made relative to GAPDH by using Northern blot analyses).

Essentially all of the PON1 protein and esterase activity detected in peritoneal macrophages obtained from PON1-Tg mice was retained in the 100,000 × *g* membrane pellet, and none was in the supernatant (Fig. 2.1B and C). These findings are consistent with previous studies showing that PON1 expressed by Chinese hamster ovary cells is retained within the plasma membrane and inefficiently secreted (30). The lack of cleavage of the N-terminal signal sequence of endogenous PON1 may be responsible for its inefficient secretion (34-36). Additional studies showed that after an 18-h incubation of peritoneal macrophages obtained from PON1-Tg mice, no PON1 protein or enzyme activity was secreted (i.e., detected in culture medium).

The expression of the PON1-Tg did not affect circulating levels of individual types of leukocytes (Fig. 2.1D and E). Furthermore, the presence of the PON1-Tg did not affect the ability of bone marrow HSCs to differentiate into monocyte/macrophages *ex vivo* in culture medium (bone marrow from both groups of mice yielded identical numbers of differentiated monocyte/macrophages (Fig. 2.1A). In addition, bone marrow-derived monocyte/macrophages obtained from control and PON1 transgenic mice expressed similar levels of mRNA encoding SRA, CD36, IL-1, and IL-12 both before and after stimulation with LPS (Fig. 2.1A). These data show that the expression of the PON1-Tg did not impair HSC differentiation or the general monocyte/macrophage function.

Engraftment and Expression of Kupffer Cells Expressing PON1-Tg

To have a fluorescent marker for engrafted HSCs, mice expressing the PON1-Tg were bred with mice expressing a GFP transgene driven by the β-actin promoter [i.e., C57BL/6-TgN (ACTbEGFP) 1Osb mice (26)]. Bone marrow from mice expressing both the GFP marker transgene and the macrophage-specific PON1-Tg was engrafted into lethally irradiated LDL-receptor-deficient mice. One month later, mice were injected with either saline (control) or $GdCl_3$ (25 mg/kg in saline). Livers from mice killed 4 weeks after treatment with $GdCl_3$ contained markedly more (i.e., 9-fold, $P < 0.02$) PON1-Tg mRNA compared with the livers injected with saline only (Fig. 2.2A). Confocal fluorescence microscopy of serial sections obtained from $GdCl_3$-treated mice stained with a rhodamine-conjugated macrophage-specific antibody Moma-2 (37) showed that the majority (67 ± 31%) (Fig. 2.2B) of hepatic macrophages (presence of Moma-2 antigen) displayed GFP fluorescence and thus were derived from engrafted HSCs (expressing the PON1-Tg and GFP transgene). The 9-fold increase in the expression of the PON1-Tg mRNA indicates that $GdCl_3$-facilitated apoptosis markedly accelerated the rate of Kupffer cell replacement and liver expression of the PON1-Tg.

Effect of Kupffer Cell Gene Expression of PON1 on Atherosclerosis Lesion Formation

To examine whether $GdCl_3$-facilitated transgenesis of PON1 exhibited a therapeutic amelioration of atherosclerosis, lethally irradiated LDL receptor-deficient

mice were injected with bone marrow from mice expressing either the GFP transgene only (control) or both the GFP transgene and PON1-Tg. One month later, mice were treated with either $GdCl_3$ or saline vehicle. Mice from all three groups were fed a chow diet for 4 weeks and then a cholesterol-enriched atherogenic diet (1.25% cholesterol, 6% fat) for the remaining 12 weeks. Leukocytes obtained from the blood of recipients 4 weeks after receiving PON1 bone marrow displayed the PON1-Tg mRNA, whereas leukocytes from mice receiving nontransgenic bone marrow did not contain detectable transgenic PON1 mRNA (Fig. 2.3A). PON1 esterase activity in plasma was similar among all groups (Fig. 2.3B). These findings support experiments showing that PON1 protein and enzyme activity are retained in the microsomal membrane fraction of peritoneal macrophages (Fig. 2.1B and C) (34-36). Consistent with the results of others (23,24), consumption of the cholesterol-enriched atherogenic diet caused a ≈30% decrease in plasma activities of PON1 (Fig. 2.3B).

When mice were fed the chow diet, plasma cholesterol levels were unchanged by the expression of the PON1-Tg or treatment with $GdCl_3$ (Fig. 2.3C). However, when mice were fed the cholesterol-enriched atherogenic diet, $GdCl_3$-treated mice exhibited a significant reduction (≈20%, $P < 0.05$) in plasma LDL cholesterol (Fig. 2.3C and D). Plasma high-density lipoprotein cholesterol levels were unaffected by $GdCl_3$ treatment. Additional data showed that $GdCl_3$ treatment of cholesterol-fed mice receiving bone marrow expressing GFP, but not PON1, had no effect on plasma LDL levels (Fig. 2.3E). Thus, the reduction in plasma LDL cholesterol required $GdCl_3$ treatment, the PON1-Tg, and the cholesterol-rich atherogenic diet.

The combined data suggested that $GdCl_3$ treatment decreased LDL cholesterol in diet-induced hypercholesterolemic mice through a mechanism dependent on the expression of the PON1-Tg by Kupffer cells. Moreover, because plasma activity of PON1 (Fig. 2.3B) was unaffected by engraftment of Kupffer cells expressing PON1 (Fig 2.3B), and the PON1-Tg protein and enzyme activity were not secreted and were retained within macrophages (Fig. 2.1), the PON1-Tg reduced plasma LDL cholesterol levels via a mechanism occurring predominantly within Kupffer cells.

Mice receiving bone marrow from PON1 transgenic mice treated with $GdCl_3$ exhibited a 50% reduction ($P < 0.0003$) in aortic sinus atherosclerotic lesions compared with mice receiving bone marrow not containing the PON1-Tg (Fig. 2.4A and B). Whereas atherosclerotic lesions appeared to be slightly less in mice receiving PON1 bone marrow without $GdCl_3$ treatment compared with mice receiving bone marrow lacking PON1-Tg expression, this difference was not statistically significant (Fig. 2.4A and B). Similar findings were obtained with regard to the surface area of atherosclerotic lesions in abdominal aorta; mice receiving bone marrow from PON1 transgenic mice treated with $GdCl_3$ exhibited a 45% reduction in abdominal aorta atherosclerotic surface lesions compared with mice receiving bone marrow not containing PON1 [in percent surface area, 1.51 ± 0.84 (control) vs. 0.84 ± 0.7 ($GdCl_3$-treated), $P < 0.006$].

To further examine the role of $GdCl_3$ treatment in the 50% reduction in atherosclerosis lesions, LDL receptor-deficient mice were engrafted with bone marrow expressing the GFP transgene but not the PON1-Tg and then treated with and without

$GdCl_3$ (Fig. 2.4C). In the absence of the PON1-Tg, $GdCl_3$ treatment had no significant effect on atherosclerosis lesion formation (Fig. 2.4C). These data clearly show that HSCs expressing the PON1-Tg are essential for $GdCl_3$ treatment to significantly reduce atherosclerosis.

After 16 weeks of cholesterol feeding, hepatic expression of transgenic PON1 mRNA was still 2-fold greater in $GdCl_3$-treated mice than in the livers of mice receiving bone marrow cells expressing the PON1-Tg but not $GdCl_3$ (Fig. 2.4D). This 2-fold difference in the expression of the PON1 transgenic mRNA observed 16 weeks after $GdCl_3$ treatment (Fig. 2.4D) is less than the 9-fold difference observed 4 weeks after $GdCl_3$ treatment (Fig. 2.2A). The smaller difference between the two groups of mice was due to an increased expression of the PON1 transgenic mRNA by the saline (control mice). These data suggest that Kupffer cells expressing the PON1-Tg were replaced in the absence of $GdCl_3$ treatment (albeit at a rate slower than with $GdCl_3$ treatment). Mice receiving HSCs expressing the PON1-Tg showed no significant reduction in atherosclerotic lesions compared with mice receiving HSCs lacking the PON1-Tg (Fig. 2.4A and B). Thus, $GdCl_3$-facilitated engraftment of Kupffer cells enhanced the rapid expression of the therapeutic PON1-Tg throughout the 16-week experiment and was responsible for both the reduction in LDL cholesterol (Fig. 2.3C and D) and the decrease in atherosclerosis (Fig. 2.4A and B).

Discussion

In this report, we demonstrate a simple method for rapidly replacing a significant fraction of liver macrophages with Kupffer cells derived from bone marrow HSCs and for permanently expressing therapeutic transgenes. This method is based on the i.v. injection of gadolinium chloride, an inorganic contrast material commonly used in humans, which is rapidly and specifically taken up by Kupffer cells. The uptake of $GdCl_3$ causes Kupffer cell apoptosis, allowing for transplanted HSCs to repopulate the liver. Using HSCs with normal expression of endogenous genes or HSCs carrying therapeutic transgenes, Kupffer cell precursors can thus act as "Trojan Horses" and provide rapid nontoxic physiologically relevant expression of therapeutic transgenes.

Our combined data supported two conclusions: (*i*) that $GdCl_3$-facilitated apoptosis was an effective method to accelerate the engraftment of Kupffer cells derived from bone marrow HSCs in mice; and (*ii*) that engrafted Kupffer cells provided a large tissue reservoir for expression of a therapeutic transgene (i.e., PON1) via the human SRA macrophage-specific promoter element (25) at levels sufficient to significantly reduce plasma LDL cholesterol levels and decrease aortic atherosclerotic lesions. This PON1-based gene therapy provided an effective treatment for reducing atherosclerosis in a murine model of homozygous familial hypercholesterolemia, a disease in humans for which the only long-term effective treatment is liver transplantation (13).

The additional finding that the transgenic PON1 protein and enzyme activity were retained within macrophages demonstrates that the therapeutic transgene acted within Kupffer cells. Kupffer cells play a critical role in removal from plasma of oxidized and chemically modified forms of LDL (14,15). Kupffer cell-specific ablation immediately after $GdCl_3$ injection blocks hepatic uptake of oxidized [^{125}I]-LDL, causing a ≈2- to 3-fold increased uptake by peripheral tissues including aorta, heart, lungs, and kidney (38). Our findings showing that Kupffer cell expression of PON1 reduced LDL cholesterol levels and atherosclerosis were predicted by the proposal that PON1 prevents atherosclerosis by metabolizing and inactivating the proatherogenic, inflammatory lipids produced by oxidative modification of LDL (16-24). Kupffer cell/PON1 transgenic disposal of proatherogenic lipid mediators associated with oxidatively modified LDL presumably prevented them from entering the arterial wall and promoting atherosclerotic lesion formation.

Our findings clearly show that $GdCl_3$-facilated Kupffer cell replacement was essential to show a significant amelioration of atherosclerosis lesions [i.e., mice receiving HSCs expressing the PON1-Tg but not treated with $GdCl_3$ showed no protection from atherosclerosis (Fig. 2.4C)]. Considering that $GdCl_3$ is relatively nontoxic and is commonly used as a contrast reagent in humans, $GdCl_3$-facilitated gene transfer may provide an important enhancement of macrophage-based gene therapies.

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Chapter Two, in full, is a reprint of the material as it appears in Proceedings of the National Academy of Science of the United States of America 2005 vol. 102, Bradshaw G; Gutierrez A; Miyake JH; Davis KR; Li AC; Glass CK; Curtiss LK; Davis RA. The dissertation author was the co-primary researcher and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

Figure 2.1: Characterization of PON1 transgenic mice.
(*A*) Expression of the PON1-Tg in peritoneal macrophages. C57BL/6 mice expressing a transgene constructed from the cDNA encoding PON1 driven by the macrophage-specific promoter elements obtained from the human SRA gene (25) or their nontransgenic littermates were injected i.p. with 1 ml of 4% thioglycolate. Four days later, abdominal macrophages were obtained (25) by flushing the peritoneum with PBS and plating the cells onto plastic culture dishes. Cells adhering to plastic culture dishes after 6 h were harvested, and mRNA was extracted and used to make cDNA. Transgenic PON1 mRNA relative to GAPDH was quantitated by SyBr green real-time PCR. Each value represents the mean ± SD of six separate plates of cells. *, Significant differences, $P < 0.001$. (*B*) Presence of immunoreactive PON1 protein. Macrophages were homogenized and separated into microsomes (100,000 × *g* pellet) and supernatant. Western blotting of cell membrane fraction (100,000 × *g* pellet) separated by SDS/PAGE by using antibody specific for mouse PON1 (39) showed an immunoreactive band having the same molecular weight as mouse PON1 in plasma (arrow). No PON1 immunoreactivity was present in the 100,000 × *g* supernatant. (*C*) PON1 enzyme activity by using phenylacetate as the substrate and the membrane fraction of peritoneal macrophages. Each value represents the mean ± SD of six separate plates of cells. *, Significant differences, $P < 0.02$. No PON1 enzyme activity was present in the 100,000 × *g* supernatant. (*D* and *E*) FACS analysis of peripheral blood cells. Blood was collected through retroorbital eye bleed from four PON transgenic mice (hatched bars) and four nontransgenic littermates (open bars). The red blood cells were lysed, and the remaining cells were incubated with APC-mac3 and PE-CD11b mouse antibodies. The cells were quantitated by FACS analysis. Data were derived from 20,000 gated events. (*D*) Analysis obtained from cells separated by fluorescent markers for: Mac3 (macrophages and not lymphocytes), CD11b antigen (mature and immature macrophages and granulocytes), and +/+ cells positive for both markers. (*E*) Separation of cells based on size and granularity; “others” are cells in blood that do not fit into the gates denoted for T/B cells or macrophages based on size and granularity. (*F-I*) Response of bone marrow-derived monocyte/macrophages to LPS. Bone marrow cells were extracted from the femurs and tibias of PON1-Tg and nontransgenic littermates. Cells were cultured in medium containing 10% FBS and 30% L-cell conditioned media. Differentiated monocytes were replated in medium containing 10% FBS with and without 2 mg/ml LPS. Cells were harvested 24 h later, and the indicated mRNAs were quantitated by SyBr real-time PCR. Each value represents the mean ± SD of six individual plates of cells.

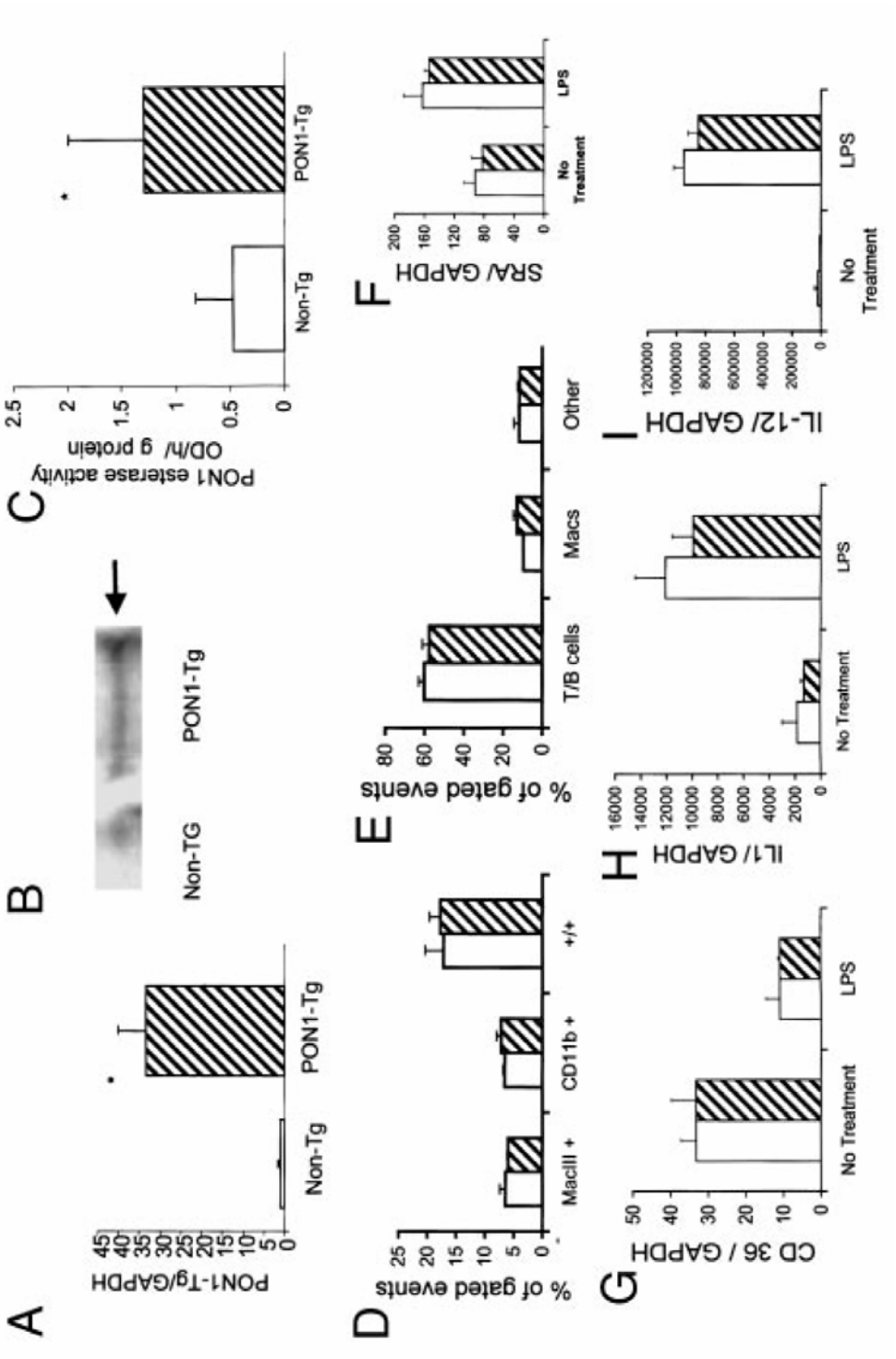
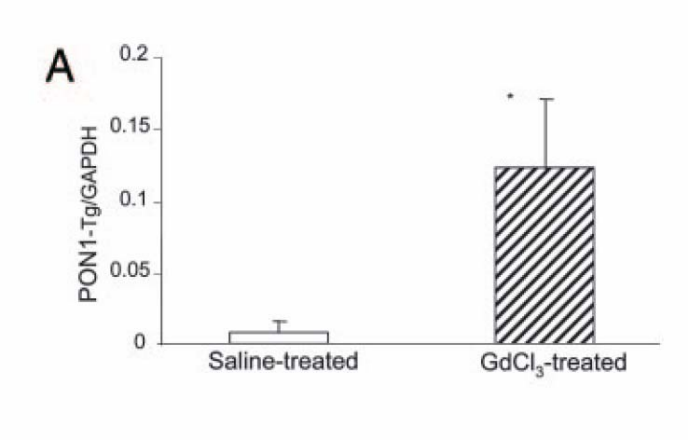

A
PON1-Tg/GAPDH
Non-Tg
PON1-Tg
B
Non-TG
PON1-Tg
C
PON1 esterase activity
OD/h/ g protein
Non-Tg
PON1-Tg
D
% of gated events
MacIII +
CD11b +
+/+
E
% of gated events
T/B cells
Macs
Other
F
SRA/ GAPDH
No Treatment
LPS
G
CD 36 / GAPDH
No Treatment
LPS
H
IL1/ GAPDH
No Treatment
LPS
I
IL-12/ GAPDH
No Treatment
LPS

Figure 2.2: Effect of $GdCl_3$ on hepatic engraftment of macrophages derived from HSCs.

Lethally irradiated LDL receptor-deficient mice (11) were injected with bone marrow HSCs expressing the PON1-Tg and GFP [derived from C57BL/6-TgN(ACTbEGFP) 1Osb mice (40)]. After 14 days of recovery, mice were injected with either $GdCl_3$ (25 mg/kg in saline) or saline only and killed after 4 weeks. (*A*) Hepatic expression of transgenic PON1 mRNA relative to GAPDH. Livers from mice treated without (saline control, open bars) or with $GdCl_3$ (hatched bars) obtained 4 weeks after treatment were used to make cDNA. The hepatic content of transgenic PON1 mRNA relative to GAPDH was quantitated by SyBr green real-time PCR. Each value represents the mean ± SD of six separate mice per group. *, Significant differences, $P < 0.02$. (*B*) Confocal fluorescent microscopic colocalization of GFP (green) and the macrophage Moma-2 antigen (detected with rhodamine red-conjugated antibody) in frozen thin sections of liver from mice 4 weeks after treatment with $GdCl_3$. Six serial sections from three separate mice per group were used to estimate fluorescence attributed to GFP (green), Moma-2 (red), and colocalization of GFP and Moma-2 (yellow) and are indicated ± SD below *A* and *B*.


A
0.2
0.15
0.1
0.05
0
PON1-Tg/GAPDH
*
Saline-treated
GdCl3-treated

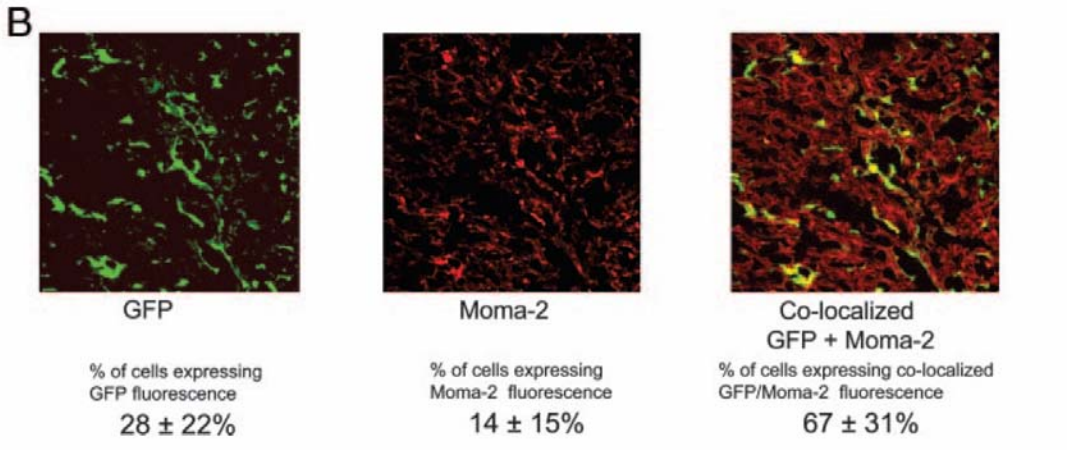

B
GFP
% of cells expressing
GFP fluorescence
28 ± 22%
Moma-2
% of cells expressing
Moma-2 fluorescence
14 ± 15%
Co-localized
GFP + Moma-2
% of cells expressing co-localized
GFP/Moma-2 fluorescence
67 ± 31%

Figure 2.3: $GdCl_3$ treatment decreases atherogenic LDL cholesterol in cholesterol-fed LDL-receptor-deficient mice engrafted with bone marrow expressing the PON1-Tg.

(*A*) RT-PCR for PON1-Tg mRNA verified expression of PON1 in peripheral blood leukocytes of recipient LDL-receptor-deficient mice 4 weeks after injection of bone marrow obtained from mice expressing both the PON1-Tg and the GFP transgene. The migration of PON1-Tg and GAPDH cDNA standards are indicated. (*B*) Plasma PON1 enzyme activity by using paraoxon as the substrate is shown as the mean ± SD for 12 separate mice per group. There were no significant differences in the plasma activity of PON1 in the three groups of mice. *, Significant difference between the activities in mice fed chow compared with their activities when fed the cholesterol-enriched diet ($P < 0.01$). Open bars represent mice receiving bone marrow expressing only the GFP transgene; filled bars represent mice receiving bone marrow expressing both the PON1-Tg and the GFP transgene and treated subsequently with saline vehicle only; and hatched bars represent mice receiving bone marrow expressing both the PON1-Tg and the GFP transgene and subsequently treated with $GdCl_3$ (25 mg/kg in saline). Time in weeks corresponds to time after treatment with $GdCl_3$ or saline vehicle only. Mice were fed a chow diet until the end of week 4. After week 4, mice were fed an atherogenic cholesterol-enriched diet for 12 weeks. (*C*) $GdCl_3$ treatment reduced plasma LDL cholesterol levels in cholesterol-fed LDL receptor-negative mice engrafted with HSCs expressing the PON1-Tg. Plasma LDL cholesterol levels are shown as the mean ± SD for 12 separate mice in each group. *, Significant differences between mice treated with $GdCl_3$ and the other two groups (not receiving $GdCl_3$), $P < 0.02$. Columns represent the same groups of mice designated in *B*.(*D*) $GdCl_3$ treatment reduced cholesterol content of the LDL fraction of plasma obtained from cholesterol-fed LDL receptor-negative mice engrafted with HSCs expressing the PON1-Tg. Red lines represent mice receiving bone marrow expressing only the GFP transgene; blue lines represent mice receiving bone marrow expressing both the PON1-Tg and the GFP transgene and treated subsequently with saline vehicle only; and green lines represent mice receiving bone marrow expressing both the PON1-Tg and the GFP transgene and subsequently treated with $GdCl_3$ (25 mg/kg in saline). Time in weeks corresponds to time after treatment with $GdCl_3$ or saline vehicle only. Mice were fed a chow diet until the end of week 4. After week 4, mice were fed an atherogenic cholesterol-enriched diet for 12 weeks. Blood was obtained at 4 and 8 weeks after beginning the cholesterol-rich diet. Plasma from all mice in the same group were pooled and subjected to FPLC separation (33). (*E*) $GdCl_3$ treatment did not reduce plasma LDL cholesterol levels in cholesterol-fed LDL receptor-negative mice engrafted with HSCs not expressing the PON1-Tg. Mice treated identically as in *C* (except they received bone marrow expressing the GFP transgene but not the PON1-Tg) were killed after being fed for 12 weeks the cholesterol-rich atherogenic diet. Plasma LDL levels are shown as the mean ± SD for 11 separate mice in each group. There were no significant differences among groups.

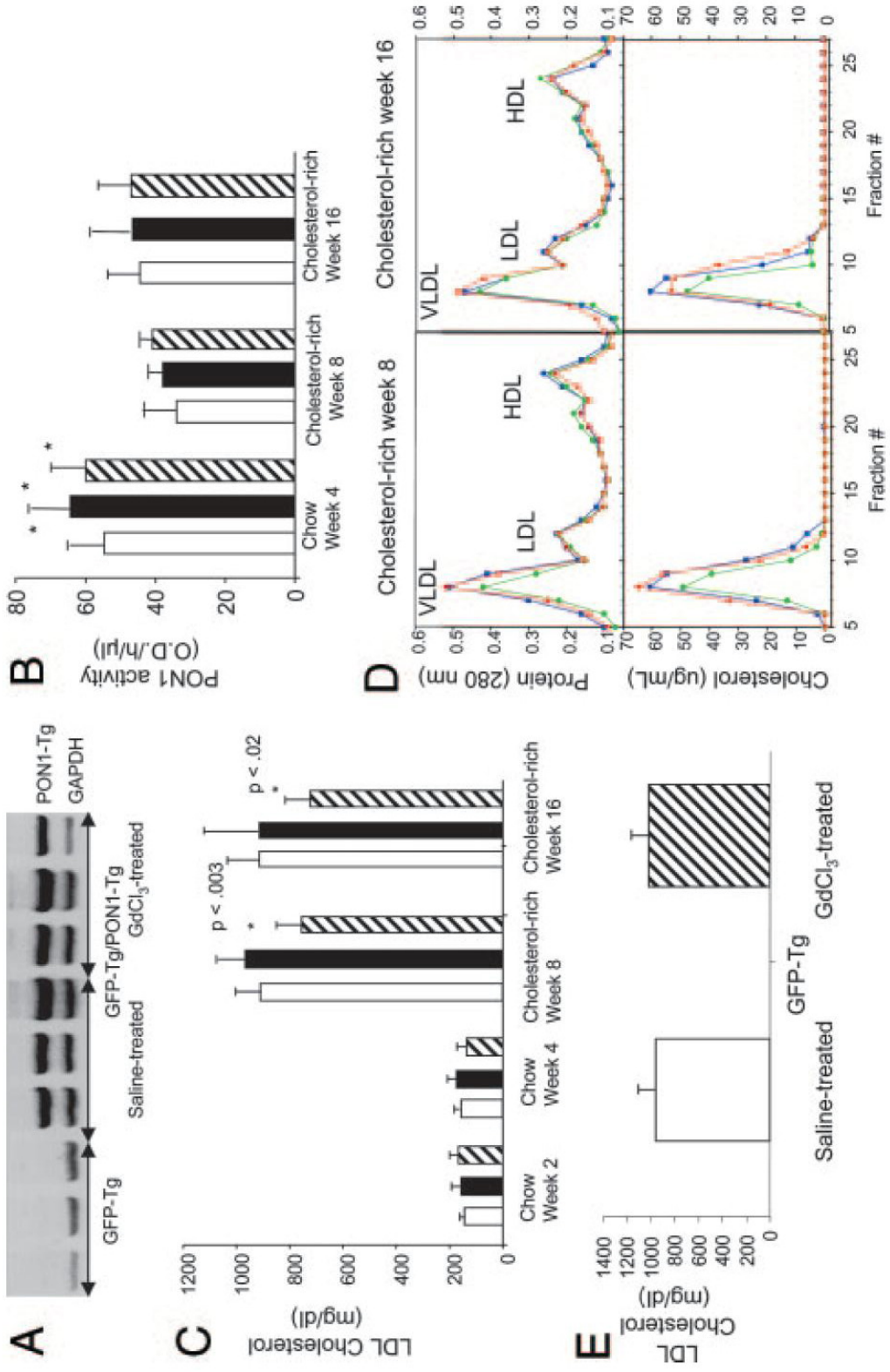
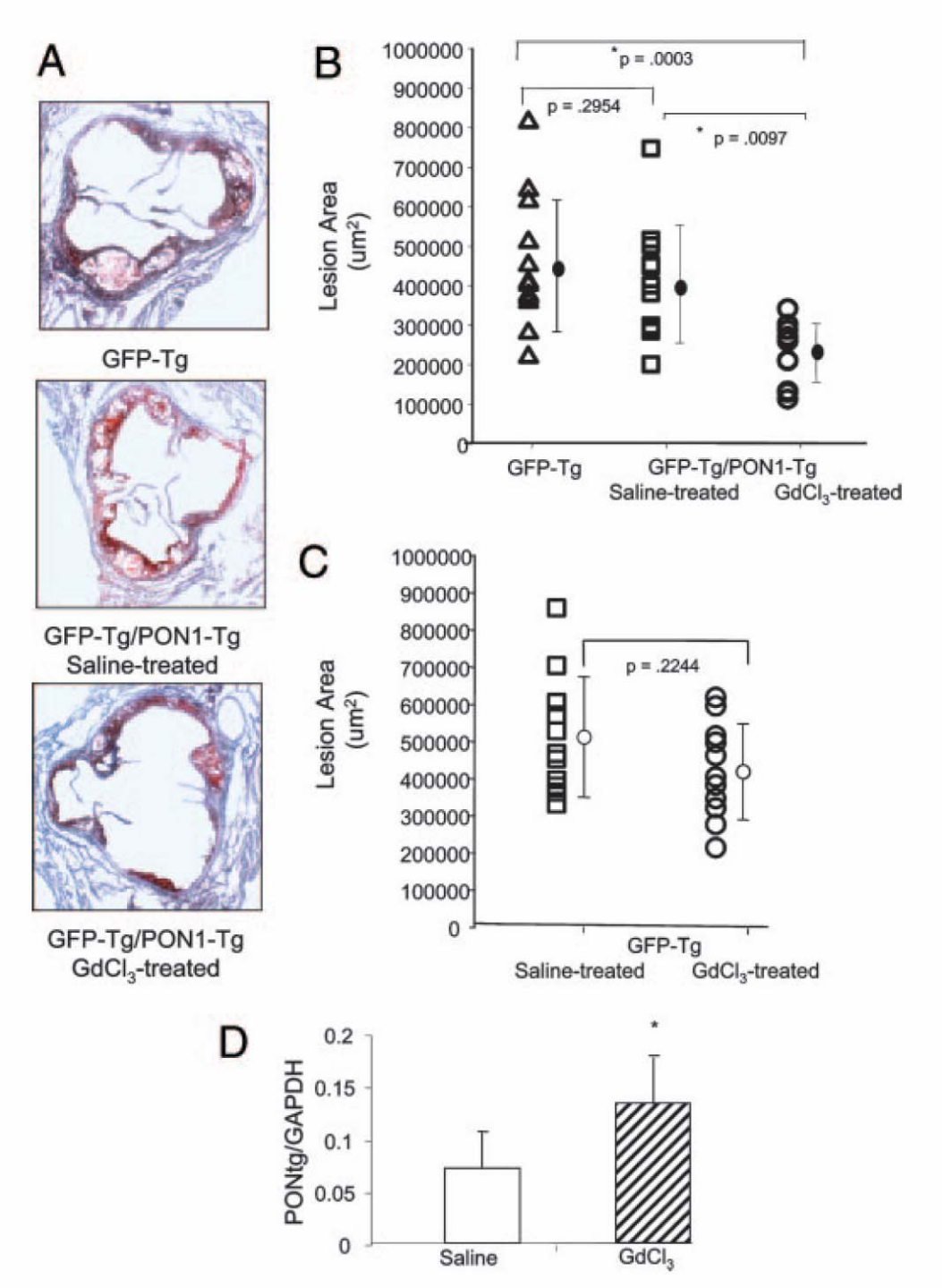

A
PON1-Tg
GAPDH
GFP-Tg
GFP-Tg/PON1-Tg
Saline-treated
GdCl3-treated
B
PON1 activity
(O.D./h/µl)
Chow
Week 4
Cholesterol-rich
Week 8
Cholesterol-rich
Week 16
C
LDL Cholesterol
(mg/dl)
p < .003
p < .02
Chow
Week 2
Chow
Week 4
Cholesterol-rich
Week 8
Cholesterol-rich
Week 16
D
Cholesterol-rich week 8
Cholesterol-rich week 16
VLDL
LDL
HDL
Protein (280 nm)
Cholesterol (ug/mL)
Fraction #
E
LDL
Cholesterol
(mg/dl)
Saline-treated
GFP-Tg
GdCl3-treated

Figure 2.4: $GdCl_3$ treatment decreased atherosclerotic lesions in LDL receptor-deficient mice engrafted with HSCs expressing PON1.
(*A*) The atherosclerotic lesion area (27,33) was quantitated by using aortic sinus frozen thin sections stained with oil red O obtained from mice killed after 12 weeks of being fed an atherogenic cholesterol-enriched diet. Representative micrographs are shown. (*B*) The mean lesion area ± SD for 12 separate mice per group is shown. Statistical differences (*P* values) among the groups are indicated. GFP-Tg represents mice receiving bone marrow expressing the GFP transgene; GFP-Tg/PON1-Tg saline treated represents mice receiving bone marrow expressing both the GFP transgene and the PON1-Tg and subsequently treated with saline only; and GFP-Tg/PON1-Tg $GdCl_3$-treated represents mice receiving bone marrow expressing both the GFP transgene and the PON1-Tg and subsequently treated with $GdCl_3$ (25 mg/kg in saline). (*C*) $GdCl_3$ treatment did not reduce atherosclerotic lesions in LDL-receptor-deficient mice engrafted with HSCs not expressing the PON1-Tg. Mice were treated identically as in *A* (except they received bone marrow expressing the GFP transgene but not the PON1-Tg), and the aortic sinus lesions were analyzed. There were 11 mice per group. There was no significant difference between groups. (*D*) Hepatic expression of transgenic PON1 mRNA relative to GAPDH. Livers from LDL receptor-deficient mice engrafted with bone marrow expressing the PON1-Tg were treated without (saline control, open bars) or with $GdCl_3$ (hatched bars), killed 12 weeks after being fed the cholesterol-rich diet and used to make cDNA. The relative hepatic content of transgenic PON1 mRNA relative to GAPDH was quantitated by SyBr green real-time PCR. Each value represents the mean ± SD of 12 separate mice in each group. *, Significant differences, $P < 0.02$.


A
GFP-Tg
GFP-Tg/PON1-Tg
Saline-treated
GFP-Tg/PON1-Tg
GdCl3-treated
B
Lesion Area (um2)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
*p = .0003
p = .2954
* p = .0097
GFP-Tg
GFP-Tg/PON1-Tg
Saline-treated
GdCl3-treated
C
Lesion Area (um2)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
p = .2244
GFP-Tg
Saline-treated
GdCl3-treated
D
PONtg/GAPDH
0.2
0.15
0.1
0.05
0
*
Saline
GdCl3

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Chapter 3

Bile Acids Decrease Hepatic Paraoxonase1 Expression and Plasma HDL Levels via FXR-Mediated Signaling of FGF Receptor 4

Abstract

Objective: To determine how dietary bile acids repress hepatic expression of paraoxonase-1.

Methods and Results: C57BL/6 mice and C3H/HeJ mice, having different susceptibilities atherosclerosis, were fed a chow diet and an atherogenic diet containing taurocholate. Compared to the more atherosclerosis-susceptible C57BL/6 mice, C3H/HeJ mice display resistance to dietary bile acid repression of hepatic paraoxonase-1 mRNA and decreased HDL cholesterol. While knockout of toll receptor 4 did not affect response to taurocholate, deletion of either FXR or FGFR4 blocked taurocholate repression of paraoxonase-1 and CYP7A1. FGF19, an activator of FGFR4 expressed in human ileum, decreased expression of both paraoxonase-1 and CYP7A1 expression by human hepatoma cells. In all mice studied, dietary taurocholate increased ileal expression of FGF15, a FXR-inducible murine homologue of human FGF19.

Conclusion: Hepatic paraoxonase-1 and CYP7A1 mRNA expression is repressed by bile acids via FXR-mediated induction of FGF15. Thus, the inability of C3H/HeJ mice to display taurocholate repression of paraoxonase-1 and CYP7A1 mRNAs was not due to a lack of induction of FGF15, but rather signaling events distal to FGF15-FGFR4 association.

Introduction

Multiple case-controlled studies of humans show an inverse relationship between the plasma activity of paraoxonase-1 (PON1), the formation of atherosclerotic lesions and myocardial infarction (1-6). Thus plasma levels of PON1 accurately predict susceptibility to atherosclerosis.

PON1 is mainly expressed in the liver (7) and displays the unusual characteristic of being secreted with an intact N-terminal signal sequence (8). PON1 exhibits multiple enzyme activities including acting as an organophosphate esterase, a carboxyl esterase (9,10), a lactonase (11, 12) and a phospholipase A2 (13, 14). The latter activity has been proposed to play an important role in inactivating the proatherogenic inflammatory lipids produced by the oxidative modification of LDL (15). Most of the PON1 present in plasma is associated with HDL, which may explain the well-established athero-protective effect of HDL (13,16,17). The anti-inflammatory properties of HDL are dependent, at least in part, on the presence of PON1 (13). Dissociation of PON1 from HDL causes the HDL particle to become atherogenic (13).

Studies using inbred strains of mice showed that a cholic-acid containing atherogenic diet reduced the hepatic expression of both PON1 and CYP7A1 mRNAs and plasma HDL cholesterol levels in atherosclerosis-susceptible C57BL/6 mice, but not in atherosclerosis-resistant C3H/HeJ mice (18). Further analysis of a subset of recombinant inbred strains derived from the B6 and C3H parental strains, showed that

the ability of the cholic-acid containing atherogenic diet to decrease hepatic PON1 mRNA expression segregated with aortic lesion development (18). In a similar subset of recombinant progeny from B6 and C3H parental strains, HDL levels were linked to three individual genetic loci also linked to the hepatic expression of CYP7A1 (19). Thus, the ability of the cholic-acid containing atherogenic diet to reduce hepatic expression of both PON1 and CYP7A1 correlated with both plasma HDL levels and atherosclerosis lesion formation.

The athero-protective effects of PON1 and CYP7A1 were further demonstrated by studies showing that transgenic expression of either PON1 (4) or CYP7A1 (20) reduced atherosclerotic lesion formation in susceptible C57BL/6 mice. Transgenic expression of PON1 reduced atherosclerotic lesion formation via the production of plasma HDL that protected LDL from oxidation (4). Transgenic expression of CYP7A1 reduced atherosclerotic lesion formation by preventing diet-induced hypercholesterolemia and reduced plasma HDL levels (20). The goal of this research was to elucidate the mechanism through which atherogenic bile acid-containing diets reduce hepatic expression of PON1 in atherosclerosis-susceptible C57BL/6 mice.

Materials and Methods

Mice and Diets

Male mice were housed in a room with a 12 h light cycle. C57BL/6 mice having both alleles of the TLR4 deleted (21) were a gift from Dr. Peter Tobias (Scripps Research Institute). The generation of mice lacking FGFR4 has been described (22, 23). C57BL/6 mice lacking FXR (24) were a gift from Dr. Frank Gonzalez (NCI). The mice were fed either a chow (no. 5015 Harlan Teklad) or an atherogenic diet composed of chow and 20% olive oil, 2% cholesterol, and 0.5% taurocholate. FXR null animals were fed the experimental diets for 5 days, due to increased mortality when fed the atherogenic diet. During the feeding periods, there were no apparent changes in appetite and/or body weight. Mice were sacrificed at mid-dark.

Plasma lipids

Mice were anesthetized with isofluorane and bled. Plasma total cholesterol and HDL cholesterol were determined (25).

Liver and Ileum RNA isolation

Following sacrifice, livers and intestines were removed and flash frozen. A length of 5 cm from the cecum was used for the ileum. RNA was isolated from

frozen tissue using the Versagene RNA Tissue Kit (Gentra Systems). cDNA was made from 4μg RNA using the iScript cDNA synthesis kit (Bio-Rad).

HepG2 Experiments

HepG2 cells, cultured as described (26), were treated with FGF19 (160 ng/mL) (a gift from Genentech) or 10ng/mL TNFα (R & D Systems) and harvested as indicated.

Real Time SybrGreen PCR Analysis

Quantitative real-time PCR, using SYBR Green was performed on an IQ-Cycler (BioRad) using primer sequences and annealing temperatures described in Supplemental Figure 3.I .

Western Blot Detection of FGFR4

Livers samples (50 μg) were separated by SDS/PAGE electrophoresis and electroblotted onto PVDF membranes. A goat anti-mouse FGFR4 antibody 1:1000 (R & D systems) was used for detection. Samples were quantitated to their respective tubulin controls using ImageJ software (NIH). Please see Supplemental Figure II for more detail.

Statistical Analysis

Statistical analysis between the groups were performed using Student's t test (double tailed, unpaired). All values are reported as mean $\pm$ S.D.

Results

TLR4 function is not involved in the resistance of C3H/HeJ mice to diet-induced repression of hepatic PON1 and CYP7A1 and decreased HDL cholesterol levels

C3H/HeJ mice lack functional TLR4 and thus LPS signaling (27). Since inflammatory cytokines TNFα and IL1, induced by bile acid activation of macrophages, block hepatic expression of CYP7A1 (26), functionally defective TLR4 might explain the inability of C3H/HeJ mice to display bile acid induction of inflammatory cytokines and repression of CYP7A1. To examine this possibility C57BL/6 *tlr4-/-* knockout mice (28) were fed either chow or the bile acid-containing atherogenic diet (20). Compared to C57BL/6, C3H/HeJ mice show a resistance to diet-induced repression of hepatic: CYP7A1 (Figure 3.1A) and PON1 (Figure 3.1B); induction of TNFα (Figure 3.1C) and to the associated decrease in HDL cholesterol levels (Figure 3.1D). Moreover, the lack of functional TLR4 did not affect the ability of the bile acid-containing atherogenic diet to cause similar changes in C57BL/6 mice (Figure 3.1A-D). Thus, the inability of atherosclerosis-resistant C3H/HeJ mice to respond to the bile acid-containing atherogenic diet does not require a functionally active TLR4.

FXR is required for diet-induced repression of hepatic PON1 and CYP7A1 mRNA levels and decreased HDL cholesterol levels

One of the major pathways through which bile acids affect gene expression is by binding to the nuclear receptor FXR (29-31). We examined the response of C57BL/6 mice lacking functional FXR nuclear receptors (24) to the bile acid-containing atherogenic diet. Because these mice display increased hepatotoxicity to bile acids, the mice were fed the bile acid diet for only 5 days; allowing all mice to remain healthy throughout the experiment. In the absence of functional FXR nuclear receptors, livers of C57BL/6 mice display no repression of CYP7A1 (Figure 3.1A) or PON1 (Figure 3.1B), lack the induction of TNFα (Figure 3.1C) and the mice display no significant decrease in HDL cholesterol levels (Figure 3.1D) in response to the bile acid-containing atherogenic diet. Previous studies have shown that FXR-/- mice exhibit increased HDL cholesterol levels (32). Our findings indicate that FXR is essential for mediating dietary bile acid repression of both CYP7A1 and PON1 and the associated decrease in HDL cholesterol.

FGF receptor 4 links hepatic expression of CYP7A1, PON1, and HDL cholesterol levels to the bile acid-containing atherogenic diet

FGFR4, containing a tyrosine kinase domain, can be activated by a number of ligands including FXR inducible FGF15 (mouse) and FGF19 (human) (23, 33-36). Mice lacking FGFR4 express unusually high levels of CYP7A1 (23,36). Additional studies show that dietary bile acids activate FGFR4 and as a result the expression of

CYP7A1 mRNA is reduced via a mechanism that appears to not require FXR inducible SHP or c-Jun N-terminal kinase (36). Expression of a mutant form of FGFR4 thought to cause constitutive activation of the tyrosine kinase domain, in mice lacking the endogenous FGFR4, resulted in constitutive repression of CYP7A1 (36). We examined regulation of PON1 expression in mice lacking FGFR4 (Figure 3.2A). FGFR4 -/- mice exhibited a phenotype similar to C3H/HeJ and FXR-/- mice, i.e. resistance to bile acid repression of CYP7A1 and PON1 mRNA (Figure 3.2A). In addition, mice lacking FGFR4 also failed to display induction of hepatic TNFα mRNA expression and decrease in HDL cholesterol, which occur in response to dietary bile acids (Figure 3.2A). These findings indicate activation of FGFR4 signaling reduces expression of both CYP7A1 and PON1, as well plasma HDL cholesterol levels via a mechanism independent of TNFα.

Murine FGF15 and its human homologue FGF19 are one of many FGF ligands that bind to and activate FGFR4 signaling (34,35,37). FGF15 gene expression in the ileum has been shown to be bile acid-inducible via activation of FXR and has been proposed to be responsible for FGFR4-mediated repression of CYP7A1 in response to bile acids (23,34). We examined the ability of the individual groups of mice to display an induction of FGF15 mRNA by the bile acid-containing atherogenic diet (Figure 3.2B). Our findings show that the bile acid-containing atherogenic diet resulted in enhanced expression of FGF15 mRNA in all mice (including bile acid-resistant C3H/HeJ mice) examined except those lacking FXR (Figure 3.2B). Our findings support those suggesting that FXR mediates the induction of FGF15 mRNA

by bile acids. Since C3H/HeJ mice displayed an induction of FGF15 mRNA similar to the induction displayed by C57BL/6 mice (Figure 3.2B), impaired FXR induction of FGF15 is not responsible for differences in response to the atherogenic diet.

FGF19 decreases the mRNA expression of CYP7A1 and PON1 by HepG2 cells

We examined the regulation of PON1 mRNA expression by human hepatoma HepG2 cells. HepG2 cells were treated with recombinant FGF19, the human homologue of FGF15 and an agonist of FGFR4 (35). Cells were also treated with or TNFα, which reduces CYP7A1 expression (26). Within 6 h both FGF19 and TNFα reduced CYP7A1 mRNA expression, but did not affect the expression of PON1 mRNA (Figure 3.3). However, after 16 h, the expression of both CYP7A1 and PON1 mRNA were significantly reduced by either FGF19 or TNFα (Figure 3.3). The extremely short half-life of CYP7A1 mRNA (38) may explain the more rapid decrease in CYP7A1 mRNA compared to PON1 mRNA. These combined data indicate that FGF15 (mouse) and FGF19 (HepG2 cells) decreased hepatic expression of both CYP7A1 and PON1 via a mechanism dependent on FGFR4.

FGFR4 mRNA, but not protein, is elevated in the livers of C3H/HeJ mice

We examined the hepatic content of FGFR4 mRNA and protein in the livers of C57BL/6 and C3H/HeJ mice fed chow or the bile acid-containing atherogenic diet. When fed either diet, FGFR4 mRNA relative to GAPDH was significantly (5-fold) greater in C3H/HeJ mice compared to C57BL/6 mice (Figure 3.IIA). When fed the

chow diet, the amount of immuno-detectable FGFR4 protein in the livers of C57BL/6 was significantly (~20%, $p<0.006$) higher than that of C3H/HeJ mice (Figure 3.IIB). However, when fed the bile acid-containing diet, the amount of FGFR4 protein in livers from C57BL/6 mice decreased (-23%, $p<0.025$ – Figure 3.IIC), whereas this decrease did not occur in the livers of resistant C3H/HeJ mice. Thus, when fed the bile acid-containing diet the amount of FGFR4 protein in the livers of C57BL/6 mice became similar to those of C3H/HeJ mice. Thus, the inability of C3H/HeJ mice to display taurocholate repression of PON-1 and CYP7A1 mRNAs was not due to a lack of induction of FGF15, but rather signaling events distal to FGF15-FGFR4 association.

Discussion

There is an agreement in results of studies of humans and experimental animals showing that the activity of plasma PON1 varies inversely with the development of atherosclerosis lesions (1-6). Additional studies showing that alteration of plasma activity of PON1 through manipulation of its genetic expression results in inverse changes in atherosclerosis lesion formation indicates that PON1 plays a causal role (3,4,18,39). Some epidemiological studies have shown correlations between the enzyme activities of PON1 and genetic polymorphisms (40). More recent analysis indicates that factors other than the PON1 gene are important determinants of plasma PON1 activity (5). Our findings provide evidence indicating that bile acids repress PON1 mRNA expression via FXR activation of ileal FGF15 which subsequently acts on hepatic FGFR4 causing decreased hepatic PON1 mRNA expression. Our findings further show that the inability of atherosclerosis-resistant C3H/HeJ mice to display taurocholate repression of paraoxonase-1 and CYP7A1 mRNAs was not due to a lack of induction of FGF15, but rather signaling events distal to FGF15-FGFR4 association.

Dietary bile acids repress PON1 and CYP7A1 by two mechanisms: activation of inflammatory cytokines (e.g. TNFα) and activation of FGFR4

Previous studies show that cholic acid is the dietary component in the atherogenic diet responsible for decreased hepatic expression of CYP7A1 and PON1

(18,26,41). Activation of FXR (29-31) and increased expression and secretion of inflammatory cytokines (e.g. TNFα) (26,42) are two of the mechanisms through which bile acids alter gene expression. Our studies showing that genetic deletion of TLR4 has no effect on bile acid repression of either CYP7A1 or PON1 (Figure 3.1) clearly indicate that the LPS signaling receptor TLR4 is not required for bile acid repression of both genes. These findings corroborate gene mapping studies indicating that the loci responsible for resistance of C3H/HeJ mice to bile acid repression of CYP7A1 does not include the *tlr4* locus on chromosome 4 (19). In addition, while dietary bile acids increased hepatic expression of TNFα in the livers of C57BL/6 mice (Figure 3.1C), this did not occur in C57BL/6 mice lacking TLR4, yet they displayed repression of CYP7A1 and PON1.

Bile acid activation of FXR induces FGF15 and represses CYP7A1 and PON1 via FGFR4 signaling

When fed the bile acid-containing diet, C57BL/6 mice lacking FXR (24) showed neither a repression of PON1 and CYP7A1 nor an induction of the inflammatory cytokine TNFα (Figure 3.1). These data clearly show that FXR is required for both bile acid repression of PON1 and CYP7A1. Several lines of evidence indicate that the FXR requirement involves induction of FGF15 by the ileum: (1) in the absence of FGFR4, dietary bile acids neither repressed PON1 nor CYP7A1 (Figure 3.2A); (2) in all mice studied except those lacking FXR, dietary bile acids induce the ileal expression of FGF15 (Figure 3.2B); (3) in human hepatoma

HepG2 cells, addition of FGF19, the assumed homologue of murine FGF15 (34,35), caused repression of both PON1 and CYP7A1 (Figure 3.3). Perhaps the most parsimonious interpretation of the combined data is that via ligand-activation of FXR, bile acids induce ileal expression of FGF15, which through FGFR4 dependent signaling caused decreased expression of both PON1 and CYP7A1. In previous studies, a diet containing 2% cholic acid, whose bile acid content is 4-fold greater than the diet used in this present study, was shown to decrease CYP7A1 mRNA expression in FGFR4 deficient mice (23). This higher diet content of cholate may have been sufficient to cause liver inflammation and induction of TNFα, a potent inhibitor of CYP7A1 mRNA expression (26).

Our findings further indicate the inability of atherosclerosis-resistant C3H/HeJ mice to respond to bile acid feeding is not caused by a lack of FXR-dependent induction of ileal FGF15 (Figure 3.2) or the hepatic content of FGFR4 protein (see Supplement Figure 3.II). The combined data suggest that the inability of C3H/HeJ mice to display taurocholate repression of PON1 and CYP7A1 mRNAs is caused by signaling events that occur distal to FGF15-FGFR4 association. Impaired signaling of the FGFR4 can explain the pleiotropic resistance of C3H/HeJ mice displayed in the inflammatory response of liver and vascular wall cells to atherogenic stimuli (43,44). FGF receptor signaling has a significant influence on arterial wall cell growth, differentiation, and susceptibility to atherosclerosis (45,46). One of several gene loci responsible for the resistance of C3H/HeJ mice to diet-induced atherosclerosis is located close to and may include FGFR4 (A.J. Lusis, unpublished).

An interesting aspect of our findings is that reduction in HDL cholesterol levels, one of the major factors responsible for the susceptibility of mice to diet-induced atherosclerosis (43,44,47), correlates with repression of PON1 and CYP7A1 (Figures 3.1 and 3.2). Constitutive expression of a CYP7A1 transgene in C57BL/6 mice prevented the decrease in HDL cholesterol and atherosclerotic lesion formation that occurs in response to the bile acid-containing atherogenic diet (26). Transgenic over-expression of PON1 is associated with the protection of HDL from oxidative stress (4,48). Our combined findings suggest that regulation of hepatic expression of PON1 and CYP7A1 by FGFR4 signaling can have a significant influence on HDL cholesterol levels and susceptibility to atherosclerosis. The finding that FXR-mediated induction of ileal production of FGF15 is an important factor controlling hepatic PON1 and CYP7A1 expression reveals how a response of the intestine to diet may influence hepatic gene expression and susceptibility to atherosclerosis.

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Chapter Three, in full, is a reprint of the material as it appears in Arteriosclerosis Thrombosis and Vascular Biology 2006 vol.26, Gutierrez A; Ratliff EP; Andres AM; Huang X; McKeehan WL; Davis RA. The dissertation author was the primary researcher and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

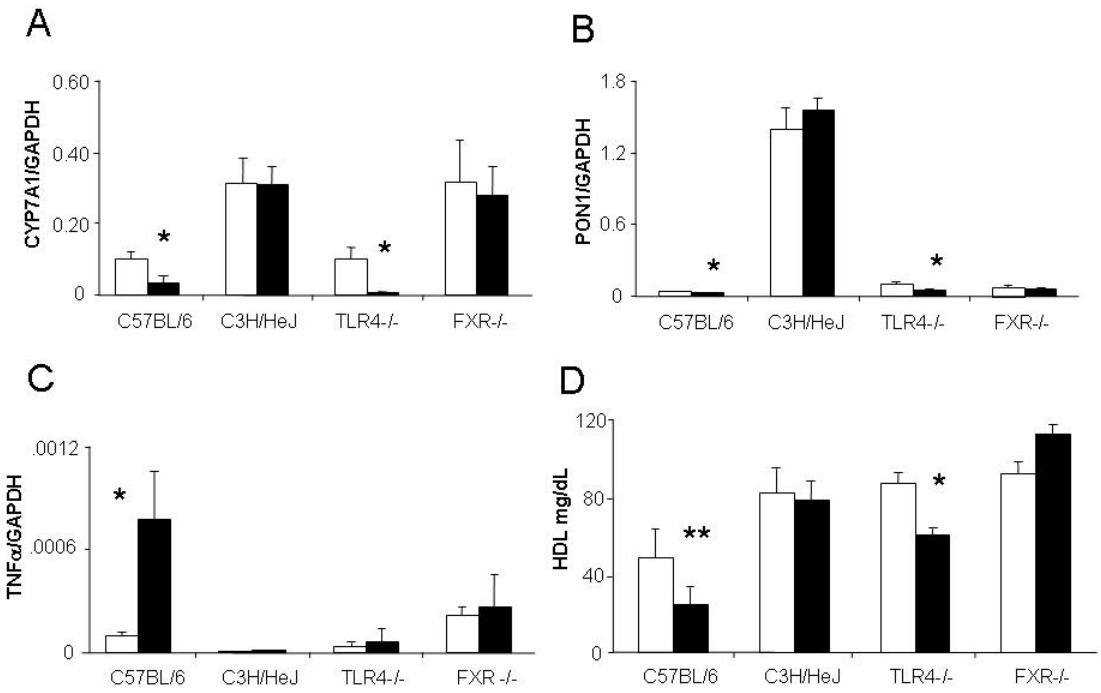


Figure 3.1: Response of C57BL/6, C3H/HeJ , C57BL/6 TLR4-/- and C57BL6 FXR-/- mice to dietary bile acid.

Mice were fed either a chow diet (open bars) or an atherogenic diet containing 0.5% taurocholate (filled bars) for two weeks, except FXR-/- mice, which were fed for 5 days. Hepatic mRNA expression of CYP7A1 (A), PON1 (B), TNFα (C) and HDL levels (D) are shown as the mean +/- S.D. for 6 mice per group. *P<0.0005, ** P< 0.02 indicate differences between chow and atherogenic diet.

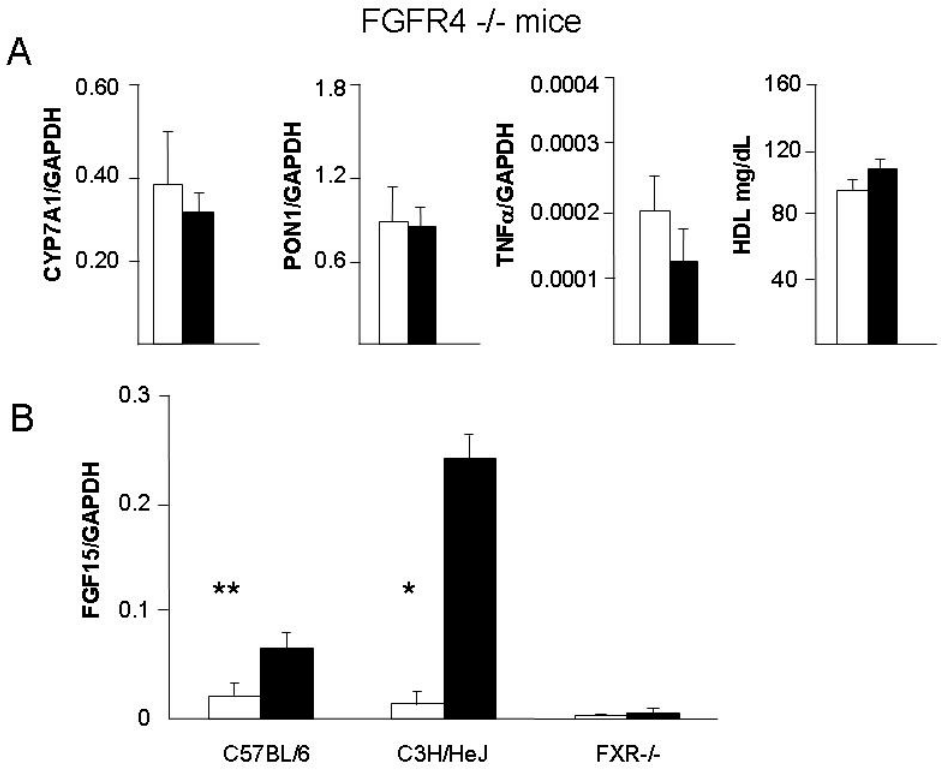


Figure 3.2: FGFR4 is required for the reduction in hepatic expression of CYP7A1, PON1 and plasma HDL cholesterol levels caused by dietary bile acid. Mice were fed either a chow diet (open bars) or an atherogenic diet containing 0.5% taurocholate (filled bars) for two weeks, except FXR-/- mice, which were fed for 5 days. (A) Hepatic mRNA expression of CYP7A1, PON1, TNFα and HDL levels are shown as the mean +/- S.D. for 6 mice per group. (B) Ileal expression of FGF15 in C57BL/6, C3H/HeJ, and FXR -/- mice are shown as the mean +/- S.D. for 6 mice per group. * $P<0.0005$, ** $P< 0.04$ indicate differences between chow and atherogenic diet.

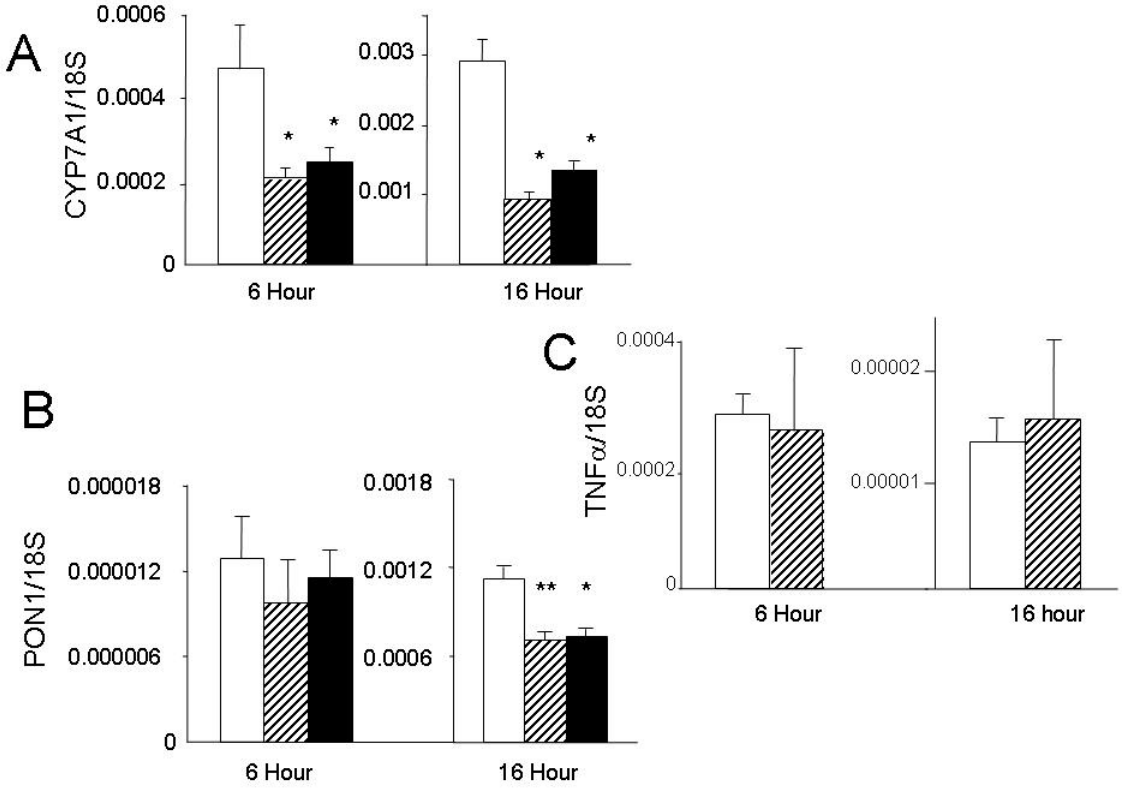
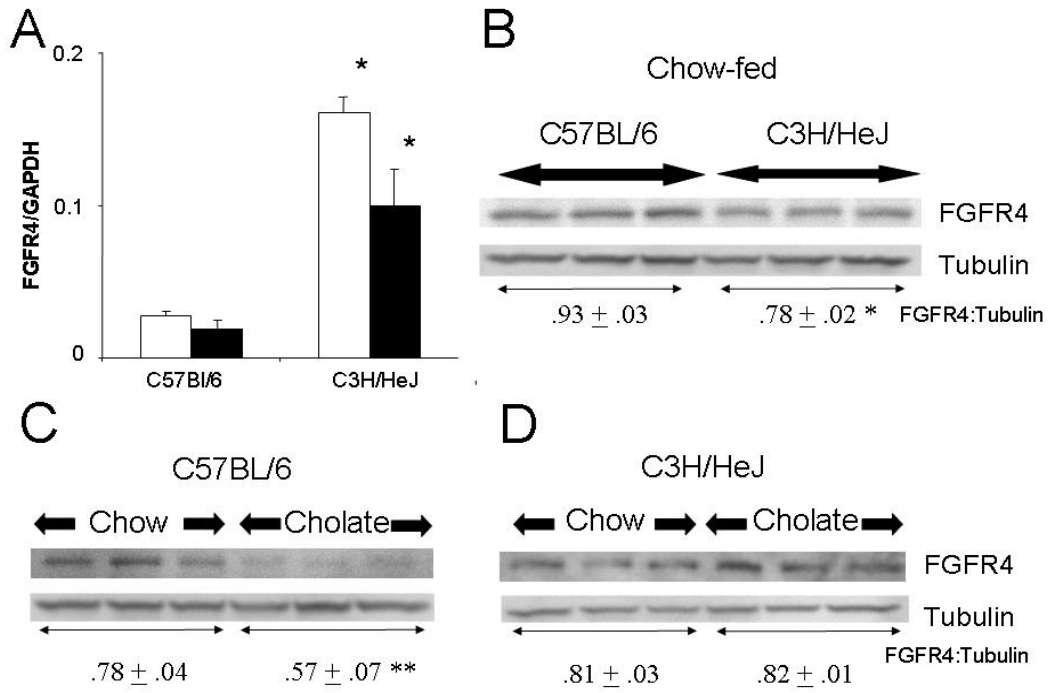


Figure 3.3: FGF19 decreases the expression of CYP7A1 and PON1 mRNA by HepG2 cells without increasing TNFα mRNA.
HepG2 cells were incubated with either 160 ng/mL FGF19 (hatched bars), 10 ng/mL TNFα (closed bars), or no treatment (open bars) for time indicated. mRNA expression of CYP7A1 (A), PON1 (B), and TNFα (C) relative to 18S RNA was determined by real-time PCR and is shown as the mean +/- S.D. of triplicate dishes. * P<0.0005, ** P< 0.02 indicate differences between no treatment and respective condition.

Gene	Organism	Annealing Temp. Degrees C	Forward Primer	Reverse Primer
CYP7A1	mouse	57.0	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
FGF15	mouse	57.1	GAGGACCAAAACGAACGAAATT	ACGTCCTTGATGGCAATCG
FGFR4	mouse	57.1	GCCTCCGACAAGGATTTGGCA	GAGTGCAGACACCCAGCAGGT
PON1	mouse	57.0	GATTGGCACTGTGTTCCAC	ATCACTGTGGTAGGCACCTT
PON1	mouse	58.9	TGGTGGTAAACCATCCAGACTC	TGTGATGGTTTTCAGATGCAAG
TNFα	mouse	62.0	CGGAGTCCGGGCAGGT	GCTGGGTAGAGAATGGATGAACA
GAPDH	mouse	55.0	GTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTG
CYP7A1	human	58.9	GAGAAGGCAAACGGGTGAAC	GGTATGACAAGGGATTTGTGATGA
PON1	human	55.0	TTGTTCACTTCGATGACTGG	CAGTGTAGTCTTTGGGGACA
PON1	human	58.9	GTGGGACCTGAGCACTTTTATG	GACCACGCTAAACCCAAATACA
TNFα	human	63.3	CCCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA
18S RNA	human	59.0	GATATGCTCATGTGGTGTTG	AATCTTCTTCAGTCGCTCCA

Supplemental Figure 3.I: Primers and annealing temperatures used for Real-Time PCR.

Primer sequences and annealing temperatures are indicated. Primer sequences were checked for specificity using the NCBI BLASTn search program. PCR conditions were 95ºC 3 min, followed by 40 cycles of 95ºC 10 sec, annealing 45 sec, and 72ºC 30 sec. PON1 PCR was performed three times with two different sets of primers which both gave the same results. PCR analysis were run in triplicate and melt curve analyses of PCR products were shown to produce a single DNA duplex. Internal standards were checked to confirm that they did not vary between the various conditions used.



Supplemental Figure 3.II: Livers from C3H/HeJ mice express more FGFR4 mRNA but less FGFR4 immunoreactive protein.
Mice were fed either a chow diet (open bars) or an atherogenic diet containing 0.5% taurocholate (filled bars) for two weeks. (A) Hepatic expression of FGFR4 mRNA relative to GAPDH are shown as the mean +/- S.D. for 6 mice per group. * $P< 0.02$ significant increase compared to C57BL/6 mice. (B-D) Liver FGFR4 protein immunodetection was performed following incubation with goat anti-mouse FGFR4 antibody 1:1000 (R & D systems). Samples were quantitated to their respective tubulin loading controls using ImageJ software (NIH). FGFR4 deficient liver samples were used as negative controls (data not shown). Values for each sample (FGFR4: α-tubulin) represent the mean +/- S.D. for 3 separate mice. * $P<0.005$, ** $P< 0.02$ indicate differences between chow and atherogenic diet.

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Chapter 4

Conclusion and Future Directions

Summary

The study presented in this dissertation attempts to examine various aspects of PON1. Initially, research focused on the ability of PON1 to be used as a therapeutic tool through gene therapy. We transplanted transgenic bone marrow expressing PON1 under the control of a macrophage-specific promoter into LDLR-knockout animals, and then treated these animals with $GdCl_3$ to replace their hepatic resident macrophages (i.e. Kupffer cells). Our research indicates that the macrophage-specific expression of PON1 in liver resident macrophages ameliorates atherosclerosis and decreases circulating plasma LDL levels in LDLR-knockout animals when challenged with a "western diet".

We further researched the regulation of PON1 expression. Our studies revealed that hepatic inflammation is not necessary to decrease hepatic mRNA expression of PON1 and illustrated that hepatic repression of PON1 due to taurocholate requires FXR and FGFR4. Using the HepG2 cell model, we demonstrate the negative regulation of PON1 and CYP7A1 expression mediated by FGF19, a specific agonist of FGFR4. We further examined the response of athero-resistant

C3H/HeJ animals to dietary taurocholate and found that these mice did not repress hepatic expression of PON1. This lack of repression involved events distal to the FGF15-FGFR4 association since C3H/HeJ animals showed an induction of FGF15, the murine homologue of FGF19, in response to taurocholate feeding.

Future Studies

The link between HDL and PON1

During the course of our studies into the regulation of PON1 in mice it was observed that plasma HDL levels correlated with PON1 plasma activity levels. A similar correlation has since been identified in humans (1). Additional studies examining how diet affected plasma PON1 protein and activity showed a direct relationship to plasma HDL levels (2). In contrast, plasma PON1 protein and enzyme activity did not correlate with hepatic mRNA levels. These data suggest that plasma PON1 levels are linked to plasma HDL levels but not necessarily to hepatic PON1 mRNA levels. Based on these findings we examined in detail the relationship between plasma PON1 protein and enzyme activity and hepatic PON1 mRNA expression.

Hepatic secretion of PON1

Preliminary data show that in mouse liver, the majority of immuno-detectable PON1 resides in the membrane fractions (Figure 4.1). Our findings are consistent with those of James' group showing that in CHO cells expressing a human PON1 transgene, the majority of PON1 was retained as an integral plasma membrane protein orientated with the C-terminus in the plasma compartment (3).

We hypothesize that the retention of an intact N-terminal signal sequence by PON1 (4-6) causes it to be integrated in the endoplasmic reticulum membrane. Following its intracellular transport through the secretory pathway, PON1 reaches the

plasma membrane where it is retained as an integral membrane protein (Figure 4.2). In order to enter the plasma compartment as a component of nascent HDL, membrane integrated PON1 has to be released from the plasma membrane, perhaps as a component of lipid vesicles (Figure 4.2). This hypothesis is consistent with our findings showing that peritoneal macrophages expressing PON1 do not secret PON1 (7) and the findings of others showing that CHO cells expressing PON1 also display an inefficient secretion of PON1, which is retained on the plasma membrane (3). The observation that PON1 secretion from CHO cells requires apo A1 and lipid (8) provides an additional clue concerning the processes responsible for PON1 secretion. We hypothesize that membrane integrated PON1 may be ejected from the surface membrane as a component of nascent HDL, containing phospholipids, free cholesterol discs and apo A1 (9,10).

We wish to elucidate the molecular mechanism responsible for the secretion of PON1 by the liver. Retention of the N-terminal signal sequence in plasma PON1 indicates that it is initially synthesized as an integral membrane protein on the sinusoidal surface of hepatocytes. Recent experiments suggest that the release of PON1 from hepatocytes is indirectly dependent on the apical membrane transporter ABCA1.

We examined plasma levels of PON1 activity in mice having a marked deficiency in HDL (i.e. ABCA1 knockout mice). ABCA1 deficiency is responsible for the very low HDL levels observed in Tangier's disease patients. Plasma levels of PON1 esterase activity (Figure 4.3A) and PON1 protein (Figure 4.3C) were reduced by

nearly 75% in ABCA1 knockout mice. In contrast, deletion of ABCA1 did not alter hepatic PON1 mRNA levels (Figure 4.3B), while the amount of PON1 protein retained within liver membranes was actually increased (Figure 4.3D). These data indicate that in the absence of ABCA1, PON1 protein accumulates in the liver and is not secreted into plasma. Thus, the decreased PON1 enzyme activities and proteins exhibited by ABCA1 knockout mice were caused by the inability to release PON1 protein from liver membranes.

Based on these data we hypothesize that PON1 excretion from liver is linked to the assembly and secretion of HDL phospholipids.

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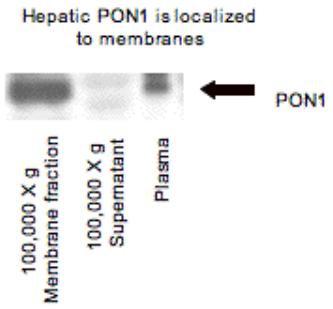

Figure 4.1: Immunoreactive PON1 is retained within membranes obtained from livers of C57BL/6 mice.
Livers were obtained from C57BL/6 mice, homongenized and fractionated by ultracentrifugation (11). An aliquot representing 0.01% of the supernatant and membrane fraction and 0.1 µl of plasma were separated by SDS/PAGE and western blotted using a goat anti-PON1 antiserum (6).

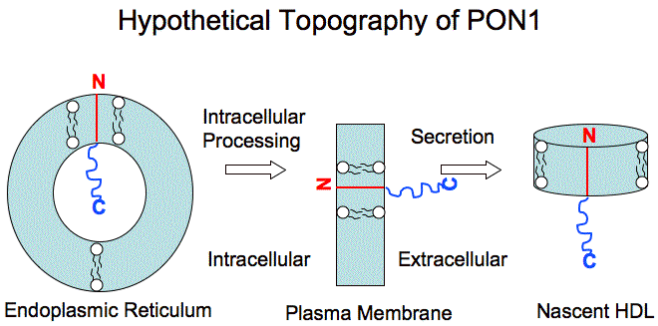

Figure 4.2: Hypothetical topography of PON1 during hepatic biogenesis.
Our hypothesis predicts that PON1 (blue) with its retained N-terminal signal sequence (red) becomes an integral membrane protein with the topography predicted by the "signal sequence hypothesis of Blobel and co-workers (12-14).

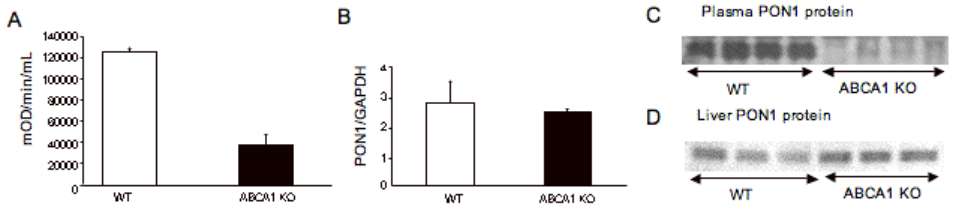

Figure 4.3: ABCA1 knockout mice display decreased plasma levels of PON1 esterase activity and protein while hepatic expression of PON1 mRNA and protein are NOT decreased.
C57BL/6/ABCA1 knockout and wild-type C57BL/6 mice were fed a chow diet. (A) Plasma was obtained and the esterase activity of PON1 was determined using phenyl acetate as the substrate. Each value represents the mean ± S.D. for 4 separate mice in each group. There was a significant difference between wild-type and ABCA1 knockout mice at $p<0.01$. (B) Hepatic mRNA expression of PON1 was assayed relative to GAPDH using real-time PCR. Each value represents the mean ± S.D. for 4 separate mice in each group. (C) Plasma was western blotted using a goat anti-PON1 antiserum. (D) Liver membranes were dissolved in buffer containing 1% Triton X-100 and 0.1% SDS and 15 µg of protein was western blotted using a goat anti-PON1 antiserum.

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