GRADUATE SCHOOL
EAST TENNESSEE STATE UNIVERSITY

East Tennessee State University
Digital Commons @ East Tennessee State University

Electronic Theses and Dissertations

Student Works

12-2004

Role of Macrophage Apoptosis in Atherosclerosis.

June Liu
East Tennessee State University

Follow this and additional works at: https://dc.etsu.edu/etd

 Part of the Medical Sciences Commons

Recommended Citation

Liu, June, "Role of Macrophage Apoptosis in Atherosclerosis." (2004). *Electronic Theses and Dissertations.* Paper 955. https://dc.etsu.edu/etd/955

This Dissertation - unrestricted is brought to you for free and open access by the Student Works at Digital Commons @ East Tennessee State University. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ East Tennessee State University. For more information, please contact digilib@etsu.edu.

Role of Macrophage Apoptosis in Atherosclerosis

A dissertation
presented to
the faculty of the Department of Biochemistry and Molecular Biology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy in Biomedical Sciences

---

by
June Liu
December 2004

Douglas P. Thewke, Chair
Balvin H.L. Chua
John J. Laffan
Antonio E. Rusinol
Michael S. Sinensky

Keywords: Apoptosis, Atherosclerosis, Macrophage, Bax, LDL Receptor, oxysterols

ABSTRACT

Role of Macrophage Apoptosis in Atherosclerosis

by

June Liu

The presence of apoptotic cells in atherosclerotic lesions has been broadly reported in the past 10 years. The majority of these apoptotic cells are macrophages. However, the pathogenic role of macrophage apoptosis in the development of atherosclerosis remains to be elucidated. Elevated expression of Bax, one of the pivotal pro-apoptotic proteins of the Bcl-2 family, has been found in human atherosclerotic plaques. Activation of Bax also occurs in free cholesterol-loaded and oxysterol treated mouse macrophages. In this study, we evaluated the influence of Bax deficiency on apoptosis in macrophage-like P388D1 cells by using small interfering RNA (siRNA) to suppress Bax expression, as well as in peritoneal macrophages isolated from Bax null mice (Bax-/-). Apoptotic activities in both cell types deficient for Bax were significantly reduced compared to that in control cells. To examine the effect of macrophage Bax deficiency on the development of atherosclerosis, fourteen 8-week-old male LDL-receptor null (LDLR-/-) mice were lethally irradiated and reconstituted with either wild type (WT) C57BL6 or Bax-/- bone marrow. Three weeks later, the mice were challenged with a Western diet for 10 weeks. No differences were found in the plasma cholesterol level between the WT and Bax-/- group. However, quantitation of cross sections from proximal aortas revealed a 49.2% increase ($P=0.0259$) in the mean lesion area of the Bax-/- group compared to the WT group. A 53% decrease in apoptotic macrophages in the Bax-/- group was found by TUNEL staining ($P<0.05$). In conclusion, Bax deficiency produces a reduction of apoptotic activity in macrophages and is associated with the accelerated atherosclerosis in LDLR null mice fed a Western diet. These results strongly support our hypothesis that macrophage apoptosis suppresses the development of atherosclerosis.

ACKNOWLEDGEMENTS

This work was supported in part by NIH grants DK058071 (Dr. Thewke's, East Tennessee State University), HL65709, HL57986 (Dr. Fazio's, Vanderbilt University), HL53989, HL65405 (Dr. Linton's, Vanderbilt University). Special thanks to Dr. Jan L. Breslow (Rockefeller University) and Dr. Patricia G. Yancey (Vanderbilt University) for critical reviews, Ms. Youmin Zhang for technical assistance, and Dr. Tao P. Zhong for sharing the videoimaging system.

CONTENTS

Page

ABSTRACT 2

ACKNOWLEDGEMENTS 3

LIST OF FIGURES 6

LIST OF ABBREVIATIONS 7

Chapter

1. INTRODUCTION 9

Apoptosis 9

Caspase 10

Two Major Apoptotic Pathways in Mammalian Cells 11

Bcl-2 Family 12

Apoptosis in Development and Pathogenesis of Diseases 16

Plasma Lipoproteins 18

LDL and LDL Receptor (LDLR) 19

Atherosclerosis 22

Pathogenesis of Atherosclerosis 24

Apoptosis in Atherosclerosis 26

Mechanisms of Apoptosis Induction During Atherosclerosis 27

OxLDL/oxysterol-induced Apoptosis 27

Free cholesterol-induced Apoptosis 31

Mouse Models of Atherosclerosis 32

Bone Marrow Transplantation (BMT) 35

Specific Aims 37

2. MATERIALS AND METHODS 39

Chapter Page

Materials 39

Mice 39

SiRNA Construct for *Bax* 39

Stable Suppression of *Bax* Gene in P388D1 Cells 41

Immunoblotting to Examine Bax Suppresion in P388D1 Cells 42

Caspase 3 Activity Assay in P388D1 Cells 43

Peritoneal Macrophage Isolation and Caspase 3 Activity Assay 44

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) 45

Bone Marrow Transplantation 46

Determination of Chimerism by Western Blotting 46

Determination of Chimerism by PCR 47

Serum Cholesterol, Triglyceride Assay, and Lipoprotein Analysis by FPLC 48

Peripheral White Blood Cell Count 49

Analysis of Atherosclerotic Lesions 49

Sectioning, Staining, and Quantitative Analysis 49

Immunohistochemistry 50

TUNEL Staining 50

Statistics 51

3. RESULTS 52

Effect of Bax Knockdown in Macrophage-like Cell Line P388D1 52

Suppression of Bax in P388D1 Cells Transfected with SiBax 53

Reduced Caspase 3 Activity in P388D1 Cells with Bax Suppression 54

Bax Deficient Mouse Peritoneal Macrophages Display Reduced Levels of Apoptosis *in vitro* upon Stimulation by Oxysterols or Staurosporine 55

Chapter Page

Complete Reconstitution of LDLR-/- Mice with WT or Bax-/- Bone Marrow.. 58

Bax Deficiency in Bone Marrow Derived Cells does not Affect the Plasma Cholesterol and Triglyceride Levels 59

Bax Deficiency in Bone Marrow Derived Cells Has no Influence on Plasma Lipoprotein Profile 61

Bax Deficiency in Bone Marrow Derived Cells Does not Affect Peripheral Lymphocyte Population 64

Bax Deficiency in Macrophages Accelerates the Development of Atherosclerosis..... 65

Deficiency of Macrophage Bax Leads to Reduced Apoptosis in Atherosclerotic Lesions 67

4. DISCUSSION 69

REFERENCES..... 76

VITA 91

LIST OF FIGURES

Page

1. Two major apoptotic pathways in mammalian cells 12
2. Structures of representative Bcl-2 family members 13
3. Pathogenesis of atherosclerosis 25
4. pSilencer 1.0-U6 Vector 41
5. Caspase 3 activity assay in R-Bax and SiBax stably transfected clones..... 53
6. Suppression of Bax in P388D1 cells by SiRNA 54
7. Caspase 3 activity assay in clone R1 and S1 55
8. Bax deficient mouse peritoneal macrophages display reduced apoptosis *in vitro* 57
9. Immunoblotting to examine the chimerism of reconstitution 58
10. PCR assay to examine the chimerism of reconstitution 59
11. Plasma total cholesterol (A) and triglyceride (B) levels 61
12. Plasma lipoprotein profiles obtained by FPLC 63
13. Peripheral lymphocyte counts 12 weeks after bone marrow transplantation 64
14. Lesion area quantitation in cross sections of proximal aortas after 10 weeks on Western diet 65
15. Immunostaining for macrophages in the lesion (A,C) and SMCs in the arterial wall and lesion cap area (B,D) in serial cross sections from proximal aorta . 67
16. In situ TUNEL staining in proximal aorta sections. 68

ABBREVIATIONS

ACAT	Acyl Coenzyme A-cholesterol Acyltransferase
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
Bax-/-	Bax Deficient
BH	Bcl-2 Homology
BMT	Bone Marrow Transplantation
CMV	Cytomegalovirus
DAB	Diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
ER	Endoplasmic Reticulum
EtOH	Ethanol
FBS	Fetal Bovine Serum
FPLC	Fast Performance Liquid Chromatography
HDL	High Density Lipoprotein
HMG-CoA	Hydroxymethyglutaryl-coenzyme A
HRP	Horseradish Peroxidase
IDL	Intermediate Density Lipoprotein
7-KC	7-Ketocholesterol
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LDLR-/-	Low Density Lipoprotein Receptor Deficient
MCSF	Macrophage Colony-Stimulating Factor
OxLDL	oxidized LDL
25-OHC	25-Hydroxycholesterol
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PVDF	Polyvinylidene Difluoride
RFU	Relative Fluorescent Unit
RISC	RNA Induced Silencing Complexes
RNAi	RNA Interference
SERCA	Sarcoplasmic-ER Ca^{2+} Adenosine Triphosphatase Pump
SiRNA	Small Interfering RNA
SMC	Smooth Muscle Cell
SREBP	Sterol Response Element Binding Protein
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling
UPR	Unfolded Protein Response
VLDL	Very Low Density Lipoprotein
WT	Wild Type

CHAPTER 1
INTRODUCTION

Apoptosis

Within each cell lineage, the control of cell number is determined by the balance between cell proliferation and cell death. Biologists are now beginning to appreciate that the regulation of cell death is just as complex as the regulation of cell proliferation. Apoptosis (also called programmed cell death), characterized by cell shrinkage, membrane blebbing, DNA fragmentation, and nuclear condensation, plays a critical role in tissue development and maintenance of homeostasis within multi-cellular organisms. When activated, this suicide program allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage.

Apoptotic cell death can be distinguished from necrotic cell death (Wyllie and others 1980; Raff 1992). Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, and is typified by an influx of water and extracellular ions, leading to rapid cell swelling and lysis. Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular environment. Therefore, *in vivo,* necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response. Apoptosis, on the other hand, can be triggered by a variety of extrinsic and intrinsic signals, generating completely opposite morphology. The apoptotic cell maintains its plasma membrane integrity. However, alterations in the plasma membrane of apoptotic cells signal neighboring phagocytic cells to engulf them and thus to complete the degradation process (Ellis and others 1991). Cells not immediately phagocytosed break down into

smaller membrane-bound fragments called apoptotic bodies, which will be digested by phagocytic cells eventually. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response.

Caspase

One key to understanding apoptosis is the activation and function of a set of proteinases, the caspases. Caspases belong to a family of cysteine proteases that selectively cleave proteins at sites just C-terminal to aspartate residues. Numerous caspases have been discovered and each has been given a variety of names: caspase-1 (ICE), caspase-2 (ICH-1, Nedd-2), caspase-3 (CPP32, Yama, apopain), caspase-4 (TX, ICH-2, ICErel-II, also homologous to murine caspase-11), caspase-5 (ICErel-III, TY), caspase-6 (Mch-2), caspase-7 (Mch-3, ICE-LAP, CMH-1), caspase-8 (MACH, FLICE, Mch-5), caspase-9 (Apaf-3, ICE-LAP6, Mch-6), caspase-10 (Mch-4), caspase-13 (ERICE), and caspase-14 (MICE) (Chang and Yang 2000).

Normally, caspases reside in cells as inactive proforms, and can be activated by proteolytic cleavage in two different ways. Caspases can be broadly catagorized as either “initiator” caspases (caspase 8, 9, etc.) or “executioner” caspases (caspase 3, 6, 7, etc.). Initiator caspases play a role in the amplification of an apoptotic signal by cleaving and activating other initator caspases, which in turn, cleave and activate the executioner caspases. This is the major way of activating executioner caspases. Another way to activate an executioner caspase is through the action of adaptor proteins, such as Apaf-1 and FADD, which bind to the prodomains of the initiator caspases. Binding of the adaptor protein promotes the activation of the initiator caspases, which can then go on to cleave and activate the executioner caspases. The executioner caspases have specific intracellular targets such as proteins of the nuclear lamina and cytoskeleton (Porter and Janicke 1999). Cleavage of these substrates leads to the demise of the cell.

Two Major Apoptotic Pathways in Mammalian Cells

Two basic pathways for apoptosis have been described, the death receptor pathway and the mitochondrial pathway (Gross and others 1999; Hengartner 2000). In the death receptor pathway, extrinsic signals, such as Fas ligand and tumor necrosis factor (TNF), bind to their receptors, Fas or TNF receptor on the plasma membrane, forming membrane-bound signaling complexes. These complexes then recruit, through adapter proteins, procaspase 8 molecules, resulting in a relatively high local concentration of zymogen. Self or mutual cleavage of procaspase 8 leads to the activation of caspse 8, which then further activates the death executioner protease - caspase 3.

In the mitochondrial apoptosis pathway, intrinsic signals such as DNA damage caused by UV radiation, stress caused by oxidants or growth factor withdrawal, can induce the mitochondrial dysfunction and release of cytochrome *c* from the intermembrane space through the combined regulation of Bcl-2 family members on the mitochondrial membrane. The detailed mechanism regulating cytochrome *c* release remains to be elucidated. Released cytochrome *c* activates Apaf-1, which in turn activates caspase 9. The apoptosome, a complex consisting of cytochrome *c*, Apaf-1, and caspase 9, then activates the common death executor – caspase 3.

The cross-talk between the two apoptotic pathways is mediated by another Bcl-2 family member, Bid (Luo and others 1998). Activated caspase 8 in the death receptor pathway can cause the cleavage of Bid. The C-terminal part of Bid, also called truncated Bid, will then translocate to the mitochondria where it stimulates the cytochrome *c* release (Fig 1).

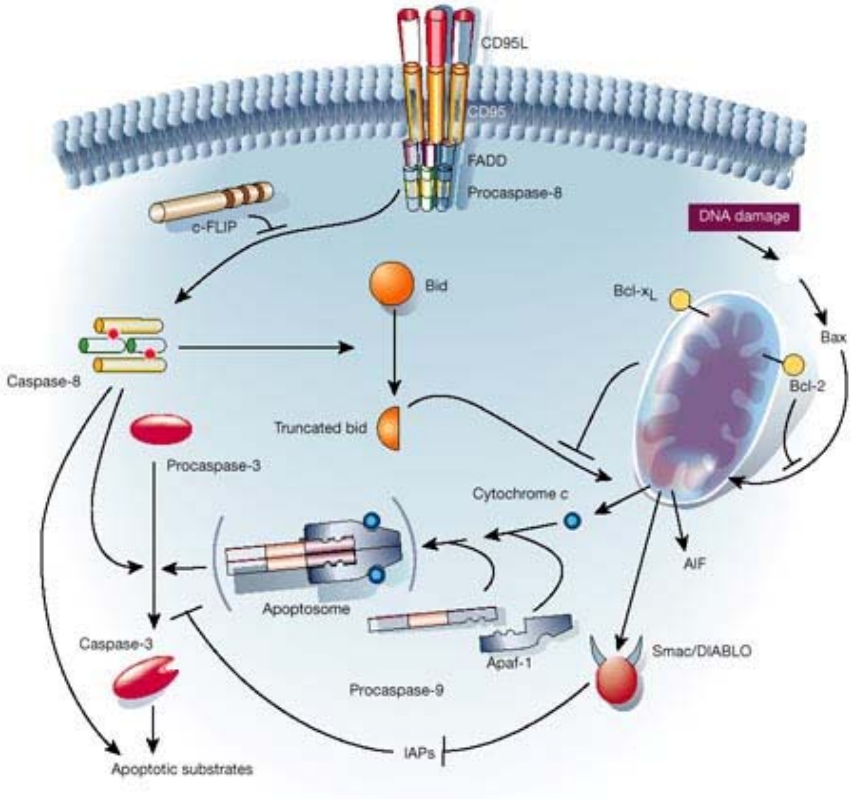


Figure 1 Two major apoptotic pathways in mammalian cells (Adapted from: Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770-776)

Bcl-2 Family

The Bcl-2 family members focus much of their effects at the level of mitochondria and play pivotal roles in deciding the fate of a cell (Gross and others 1999). Based on their intracellular functions, Bcl-2 family proteins can be divided into two opposite subtypes: anti-apoptotic proteins such as Bcl-2 and Bcl-x_L, and pro-apoptotic proteins such as Bax and Bak. Indeed, the ratio between these two subsets determines, at least in part, the susceptibility of cells to a death signal (Oltvai and others 1993).

Bcl-2 family members have four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3, and BH4. Many of the anti-apoptotic members display sequence

conservation in all four domains. However, many pro-apoptotic members, so-called BH3-domain-only members, have sequence homology only in the BH3 domain. To date, all the BH3-domain-only members are pro-apoptotic, suggesting a critical death function of the BH3 domain. Multidomain pro-apoptotic members, such as Bax and Bak, have sequence homology in BH1, BH2, as well as BH3 (Fig 2).

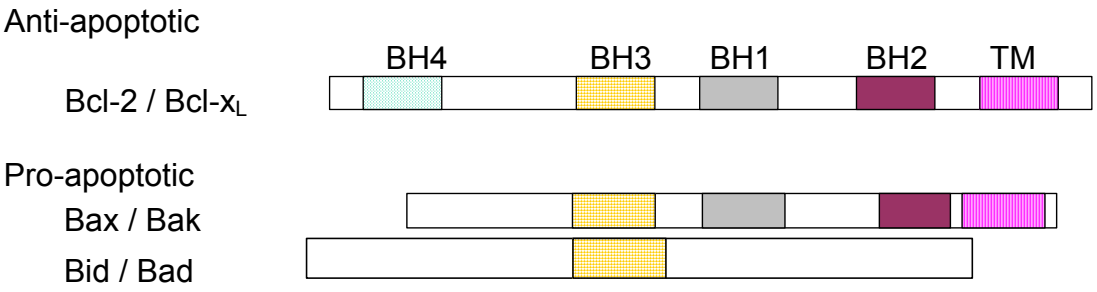


Figure 2 Structures of representative Bcl-2 family members. TM: terminal hydrophobic domain. (Modified from Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770-776)

Two major anti-apoptotic proteins in the family, Bcl-2 and Bcl-x_L, normally reside in the mitochondrial membrane. Over-expression of Bcl-2 in a variety of cells protects them from different death stimuli (Vaux and others 1988; McDonnell and others 1989; Sentman and others 1991). Bcl-x_L deficiency leads to massive cell death of immature hematopoietic cells in mice (Motoyama and others 1995).

Among pro-apoptotic members, Bak has been reported to be a membrane protein localized in the mitochondrial membrane (Griffiths and others 1999). Controversy remains in terms of the subcellular localization of Bax due to lack of direct evidence. One hypothesis is that in healthy cells, Bax is predominantly a cytosolic monomer. Upon

stimulation, it undergoes conformational changes, translocates to the outer mitochondrial membrane or endoplasmic reticulum (ER) where it forms homodimers (Hsu and others 1997; Antonsson and others 2001; Zong and others 2003). However, it remains unclear whether the dimerization of Bax occurs in the cytosol or is coincident with membrane insertion. An alternative hypothesis is that formation of heterodimers of Bax and Bcl-2 in the mitochondrial membrane prevents cell death under normal growth conditions (Oltvai and others 1993; Yin and others 1994). In the presence of survival factors, phosphorylation of Bad, one of the BH3-domain only pro-apoptotic proteins, by Akt/protein kinase B results in the binding of phosphorylated Bad to protein 14-3-3 in the cytosol. In the absence of survivial factors, dephosphorylation of Bad leads to the heterodimerization of Bad with Bcl-2 or Bcl-xL on the mitochondrial membrane, and displaces Bax (Yang and others 1995a; Zha and others 1996; Datta and others 2002; Won and others 2003), which will then form homodimers on the mitochondrial membrane.

Despite of the controversy, the ultimate function of pro-apoptotic proteins Bax or Bak is that they permeabilize the outer mitochondrial membrane, allowing the efflux of cytochrome *c* and other apoptosis regulatory proteins into the cytosol (Manon and others 1997; Gross and others 1999; Martinou and Green 2001). The presence of excessive Bcl-2 or Bcl-x_L can inhibit the activation of Bax following a death signal (Gross and others 1998). Expression of the multidomain pro-apoptotic proteins, Bax and/or Bak, are required for the activity of the mitochondrial apoptosis pathway (Wei and others 2001). Suggestive of the central physiological role for Bax in programmed cell death, Bak null mice fail to show any developmental defects (Lindsten and others 2000); however Bax deficient mice displayed hyperplasia of thymocytes and B cells as well as abnormalities in

the development of the reproductive system (Knudson and others 1995). When Bak deficient mice were mated to Bax deficient mice to create double knockout mice, the majority of Bax-/-Bak-/- mice died perinatally with fewer than 10% surviving into adulthood. The surviving Bax-/-Bak-/- mice displayed multiple developmental defects such as persistence of interdigital webs, and accumulation of excess cells with both the central nervous and hematopoietic systems. Mouse lymphocytes deficient in both Bax and Bak were resistant to death induced by growth factor withdrawal (Lindsten and others 2000). Bax-/-Bak-/- mouse embryonic fibroblasts have also been shown to be resistant to multiple apoptotic stimuli that act through disruption of mitochondrial function: staurosporine, UV radiation, growth factor deprivation, and the ER stress stimuli thapsigargin and tunicamycin (Wei and others 2001). Thus, Bax and Bak have overlapping roles in the regulation of apoptosis during mammalian development and tissue homeostasis, and the activation of Bax or Bak appears to be an essential gateway to mitochondrial dysfunction in apoptosis (Wei and others 2001; Zong and others 2003).

Most recently, a large body of research has been directed to the regulation of ER Ca^{2+}, the main intracellular Ca^{2+} storage, by Bcl-2 family members (Nutt and others 2002; Scorrano and others 2003; Zong and others 2003). Alterations in intracellular Ca^{2+} homeostasis have been implicated in the control of apoptosis. Depletion of the ER Ca^{2+} pool has been believed to be an early event in apoptosis (Liu and others 1996; McConkey and Orrenius 1997). Close contacts exist between mitochondrial and the sites of ER Ca^{2+} release, so that ER Ca^{2+} release leads to rapid Ca^{2+} accumulation in mitochondria (Rizzuto and others 1998; Csordas and others 1999). Mitochondral Ca^{2+} uptake promotes cytochrome *c* release in cells exposed to the proapoptotic agent staurosporine (Szalai

and others 1999; Pacher and Hajnoczky 2001). Bcl-2 has been shown to regulate both the mitochondrial and ER Ca^{2+} homestasis (Murphy and others 1996; Foyouzi-Youssefi and others 2000), and prevents Ca^{2+} -induced cytochrome *c* release (Shimizu and others 1998). Overexpression of Bcl-2 protects cells from death induced by thapsigargin, an irreversible inhibitor of the sarcoplasmic-ER Ca^{2+} adenosine triphosphatase pump (SERCA) responsible for the uptake of Ca^{2+} from the cytosol into the ER lumen (Lam and others 1994). On the other hand, Bax and Bak have also been believed to localize to the ER. By increasing the ER Ca^{2+} load and Ca^{2+} transfer from ER to mitochondria, Bax and Bak control another check-point for apoptosis (Breckenridge and others 2003; Oakes and others 2003). A detailed mechanism for the regulation of ER Ca^{2+} by Bcl-2 family members has yet to be elucidated.

Apoptosis in Development and Pathogenesis of Diseases

The fundamental roles of apoptosis in mammalian development have been well described from the formation of the inner and outer cell mass in the blastocyst to the ongoing cell death in adulthood that maintains homeostasis of cell number by balancing mitotic cell production (Coucouvanis and Martin 1995; Brison and Schultz 1997). It is broadly involved in the formation of tubes, the separation of the digits, the remodeling of bone, and the involution of the mammary glands. In the haemopoietic system, the excess cells at each stage of development are culled by apoptosis. In the thymus, cells that fail to correctly rearrange their antigen receptors are also removed by apoptosis (Nossal 1994).

Failure of cells to undergo apoptosis is involved in the pathogenesis of a variety of human diseases, including cancer, autoimmune disease, and certain viral infections, which are all characterized by the excessive accumulation of cells (Williams and others

1990; Raff and others 1993; Hoffman and Liebermann 1994). For example, the p53 gene product is required for cells to initiate apoptosis in response to genotoxic damage (Lowe and others 1993). Deficiency of p53 in tumors, such as fibrosarcoma, underlies the enhanced resistance to chemotherapeutic agents and radiation (Lowe and others 1994). The *Bcl-2* gene was first discovered in most human follicular lymphomas (Bakhshi and others 1985; Tsujimoto and others 1985). Initially viewed as an oncogene, *Bcl-2* overexpression prevents cells from initiating apoptosis in response to a number of stimuli (Vaux and others 1988; Hockenbery and others 1990).

Failure to remove autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease. For example, cell surface receptor Fas, a member of the TNF receptor family, is critical in regulating cell death in lymphocytes. Mutations in the Fas receptor and in the Fas ligand have been reported to attribute to two forms of hereditary autoimmune disease (Suda and others 1993).

In viral infection, the disruption of cell physiology can cause an infected cell to undergo apoptosis. A number of viruses have developed mechanisms to disrupt the normal regulation of apoptosis within the infected cell. For example, an effective adenoviral infection depends on the function of the E1B 19-kD protein (Rao and others 1992). The E1B protein has been shown to block apoptosis directly, and its function can be replaced in adenovirus by *Bcl-2*.

In addition, an accelerated rate of cell death may play a role in the progression of a large number of diseases characterized by cell loss, such as neurodegenerative disorders, AIDS, and osteoporosis. A wide variety of neurological diseases, such as Alzheimer’s disease, Parkinson’s disease, and various forms of cerebellar degeneration that are characterized by the gradual loss of specific sites of neurons may also have an apoptotic component in their pathologies (Heintz 1993; Isacson 1993). In these diseases,

excessive cell death results in specific disorders of movement and central nervous system function. Oxidative stress, calcium toxicity, mitochondrial defects, excitatory toxicity, and deficiency of survival factors have all been postulated to contribute to the pathogenesis of these disorders (Choi 1992). Each of these pathways predisposes neurons to apoptosis, either *in vitro* or *in vivo*.

Plasma Lipoproteins

Plasma lipoproteins are the primary carriers of lipids in the circulation. Lipoproteins can be divided into six major classes and two specialized classes. Four of the major classes of lipoproteins — very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) — are derived from the liver and are present in plasma from both fasted and nonfasted subjects. The other two major classes — chylomicrons and chylomicron remnants — are derived from the small intestine and are found in the plasma only after a fatty meal. Like LDL, the specialized classes of lipoproteins, β-VLDL and lipoprotein(a), are significant because they are positively correlated with coronary heart disease and atherosclerosis.

Due to the distinct compositions of different percentages of lipids and proteins, lipoproteins can be separated by sequential ultracentrifugation using salt solutions with increasing densities. Lipoproteins with a greater proportion of lipids, especially neutral lipids (triglycerides and cholesteryl esters), are less dense and float at a lower density layer upon ultracentrifugation. For example, VLDLs float to the top of the tube when plasma is ultracentrifuged. After the VLDL is removed, the remaining is raised to d = 1.019 g/ml with salt and the ultracentrifugation is repeated to isolate IDL. The same

process is repeated to isolate LDL and HDL. The density of lipoprotein particles is also inversely related to their size (Table 1).

Table 1 Characteristics of human plasma lipoproteins

Lipoprotein	Density (g/ml)	Diameter (Å)	Apoproteins	Major lipids
VLDL	< 1.006	300 – 700	B-100, E, C	Triglycerides
IDL	1.006 - 1.019		B-100, E	Cholesterol, triglycerides
LDL	1.019 - 1.063	200	B-100	Cholesterol
HDL	1.063 - 1.21	80 – 130	A-I, A-II, C	Phospholipid, cholesterol
Chylomicron	< 0.95	> 1,000	B-48, E, A, C	Triglycerides
Chylomicron Remnant	<0.95	> 1,000	B-48, E	Cholesterol, triglycerides

(Modified from Thomas L. Innerarity. 1991. Plasma Lipoproteins. In: Encyclopedia of Human Biology, Volume 6. Academic Press, Inc. p 23-35.)

LDL and LDL Receptor (LDLR)

LDL, the end product of the lipolysis of VLDL and IDL by lipoprotein lipase, is the major cholesterol-transporting lipoprotein in humans, carrying about two-thirds of the total plasma cholesterol. The lipid composition of LDL is ~35% cholesteryl ester, ~12% cholesterol, ~8% triglycerides, and 20% phospholipids. ApoB100, the solo apolipoprotein crisscrossing LDL surface, contributes most if not all of the remaining 25%. The apoB100 on the surface of these particles is recognized by hepatic and extrahepatic LDLRs. This interaction is responsible for ~75% of the clearance of LDL from the plasma, principally through the liver (Havel and Kane 1989). Elevated plasma cholesterol concentrations,

especial LDL concentrations, are strongly correlated with atherosclerotic coronary heart disease (Watanabe and others 1985; Hobbs and others 1992).

Two isoforms of B apoproteins exist: apoB100 and apoB48 (Anant and Davidson, 2001). Both of them are derived from the same gene; however, apoB100 is produced in liver, while apoB48 is the result of apoB mRNA editing that occurs only in the intestine. A single cytosine is changed to a uracil, which changes the codon 2153 from a CAA (glutamine) to the translational termination codon UAA. As a result, a shortened apoB (apoB48), approximately 48% of the N-terminus of apoB100, is produced in the intestine. Therefore, apoB48 is unique to chylomicrons and chylomicron remnants. These two B proteins also differ in function (Brown and others 2000). ApoB100 is involved in LDLR-meidated clearance of lipoprotein because it has a receptor-binding domain in its C-terminal segment. Because apoB48 does not contain this portion of apoB, it does not bind to LDLR well, if at all. Finally, apoB100 is required for the synthesis of VLDL by liver, while apoB48 is required for the synthesis of chylomicron by small intestine.

LDLR is a transmembrane protein residing in the plasma membrane (Brown and Goldstein 1986), responsible for clearing LDL and other lipoproteins from the plasma through the binding to apoB100 or apoE. It is expressed by all cell types studied in humans, mice, rabbits, dogs, etc. Due to the large mass, the liver is responsible for about 70% of the total body LDLR-mediated uptake of LDL, with the remaining one-third being cleared by extrahepatic tissues (Pittman and others 1982). Lipoproteins bound to the receptor are internalized via clathrin-coated pits on the plasma membrane. The coated pits ‘pinch off ’ to form coated vesicles, then fuse together, lose their clathrin protein coat, and become endosomes. Proton pumps acidify the endosomes, causing lipoprotein

release from the receptor. The receptor is then recycled to the cell surface while the lipoproteins are delivered to lysosomes, where enzymes degrade the apoproteins into their constituent amino acids and hydrolyze the cholesteryl esters to unesterified cholesterol. The released cholesterol is used for the synthesis of cell membranes, and in certain specialized cells it is a precursor for sterol end products. For example, in the adrenal gland, cholesterol is converted into steroid hormones; while in the liver, it is converted into bile acids (Brown and Goldstein 1986).

By 1) adjusting the number of LDLRs on their surface, 2) regulating the synthesis of hydroxymethyglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme involved in cholesterol biosynthesis, 3) regulating the activity of acyl coenzyme A-cholesterol acyltransferase (ACAT), an enzyme that re-esterifies cholesterol into cholesteryl esters stored in the cytoplasm as lipid droplets, cells maintain the amount of intracellular cholesterol sufficient to perform various functions, but not so high as to overload the cell.

In addition to controlling intracellular cholesterol, LDLRs regulate plasma LDL levels and determine, in part, how much VLDL is converted to LDL. The importance of receptor-mediated LDL clearance is demonstrated by two genetic defects: familial hypercholesterolemia, in which the LDLR is defective, and familial defective apoB100, in which the ligand (apoB100) is defective.

In familial hypercholesterolemia, individuals who inherit one copy of the mutant LDLR gene possess LDL levels about twice that of normal, and are more susceptible to heart attacks than normal individuals. Those who inherit two defective LDLR alleles (homozygotes) have circulating LDL levels six to eight times higher than normal and

usually have heart attacks before the age of 20. Massive accumulation of LDL in the blood leads to two and half times longer survival of the LDL particles than that in the normal individuals because the LDL particles must be removed by a metabolic pathway that is not as efficient as the LDLR pathway. In addition to the slower removal of LDL, about twice as much LDL is produced in familial hypercholesterolemic homozygotes. Thus, the build-up of LDL in the plasma is due to both a slower utilization and increased production of LDL particles (Goldstein and Brown 1979).

In the second genetic disorder – familial defective apoB100, a single amino acid substitution in the receptor-binding domain of apoB100 abolishes the ability of LDL to bind to the LDLR (Weisgraber and others 1988; Soria and others 1989). Individuals heterozygous for this disorder have one population of normal LDL and one that binds poorly, if at all, to LDLR. The abnormal LDL accumulates in the plasma to levels 50-70% above normal. In contrast to familial hypercholesterolemic homozygotes, however, there is no overproduction of LDL, and consequently, the LDL concentrations are not as elevated. These two genetic disorders of the LDLR system clearly demonstrate the physiological significance of the LDLR pathway in regulating plasma LDL levels.

Atherosclerosis

Atherosclerosis, the primary cause of heart disease and stroke, is responsible for around 50% of all deaths in western societies (Lusis 2000). It is a progressive disease characterized by the accumulation of lipids and fibrous elements in the artery wall.

A variety of risk factors are associated with atherosclerosis. Genetic risk factors include hypertension, diabetes, hyperlipidemia, and obesity etc. Environmental factors such as high-fat diet, smoking, and lack of excercises, etc. are frequently observed in the populations with atherosclerosis. Certain viral infections and bacterial infections have also been implicated in atherosclerosis, such as human cytomegalovirus (CMV) (Speir and others 1994; Zhou and others 1996) and *Chlamydia pneumoniae* (Campbell and others 1998; Grayston 2000; Saikku 2000). *Chlamydia pneumoniae* is an obligate intracellular bacterium which frequently causes airway infections in humans and has been implicated in chronic inflammatory diseases in addition to atherosclerosis. CMV is a member of the herpes virus group. Infection of CMV results in a variety of disorders which depend largely on the immune status of the host.

Generally, atherosclerosis is characterized by three different stages: fatty streaks, fibrous plaques, and advanced lesions. The early lesion of atherosclerosis, fatty streak, consists of subendothelial accumulation of cholesterol-rich macrophages, also called ‘foam cells’. Though it is not clinically significant, fatty streaks are the precursors of more advanced lesions – fibrous lesions. Typical fibrous lesions have a lipid-rich ‘necrotic core’ covered by a fibrous cap consisting smooth muscle cells (SMCs) and the extracellular matrix generated by SMCs. In the most advanced stage, plaques become more complex, with calcification in the necrotic core, ulceration at the luminal surface, and haemorrhage from small vessels that grow into the lesion from the media of the blood vessel wall. Plaque rupture occurs when the mechanical stresses in the fibrous cap exceed a critical level that the cap tissue can withstand, leading to the formation of thrombus, one of the life-threatening consequences of atherosclerosis.

Pathogenesis of Atherosclerosis

A normal large artery consists of three morphologically distinct layers (Fig 3A). Intima, the innermost layer, is bounded by a monolayer of endothelial cells on the luminal side and a sheet of elastic fibres, the internal elastic lamina, on the peripheral side. The normal intima consists of extracellular connective tissue matrix, primarily proteoglycans and collagen. The media, middle layer, consists of SMCs. The adventitia, the outer layer, consists of connective tissues with interspersed fibroblasts and SMCs.

Atherosclerotic lesions tend to occur at the sites where the direction of blood flow changes. The primary event is the accumulation of LDL particles and their aggregates in the subendothelial matrix. Trapped LDL undergoes modifications such as oxidation, aggregation, lipolysis, and proteolysis. Modified LDL can stimulate the endothelial cells to produce a number of pro-inflammatory molecules, including adhesion molecules and growth factors such as macrophage colony-stimulating factor (MCSF), which mediates the recruitment of monocytes and lymphocytes to the artery wall. Mice deficient in both MCSF and apolipoprotein E have dereased atherosclerosis, which proves the critical role of MCSF and macrophage recruitment in the development of atherosclerosis (Smith and others 1995). After they migrate across the single layer of endothelial cells, monocytes proliferate and differentiate into macrophages that can continuously uptake modified LDL by scavenger receptors, which are not regulated by the intracellular content of cholesterol, forming lipid-rich foam cells (Gerrity 1981) (Fig 3B).

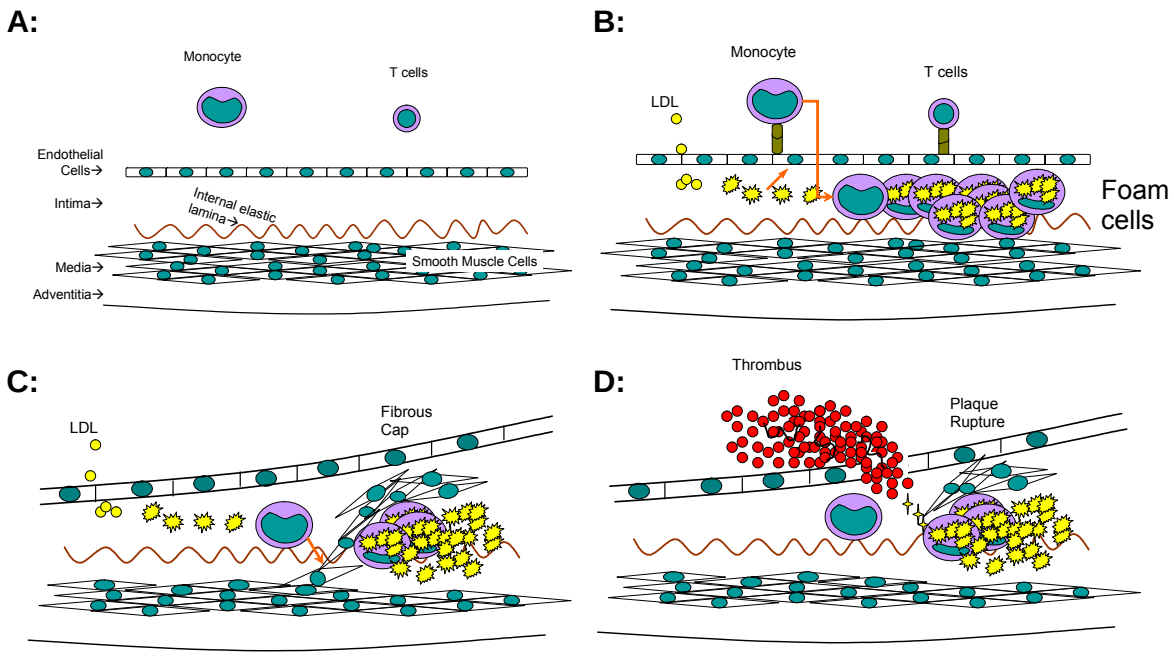


Figure 3 Pathogenesis of atherosclerosis. A: normal healthy artery wall; B: fatty streak consisting lipid-rich foam cells; C: fibrous plaque consisting a necrotic core covered by a fibrous cap; D: ruptured plaque with formation of thrombus in the artery.

Cytokines and growth factors secreted by macrophages and T cells can lead SMCs to migrate and accumulate in the intima (Schonbeck and others 2000). The SMC-derived extracellular matrix, together with SMC, forms the fibrous cap. Underneath is the so-called lipid core, consisting of a growing mass of extracellular lipids due to the death of foam cells. The shoulder region of the plaque is generally rich in foam cells (Fig 3C).

The development of thrombus-mediated acute coronary events depends primarily on the composition and vulnerability of a plaque. Vulnerable plaques generally have very thin fibrous caps and increased numbers of inflammatory cells. Once the fibrous cap ruptures, exposure of the extracellular contents of the necrotic core such as tissue factor, a key protein in the initiation of the coagulation cascade, to the blood leads to the formation of

thrombus or blood clot (Fig 3D), resulting in myocardial infarction or stroke – the most important clinical complication. Rupture frequently occurs at the lesion edges, which are rich in foam cells, suggesting a significant role of foam cells in thrombosis (Lusis 2000).

Apoptosis in Atherosclerosis

Macrophages are involved in all stages of atherosclerosis. The expression of a variety of genes and their proteins which affect lipoprotein metabolism and cholesterol accumulation in macrophages has been shown to influence the progression of atherosclerosis (Linton and Fazio 2001; Linton and Fazio 2003). In addition, upon activation, macrophages generate a large number of cytokines and growth factors that regulate lesion development in both paracrine and autocrine manners (Frostegard and others 1999; Seshiah and others 2002; Linton and Fazio 2003).

Macrophages in atherosclerotic lesions have been shown to have cellular characteristics of both necrosis and apoptosis (Hegyi and others 1996). The pathophysiological consequences of these two different types of cell death are still under investigation. All three of the major cell types found in atherosclerotic lesions (e.g. macrophages, SMCs, and endothelial cells) can undergo apoptosis. Given the complexity of atherosclerotic plaque, apoptosis in these different cell types may play different roles in atherogenesis (Kockx and Herman 2000; Lusis 2000).

The majority of apoptotic cells in atherosclerotic lesions are macrophages localized near the necrotic areas of advanced lesions (Ball and others 1995). Overexpression of Bax and other related apoptotic proteins has been found in all stages of human atherosclerotic plaques (Kockx and others 1998; Martinet and others 2002; Saxena and

others 2002). *In vitro*, Bax activation has been shown to occur during apoptosis resulting from free cholesterol loading of macrophages (Yao and Tabas 2001) or after treatment of macrophages with oxysterols which are cytotoxic components of oxidized LDL (Panini and Sinensky 2001).

Although foam cell apoptosis has long been hypothesized to contribute to the development of the lipid core (Ball and others 1995; Hegyi and others 1996), the role of macrophage apoptosis in atherosclerosis remains unclear. Macrophage death may reduce the production of growth factors and inflammatory cytokines, which in turn may impede the development of atherosclerosis. On the other hand, loss of macrophages may promote pro-atherogenic factors such as decreased production of apoE and reduced scavenging of toxic substances (i.e. oxidized LDL (oxLDL)) (Linton and others 1995; Nicholson and others 2001; Platt and others 2002).

Mechanisms of Apoptosis Induction during Atherosclerosis

Possible causes of cell death in atherosclerotic lesions include exposure to oxidized lipoproteins, cholesterol-induced cytotoxicity (Warner and others 1995; Tabas and others 1996), growth factor deprivation (Chin and others 1999), and exposure to other arterial wall factors such as inflammatory cytokines and nitric oxide (Mitchinson and others 1996; Lopez-Collazo and others 1998).

OxLDL/oxyserol-induced Apoptosis OxLDL can be generated *in vivo* by at least three classes of mechanisms: 1) autoxidation in the presence of transition metals (Morel and others 1983; Lamb and others 1995); 2) cell-mediated oxidation (Morel and others 1984; Steinbrecher and others 1984); and 3) plasma enzyme-mediated oxidation

(Parthasarathy and others 1985; Daugherty and others 1994; Yla-Herttuala and others 1995).

It has been proposed that oxidative modifications of LDL increase its atherogenicity and that the resulting oxLDL, but not the native LDL, plays a pivotal role in the development of atherosclerotic lesions. OxLDL is involved in many events that are associated with atherosclerosis: it induces the expression of adhesion molecules on endothelial cells (Kume and others 1992) which will recruit monocytes or lymphocytes from the blood, the transformation of macrophages and smooth muscle cells to foam cells (Henriksen and others 1981), the production of various proinflammatory cytokines and growth factors by almost all vascular cells (Kume and Gimbrone 1994; Nakano and others 1994), the proliferation and migration of vascular SMCs (Quinn and others 1988; Yui and others 1993; Auge and others 1996;), the retardation of endothelial regeneration (Murugesan and Fox 1996), and it increases the procoagulant activity on the vascular cells (Aupeix and others 1996). These changes are suggested to eventually result in the formation of atheromatous lesions.

OxLDL also exhibits a dramatic cytotoxic effect on several vascular cell types, including SMCs, macrophages, and endothelial vascular cells. It has been well established that vascular cells - particularly macrophages (Reid and others 1993; Hardwick and others 1996), and endothelial cells (Escargueil-Blanc and others 1997) - undergo apoptosis after uptake of circulating oxLDL (Mitchinson and others 1996). OxLDL induces both the morphological changes and DNA fragmentation characteristic of apoptosis in cultured smooth muscle cells, macrophages, endothelial cells, and lymphoid cells (Dimmeler and others 1997; Escargueil-Blanc and others 1998; Brown and Jessup 1999). The cytotoxic effect of oxLDL depends on concentration and oxidative

modification. High concentrations of oxLDL are pro-apoptotic, whereas low concentrations are mitogenic for vascular SMCs. Regarding the degree of oxidation, Siow and others (1999) provided evidence that moderately oxLDL, with its high lipid hydroperoxide content, seems to be more cytotoxic than mildly or highly oxLDL (Siow and others 1999; Napoli and others 2000). In contrast, protein modifications do not seem to be necessary for LDL-induced cytotoxicity.

The oxidation of LDL by incubation with cells results in marked changes that include an increased lysolecithin content; a decreased content of polyunsaturated fatty acids associated with an abundance of products of the peroxidation of these acids; a fragmentation of apolipoprotein B100, an increased density, and an significant increase in the oxysterol levels (Steinbrecher and others 1984). Oxysterols have been demonstrated to exist both in oxLDL (Colles and others 1996) and in atherosclerotic plaque (Carpenter and others 1993). Various oxysterols in oxLDL have been shown to induce apoptosis in endothelial cells (Harada-Shiba and others 1998), monocytic cell lines (Aupeix and others 1995), thymocytes (Christ and others 1993), and smooth muscle cells (Ares and others 1997). Therefore, the cytotoxicity of oxLDL can best be accounted for by the formation of these oxysterols.

Oxysterols are 27-carbon products of cholesterol oxidation (Brown and Jessup 1999). Various oxysterols have been detected in appreciable quantities in human tissue and fluids, including human plasma, atherogenic lipoproteins, and atherosclerotic plaque. How these oxysterols originate *in vivo* is not fully known. Some researchers claim that they arise principally from dietary sources while others contend that they are generated by non-enzymatic oxidation *in vivo* (Brown and Jessup 1999).

Of the various oxysterols, 7-ketocholesterol has been reported to be the predominant sterol in oxLDL (Zhang and others 1990; Jialal and others 1991; Brown and others 1996; Chang and others 1997). To lesser contents, 7α-OH-cholesterol and 7β-OH cholesterol

are also major oxysterols detected in oxLDL. 25-hydroxycholesterol (25-OHC) was detected at a very low level in oxLDL, however it is the most extensively studied due to its ability to inhibit HMG-CoA reductase activity (Smith and Johnson 1989), reduce transcription of LDL receptor (Sudhof and others 1987), stimulate cellular cholesterol esterification by the activation of ACAT (Brown and others 1975; Zhang and others 1990), and induce apoptosis in a variety of different cell types (Smith and Johnson 1989). 25-OHC down regulates HMG-CoA reductase and the LDL receptor by blocking the proteolytic activation of two sterol-regulated transcriptional factors, sterol response element binding protein-1 (SREBP-1) and SREBP-2 (Yang and others 1995b). It has been found in human atheromatous plaques and aortic tissues (Hodis and others 1991) and proposed to disrupt endothelial cell barrier function, disorganize cytoskeletal protein, and inhibit gap junctional communication. As a model compound, 25-OHC has been used to induce apoptosis in monocyte, macrophage, and lymphoid cell lines in the range of 1-10 μM. 7-ketocholesterol has also been shown to induce apoptosis in vascular endothelial and smooth muscle cells (Nishio and Watanabe 1996; Lizard and others 1998), as well as macrophages (Liao and others 2000; Geng and others 2003).

The relative toxicities of oxysterols to cells *in vitro* depend very much on the conditions under which exposure is conducted, the cell type, and even the species. For example, the concentration of oxysterol required to achieve detectable toxicity is higher in the presence of serum or lipoproteins than in serum-free media; this is probably due to the capacity of lipoproteins to absorb oxysterols and reduce their effective concentration as well as direct antagonism of oxysterol toxicity by cholesterol (Clare and others 1995; Colles and others 1996; Nishio and Watanabe 1996).

Oxysterols induce apoptosis in many cell types including vascular cells such as endothelial cells (Ramasamy and others 1992), macrophages, SMC, and lymphocytes.

Evidence includes oxysterol-stimulated DNA fragmentation (Christ and others 1993; Aupeix and others 1995; Clare and others 1995; Ares and others 1997), decreased expression of Bcl-2 (Nishio and Watanabe 1996), activation of Bax (Rusinol and others 2004), activation of the protease caspase 3 (a key mediator of apoptotic cell death) (Ares and others 1997). Caspase 3 inhibition partially blocked apoptosis induced by oxysterol (Nishio and Watanabe 1996).

Oxysterol induced apoptosis proceeds partially through the mitochondrial death pathway (Panini and Sinensky 2001). It has been demonstrated lately (Rusinol and others 2004) that oxysterols induce the degradation of the prosurvival protein kinase Akt (protein kinase B), which leads to the activation of proapoptotic proteins Bim and Bad and down-regulation of the antiapoptotic Bcl-x_L. These responses would be expected to activate the proapoptotic protein Bax and Bak, leading to the release of cytochrome *c*.

Free Cholesterol-induced Apoptosis As stated above, the toxicity of oxLDL/oxysterols is partially accounted for the induction of apoptosis in atherosclerotic lesions. In addition, free cholesterol accumulation in foam cells has also been reported to be cytotoxic (Ross 1995; Yao and Tabas 2000; Yao and Tabas 2001). Macrophages in atherosclerotic lesions have been shown to accumulate large amounts of free cholesterol (Shio and others 1979; Rapp and others 1983; Small and others 1984; Lundberg 1985). Free cholesterol loading of cultured macrophages is a potent inducer of cell death (Warner and others 1995; Tabas and others 1996) probably through the inhibition of certain critical plasma membrane enzymes (Papahadjopoulos 1974; Yeagle 1991; Kellner-Weibel and others 1998). In fact, Papahadjopoulos demonstrated 30 years ago

that free cholesterol-mediated inhibition of two critical plasma membrane enzymes, (Na^+ K^+)-ATPase and adenylate cyclase, led to cellular death and proposed that these events may play an important role in the development of atherosclerosis.

Most recently, free cholesterol loading in cultured mouse peritoneal macrophages has been reported to induce apoptosis by activating both the death receptor pathway (Yao and Tabas 2000) and the mitochondrial pathway (Yao and Tabas 2001). Increased level of Bax protein in both cellular and mitochondrial fractions has been observed as early as 4 h after free cholesterol loading in cells (Yao and Tabas 2001).

In addition, ER has also been reported to be the site of free cholesterol-induced cytotoxicity in macrophages (Feng and others 2003). Free cholesterol loading leads to the depletion of ER Ca^{2+} storage, an event known to induce the unfolded protein response (UPR). Activation of UPR results in the expression of the cell death effector CHOP, a transcription factor activated during the cell responses to injury associated with ER stress (Zinszner and others 1998; Oyadomari and others 2002). In the presence of selective inhibitor of cholesterol trafficking to the ER, the UPR, caspse-3 activation, and apoptosis are markedly inhibited. Consistently, *Chop-/-* macrophages are protected from cholesterol-induced apoptosis.

Mouse Models of Atherosclerosis

Mice are highly resistant to atherosclerosis. On a low cholesterol, low fat diet, they typically have cholesterol levels of <100 mg/dl, mostly contained in the antiatherogenic HDL fraction, and they do not develop lesions. However, when they are fed a very high cholesterol, high fat diet, their plasma cholesterol levels rise by a factor of two to three,

with the majority now in the non-HDL fraction (Breslow 1996). After months on this diet, certain inbred strains, such as C57BL6, develop several layers of foam cells in the subendothelial space near the aortic valve leaflets. Although promising, this model has two problems. First, lesions often occur at the branch points of major vessels and progress to the fibrous plaque stage in humans, but only occur in the region of aortic valve leaflets in C57BL6 mice and do not progress past the fatty streak stage. Second, the diet required to induce atherosclerosis is unphysiological, as it contains 10 to 20 times the cholesterol of a Western-type diet.

The apolipoprotein E deficient mouse (ApoE-/-) was the first model generated with these two problems resolved (Plump and others 1992; Zhang and others 1992). ApoE, which is made primarily in the liver, is a surface constituent of almost all lipoprotein particles other than LDL, and it is a ligand for lipoprotein recognition and clearance by lipoprotein receptors LDLR and chylomicron remnant receptor. In apoE-/- mice, accumulations of chylomicron and very low density lipoprotein (VLDL) remnants are the major stimuli for atherosclerosis. These mice develop not only fatty streaks but also widespread fibrous plaque lesions spontaneously at vascular sites typically affected in human atherosclerosis (aortic valve leaflet regions and branch points of major vessels). The apoEs produced by bone marrow derived macrophages are sufficient to inhibit the development of atherosclerosis in apoE-/- mice (Linton and others 1995).

The LDLR deficient mouse (LDLR-/-) is another frequently used model in atherosclerosis studies (Ishibashi and others 1994). As described before, LDLR is expressed by all cell types so far studied. The liver is responsible for about 70% of the total body LDLR-mediated uptake of lipoproteins. Cell surface LDL receptors recognize

apolipoprotein B on LDL and apoE on IDL and remove these lipoproteins from the circulation. LDLR activity is also expressed by human monocyte-derived macrophages (Traber and Kayden 1980) and arterial macrophage-derived foam cells (Jaakkola and others 1989). However, LDLR expressed by macrophages has no significant effect on the development of atherosclerosis (Boisvert and others 1997; Fazio and others 1997b; Herijgers and others1997). LDLR-/- mice have increased plasma cholesterol, with most of the increase in the IDL and LDL lipoprotein fractions. However, unlike apoE-/- mice, LDLR-/- mice do not develop atherosclerosis on low-cholesterol, low-fat diet. While fed a Western diet (0.15% cholesterol and 21% fat), they produce massive fatty streak lesions with lipid-rich necrotic core covered with foam cells and/or fibrous caps which mainly localize in the aortic valve leaflet regions.

There are two versions of apolipoprotein B as described above, apo B100 and apo B48. In human, apoB100 is produced by liver and secreted in VLDL, while apoB48 is generated by small intestine and secreted in chylomicron. The apoB100-containing VLDLs give rise to apoB100-containing IDLs, which are cleared from plasma almost entirely by LDLR (Brown and Goldstein 1983; Havel 1987). The apoB48-containing chylomicrons are hydrolysed into chylomicron remnant and cleared by chylomicron remnant receptor (Scott 1989; Chan 1992; Higuchi and others 1992). In LDLR deficiency, the apoB100-containg IDLs remain in the the plasma where they are converted into LDL. Mouse livers, unlike those of humans, produce both apo B100 and apo B48, which are incorporated into VLDL (Scott 1989; Chan 1992; Higuchi and others 1992). The apoB48-containing VLDLs, like intestinal chylomicrons, generates apoB48-containing IDLs that can bind to chylomicron remnant receptors, and rapidly cleared even in the

absence of LDLR (Kita and others 1982; Rubinsztein and others 1990). Thus, the LDLR-/- mice do not give rise to appreciable amounts of plasma LDL as happens in human homozygous for *LDLR* gene described above.

Bone Marrow Transplantation (BMT)

The possibility of BMT came first from studies in the mouse. At the end of World War II following the atomic bomb explosions, there was a great deal of interest in how radiation damages living organisms. It became recognized that the marrow is the organ most sensitive to radiation and that death following low-lethal exposures was due to marrow failure.

Early transplantation studies documented the ability of bone marrow cells to repopulate lethally irradiated animals (Lorenz and others 1951). Subsequent studies have shown that approximately 10^5 nucleated marrow cells are necessary to reestablish hematopoiesis in murine-irradiated hosts (Boggs and others 1982; Harrison and Astle 1982). Marrow grafts are traditionally established by infusing unmanipulated suspensions of aspirable, donor-derived marrow cells into the respective recipient. The marrow suspension consists of a complex mixture of cells that includes the essential population of repopulating cells as well as cells that are part of the regulatory network of hematopoiesis, such as T lymphocytes. Marrow given intravenously was just as effective in repopulating the marrow spaces as marrow given by any other route (Van Bekkum and others 1956). The marrow cellularity in recipient increases rapidly over the following 2 to 4 weeks after BMT, and shows morphological evidence of all myeloid components (van den Berg and others 1990).

Conditioning programs serve to suppress the recipient's immune system for acceptance of the marrow graft and to eradicate the recipient's underlying disease that made treatment by BMT necessary. Total body irradiation has been the most commonly used conditioning program for BMT. Mice not given a marrow infusion usually die from complications of marrow failure within 2 weeks after a lethal dosage of irradiation. Consistent survival following lethal doses of total body irradiation can be obtained by giving an infusion of marrow, fresh or cryopresserved (Mannick and others 1960; Cavins and others 1962).

Complete engraftment can be sustained by a limited number of hematopoietic clones. Introduction of genetic markers into individual hematopoietic stem cells provided a method of tracing their progeny (Lemischka and others 1986). A period of instability in the clonal composition of the graft was observed early after BMT. However, the clonal composition of the graft stabilized after some weeks and remained constant for the life span of the animals. In addition, the marrow of engrafted animals appears to contain repopulating cells at a number that is sufficient to reestablish hematopoiesis in a second generation of recipients. These observations suggest that murine hematopoietic clones can be expanded to function for at least two successive generations of BMT recipients (Jordan and Lemischka 1990).

In bone marrow derived cells, macrophages affect the development of atherosclerosis most. Recently, BMT has been broadly used to study the physiological or pathological functions of macrophages and macrophage gene expression in atherogenesis (Linton and others 1995; Herijgers and others 1997). By repopulating the recipient mice with the donor marrow either deficient in or overexpressing a gene of interest, the significant

functions of many proteins produced by macrophages have been defined. For example, apoE produced by bone marrow derived macrophages has been found to be sufficient to inhibit atherosclerosis in apoE-/- mice (Linton and others 1995). The *in vivo* effects on atherogenesis of many other key genes have also been defined through BMT, such as ACAT1 (Fazio and others 2001), lipoprotein lipase (Babaev and others 1999), NF-κB (Kanters and others 2003), etc.

As described above, LDLRs expressed by bone marrow derived macrophages have no significant effect on the development of atherosclerosis in LDLR-/- mice (Boisvert and others 1997; Fazio and others 1997b; Herijgers and others 1997); however, apoEs produced by bone marrow derived macrophages are sufficient to inhibit the development of atherosclerosis in apoE-/- mice (Linton and others 1995). Thus, in the studies of atherosclerosis, LDLR-/- mice are more often chosen as the model than apoE-/- mice because LDLR-/- mice can be directly reconstituted by bone marrow isolated from donor mice of interest. In certain cases that apoE-/- mice are appreciated, most often apoE-/- mice have to be interbred with the mice of interest, generating springs that carrying the genotypes of both parents, which is time-consuming and highly unefficient.

Specific Aims

The goal of the present studies is to determine the consequences of macrophage apoptosis in atherogenesis. Because of the well established and accepted role of Bax in apoptosis, as well as the specific observations implicating Bax activation in macrophage apoptosis associated with atherogenesis, we chose to examine whether Bax deficiency

will produce resistance, at least partially, to oxysterol-induced macrophage apoptosis *in vitro* by inhibiting Bax expression in cultured macrophage cell line P388D1 using siRNA. We also sought to determine if primary macrophages isolated from Bax-/- mice were resistant to the induction of apoptosis by oxysterols. The *in vivo* effect of Bax deficiency in macrophages on the development of atherosclerosis was investigated by transplanting atherosclerosis susceptible mice with Bax positive (WT) or deficient (Bax-/-) bone marrow. Because the donor mice are positive for both apoE and LDLR, LDLR-/- mice were chosen as the model for the current study due to the reasons described above.

CHAPTER 2

MATERIALS AND METHODS

Materials

Cell culture media and supplements were obtained from BioWhitaker (Walkersville, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Oxysterols were from Steraloids (Newport, RI). All the other reagents were obtained from Fisher Scientific and were of the highest quality available.

Mice

Both male LDLR-/- recipient mice (B6.129S7-Ldlr^{tm1Her}) and Bax-/- donor mice (B6.129X1-Bax^{tm1Sjk}) are from The Jackson Laboratory (Bar Harbor, ME) and on C57BL6 background. All mice were maintained in microisolator cages on autoclaved rodent chow containing 4.5% fat (Purina Mills Inc., St. Louis, MO) and autoclaved acidified water (pH 2.8). Experimental protocols were performed according to the regulations of Vanderbilt University's Animal Care Committee and East Tennessee State University Animal Care Committee.

SiRNA Construct for *Bax*

To suppress the expression of *Bax*, a plasmid that generates siRNAs targeting Bax mRNA for degradation, SiBax, was constructed using pSilencer1.0 U6 vector (Ambion

Inc., Austin, TX) (Fig 4). Two complementary oligonucleotides were annealed and cloned into the ApaI/EcoRI-digested pSilencer: Bax-Forward, ACTGGTGCTCAAGGCCCTGTT CAAGAGACAGGGCCTTGAGCACCAGTTTTTTT; and Bax-Reverse, AATTAAAAAAAC TGGTGCTCAAGGCCCTGTCTCTTGAACAGGGCCTTGAGCACCAGTGGCC. To make the negative control vector R-Bax, two complementary oligonucleotides with a randomized Bax target sequence were annealed and cloned into the ApaI/EcoRI-digested pSilencer: R-Bax-Forward, ACCGCTCGAGCGTGCTAGTTTCAAG AGAACTAGCACGCTCGAGCGGTTTTTTT; and R-Bax-Reverse, AATTAAAAAAACCG CTCGACGGTGCTAGTTCTCTTGAAACTAGCACGCTCGAGCGGTGGCC. The ligation reactions were performed using a Fast-Link DNA ligation kit (Epicentre, Madison, WI) according to manufacturer's instructions. A portion of the ligation products was used to transform competent JM109 cells (Promega, Madison, WI). The recombinant cells were selected on LB plates containing 50 µg/ml ampicillin. Selected colonies were grown in 100 ml LB medium containing 50 µg/ml ampicillin. Plasmids were purified using a QIAfilter plasmid midi kit (Qiagen, Valencia, CA) as directed. The plasmids were then sequenced (Molecular Biology Core Facility at ETSU) using T3 primer to verify the proper construction.

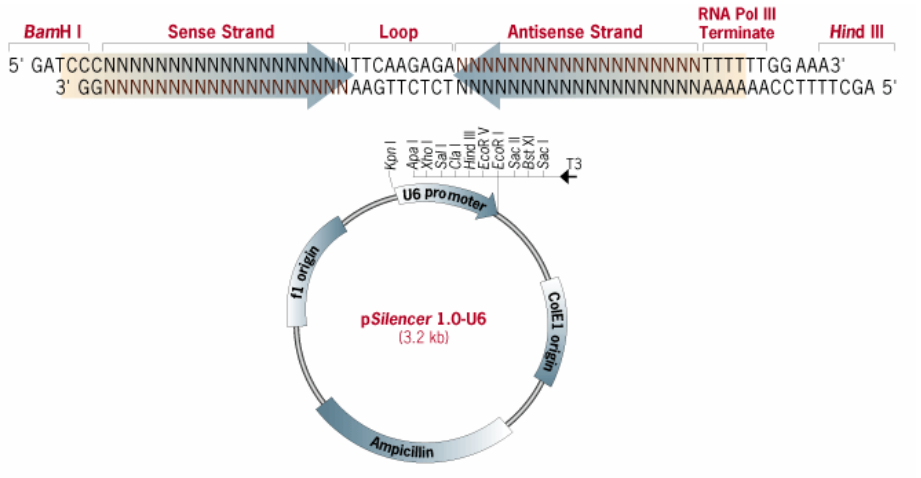


Figure 4 pSilencer 1.0-U6 Vector. A representative insert is shown with stem region in the hairpin structure shaded. (http://www.ambion.com/techlib/misc/vectors/1.0_U6.html)

Stable Suppression of *Bax* gene in P388D1 Cells

All cell cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. P388D1 cells, a mouse lymphoblast cell line with macrophage-like morpholgy, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Stable suppression of *Bax* gene was achieved by cotransfecting P388D1 cells (2 x 10^6) with a plasmid vector carrying the neomycin-resistant gene, pEGFP (Clontech, Palo Alto, CA), and either SiBax or R-Bax using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Stable clones were first selected by culturing single cell/well with 100 µl medium containing 1 mg/ml G418 in 96-well microculture plate for 7 days, followed by 14 days in 0.25 mg/ml G418. The integration of the pSilencer vector into host genomes of the G418 resistant clones was then screened

by polymerase chain reaction (PCR) on a PCR Express thermocycler (Hybaid) using primers complementary to the vector sequences: pSi-Forward, CTCCCCGTCGTGTAGA TAACTACG; pSi-Reverse, GTCGCCGCATACACTATTCTCAG.

The PCR reaction solution contained ~100 cells per reaction, 200 μM dNTP mix, 0.2 μM of each primer, 1.25 units of ExTaq DNA polymerase in 1x ExTaq DNA polymerase buffer (Takara Bio, Inc., Shiga, Japan). After 30 PCR cycles (94 °C for 1 min, 57°C for 45 sec, 72°C for 1 min), the 500-bp PCR product was separated by gel electrophoresis on 1.0% agarose gel, and then extracted using gel extraction kit (Qiagen, Valencia, CA). To further confirm the PCR product was derived from the integrated vector sequence, the PCR product was subjected to restriction analysis using restriction enzyme PvuI, whose restriction site was included in the original pSilencer vector. The digestion process was expected to generate two fragments, 374 bp and 126 bp.

PCR positive and G418-resistant SiBax clones were then subjected to selection in medium containing 10 μg/ml 25-OHC for 68 h. The surviving SiBax cells were expanded and maintained in medium containing 0.5 mg/ml G418.

Immunoblotting to Examine Bax Suppresion in P388D1 cells

The suppression of Bax expression was determined by immunoblotting whole cell lysates prepared from the isolated SiBax clones and G418-resistant R-Bax clones. P388D1 cells were collected by centrifugation at 100 x *g* for 5 min. After rinsing with cold phosphate buffered saline (PBS), the cells were treated with lysis buffer containing 20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, and 1 μg/ml

leupeptin. Unbroken cells and membrane debris were removed by centrifugation at 10,000 x *g* for 10 min, and the protein concentration of the supernatant was determined by micro-BCA assay (Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE on 4-12% NuPage gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp.). The blots were blocked in 3% non-fat milk in PBS and then probed using antibodies specific for Bax (Cell Signaling Tech., Inc., Beverly, MA) at 1:1000 dilution, Bak (Cell Signaling Tech., Inc.) at 1:2000 dilution, Akt (Santa Cruz Biotehnology, Santa Cruz, CA) at 1:2000 dilution, and horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:5000 dilution. The protein bands were visualized by enhanced chemiluminescence using SuperSignal® West Pico chemiluminescent substrate (Pierce) as directed.

Caspase 3 Activity Assay in P388D1 cells

P388D1 cells were seeded at 2 x 10^6/well in 12-well culture plates and cultured in DMEM medium supplemented with 5% FBS plus 10 µg/ml 25-OHC or an equivalent volume of ethanol alone (control). Following a 16-h incubation, cells were collected by centrifugation at 1000 x *g* for 5 min, washed with PBS, resuspended in lysis buffer (10 mM Tris, pH7.5, 130 mM NaCl, 1% Triton X-100, 10 mM sodium P_i, and 10 mM sodium PP_i). After 10 min incubation on ice, unlysed cells and membrane fraction were removed by centrifugation at 12,000 x *g* for 20 min at 4 °C. The sample protein concentration was determined using micro-BCA kit (Pierce). Caspase 3 activity was assayed by incubating equivalent protein amounts of each sample in caspase assay buffer (20 mM Hepes, pH7.5, 10% glycerol, and 2 mM dithiothreitol) containing 5 µM caspase 3 substrate

(Ac-DEVD-7-amino-4-trifluoromethyl coumarin, BioMol, Plymouth Meeting, PA) at 37 °C for 2.5 h in the presence or absence of 100 μM caspase 3 inhibitor DEVD-CHO (BioMol). Liberated 7-amino-4-trifluoromethyl coumarin was measured using a spectrofluorometer (FluroMax 3 equipped with a microplate reader, Jobin-Yvon Inc.) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm. For each sample, the net caspase-3 activity was determined by subtracting the relative fluorescent light units (RFUs) obtained in the absence of DEVD-CHO from the relative RFUs obtained in the presence of DEVD-CHO, and normalized to the protein content of the sample. The results were presented as the mean±S.D. of triplicate samples.

Peritoneal Macrophage Isolation and Caspase 3 Activity Assay

Mouse peritoneal macrophages were isolated 3 days after intraperitoneal injection with 1.5 ml of 6% thioglycollate. Cells were seeded in DMEM at 1 x 10^6/well in 12-well culture plates. The nonadherent cells were washed away with PBS 2 hrs after seeding, and the culture medium was changed to DMEM+5% FBS. After the overnight incubation, cells were treated with 10 μg/ml 7-KC dissolved in ethanol in the culture medium for 12 hrs. Each treatment was performed in triplicate. Control groups were treated with an equal volume of ethanol. The caspase 3 activity was assayed using CPP32/Caspase-3 fluorometric protease assay kit from Chemicon International Inc. (Temecula, CA) according to the manufacturer's directions. For each sample, 100 μl of cell lysate was incubated with 100 μl reaction buffer plus 1 μl of caspase 3 substrate (Ac-DEVD-7-amino-4-trifluoromethyl coumarin) in a 96-well plate at 37 °C for 1.5 hrs. The relative fluorescent unit (RFU) of each sample generated by 7-amino-4-trifluoromethyl

coumarin was read by Fusion spectrofluorometer (Perkin-Elmer, Boston, MA) at an excitation wavelength wavelength of 400 nm and an emission wavelength of 485 nm. A mixture of 100 µl cell lysis buffer and 100 µl reaction buffer plus 1 µl substrate was used for a blank reading, which was subtracted from all the experimental readings. For each sample, the final RFU was then normalized to the protein concentration of the sample determined using micro-BCA kit (Pierce). The data were presented as the mean±S.D. of triplicate treatments.

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL)

Mouse peritoneal macrophages were cultured at 500,000 cells/chamber in DMEM supplemented with 5% FBS in 4-chamber Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL). Following the incubation with either 10 µg/ml 25-OHC or 0.5 µg/ml staurosporine (a protein kinase inhibitor), cells were fixed in 4% paraformaldehyde in PBS and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate on ice for 5 min. After incubation with a 50 µl mixture of deoxynucleotidyl transferase and nucleotide containing fluorescein-labeled dUTP (ApoAlert DNA fragmentation assay kit, Clontech, Palo Alto, CA) at 37 °C for 1 h in a humidified chamber, cells were washed with PBS twice and counterstained with propidium iodide (PI). TUNEL positive cells and PI staining positive cells were counted in 10 viewfields (400x) under fluorescent microscope with GFP and rodamine filters. The data were expressed as the mean percentage of TUNEL positive cell number per field / PI staining positive cell number per field ± S.D.

Bone Marrow Transplantation

To study the *in vivo* effect of Bax deficiency in macrophages on the development of atherosclerosis, LDLR-/- mice were reconstituted with bone marrow isolated from either WT or Bax-/- donor mice. One week before and 2 weeks after transplantation, all recipient LDLR-/- mice were given acidified water containing 5 mg/L neomycin and 25000 units/L polymyxin B sulfate (Monarch Pharmaceuticals, Bristol, TN). Eight-week-old male LDLR-/- mice received 10 Gy whole body irradiation from a cesium gamma source and were transplanted with $5x10^6$ bone marrow cells from WT C57BL6 or Bax-/- donor mice as described (Linton and others 1995). Briefly, the bone marrows from femurs and tibias of donor mice were flushed with RPMI 1640 medium (Gibco, Invitrogen) supplemented with 2% FBS (Hyclone, Logan, UT) and heparin 5 units/ml. Single cell suspension was prepared by passing the bone marrow through a 27 gage needle several times. After washing with fresh RPMI 1640 plus 2% FBS, bone marrow cells were resuspended in RPMI 1640 without FBS. Five million bone marrow cells in 0.2 ml RPMI media were injected into the retro-orbital venous plexus of each recipient mouse. Three weeks later they were fed a Western diet (21% fat and 0.15% cholesterol; Harlan Teklad, Madison, WI) for 10 weeks.

Determination of Chimerism by Western blotting. Before sacrifice, peritoneal macrophages were collected from 4 recipient mice randomly chosen from each group. Whole cell lysate was prepared in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail 1% (Sigma). Protein concentration was determined by modified Lowry

assay using D_c protein assay reagent (Bio-Rad). Protein samples (20 µg each) were separated on a NuPage 10% Bis-Tris Gel, and transferred onto Hybond-P PVDF membrane (Amersham Biosciences). Bax protein was detected using rabbit polyclonal anti-Bax (Upstate Biotech, Lake Placid, NY) at 1:1000 dilution and HRP-conjugated goat anti-rabbit IgG (Upstate Biotech) at 1:5000 dilution. ECL reagents (Amersham Biosciences) were used for the visualization. The same blot was stripped in a buffer containing 2% SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, at 55 °C for 10 min and reprobed for beta-actin using rabbit polyclonal anti-beta actin (Abcam, Cambridge, MA.) and HRP-conjugated goat anti-rabbit IgG both at 1:5000 dilution.

Determination of Chimerism by PCR. Genomic DNA extracted from whole blood 8 weeks after transplantation was subjected to PCR using primers for wild type or mutant LDLR gene. We took advantage of the fact that the donor mice (wild type or Bax-/-) were LDLR+/+, while the recipients in both groups were LDLR-/-. Eight weeks after bone marrow transplantation, 6 or 7 mice from each group were randomly picked. After overnight fasting, 100 µl of blood was drawn by retro-orbital venous plexus puncture under anesthesia. The genomic DNA was extracted using PUREGENE DNA purification system (Gentra Systems, Minneapolis, MN). The PCR reactions (94°C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min, 30 cycles) were carried out on a thermocycler (Applied Biosystems, Foster City, CA) using LDLR+/+ specific downstream primer 5'-CGCAGTGCTCCTCATCTGACTTGTCTTGTTGT-3', LDLR-/- specific downstream primer 5'-AGGTGAGATGACAGGAGATC-3'. The common upstream primer was: 5'-ACCCCAAGACGTGCTCCCAGGATGA-3'. Each reaction contained 50 ng DNA

template, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 2.5 units of recombinant DNA Taq polymerase, and 1x PCR buffer. All the PCR reagents were from Invitrogen. DNA samples from WT C57BL6 and LDLR-/- mice were used as the controls. A 400 bp PCR product was expected from the WT *LDLR* gene, while an 800 bp PCR product was expected from the interrupted *LDLR*.

Serum Cholesterol, Triglyceride Assay, and Lipoprotein Analysis by FPLC

Blood samples were collected by retro-orbital venous plexus puncture under anesthesia after overnight fasting. Serum was separated by centrifugation at 10,000 x *g* for 10 min at 4 °C. Total cholesterol and triglycerides were determined using Cholesterol Reagent and Triglycerides GPO reagent kits (Raichem, San Diego, CA). Serum samples were diluted at 1:100 in diH_2O. For each sample, 100 µl sample dilution was mixed with 100 µl Cholesterol Reagent or Triglycerides GPO reagent in 96-well plate. For standard solutions, 6 different concentrations were provided: 0.5, 1.0, 1.5, 3.0, 6.0, and 10.0 mg/dl. Following 10-min incubation at 37 °C, the sample absorbance was read using a spectrophotometer at 500 nm for cholesterol and 520 nm for triglycerides. Each sample was assayed in triplicate.

To analyze the serum lipoprotein profiles, several serum samples in each group were randomly chosen and subjected to gel filtration chromatography as described (Fazio and others 1997a). A Superose 6 10/300 GL column (Amersham Biosciences, Piscataway, NJ) on a fast performance liquid chromatography (FPLC) system model 600 (Waters Chromatography, Milford, MA) was used. One hundred microliter of each serum sample before the Western diet or 70 µl after the Western diet was loaded into the column.

Lipoproteins were separated using a buffer containing 0.15 M NaCl, 0.01 M Na_2HPO_4, 1 mM EDTA (pH 8.0), at a flow rate of 0.5 mL/min. Forty 0.5 ml fractions were collected and analyzed for cholesterol concentrations as described above, and the cholesterol concentrations were then divided by the total sample volume loaded into the column for comparison between the different time points.

Peripheral White Blood Cell Count

Blood smears were prepared from tail blood 12 weeks after bone marrow transplantation under anesthesia, and subjected to Quick-Dif staining (Vanderbilt Histochemistry Core Lab). White blood cells were differentiated into lymphocyte, monocyte, and granulocyte by scoring a minimum of 200 cells under a light microscope.

Analysis of Atherosclerotic Lesions

Sectioning, staining, and quantitative analysis. After 10 weeks on Western diet, mice were sacrificed and perfused with 20 ml of PBS through the left ventricle. The heart with proximal aorta attached was embedded in OCT compound and snap-frozen in liquid nitrogen. All samples were stored at -80 °C before sectioning. Every other 10-μm cross section was collected starting from the end of the aortic sinus as described (Paigen and others 1987). Fifteen cryosections from each mouse were stained with Oil Red O (Sigma) to visualize the lipid contents and counterstained with Mayer's hematoxylin (Electron Microscopy Sciences, Hatfield, PA). Quantitative analysis of the lesion area was performed blindly using a KS300 Imaging Version 3.0 connected to a Carl Zeiss Vision microscope (Carl Zeiss Vision GmbH, Germany).

Immunohistochemistry. Serial 5 μm cryosections from proximal aortas were fixed in acetone at -20 °C for 10 min and stained at 4 °C overnight with either rat anti-mouse macrophages/monocyte marker MOMA-2 (Accurate Chemical & Scientific Corp., Westbury, NY) diluted at 1:1000 in the blocking buffer (5% mouse serum in PBS), or smooth muscle alpha actin epitope specific rabbit antibody (Lab Vision, Fremont, CA) diluted at 1:200 in the blocking buffer (5% goat serum in PBS). After briefly rinsing with PBS, the sections were then incubated with either biotinylated mouse anti-rat IgG_{2b} antibody (BD Biosciences Pharmingen, San Diego, CA) at 1:200 dilution in the blocking buffer or biotin labeled goat anti-rabbit IgG polyclonal antibody (BD Biosciences Pharmingen) at 1:200 dilution at room temperature for 30 min. Following the inactivation of endogeneous peroxidase in 0.3% H_2O_2/methanol at room temperature for 30 min, the sections were incubated with 50 μl streptavidin-HRP complex (BD Biosciences Pharmingen) at room temperature for 30 min, developed with diaminobenzidine (DAB) enhanced liquid substrate system (Sigma) at room temperature for 1-2 min, and then counterstained with methyl green (Vector Labs, Burlingame, CA) at 55 °C for 5 min. After brief rinsing in PBS, the sections were air-dried and mounted in Permount (Fisher Scientific). The staining was analyzed using AxioVision 3.1 connected with Axioplan2 imaging microscope (Carl Zeiss Vision GmbH, Germany).

TUNEL staining. An *in situ* cell death detection POD kit (Roche Applied Science, Indianapolis, IN) was used with slight modification. Five-micron cryosections were pretreated with 3% citric acid to eliminate free calcium in order to remove false staining of calcium-containing matrix vesicles (Kockx and others 1996). Then the sections were fixed

in 4% paraformaldehyde in PBS, and permeabilized in 0.1% Triton X-100, 0.1% sodium citrate on ice for 5 min. After incubation with deoxynucleotidyl transferase (5 µl) and fluorescein-labeled nucleotide mixture (45 µl) in a humidified chamber at 37 °C for 1 h, sections were treated with anti-fluorescein antibody conjugated with HRP in a dark humidified chamber at 37 °C for 30 min. The sections were then developed with DAB and counterstained with methyl green as described above. Four serial sections from each mouse were stained and the data for each individual mouse were expressed as the average TUNEL positive cell number per mm^2 lesion area. For each group, the data were expressed as mean±SD.

Statistics

Nonparametric Mann-Whitney test was used to measure the statistical differences in lesion area. Student's *t* test assuming two samples with equal variances was used in other analyses. $P<0.05$ was considered to be statistically significant.

CHAPTER 3

RESULTS

Effect of Bax Knockdown in Macrophage-like Cell Line P388D1

The goal of the present studies is to determine the consequences of macrophage apoptosis in atherogenesis. Because of the well established and accepted role of Bax in apoptosis, as well as the specific observations implicating Bax activation in macrophage apoptosis resulting from free cholesterol loading as well as treatment with oxLDL or oxysterols, we chose to examine whether Bax deficiency will produce resistance, at least partially, to oxysterol-induced macrophage apoptosis *in vitro* by inhibiting Bax expression in cultured macrophage-like cell line P388D1 using siRNA. The single-stranded SiBax transcript contains 20-nucleotide complementary sequences (bold): 5'-**ACTGGTGCTCA AGGCCCTGT**TCAAGAG**ACAGGGCCTTGAGCACCAGT**TTTTTT-3'. It forms a hairpin structure with the complementary sequence in the stem region. Upon cleavage by Dicer, the stem region will then form a functional double-straned siRNA, which would be expected to bind and degrade the target Bax mRNA. The double-stranded small RNA formed by the cleavage of R-Bax transcript (5'-**ACCGCTCGAGCGTGCTAGTT**TCAAG AG**AACTAGCACGCTCGAGCGGT**TTTTTT-3') is not expected to interfere with Bax expression due to its sequence nonspecificity.

Stably transfected P388D1 cells were selected as described in Materials and Methods. Several G418 resistant clones, three transfected with SiBax (S1, 7, 14) and two transfected with R-Bax (R1, 2), were selected. Caspase 3 activity was examined after a 16-h treatment with 10 μg/ml 25-OHC. The increase of caspase 3 activity was

significantly lower in clone S1, 7, and 14, compared to that in clone R1 and R2, indicating decreased apoptotic activities in clone S1, 7, and 14 (Fig 5).

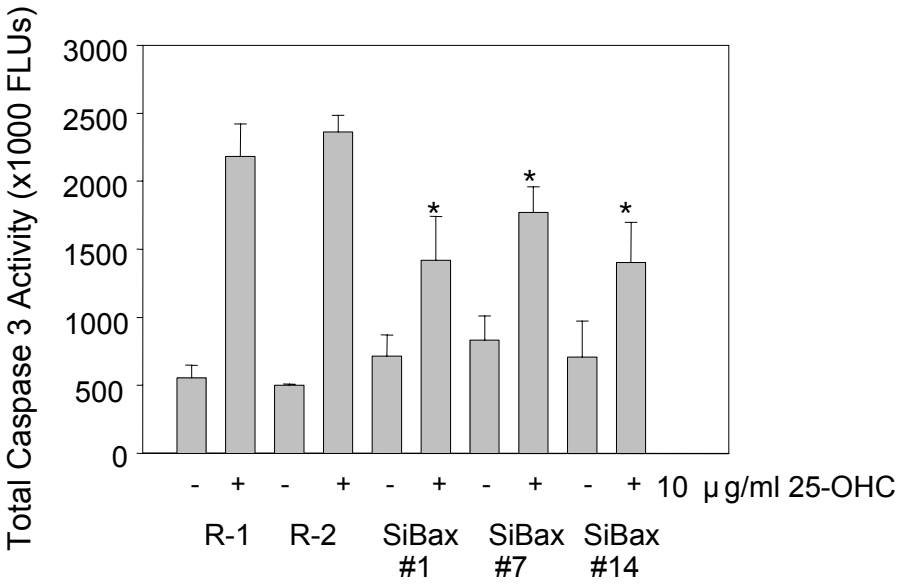


Figure 5 Caspase 3 activity assay in R-Bax and SiBax stably transfected clones. Cells were treated with 10 µg/ml 25-OHC for 16 h. Each treatment was performed in triplicate, and data were presented as mean±S.D. *P< 0.05 compared to R1 treated with 25-OHC.

Suppression of Bax in P388D1 Cells Transfected with SiBax

Clones S1 and R1 were selected based upon the caspase 3 assay results and examined for the suppression of Bax protein by immunoblotting (Fig 6). The expression of Bax was significantly reduced in clone S1 comparing with that in clone R1. As expected, suppression of Bax did not affect the expression of other proteins such as Akt/ protein kinase B or Bak in either of these clones, which indicates the high specificity of the insert sequence in SiBax.

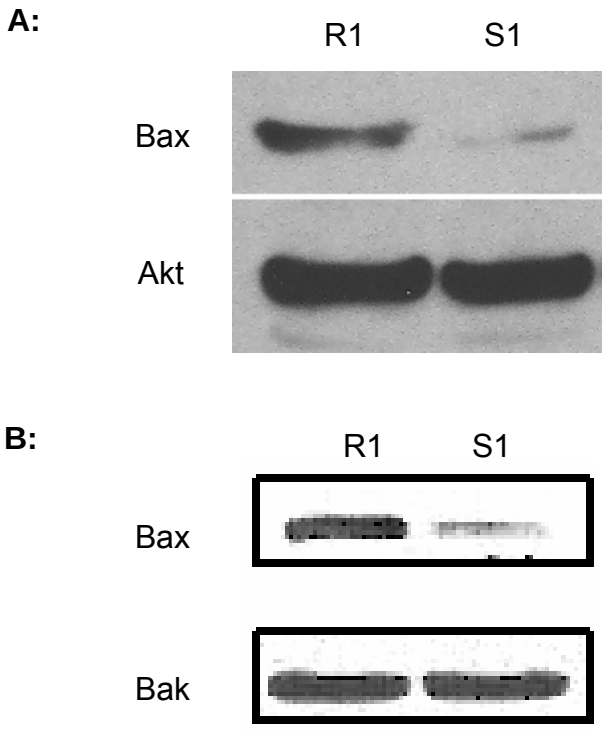


Figure 6 Suppresion of Bax in P388D1 cells by siRNA. Whole cell lysates from clone R1 and S1 were resolved by SDS-Page on 4-12% NuPage gel, and probed for Bax and Akt (A) or for Bax and Bak (B). Neither Akt nor Bak expression level was affected in clone S1 with significant Bax suppression comparing to that in clone R1.

Reduced Caspase 3 Activity in P388D1 Cells with Bax Suppression

Because the G418 resistant SiBax clones were only partially resistant to the induction of caspase activity by 25-OHC, clone S1 was further selected by incubating with 10 μg/ml 25-OHC for 68 h. The surviving cells were expanded and assayed for the caspase 3 activity assay. Clone R1 and the oxysterol selected clone S1 were cultured in medium containing increasing amounts of 25-OHC for 16 h. Control cells received an equal amount of vehicle only (ethanol). Caspase 3 activity was determined as described in

Materials and Methods. Induction of apoptosis by 25-OHC was greatly attenuated in clone S1 comparing with that in clone R1 regardless of the normal expression of Bak (Fig 7). About 5.5 fold increase was observed in R1 cells treated with 20 μg/ml 25-OHC, however, almost no induction was found in S1 cells under the same treatment.

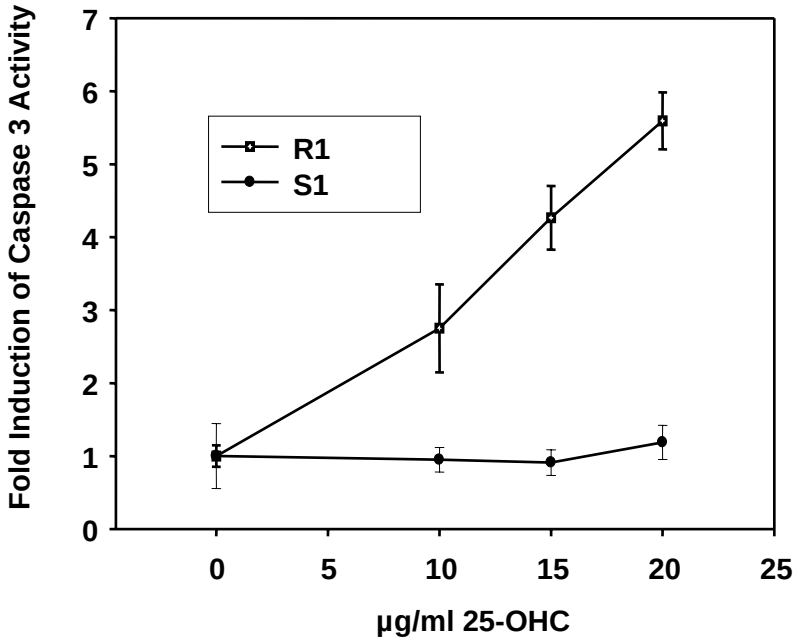


Figure 7 Caspase 3 activity assay in clone R1 and S1. After 68-hour selction in 10 μg/ml 25-OHC, cells were treated for 16 h with increasing amounts of 25-OHC. Each treatment was done in triplicate, and the data presented as the mean ± S.D. of the fold increase in caspase 3 activity compared to the untreated cells.

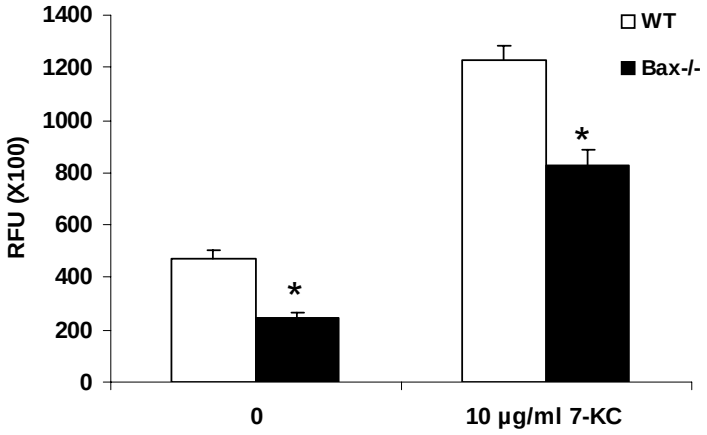
Bax Deficient Mouse Peritoneal Macrophages Display Reduced Levels of Apoptosis *in vitro* upon Stimulation by Oxysterols or Staurosporine

Our siRNA experiments successfully demonstrated that suppression of Bax leads to decreased apoptotic activity in response to 25-OHC in P388D1 cells, a mouse

lymphoblast cell line with macrophage-like morpholoIgy. In order to directly examine the impact of Bax deficiency on apoptosis in mouse macrophages, peritoneal macrophages were isolated from WT C57BL6 and Bax knockout mice. The caspase 3 activity was assayed after 12 h treatment with 10 μg/ml 7-ketocholesterol, one of the major cytotoxic components of oxLDL (Panini and Sinensky 2001). The caspase 3 activity was significantly lower in Bax null macrophages treated with 7-ketocholesterol compared to that in WT macrophages treated with 7-ketocholesterol ($P<0.05$) (Fig 8A).

To further determine the effect of Bax deficiency on apoptosis, mouse peritoneal macrophages were treated with either 25-OHC or staurosporine and subjected to TUNEL staining, an alternative technique to identify apoptotic cells. Consistent with the results from caspase 3 activity assay, Bax null macrophages also showed less TUNEL positive cells compared with the WT cells after treatment with either 25-OHC or staurosporine (Fig 8B, $P<0.05$). Therefore, consistent with prior studies with Bax knockdown P388D1 cells (Fig 5 and 7), Bax null mouse peritoneal macrophages are also partially resistant to apoptosis induced by oxysterols. They are also partially resistant to staurosporine, which also acts through the mitochondrial pathway. This is consistent with previous report that Bax deficient mouse embryonic fibroblast cells shows partial resistance to the apoptosis induction by staurosporine (Wei and others 2001).

A: Caspase 3 activity assay



B: TUNEL assay

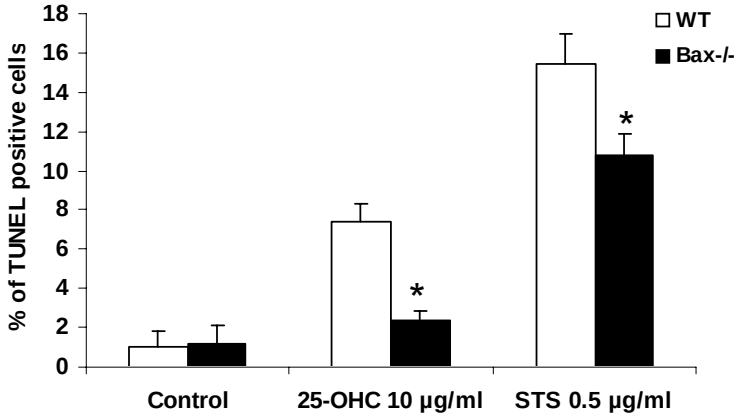


Figure 8 Bax deficient mouse peritoneal macrophages display reduced apoptosis *in vitro*. A: Caspase 3 activity assay after 12 h treatment with 7-ketocholesterol (7-KC). Relative fluorescent unit (RFU) was normalized to protein concentration of the sample. Data represent mean±SD of three independent experiments. B: TUNEL staining after the treatment with 25-OHC for 40 h or staurosporine (STS) for 21 h. Each bar represents the mean value of 10 viewfields under 400x magnification. Data represent the mean±SD, *P<0.05 compared to the WT group.

Complete Reconstitution of LDLR-/- Mice with WT or Bax-/- Bone Marrow

As expected, our *in vitro* studies show that Bax deficiency leads to significantly reduced apoptosis in mouse peritoneal macrophages in response to agents that activate the mitochondrial death pathway. To investigate the consequences of macrophage apoptosis in the development of atherosclerosis *in vivo*, 8-week old LDLR-/- mice (male, n=14 in each group) were lethally irradiated and transplanted with $5x10^6$ bone marrow cells isolated from WT or Bax null donor mice (male). The reconstitution of recipient mice with donor bone marrow-derived hematopoietic cells was determined by immunoblotting 13 weeks post bone marrow transplantation. Bax protein was detected in the peritoneal macrophages isolated from the WT group, but not in those from the Bax-/- group (Fig 9).

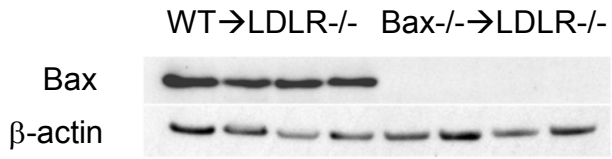


Figure 9 Immunoblotting to examine the chimerism of reconstitution. Whole cell lysates were prepared from peritoneal macrophages isolated from four recipient mice randomly chosen from each group 13 wks after bone marrow transplantation and separated by SDS-PAGE. Immunoblotting was performed as described above. The same blot was stripped and reprobed for β-actin as the loading control.

The level of reconstitution was also determined by PCR of the LDLR gene as both sets of donor bone marrow cells were LDLR positive. Consistently, 8 weeks after the bone marrow transplantation, a 400 bp PCR product of the wild type LDLR gene was detected in the genomic DNA extracted from the whole blood of all mice from both groups. In

addition, the 800 bp PCR product of the mutant LDLR gene in both groups was below the detectable level after 30 PCR cycles indicating a complete reconstitution of the recipient hematopoietic cells by the donor bone marrow cells (Fig 10).

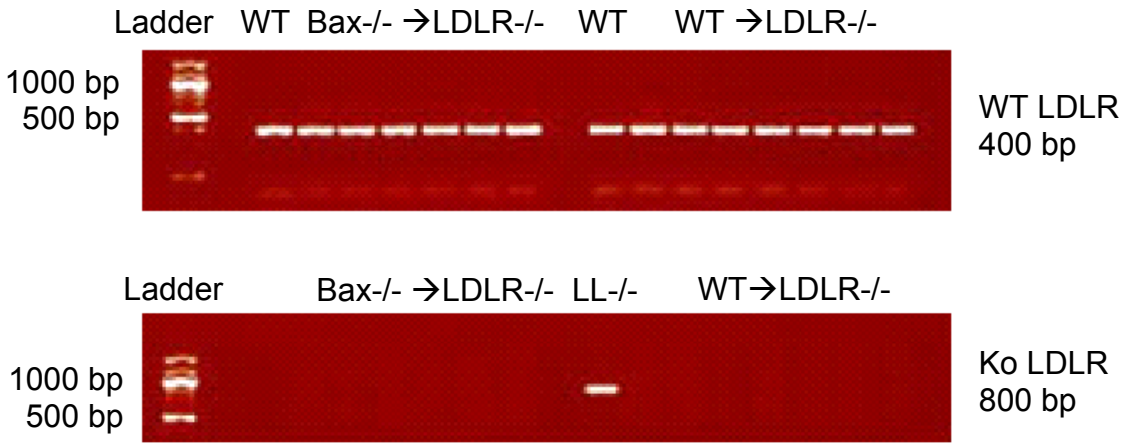


Figure 10 PCR assay to examine the chimerism of reconstitution. DNA samples from 6 mice in the Bax-/- transplantation group and 7 mice in the WT transplantation group were subjected to PCR assay using primers either for the WT LDLR gene (400 bp product) or the knockout (Ko) LDLR gene (800 bp product). For detecting WT LDLR gene, genomic DNA from WT C57BL6 mouse was used as the positive control (WT). For detecting Ko LDLR gene, genomic DNA from LDLR-/- mouse was used as the positive control (LL-/-).

Bax Deficiency in Bone Marrow Derived Cells Does not Affect the Plasma Cholesterol and Triglyceride Levels

Macrophages, native or bone marrow graft derived, express a variety of proteins that affect lipoprotein clearance and cholesterol metabolism through a number of different pathways, and they have been shown to influence the progression of atherosclerosis

(Linton and Fazio 2001; Linton and Fazio 2003). To examine whether Bax deficiency in bone marrow derived cells, especial macrophages, would affect cholesterol metabolism in the host, the plasma lipid parameters were assessed. Plasma samples were collected before the start of Western diet as the baseline and every 4 weeks thereafter. As shown below, Bax deficiency in macrophages did not affect the plasma cholesterol (Fig 11A) and triglyceride levels (Fig 11B). Both groups displayed a gradual increase in the plasma cholesterol level as time went by, from ~200 mg/dl before Western diet to ~600-700 mg/dl 8 wks after. The changes in plasma triglyceride levels were less dramatic than those observed in plasma cholesterol. In both groups, the average plasma triglyceride level was around 80 mg/dl before BMT and increased to 110 mg/dl 8 wks after BMT. This was expected because LDL is the major cholesterol carrier in the plasma as shown in Table 1. As a result, the accumulation of LDL in the plasma will mainly affect the cholesterol concentration in the plasma.

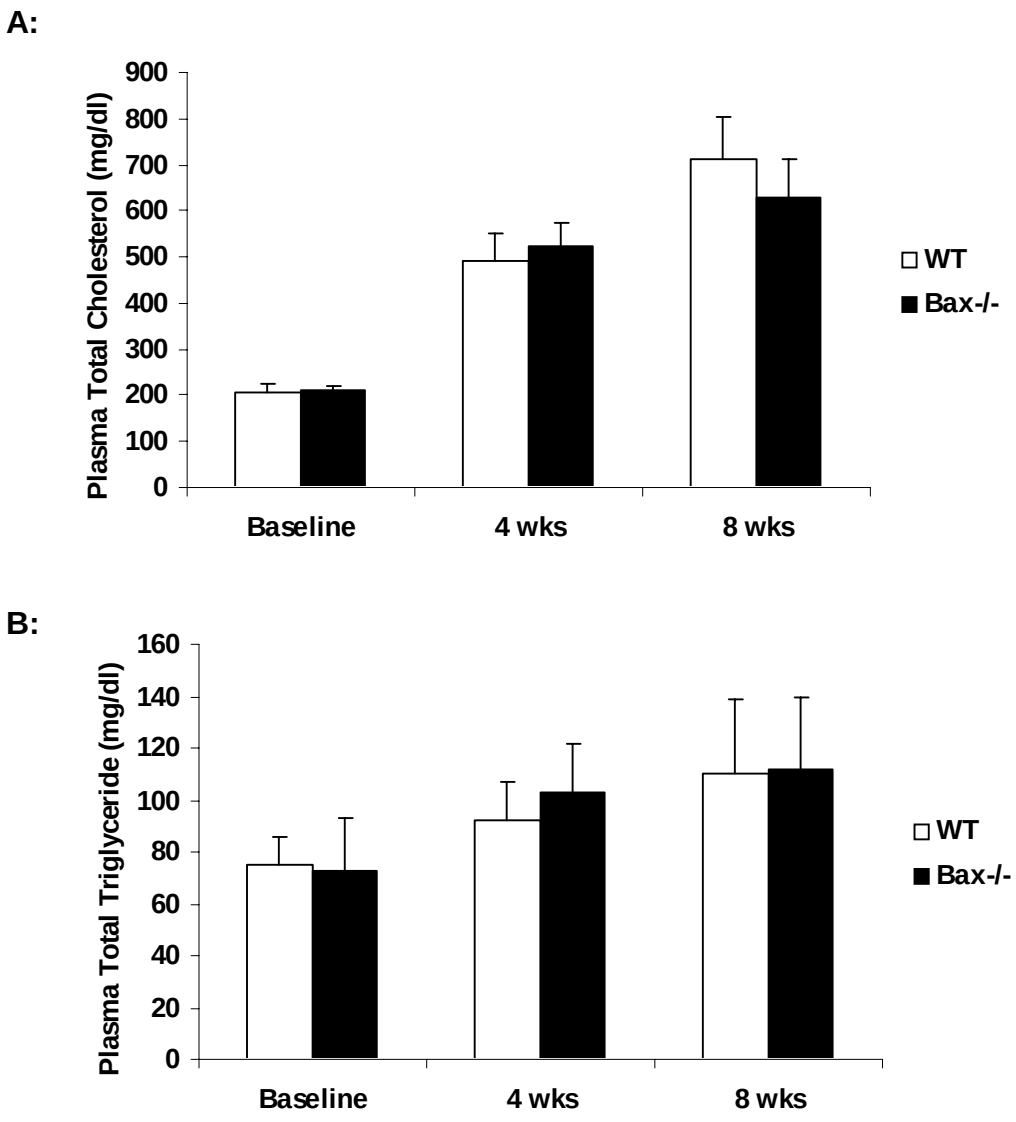


Figure 11 Plasma total cholesterol (A) and triglyceride (B) levels. Data were collected before (baseline) and every 4 wks after the beginning of Western diet. P>0.05 compared to WT group.

Bax Deficiency in Bone Marrow Derived Cells Has no Influence on Plasma Lipoprotein Profile

Elevated plasma cholesterol concentrations, especial LDL concentrations, are strongly correlated with atherosclerotic disease. Shifting between different serum lipoprotein particles can also significantly affect the incidence of coronary heart disease and the rate of progression of coronary lesions (Goldstein and Brown 1979; Hobbs and others 1992). To examine whether Bax deficiency in bone marrow derived cells would affect the plasma lipoprotein profile, plasma samples from both the control and Bax-/- groups were analysed by FPLC before and every 4 wks after the beginning of Western diet. Before Western diet, almost half of the lipoprotein particles (25 mg/dl) were anti-atherogenic HDL in both groups, leaving the rest mainly in LDL (20 mg/dl) with very low level of VLDL (<5 mg/dl). After 4 weeks of Western diet, both VLDL and LDL fractions increased dramatically with the peak value between 50-60 mg/dl. With the profile shifting towards the non-HDL fractions, the plasma lipoproteins became more atherogenic (Breslow 1996). After another 4 weeks of Western diet, the peak value of LDL fraction remained at around 60 mg/dl, while the peak value of VLDL accelerated to over 70 mg/dl. Western diet did not affect the HDL fraction, and its peak value remained at the same level as that before the diet. However, as shown below, no differences were found between the two groups (Fig 12), indicating Bax deficiency in bone marrow derived cells does not affect the lipoprotein clearance, and consequently, the shift in lipoprotein profiles induced by Western diet.

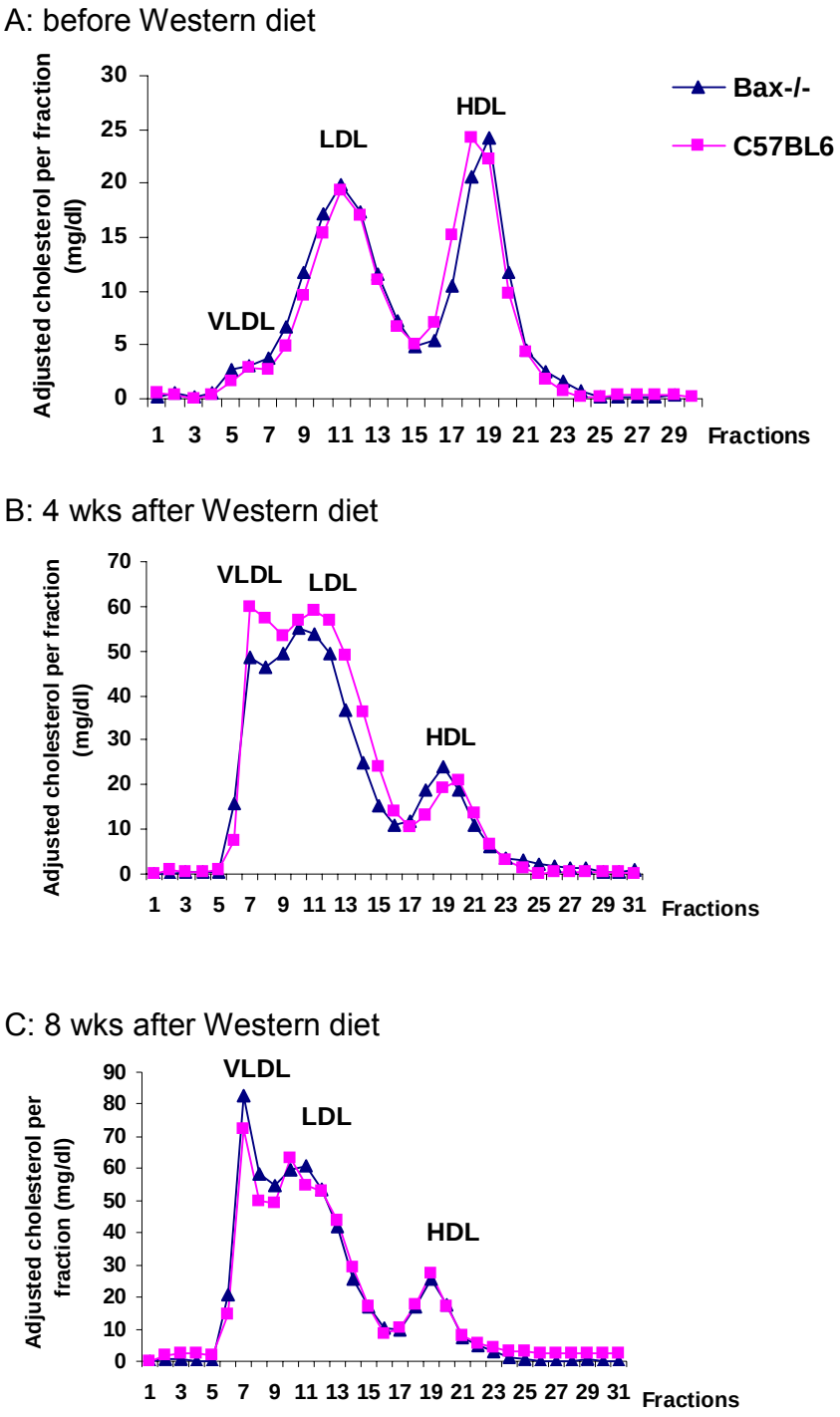


Figure 12 Plasma lipoprotein profiles obtained by FPLC. Plasma samples from individual mice were analysed. The results shown are representative curves from each group.

Bax Deficiency in Bone Marrow Derived Cells Does not Affect Peripheral Lymphocyte Population

Bax deficient mice have been reported to have selective hyperplasia of lymphoid tissues (Knudson and others 1995). The influence of lymphocytes on atherosclerosis has been reported by many groups (Daugherty and others 1997; Major and others 2002; Pinderski and others 2002; Tanigawa and others 2003). To determine whether Bax deficiency in bone marrow graft would increase the peripheral lymphocyte number, we examined the peripheral leukocytes in the blood smears obtained from all recipient mice by Quick-dif staining 12 weeks after bone marrow transplantation. Differential white blood cell counts revealed no differences in the peripheral lymphocyte populations between two groups ($P>0.05$). In the control group, about 81.3±2.9 % peripheral leukocytes were lymphocytes, compared to 79.5±8.7 % in the Bax-/- group. Thus, Bax deficiency in bone marrow derived cells did not affect the peripheral lymphocyte population (Fig 13).

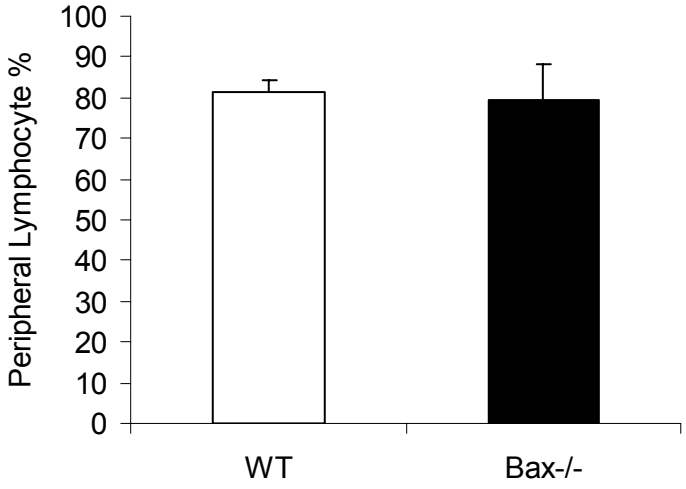


Figure 13 Peripheral lymphocyte counts 12 weeks after bone marrow transplantation. No differences were found between the WT and Bax-/- groups. $P>0.05$.

Bax Deficiency in Macrophages Accelerates the Development of Atherosclerosis

To examine the influence of Bax deficiency on the development of atherosclerosis, all recipient mice were sacrificed after 10 weeks on a Western diet. The atherosclerotic lesions in the proximal aortas were stained with Oil Red O. Quantitative analysis of the extent of atherosclerosis revealed a 49.2% increase in the mean cross sectional lesion area (μm^2±SEM) in the proximal aortas of Bax-/- group (233,800±20,489 μm^2) compared with that in the control group (156,700±20,243 μm^2) (Fig 14, P=0.0259). Thus, LDLR-/- mice reconstituted with bone marrow null for Bax display significantly higher degree of atherosclerotic lesion formation.

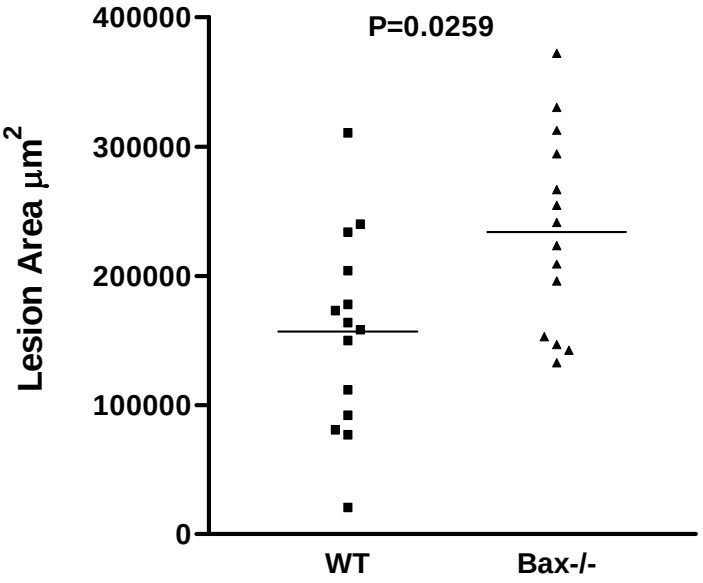


Figure 14 Lesion area quantitation in cross sections of proximal aortas after 10 weeks on Western diet. A 49.2% increase in the mean cross sectional lesion area was found in the Bax-/- group compared to that in the WT group. Each data point represents the mean lesion area per cross section of 15 sections per mouse, line represents the mean value of each group, n=14 mice per group, P=0.0259 by nonparametric Mann-Whitney test.

To further characterize the atherosclerotic plaques, macrophages and SMCs in serial cross sections of the proximal aortas from both groups were stained with cell type specific antibodies (Fig 15). Immunocytochemical analysis demonstrated that the lesions contained predominantly macrophage-derived foam cells. Lesions were classified into two types based on the prior literature (van Vlijmen and others 1994; Breslow 1996; Kanters and others 2003): early lesions mainly composed of macrophage derived foam cells and advanced lesions characterized by the presence of SMCs and collagen in the cap area. Interestingly, 31% of the lesions in the Bax-/- group, compared to only 17% in the control group, were advanced lesions with fibrous caps. These fibrous plaques from both groups were larger in size compared to those without SMC staining in the cap region, indicating they were in a more advanced stage. These lesions often contained necrotic cores while observing under higher magnification. Taken together, our studies show that Bax deficiency in bone marrow derived cells not only stimulates the formation of atherosclerosis in LDLR null mice fed a Western diet but may also accelerate the progression to more advanced lesions as well.

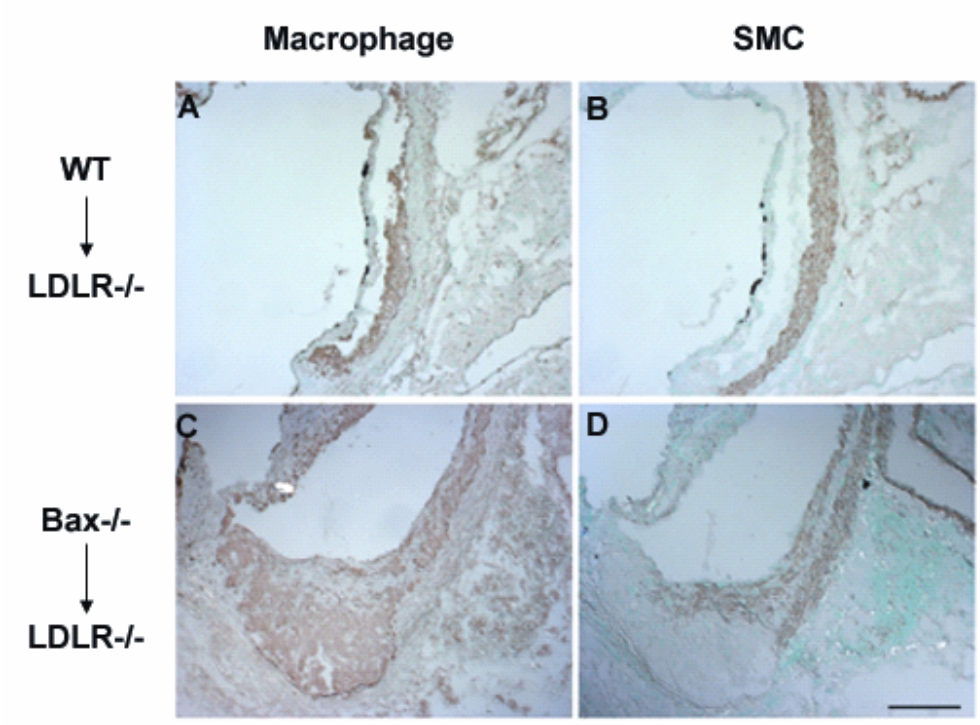


Figure 15 Immunostaining for macrophages in the lesion (A,C) and SMCs in the arterial wall and lesion cap area (B,D) in serial cross sections from proximal aorta. A,B: serial cross sections from WT group with a smaller lesion consisting mainly of macrophage derived foam cells. C,D: serial cross sections from Bax-/- group showing a larger lesion consisting of both macrophage-derived foam cells and SMCs in the cap region. Scale bar represents 200 μm.

Deficiency of Macrophage Bax Leads to Reduced Apoptosis in Atherosclerotic Lesions

As described in figure 8, Bax null macrophages display reduced levels of apoptosis *in vitro*. To investigate whether the increased lesion area in the Bax null transplantation group was associated with decreased apoptotic activity *in vivo*, modified TUNEL was carried out on cross sections of proximal aortas. The majority of apoptotic cells in the lesions were macrophages. Consistent with the proposed role for Bax in macrophage

apoptosis, the number of apoptotic macrophages was decreased by 53% ($P<0.05$) in the Bax-/- group (15.3±9.9 per mm^2) compared to that of the control group (32.5±10.8 per mm^2, Fig 16). Thus, the increased average lesion size (~50%) of Bax-/- group is associated with the reduced apoptotic activity (53%) in the lesional macrophages in this group.

Interestingly, the SMCs in the proximal aorta from the Bax-/- group also had reduced apoptosis compared to those from the WT group. About 8.6±6.3 apoptotic SMCs per mm^2 were observed in the Bax-/- group, while 25.6±10.3 apoptotic SMCs per mm^2 were observed in the control group (Fig 16C, $P<0.05$). This difference could be due to the altered interactions between macrophages and SMCs in the Bax-/- group, or the origination of SMCs from Bax-/- bone marrow. This will be discussed in greater detail in the discussion section.

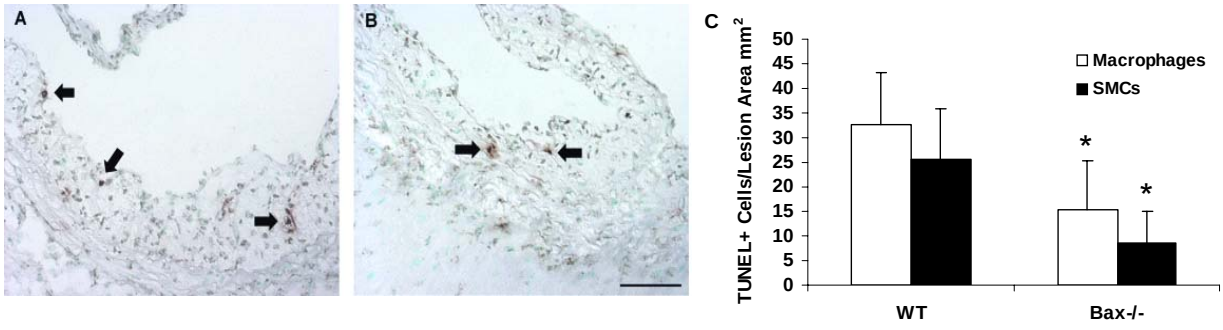


Figure 16 *In situ* TUNEL staining in proximal aorta sections. Sections from the control group (A) and the Bax-/- group (B) were labeled as described above and counterstained with methyl green. Arrows indicate TUNEL positive nuclei (brown), Scale bar represents 200 μm. C: Quantitation of TUNEL positive macrophages and SMCs per mm^2 lesion area, n=14, *$P<0.05$ compared to WT group.

CHAPTER 4

DISCUSSION

Macrophages are one of the most important cell types in atherosclerotic lesions, not only because the lesions are mainly composed of macrophages, as observed in figure 15, but also because macrophages can generate a variety of protein molecules (such as apoE, apoAI, and ACAT etc.) that affect lipoprotein clearance and cholesterol metabolism, which are two significant factors influencing the progression of atherosclerosis.

The evidence of apoptosis in atherosclerotic lesions has been broadly reported, and apoptosis mainly happens in the macrophage population in the lesion (Ball and others 1995; Lusis 2000). Possible causes of macrophage death in atherosclerotic lesions include exposure to oxidized lipoproteins, cholesterol-induced cytotoxicity (Warner and others 1995; Tabas and others 1996), growth factor deprivation (Chin and others 1999), and exposure to other arterial wall factors such as inflammatory cytokines and nitric oxide (Mitchinson and others 1996; Lopez-Collazo and others 1998). However, it remains unclear whether the apoptotic events in the lesion will prevent or facilitate the development of atherosclerosis due to lack of direct evidences.

In the current study, our goal was to determine the influence of macrophage apoptosis in atherogenesis, which was achieved through two steps: 1) we chose one of the proapoptotic proteins - Bax as our experimental target and examined the effect of Bax knockdown on apoptosis induction in macrophages; 2) we reconstituted the LDLR-/- mice, an atherogenesis-prone model, with bone marrow isolated from either WT or Bax null mice, and assessed the development of atherosclerosis in both groups.

Bax was chosen because of the well accepted role of Bax in the mitochondrial apoptosis pathway, as well as the specific observations implicating Bax activation in association with macrophage apoptosis resulting from free cholesterol loading, and oxLDL or oxysterol treatment (Yao and Tabas 2000; Yao and Tabas 2001; Rusinol and others 2004). Although Bax deficient mouse embryonic fibroblast cells have been shown to be partially resistant to the induction of apoptosis by staurosporine (Wei and others 2001), no observations have been reported in terms of the effect of Bax deficiency on apoptosis induction in macrophages. To seek direct answers, we chose to use 1) a macrophage-like cell line P388D1 with Bax expression suppressed by specific siRNA, and 2) mouse peritoneal macrophages isolated from Bax deficient mice. Both cell types showed decreased apoptotic activities upon the stimulation by oxysterols compared to control cells (Fig 7,8), demonstrating that Bax deficiency in macrophages provided partial resistance to apoptosis induction *in vitro.* Partial resistance was expected because the functions of Bax have been shown to be redundant with Bak and only cells lacking both Bax and Bak are totally defective in the mitochondrial pathway (Wei and others 2001).

The reconstitution of LDLR-/- mice with Bax null bone marrow resulted in a significant increase in mean lesion area as compared with the mice reconstituted with wild type bone marrow (Fig 14,15). As expected, fewer apoptotic macrophages were found in the Bax-/- group (Fig 16), which was not only consistent with our *in vitro* experimental results, but also consistent with our hypothesis that the increase in lesion size is due to decreased apoptotic activity in the macrophage. In addition, higher numbers of advanced lesions with fibrous caps were found in the Bax-/- group (Fig 15). Our data demonstrate that macrophage apoptosis plays a protective role in the development of atherosclerosis. This

is the first report that directly demonstrates a link between the mitochondrial apoptotic pathway and atherogenesis.

Induction of apoptosis by oxysterol is mediated via the mitochondrial death pathway and most probably occurs within atherosclerotic lesions (Panini and Sinensky 2001). However, because of the central role that Bax plays in the mitochondrial apoptosis pathway, the reduction of apoptosis in atherosclerosis lesions observed in mice transplanted with Bax-/- bone marrow would be expected for a broad spectrum of apoptotic inducers. For this reason, our observations are more relevant to the role of macrophage apoptosis in atherogenesis than to the role of any specific physiological apoptotic inducer.

Some prior studies have attempted to determine the role of apoptosis in atherogenesis by means of p53 null mice. Macrophages deficient in the tumor suppresser protein p53 have been reported to enhance atherosclerosis in apoE*3-Leiden transgenic mice, which was correlated with a decrease in apoptosis in the p53 deficient mice (van Vlijmen and others 2001). Increased atherosclerosis has also been reported in another study using p53-/-apoE-/- mice, although the authors did not find significant change in apoptotic cell numbers but, rather, an increase in cell proliferation (Guevara and others 1999). These contradictory results probably reflect the multiple functions of p53 which acts to integrate a number of cellular signals to regulate either cell cycle arrest or apoptosis rather than acting as a specific regulator of the mitochondrial death pathway (Bennett 1999; Haupt and others 2003). In contrast, Bax deficiency directly affects the mitochondrial pathway, which can be stimulated by various signals including the activation of p53.

In another study of the role of apoptosis in atherosclerosis, the *bcl-x_L* expression, one of the anti-apoptotic Bcl-2 family members, in neointimal cells in carotid arteries of rabbits was inhibited by transfection with anti-*bcl-x* antisense oligonucleotides (Pollman and others 1998). The reduced apoptosis induction within intimal cells was observed, as well as an acute regression of lesions in carotid arteries, which is consistent with the results of our study. However, under these experimental conditions, the cell types that took up the antisense oligonucleotides were uncertain. In contrast, the current study is clearly focused on lesion macrophages derived from Bax null donor bone marrow. Our results more specifically demonstrate the effect of the mitochondrial apoptotic pathway in lesion macrophages on the development of atherosclerosis.

Bcl-2 family proteins also affect the survival of lymphocytes (Cory 1995; Cheng and others 2004). Bax deficient mice have been reported to have selective hyperplasia of lymphoid tissues (Knudson and others 1995). The influence of lymphocytes on the development of atherosclerosis, a chronic inflammatory disease, has been reported by many groups (Daugherty and others 1997; Major and others 2002; Pinderski and others 2002; Tanigawa and others 2003). In searching for any additional mechanistic explanation for increased lesion area in mice transplanted with Bax-/- bone marrow, we examined the peripheral leukocytes in all recipient mice by Quick-dif staining. No differences were found in the peripheral lymphocyte content between the two groups. However, increased inflammation in Bax-/- group cannot be excluded since lesions in this group contain higher number of macrophages, one of the mediators of inflammatory response in atherosclerotic lesions (Libby 2002; Linton and Fazio 2003), compared to lesions in the WT group.

Although the reduced apoptotic activity in lesional macrophages provides an explanation for the increased atherosclerotic lesion size, there are a number of other pro-atherogenic aspects of lesion macrophages. Mice deficient in both macrophage colony-stimulating factor (MCSF) and apoE (op/apoE) have smaller proximal aortic lesions at earlier stages of lesion progression and decreased blood monocyte differentials (Smith and others 1995). Consistent with this observation, intraperitoneal administration of antibody for the MCSF receptor prevents the initial events of atherogenesis (Murayama and others 1999). Therefore, a lowered level of apoptosis in Bax deficient macrophages would also be expected to be pro-atherogenic by certain macrophage dependent processes such as increased number and/or functions of monocyte-derived macrophages, increased growth factor production, cell proliferation, or foam cell accumulation (Linton and Fazio 2003).

Recently, *Chlamydia pneumoniae* has been reported to inhibit apoptosis in THP-1 cells (Carratelli and others 2002) and HeLa cells (Fischer and others 2001). Reduction of cytochrome *c* release was characterized in *Chlamydia pneumoniae* infected Hela cells. Human CMV immediate-early proteins have also been reported to inhibit apoptosis occurence in the coronary artery smooth muscle cells by antagonizing the function of p53 protein. The reduced apoptotic activity in vascular cells following the viral/bacterial infection thus may provide an explanation for the pro-atherogenic roles of *Chlamydia pneumoniae* and CMV in humans, which is consistent with our demonstration that macrophage apoptosis is an anti-atherogenic event.

Our observation that mice reconstituted with Bax null bone marrow have decreased SMC apoptosis is also of interest. Intermediate plaques are often covered by a fibrous

cap consisting of SMCs and interstitial collagen fibers synthesized by SMCs. Loss of SMCs in the cap area will lead to a decrease in the production of the interstitial collagen fibers and, consequently, thinning of the fibrous cap. Plaque rupture occurs when the mechanical stresses in the fibrous cap exceed a critical level that the cap tissue can withstand. The interaction between macrophages and SMCs has long been controversial. For example, platelet-derived growth factor (PDGF) produced by macrophages has been shown to induce the migration and proliferation of SMCs (Shimokado and others 1988; Ross and others 1990; Shen and others 2001). However, a number of other studies have demonstrated that macrophage-derived factors could kill SMCs (Boyle and others 2002; Seshiah and others 2002; Boyle and others 2003). Our data showed increased SMC content in the cap and shoulder region of the lesions and reduced SMC apoptosis in the lesions of LDLR-/- mice reconstituted with Bax null bone marrow. Although the origin of these SMCs has not yet determined, the possibility that these SMCs are derived from Bax deficient bone marrow can not be excluded according to the recent reports (Sata and others 2002; Caplice and others 2003). The increased SMC content in the lesions of the Bax-/- group may also be due to a stimulation of SMC migration caused by Bax deficiency in bone marrow derived macrophages or lymphocytes. The mechanism by which this may occur is unclear at present. Because the increased SMCs and extracellular matrix content in fibrous plaque have been suggested to be critical factors in increasing plaque stability in human atherosclerosis (Libby and Aikawa 2002), it will be very interesting to investigate whether apoptosis inhibition in vascular SMCs could benefit the plaque stability in atherosclerosis.

In summary, our studies show that Bax deficiency in macrophages leads to reduced apoptosis both *in vitro* and *in vivo*. In atherosclerotic lesions, the reduced apoptotic activity in macrophages due to Bax deficiency promotes the development of atherosclerosis in LDLR null mice on a Western diet. Analogous to other apoptosis-associated diseases, such as cancer, autoimmune disease, and certain viral infections, failure to undergo apoptosis in atherosclerotic lesions leads to the excessive accumulation of cells, indicating that macrophage apoptosis provides a critical self-defense mechanism in suppressing atherosclerosis..

Mitochondria-targeting pro-apoptotic drugs have been under broad development for a wide range of applications, from cancer chemotherapy, HIV, to organ transplantation (Zangemeister-Wittke and Ziegler 1998; Morisaki and Katano 2003). Our data indicate that the mitochondrial apoptosis pathway in vascular macrophages may be an attractive target for the prevention and treatment of atherosclerosis.

REFERENCES

Anant S, Davidson NO. 2001. Molecular mechanisms of apolipoprotein B mRNA editing. Curr Opin Lipidol 12(2):159-65.

Antonsson B, Montessuit S, Sanchez B, Martinou JC. 2001. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem 276(15):11615-23.

Ares MP, Porn-Ares MI, Thyberg J, Juntti-Berggren L, Berggren PO, Diczfalusy U, Kallin B, Bjorkhem I, Orrenius S, Nilsson J. 1997. Ca2+ channel blockers verapamil and nifedipine inhibit apoptosis induced by 25-hydroxycholesterol in human aortic smooth muscle cells. J Lipid Res 38(10):2049-61.

Auge N, Andrieu N, Negre-Salvayre A, Thiers JC, Levade T, Salvayre R. 1996. The sphingomyelin-ceramide signaling pathway is involved in oxidized low density lipoprotein-induced cell proliferation. J Biol Chem 271(32):19251-5.

Aupeix K, Toti F, Satta N, Bischoff P, Freyssinet JM. 1996. Oyxsterols induce membrane procoagulant activity in monocytic THP-1 cells. Biochem J 314 (Pt 3):1027-33.

Aupeix K, Weltin D, Mejia JE, Christ M, Marchal J, Freyssinet JM, Bischoff P. 1995. Oxysterol-induced apoptosis in human monocytic cell lines. Immunobiology 194(4-5):415-28.

Babaev VR, Fazio S, Gleaves LA, Carter KJ, Semenkovich CF, Linton MF. 1999. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. J Clin Invest 103(12):1697-705.

Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. 1985. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41(3):899-906.

Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ. 1995. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. Atherosclerosis 114(1):45-54.

Bennett MR. 1999. Mechanisms of p53-induced apoptosis. Biochem Pharmacol 58(7):1089-95.

Boggs DR, Boggs SS, Saxe DF, Gress LA, Canfield DR. 1982. Hematopoietic stem cells with high proliferative potential. Assay of their concentration in marrow by the frequency and duration of cure of W/Wv mice. J Clin Invest 70(2):242-53.

Boisvert WA, Spangenberg J, Curtiss LK. 1997. Role of leukocyte-specific LDL receptors on plasma lipoprotein cholesterol and atherosclerosis in mice. Arterioscler Thromb Vasc Biol 17(2):340-7.

Boyle JJ, Weissberg PL, Bennett MR. 2002. Human macrophage-induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas-L interactions. Arterioscler Thromb Vasc Biol 22(10):1624-30.

Boyle JJ, Weissberg PL, Bennett MR. 2003. Tumor necrosis factor-alpha promotes macrophage-induced vascular smooth muscle cell apoptosis by direct and autocrine mechanisms. Arterioscler Thromb Vasc Biol 23(9):1553-8.

Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. 2003. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22(53):8608-18.

Breslow JL. 1996. Mouse models of atherosclerosis. Science 272(5262):685-8.

Brison DR, Schultz RM. 1997. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biol Reprod 56(5):1088-96.

Brown AJ, Dean RT, Jessup W. 1996. Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. J Lipid Res 37(2):320-35.

Brown AJ, Jessup W. 1999. Oxysterols and atherosclerosis. Atherosclerosis 142(1):1-28.

Brown MS, Dana SE, Goldstein JL. 1975. Cholesterol ester formation in cultured human fibroblasts. Stimulation by oxygenated sterols. J Biol Chem 250(10):4025-7.

Brown MS, Goldstein JL. 1983. Lipoprotein receptors in the liver. Control signals for plasma cholesterol traffic. J Clin Invest 72(3):743-7.

Brown MS, Goldstein JL. 1986. A receptor-mediated pathway for cholesterol homeostasis. Science 232(4746):34-47.

Campbell LA, Kuo CC, Grayston JT. 1998. Chlamydia pneumoniae and cardiovascular disease. Emerg Infect Dis 4(4):571-9.

Caplice NM, Bunch TJ, Stalboerger PG, Wang S, Simper D, Miller DV, Russell SJ, Litzow MR, Edwards WD. 2003. Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation. Proc Natl Acad Sci U S A 100(8):4754-9.

Carpenter KL, Taylor SE, Ballantine JA, Fussell B, Halliwell B, Mitchinson MJ. 1993. Lipids and oxidised lipids in human atheroma and normal aorta. Biochim Biophys Acta 1167(2):121-30.

Carratelli CR, Rizzo A, Catania MR, Galle F, Losi E, Hasty DL, Rossano F. 2002. Chlamydia pneumoniae infections prevent the programmed cell death on THP-1 cell line. FEMS Microbiol Lett 215(1):69-74.

Cavins JA, Kasakura S, Thomas ED, Ferrebee JW. 1962. Recovery of lethally irradiated dogs following infusion of autologous marrow stored at low temperature in dimethylsulphoxide. Blood 20:730-4.

Chan L. 1992. Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins. J Biol Chem 267(36):25621-4.

Chang HY, Yang X. 2000. Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol Biol Rev 64(4):821-46.

Chang YH, Abdalla DS, Sevanian A. 1997. Characterization of cholesterol oxidation products formed by oxidative modification of low density lipoprotein. Free Radic Biol Med 23(2):202-14.

Cheng N, Janumyan YM, Didion L, Van Hofwegen C, Yang E, Knudson CM. 2004. Bcl-2 inhibition of T-cell proliferation is related to prolonged T-cell survival. Oncogene 23(21):3770-80.

Chin BY, Petrache I, Choi AM, Choi ME. 1999. Transforming growth factor beta1 rescues serum deprivation-induced apoptosis via the mitogen-activated protein kinase (MAPK) pathway in macrophages. J Biol Chem 274(16):11362-8.

Choi DW. 1992. Excitotoxic cell death. J Neurobiol 23(9):1261-76.

Christ M, Luu B, Mejia JE, Moosbrugger I, Bischoff P. 1993. Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. Immunology 78(3):455-60.

Clare K, Hardwick SJ, Carpenter KL, Weeratunge N, Mitchinson MJ. 1995. Toxicity of oxysterols to human monocyte-macrophages. Atherosclerosis 118(1):67-75.

Colles SM, Irwin KC, Chisolm GM. 1996. Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7 beta-hydroperoxycholesterol. J Lipid Res 37(9):2018-28.

Cory S. 1995. Regulation of lymphocyte survival by the bcl-2 gene family. Annu Rev Immunol 13:513-43.

Coucouvanis E, Martin GR. 1995. Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell 83(2):279-87.

Csordas G, Thomas AP, Hajnoczky G. 1999. Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. Embo J 18(1):96-108.

Datta SR, Ranger AM, Lin MZ, Sturgill JF, Ma YC, Cowan CW, Dikkes P, Korsmeyer SJ, Greenberg ME. 2002. Survival factor-mediated BAD phosphorylation raises the mitochondrial threshold for apoptosis. Dev Cell 3(5):631-43.

Daugherty A, Dunn JL, Rateri DL, Heinecke JW. 1994. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest 94(1):437-44.

Daugherty A, Pure E, Delfel-Butteiger D, Chen S, Leferovich J, Roselaar SE, Rader DJ. 1997. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/- mice. J Clin Invest 100(6):1575-80.

Dimmeler S, Haendeler J, Galle J, Zeiher AM. 1997. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. Circulation 95(7):1760-3.

Ellis RE, Jacobson DM, Horvitz HR. 1991. Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans. Genetics 129(1):79-94.

Escargueil-Blanc I, Andrieu-Abadie N, Caspar-Bauguil S, Brossmer R, Levade T, Negre-Salvayre A, Salvayre R. 1998. Apoptosis and activation of the sphingomyelin-ceramide pathway induced by oxidized low density lipoproteins are not causally related in ECV-304 endothelial cells. J Biol Chem 273(42):27389-95.

Escargueil-Blanc I, Meilhac O, Pieraggi MT, Arnal JF, Salvayre R, Negre-Salvayre A. 1997. Oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway. Prevention by aurintricarboxylic acid. Arterioscler Thromb Vasc Biol 17(2):331-9.

Fazio S, Babaev VR, Murray AB, Hasty AH, Carter KJ, Gleaves LA, Atkinson JB, Linton MF. 1997a. Increased atherosclerosis in mice reconstituted with apolipoprotein E null macrophages. Proc Natl Acad Sci U S A 94(9):4647-52.

Fazio S, Hasty AH, Carter KJ, Murray AB, Price JO, Linton MF. 1997b. Leukocyte low density lipoprotein receptor (LDL-R) does not contribute to LDL clearance in vivo: bone marrow transplantation studies in the mouse. J Lipid Res 38(2):391-400.

Fazio S, Major AS, Swift LL, Gleaves LA, Accad M, Linton MF, Farese RV, Jr. 2001. Increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages. J Clin Invest 107(2):163-71.

Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, Fisher EA and others. 2003. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol 5(9):781-92.

Fischer SF, Schwarz C, Vier J, Hacker G. 2001. Characterization of antiapoptotic activities of Chlamydia pneumoniae in human cells. Infect Immun 69(11):7121-9.

Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, Krause KH. 2000. Bcl-2 decreases the free Ca2+ concentration within the endoplasmic reticulum. Proc Natl Acad Sci U S A 97(11):5723-8.

Frostegard J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, Hansson GK. 1999. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. Atherosclerosis 145(1):33-43.

Geng YJ, Phillips JE, Mason RP, Casscells SW. 2003. Cholesterol crystallization and macrophage apoptosis: implication for atherosclerotic plaque instability and rupture. Biochem Pharmacol 66(8):1485-92.

Gerrity RG. 1981. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. Am J Pathol 103(2):181-90.

Goldstein JL, Brown MS. 1979. The LDL receptor locus and the genetics of familial hypercholesterolemia. Annu Rev Genet 13:259-89.

Grayston JT. 2000. Background and current knowledge of Chlamydia pneumoniae and atherosclerosis. J Infect Dis 181 Suppl 3:S402-10.

Griffiths GJ, Dubrez L, Morgan CP, Jones NA, Whitehouse J, Corfe BM, Dive C, Hickman JA. 1999. Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. J Cell Biol 144(5):903-14.

Gross A, Jockel J, Wei MC, Korsmeyer SJ. 1998. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. Embo J 17(14):3878-85.

Gross A, McDonnell JM, Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13(15):1899-911.

Guevara NV, Kim HS, Antonova EI, Chan L. 1999. The absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo. Nat Med 5(3):335-9.

Harada-Shiba M, Kinoshita M, Kamido H, Shimokado K. 1998. Oxidized low density lipoprotein induces apoptosis in cultured human umbilical vein endothelial cells by common and unique mechanisms. J Biol Chem 273(16):9681-7.

Hardwick SJ, Hegyi L, Clare K, Law NS, Carpenter KL, Mitchinson MJ, Skepper JN. 1996. Apoptosis in human monocyte-macrophages exposed to oxidized low density lipoprotein. J Pathol 179(3):294-302.

Harrison DE, Astle CM. 1982. Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure. J Exp Med 156(6):1767-79.

Haupt S, Berger M, Goldberg Z, Haupt Y. 2003. Apoptosis - the p53 network. J Cell Sci 116(Pt 20):4077-85.

Havel RJ. 1987. Lipid transport function of lipoproteins in blood plasma. Am J Physiol 253(1 Pt 1):E1-5.

Havel RJ, Kane JP. 1989. Introduction: Structure and metabolism of plasma lipoproteins. In: Scriver CR, Beaudet AL, Sly WS, Dalle D, editors. The Metabolic Basis of Inherited Disease. 6th ed. New York: McGraw-Hill. p 1129-38.

Hegyi L, Skepper JN, Cary NR, Mitchinson MJ. 1996. Foam cell apoptosis and the development of the lipid core of human atherosclerosis. J Pathol 180(4):423-9.

Heintz N. 1993. Cell death and the cell cycle: a relationship between transformation and neurodegeneration? Trends Biochem Sci 18(5):157-9.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407(6805):770-6.

Henriksen T, Mahoney EM, Steinberg D. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. Proc Natl Acad Sci U S A 78(10):6499-503.

Herijgers N, Van Eck M, Groot PH, Hoogerbrugge PM, Van Berkel TJ. 1997. Effect of bone marrow transplantation on lipoprotein metabolism and atherosclerosis in LDL receptor-knockout mice. Arterioscler Thromb Vasc Biol 17(10):1995-2003.

Higuchi K, Kitagawa K, Kogishi K, Takeda T. 1992. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. J Lipid Res 33(12):1753-64.

Hobbs HH, Brown MS, Goldstein JL. 1992. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat 1(6):445-66.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348(6299):334-6.

Hodis HN, Crawford DW, Sevanian A. 1991. Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. Atherosclerosis 89(2-3):117-26.

Hoffman B, Liebermann DA. 1994. Molecular controls of apoptosis: differentiation/growth arrest primary response genes, proto-oncogenes, and tumor suppressor genes as positive & negative modulators. Oncogene 9(7):1807-12.

Hsu YT, Wolter KG, Youle RJ. 1997. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A 94(8):3668-72.

Isacson O. 1993. On neuronal health. Trends Neurosci 16(8):306-8.

Ishibashi S, Goldstein JL, Brown MS, Herz J, Burns DK. 1994. Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. J Clin Invest 93(5):1885-93.

Jaakkola O, Yla-Herttuala S, Sarkioja T, Nikkari T. 1989. Macrophage foam cells from human aortic fatty streaks take up beta-VLDL and acetylated LDL in primary culture. Atherosclerosis 79(2-3):173-82.

Jialal I, Freeman DA, Grundy SM. 1991. Varying susceptibility of different low density lipoproteins to oxidative modification. Arterioscler Thromb 11(3):482-8.

Jordan CT, Lemischka IR. 1990. Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes Dev 4(2):220-32.

Kanters E, Pasparakis M, Gijbels MJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJ, Clausen BE, Forster I, Kockx MM, Rajewsky K and others. 2003. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest 112(8):1176-85.

Kellner-Weibel G, Jerome WG, Small DM, Warner GJ, Stoltenborg JK, Kearney MA, Corjay MH, Phillips MC, Rothblat GH. 1998. Effects of intracellular free cholesterol accumulation on macrophage viability: a model for foam cell death. Arterioscler Thromb Vasc Biol 18(3):423-31.

Kita T, Goldstein JL, Brown MS, Watanabe Y, Hornick CA, Havel RJ. 1982. Hepatic uptake of chylomicron remnants in WHHL rabbits: a mechanism genetically

distinct from the low density lipoprotein receptor. Proc Natl Acad Sci U S A 79(11):3623-7.

Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. 1995. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270(5233):96-9.

Kockx MM, De Meyer GR, Muhring J, Jacob W, Bult H, Herman AG. 1998. Apoptosis and related proteins in different stages of human atherosclerotic plaques. Circulation 97(23):2307-15.

Kockx MM, Herman AG. 2000. Apoptosis in atherosclerosis: beneficial or detrimental? Cardiovasc Res 45(3):736-46.

Kockx MM, Muhring J, Bortier H, De Meyer GR, Jacob W. 1996. Biotin- or digoxigenin-conjugated nucleotides bind to matrix vesicles in atherosclerotic plaques. Am J Pathol 148(6):1771-7.

Kume N, Cybulsky MI, Gimbrone MA, Jr. 1992. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 90(3):1138-44.

Kume N, Gimbrone MA, Jr. 1994. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J Clin Invest 93(2):907-11.

Lam M, Dubyak G, Chen L, Nunez G, Miesfeld RL, Distelhorst CW. 1994. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca2+ fluxes. Proc Natl Acad Sci U S A 91(14):6569-73.

Lamb DJ, Mitchinson MJ, Leake DS. 1995. Transition metal ions within human atherosclerotic lesions can catalyse the oxidation of low density lipoprotein by macrophages. FEBS Lett 374(1):12-6.

Lemischka IR, Raulet DH, Mulligan RC. 1986. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45(6):917-27.

Liao HS, Kodama T, Geng YJ. 2000. Expression of class A scavenger receptor inhibits apoptosis of macrophages triggered by oxidized low density lipoprotein and oxysterol. Arterioscler Thromb Vasc Biol 20(8):1968-75.

Libby P. 2002. Inflammation in atherosclerosis. Nature 420(6917):868-74.

Libby P, Aikawa M. 2002. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. Nat Med 8(11):1257-62.

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K and others. 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell 6(6):1389-99.

Linton MF, Atkinson JB, Fazio S. 1995. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. Science 267(5200):1034-7.

Linton MF, Fazio S. 2001. Class A scavenger receptors, macrophages, and atherosclerosis. Curr Opin Lipidol 12(5):489-95.

Linton MF, Fazio S. 2003. Macrophages, inflammation, and atherosclerosis. Int J Obes Relat Metab Disord 27 Suppl 3:S35-40.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86(1):147-57.

Lizard G, Gueldry S, Sordet O, Monier S, Athias A, Miguet C, Bessede G, Lemaire S, Solary E, Gambert P. 1998. Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. Faseb J 12(15):1651-63.

Lopez-Collazo E, Hortelano S, Bosca L. 1998. Interferon-alpha/beta inhibits the apoptosis induced by lipopolysaccharide and interferon-gamma in murine peritoneal macrophages. J Interferon Cytokine Res 18(7):461-7.

Lorenz E, Uphoff D, Reid TR, Shelton E. 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst 12(1):197-201.

Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T. 1994. p53 status and the efficacy of cancer therapy in vivo. Science 266(5186):807-10.

Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 362(6423):847-9.

Lundberg B. 1985. Chemical composition and physical state of lipid deposits in atherosclerosis. Atherosclerosis 56(1):93-110.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94(4):481-90.

Lusis AJ. 2000. Atherosclerosis. Nature 407(6801):233-41.

Major AS, Fazio S, Linton MF. 2002. B-lymphocyte deficiency increases atherosclerosis in LDL receptor-null mice. Arterioscler Thromb Vasc Biol 22(11):1892-8.

Mannick JA, Lochte HL, Jr., Ashley CA, Thomas ED, Ferrebee JW. 1960. Autografts of bone marrow in dogs after lethal total-body radiation. Blood 15:255-66.

Manon S, Chaudhuri B, Guerin M. 1997. Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett 415(1):29-32.

Martinet W, Schrijvers DM, De Meyer GR, Thielemans J, Knaapen MW, Herman AG, Kockx MM. 2002. Gene expression profiling of apoptosis-related genes in human atherosclerosis: upregulation of death-associated protein kinase. Arterioscler Thromb Vasc Biol 22(12):2023-9.

Martinou JC, Green DR. 2001. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol 2(1):63-7.

McConkey DJ, Orrenius S. 1997. The role of calcium in the regulation of apoptosis. Biochem Biophys Res Commun 239(2):357-66.

McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP, Korsmeyer SJ. 1989. bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57(1):79-88.

Mitchinson MJ, Hardwick SJ, Bennett MR. 1996. Cell death in atherosclerotic plaques. Curr Opin Lipidol 7(5):324-9.

Morel DW, DiCorleto PE, Chisolm GM. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis 4(4):357-64.

Morel DW, Hessler JR, Chisolm GM. 1983. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. J Lipid Res 24(8):1070-6.

Morisaki T, Katano M. 2003. Mitochondria-targeting therapeutic strategies for overcoming chemoresistance and progression of cancer. Curr Med Chem 10(23):2517-21.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, et al. 1995. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 267(5203):1506-10.

Murayama T, Yokode M, Kataoka H, Imabayashi T, Yoshida H, Sano H, Nishikawa S, Kita T. 1999. Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice. Circulation 99(13):1740-6.

Murphy AN, Bredesen DE, Cortopassi G, Wang E, Fiskum G. 1996. Bcl-2 potentiates the maximal calcium uptake capacity of neural cell mitochondria. Proc Natl Acad Sci U S A 93(18):9893-8.

Murugesan G, Fox PL. 1996. Role of lysophosphatidylcholine in the inhibition of endothelial cell motility by oxidized low density lipoprotein. J Clin Invest 97(12):2736-44.

Nakano T, Raines EW, Abraham JA, Klagsbrun M, Ross R. 1994. Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. Proc Natl Acad Sci U S A 91(3):1069-73.

Napoli C, Quehenberger O, De Nigris F, Abete P, Glass CK, Palinski W. 2000. Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. Faseb J 14(13):1996-2007.

Nicholson AC, Han J, Febbraio M, Silversterin RL, Hajjar DP. 2001. Role of CD36, the macrophage class B scavenger receptor, in atherosclerosis. Ann N Y Acad Sci 947:224-8.

Nishio E, Watanabe Y. 1996. Oxysterols induced apoptosis in cultured smooth muscle cells through CPP32 protease activation and bcl-2 protein downregulation. Biochem Biophys Res Commun 226(3):928-34.

Nossal GJ. 1994. Negative selection of lymphocytes. Cell 76(2):229-39.

Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, Swisher SG. 2002. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores. J Biol Chem 277(11):9219-25.

Oakes SA, Opferman JT, Pozzan T, Korsmeyer SJ, Scorrano L. 2003. Regulation of endoplasmic reticulum Ca2+ dynamics by proapoptotic BCL-2 family members. Biochem Pharmacol 66(8):1335-40.

Oltvai ZN, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74(4):609-19.

Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M. 2002. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 109(4):525-32.

Pacher P, Hajnoczky G. 2001. Propagation of the apoptotic signal by mitochondrial waves. Embo J 20(15):4107-21.

Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. 1987. Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 68(3):231-40.

Panini SR, Sinensky MS. 2001. Mechanisms of oxysterol-induced apoptosis. Curr Opin Lipidol 12(5):529-33.

Papahadjopoulos D. 1974. Cholesterol and cell membrane function: a hypothesis concerning etiology of atherosclerosis. J Theor Biol 43(2):329-37.

Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL, Steinberg D. 1985. Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein. Proc Natl Acad Sci U S A 82(9):3000-4.

Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA. 2002. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. Circ Res 90(10):1064-71.

Pittman RC, Carew TE, Attie AD, Witztum JL, Watanabe Y, Steinberg D. 1982. Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. J Biol Chem 257(14):7994-8000.

Platt N, Haworth R, Darley L, Gordon S. 2002. The many roles of the class A macrophage scavenger receptor. Int Rev Cytol 212:1-40.

Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. 1992. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 71(2):343-53.

Pollman MJ, Hall JL, Mann MJ, Zhang L, Gibbons GH. 1998. Inhibition of neointimal cell bcl-x expression induces apoptosis and regression of vascular disease. Nat Med 4(2):222-7.

Porter AG, Janicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6(2):99-104.

Quinn MT, Parthasarathy S, Steinberg D. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. Proc Natl Acad Sci U S A 85(8):2805-9.

Raff MC. 1992. Social controls on cell survival and cell death. Nature 356(6368):397-400.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. Science 262(5134):695-700.

Ramasamy S, Boissonneault GA, Hennig B. 1992. Oxysterol-induced endothelial cell dysfunction in culture. J Am Coll Nutr 11(5):532-8.

Rao L, Debbas M, Sabbatini P, Hockenbery D, Korsmeyer S, White E. 1992. The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc Natl Acad Sci U S A 89(16):7742-6.

Rapp JH, Connor WE, Lin DS, Inahara T, Porter JM. 1983. Lipids of human atherosclerotic plaques and xanthomas: clues to the mechanism of plaque progression. J Lipid Res 24(10):1329-35.

Reid VC, Mitchinson MJ, Skepper JN. 1993. Cytotoxicity of oxidized low-density lipoprotein to mouse peritoneal macrophages: an ultrastructural study. J Pathol 171(4):321-8.

Rizzuto R, Pinton P, Carrington W, Fay FS, Fogarty KE, Lifshitz LM, Tuft RA, Pozzan T. 1998. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. Science 280(5370):1763-6.

Ross R. 1995. Cell biology of atherosclerosis. Annu Rev Physiol 57:791-804.

Ross R, Masuda J, Raines EW, Gown AM, Katsuda S, Sasahara M, Malden LT, Masuko H, Sato H. 1990. Localization of PDGF-B protein in macrophages in all phases of atherogenesis. Science 248(4958):1009-12.

Rubinsztein DC, Cohen JC, Berger GM, van der Westhuyzen DR, Coetzee GA, Gevers W. 1990. Chylomicron remnant clearance from the plasma is normal in familial hypercholesterolemic homozygotes with defined receptor defects. J Clin Invest 86(4):1306-12.

Rusinol AE, Thewke D, Liu J, Freeman N, Panini SR, Sinensky MS. 2004. AKT/protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. J Biol Chem 279(2):1392-9.

Saikku P. 2000. Chlamydia pneumoniae in atherosclerosis. J Intern Med 247(3):391-6.

Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. 2002. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 8(4):403-9.

Saxena A, McMeekin JD, Thomson DJ. 2002. Expression of Bcl-x, Bcl-2, Bax, and Bak in endarterectomy and atherectomy specimens. J Pathol 196(3):335-42.

Schonbeck U, Sukhova GK, Shimizu K, Mach F, Libby P. 2000. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. Proc Natl Acad Sci U S A 97(13):7458-63.

Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ. 2003. BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science 300(5616):135-9.

Scott J. 1989. The molecular and cell biology of apolipoprotein-B. Mol Biol Med 6(1):65-80.

Sentman CL, Shutter JR, Hockenbery D, Kanagawa O, Korsmeyer SJ. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67(5):879-88.

Seshiah PN, Kereiakes DJ, Vasudevan SS, Lopes N, Su BY, Flavahan NA, Goldschmidt-Clermont PJ. 2002. Activated monocytes induce smooth muscle cell death: role of macrophage colony-stimulating factor and cell contact. Circulation 105(2):174-80.

Shen CM, Mao SJ, Huang GS, Yang PC, Chu RM. 2001. Stimulation of smooth muscle cell proliferation by ox-LDL- and acetyl LDL-induced macrophage-derived foam cells. Life Sci 70(4):443-52.

Shimizu S, Eguchi Y, Kamiike W, Funahashi Y, Mignon A, Lacronique V, Matsuda H, Tsujimoto Y. 1998. Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. Proc Natl Acad Sci U S A 95(4):1455-9.

Shimokado K, Tsukada T, Numano F. 1988. [Platelet-derived growth factor and mechanism of cell proliferation of arterial smooth muscle: macrophage-derived growth factor and arteriosclerosis]. Nippon Rinsho 46(3):600-7.

Shio H, Haley NJ, Fowler S. 1979. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. III. Intracellular localization of cholesterol and cholesteryl ester. Lab Invest 41(2):160-7.

Siow RC, Richards JP, Pedley KC, Leake DS, Mann GE. 1999. Vitamin C protects human vascular smooth muscle cells against apoptosis induced by moderately oxidized LDL containing high levels of lipid hydroperoxides. Arterioscler Thromb Vasc Biol 19(10):2387-94.

Small DM, Bond MG, Waugh D, Prack M, Sawyer JK. 1984. Physicochemical and histological changes in the arterial wall of nonhuman primates during progression and regression of atherosclerosis. J Clin Invest 73(6):1590-605.

Smith JD, Trogan E, Ginsberg M, Grigaux C, Tian J, Miyata M. 1995. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. Proc Natl Acad Sci U S A 92(18):8264-8.

Smith LL, Johnson BH. 1989. Biological activities of oxysterols. Free Radic Biol Med 7(3):285-332.

Soria LF, Ludwig EH, Clarke HR, Vega GL, Grundy SM, McCarthy BJ. 1989. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. Proc Natl Acad Sci U S A 86(2):587-91.

Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE. 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 265(5170):391-4.

Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci U S A 81(12):3883-7.

Suda T, Takahashi T, Golstein P, Nagata S. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75(6):1169-78.

Sudhof TC, Russell DW, Brown MS, Goldstein JL. 1987. 42 bp element from LDL receptor gene confers end-product repression by sterols when inserted into viral TK promoter. Cell 48(6):1061-9.

Szalai G, Krishnamurthy R, Hajnoczky G. 1999. Apoptosis driven by IP(3)-linked mitochondrial calcium signals. Embo J 18(22):6349-61.

Tabas I, Marathe S, Keesler GA, Beatini N, Shiratori Y. 1996. Evidence that the initial up-regulation of phosphatidylcholine biosynthesis in free cholesterol-loaded macrophages is an adaptive response that prevents cholesterol-induced cellular necrosis. Proposed role of an eventual failure of this response in foam cell necrosis in advanced atherosclerosis. J Biol Chem 271(37):22773-81.

Tanigawa T, Kitamura A, Yamagishi K, Sakurai S, Nakata A, Yamashita H, Sato S, Ohira T, Imano H, Shimamoto T and others. 2003. Relationships of differential leukocyte and lymphocyte subpopulations with carotid atherosclerosis in elderly men. J Clin Immunol 23(6):469-76.

Traber MG, Kayden HJ. 1980. Low density lipoprotein receptor activity in human monocyte-derived macrophages and its relation to atheromatous lesions. Proc Natl Acad Sci U S A 77(9):5466-70.

Tsujimoto Y, Gorham J, Cossman J, Jaffe E, Croce CM. 1985. The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science 229(4720):1390-3.

Van Bekkum DW, Vos O, Weyzen WW. 1956. [Homografts and heterografts of hemopoietic tissue in mice]. Rev Hematol 11(5):477-85.

van den Berg H, Kluin PM, Vossen JM. 1990. Early reconstitution of haematopoiesis after allogeneic bone marrow transplantation: a prospective histopathological study of bone marrow biopsy specimens. J Clin Pathol 43(5):365-9.

van Vlijmen BJ, Gerritsen G, Franken AL, Boesten LS, Kockx MM, Gijbels MJ, Vierboom MP, van Eck M, van De Water B, van Berkel TJ and others. 2001. Macrophage p53 deficiency leads to enhanced atherosclerosis in APOE*3-Leiden transgenic mice. Circ Res 88(8):780-6.

van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, van der Boom H, HogenEsch H, Frants RR, Hofker MH, Havekes LM. 1994. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. J Clin Invest 93(4):1403-10.

Vaux DL, Cory S, Adams JM. 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335(6189):440-2.

Warner GJ, Stoudt G, Bamberger M, Johnson WJ, Rothblat GH. 1995. Cell toxicity induced by inhibition of acyl coenzyme A:cholesterol acyltransferase and accumulation of unesterified cholesterol. J Biol Chem 270(11):5772-8.

Watanabe Y, Ito T, Shiomi M. 1985. The effect of selective breeding on the development of coronary atherosclerosis in WHHL rabbits. An animal model for familial hypercholesterolemia. Atherosclerosis 56(1):71-9.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ. 2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 292(5517):727-30.

Weisgraber KH, Innerarity TL, Newhouse YM, Young SG, Arnold KS, Krauss RM, Vega GL, Grundy SM, Mahley RW. 1988. Familial defective apolipoprotein B-100: enhanced binding of monoclonal antibody MB47 to abnormal low density lipoproteins. Proc Natl Acad Sci U S A 85(24):9758-62.

Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DR. 1990. Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343(6253):76-9.

Won J, Kim DY, La M, Kim D, Meadows GG, Joe CO. 2003. Cleavage of 14-3-3 protein by caspase-3 facilitates bad interaction with Bcl-x(L) during apoptosis. J Biol Chem 278(21):19347-51.

Wyllie AH, Kerr JF, Currie AR. 1980. Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306.

Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. 1995a. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 80(2):285-91.

Yang J, Brown MS, Ho YK, Goldstein JL. 1995b. Three different rearrangements in a single intron truncate sterol regulatory element binding protein-2 and produce

sterol-resistant phenotype in three cell lines. Role of introns in protein evolution. J Biol Chem 270(20):12152-61.

Yao PM, Tabas I. 2000. Free cholesterol loading of macrophages induces apoptosis involving the fas pathway. J Biol Chem 275(31):23807-13.

Yao PM, Tabas I. 2001. Free cholesterol loading of macrophages is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway. J Biol Chem 276(45):42468-76.

Yeagle PL. 1991. Modulation of membrane function by cholesterol. Biochimie 73(10):1303-10.

Yin XM, Oltvai ZN, Korsmeyer SJ. 1994. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369(6478):321-3.

Yla-Herttuala S, Luoma J, Viita H, Hiltunen T, Sisto T, Nikkari T. 1995. Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. J Clin Invest 95(6):2692-8.

Yui S, Sasaki T, Miyazaki A, Horiuchi S, Yamazaki M. 1993. Induction of murine macrophage growth by modified LDLs. Arterioscler Thromb 13(3):331-7.

Zangemeister-Wittke U, Ziegler A. 1998. Bcl-2 antisense therapy for cancer: the art of persuading tumour cells to commit suicide. Apoptosis 3(2):67-74.

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell 87(4):619-28.

Zhang HF, Basra HJ, Steinbrecher UP. 1990. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. J Lipid Res 31(8):1361-9.

Zhang SH, Reddick RL, Piedrahita JA, Maeda N. 1992. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 258(5081):468-71.

Zhou YF, Leon MB, Waclawiw MA, Popma JJ, Yu ZX, Finkel T, Epstein SE. 1996. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. N Engl J Med 335(9):624-30.

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12(7):982-95.

Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, Thompson CB. 2003. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 162(1):59-69.

VITA

JUNE LIU

Education:
Shanghai Medical University School of Pharmacy, Shanghai, China; Pharmacology, B.S., 1998
East Tennessee State University, Johnson City, Tennessee; Biochemistry and Molecular Biology, Ph.D., 2004

Professional Experiences:
Assistant Editor, Acta Pharmacologica Sinica; Chinese Academy of Sciences Shanghai Institute of Materia Medica, Shanghai, 1998 – 2000
Graduate Assistant, East Tennessee State University, College of Medicine, 2000 – 2004
Visiting Student, Vanderbilt University Medical Center, 2003 – 2004

Publications:
Liu J, Thewke DP, Su YR, Linton MF, Fazio S, Sinensky MS. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in LDLR null mice. Arterioscler Thromb Vasc Biol 2004 Oct 21 [Epub ahead of print].
Rusinol AE, Thewke D, Liu J, Freeman N, Panini SR, Sinensky MS. AKT/protein kinase B regulation of BCL family members during oxysterol-induced apoptosis. J Biol Chem 2004; 279:1392-9.
Freeman N, Rusinol AE, Liu J, Linton M, Fazio S, Sinensky MS, Thewke DP. Acyl-CoA:Cholesterol acyltransferase inhibition prevents oxidized LDL/Oxysterol-induced apoptosis in macrophages. J Lipid Res, in revision.
Yang S, Liu J, Zhang AZ, Jiang MH. Biphasic manner of melittin on isolated guinea pig atria. Acta Pharmacol Sin 2000; 21: 221-4.

Honors and Awards:
Award for Young Investigator, Kern Aspen Lipid Conference, Aug 2004, Aspen, CO.
Award for Outstanding Academic Achievement, 2001 – 2002, ETSU.
Renmin Scholarship, 1996, 1995, 1994, SMU.