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Modification of Low Density Lipoprotein and the Effects of Modified Low Density Lipoproteins on the Endocytic and Secretory Repertoire of the Macrophage

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Modification of Low Density Lipoprotein and the Effects of Modified Low
Density Lipoproteins on the
Endocytic and Secretory Repertoire of the Macrophage

A thesis submitted to the faculty of the Rockefeller University in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

by

Ruth Rebecca Montgomery, B.A.

April 1987
The Rockefeller University
New York

This thesis is dedicated with love to my grandfather

WILLARD FOSTER BARBER

who has shown his family and friends the value and wonder of education. I thank him for teaching me determination, and for honoring me with his pride and affection.

Written on the day of his 78th birthday

March 21, 1987

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Of Zanvil Cohn so many students have written so many praises that all the superlatives have become ordinary. I want to add my thanks to those of

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Summary

This study examines two aspects of the complex interaction between modified low density lipoprotein (LDL) and macrophages in the initiation and development of the atherosclerotic lesion. The first of these concerns the observed biological modification of LDL that leads to its subsequent enhanced uptake by macrophages, and the role of oxidation in that process. The second aspect of this thesis studies the effects of modified LDL on the functions of macrophages.

Studies on the oxidation of LDL showed that LDL isolated from human plasma anticoagulated with EDTA (EDTA-LDL) was 4-fold more resistant to oxidation by reagent H_2O_2 , as measured by the thiobarbituric acid (TBA) assay, than LDL prepared from plasma anticoagulated with citrate (CDP-LDL). The LDLs required 1-3 mM H_2O_2 for maximal oxidation by this assay, and ED_{50} s were 1.7×10^{-3} M for EDTA-LDL and 4.5×10^{-4} M for CDP-LDL. Oxidation was enhanced 2.3-fold by Cu^{2+} ions. Rabbit endothelial cell line monolayers released three orders of magnitude less H_2O_2 than was required to oxidize LDL and failed to induce TBA reactivity in either EDTA-LDL or CDP-LDL after a 24-hour coincubation. However, this LDL was subsequently degraded by mouse macrophages more rapidly than untreated LDL. Freshly isolated human monocytes (2×10^6 cells per ml), with or without phorbol myristate acetate (100 ng/ml) to trigger the respiratory burst, did not oxidize LDL in the TBA assay, despite producing large amounts of reactive oxygen intermediates. EDTA-LDL, CDP-LDL, and acetoacetylated LDL failed to trigger H_2O_2 release from human monocytes or macrophages. These results separate oxidation of LDL as measured by TBA assay from the modification

of LDL by a rabbit aortic endothelial cell line that leads to its subsequent enhanced degradation by macrophages.

Mouse peritoneal macrophages were lipid-loaded by three regimens modeling loading through the scavenger receptor (Ac-LDL cells), by extracellular matrix-bound LDL (DS-LDL cells), and conditions of reduced cholesterol acceptors in the medium (LS/OI cells). Increased cholesterol levels in all three regimens were measured by cholesterol determination, Oil Red O staining of fixed cells, and extraction of Oil Red O from cells and determination of OD₅₃₀. Lipid-laden cells were equal to control macrophages in binding and ingesting immunoglobulin-coated sheep erythrocytes, reflecting Fc-mediated endocytosis. Cellular proteins appeared largely unchanged, but alterations in the secreted protein profile were observed by metabolic labeling with ³⁵S-methionine. DS-LDL loaded cells and LS/OI loaded cells showed increases in a 68 kD protein, and decreases in apo E secretion. Ac-LDL loaded cells showed significant enhancement in apo E secretion. The lipid-laden cells were compared to control cells for three secretory functions of macrophages that could be important in the atherosclerotic plaque. They were still capable of producing all secretory products examined, but the quantities of H₂O₂ and arachidonic acid metabolites are reduced in some cases, and plasminogen activator appeared to be increased. However, we have lipid-loaded the cells for only two days, and foam cells in vivo may be exposed to these conditions for months or years.

The different loading regimens result in different changes, which we have separated, but in vivo the cells would be exposed to a complex mixture of native and biologically modified LDL, and LDL complexed with glycosaminoglycans and with proteins of the extracellular matrix. It is

premature to predict what changes in the macrophage's large repertoire would occur in vivo.

Table of Contents

	Page
List of Figures and Tables	vii
Chapter 1 Introduction	1
General Introduction	1
The Fatty Streak, Monocytes, and LDL	4
Foam Cells and the Atherosclerotic Lesion	10
Chapter 2 Materials and Methods	15
LDL Preparation and Modifications	15
Preparation of LDL	15
Acetylation and Acetoacetylation	16
Dextran Sulfate LDL Complexes	16
H ₂ O ₂ Oxidation of LDL	16
Endothelial Cell Modification of LDL	17
Cell Culture	17
Endothelial Cells	17
Human Monocyte/Macrophage Culture	18
Mouse Peritoneal Macrophage and Foam Cell Culture	18
Assays	19
H ₂ O ₂ Assay	19
Thiobarbituric Acid Assay of Lipid Peroxidation	19
Cholesterol Assay	19
Oil Red O Staining and Extraction	20
Thin Layer Chromatography of Foam Cell Lipids	21
Phagocytosis of Immunoglobulin G-coated	
Sheep Erythrocytes	22
Fibrinolysis by Macrophages and Foam Cells	22
³⁵ S-Methionine Secreted Protein Profile	23
Immunoprecipitation of Apolipoprotein E	23
Release of ³ H-20:4 and 20:4 Metabolite Profile	24
Interleukin 1 Release	25
Chapter 3 Effects of Reagent and Cell-generated H ₂ O ₂ on the Properties of LDL	26

Introduction	26
Results	27
Characterization of H ₂ O ₂ -oxidized LDL	27
Coincubation of LDLs with Endothelial Cell Monolayers	29
EC-LDL Degradation by Murine Macrophages	29
Determination of Endothelial Cell H ₂ O ₂ and O ₂ ⁻ Release	30
Monocyte Oxidation of LDL	30
Monocyte/Macrophage H ₂ O ₂ Release with LDLs as Triggers	31
Discussion	32
Chapter 4 The Endocytic and Secretory Repertoire of the Lipid-loaded Macrophage: An in vitro Model for Foam Cells	35
Introduction	35
Cholesterol Accumulation	35
Morphology of Foam Cells	37
Phagocytosis	39
³⁵ S-Methionine Protein Profile	40
Apolipoprotein E	42
Fibrinolysis	45
H ₂ O ₂ Release	48
Arachidonic Acid Release	50
Interleukin 1	53
Response to γ Interferon	54
Chapter 5 Discussion and Conclusions	56
References	61

List of Figures and Tables

Chapter 1 Introduction

Chapter 2 Materials and Methods

Figure 1. Preparation of LDL from plasma

Figure 2. 3-10% gradient SDS PAGE of purification of LDL

Figure 3. 1% agarose gel of LDL, Ac-LDL, DS-LDL

Chapter 3 Effects of Reagent and Cell-generated H_2O_2 on the Properties of LDL

Figure 1. Characterization of H_2O_2 -oxidized LDL

Figure 2. EC modification of LDL

Figure 3. Mononuclear leukocyte oxidation of LDL

Table 1. Effect of additions to reagent H_2O_2 on oxidation of LDL

Table 2. Mouse macrophage degradation of LDL

Table 3. Mouse macrophage degradation of LDL with anti-oxidants

Table 4. Release of H_2O_2 by endothelial cells

Table 5. LDLs as triggers for H_2O_2 release from human monocytes and macrophages

Chapter 4 The Endocytic and Secretory Repertoire of the Lipid-loaded Macrophage: An in vitro Model for Foam Cells

Figure 1. Photographs of Oil Red O stained macrophages

Figure 2. Time course of cholesterol accumulation by foam cells

Figure 3. TLC of foam cell lipids

Figure 4. SDS PAGE of ^{35}S -methionine labeled cellular proteins

Figure 5. SDS PAGE of ^{35}S -methionine labeled secreted proteins

Figure 6. SDS PAGE of ^{35}S -methionine labeled secreted proteins fed doses of DS-LDL

Figure 7. SDS PAGE of immunoprecipitation of apoprotein E

Figure 8. Fibrinolytic activity of foam cells

Figure 9. H_2O_2 production by control, DS-LDL fed cells in response to doses of PMA

Figure 10. Photographs of Oil Red O Stained macrophages after IFN

Table 1. Cholesterol levels of foam cells

Table 2. OD_{530} of Oil Red O extracted from stained foam cells

- Table 3. Phagocytosis of EIgG by foam cells
- Table 4. H₂O₂ release by foam cells
- Table 5. H₂O₂ release by foam cells in response to doses of DS-LDL
- Table 6. 20:4 release by foam cells to zymosan
- Table 7. 20:4 release by foam cells to A23187
- Table 8. Distribution of ³H-20:4 in foam cells
- Table 9. 20:4 metabolite profile

Chapter 5 Discussion and Conclusions

Chapter 1 Introduction

General Introduction

Arteriosclerosis is a progressive disease that is the cause for half the deaths in this country every year (1). It consists of a plaque in the wall of a large muscular artery and is characterized by the proliferation of smooth muscle cells (SMC), accumulation of lipids and extracellular matrix materials, as well as the presence of lipid-laden foam cells, necrotic debris, and a fibrous cap (2). This lesion can occlude blood flow to the heart or brain, causing heart attack or stroke. Risk factors for the disease include hypertension, high blood cholesterol levels, smoking, diabetes mellitus, obesity, lack of exercise, stress, oral contraceptives, and a family history of the disease (3). A major research focus is the pathogenesis of its initiation and development.

Some studies of the plaque propose a model of atherosclerosis as a benign SMC neoplasm. This model is supported by: observations of a monoclonality of glucose-6-phosphate dehydrogenase isoforms in arterial tissue (4, 5); the isolation from arterial tissue of viruses (6) and an oncogene activity that can transform NIH 3T3 cells (7); and the demonstration of elevated platelet-derived growth factor B chain (the sis gene) gene transcripts in atherosclerotic lesions (8). Taken together, these findings (4-11) describe a very interesting theory which may add to our understanding of the factors involved in plaque development. The monoclonal theory has not, however, accounted for the development of the fatty streak into an advanced plaque, or explained how some fatty streaks may regress.

The more widely-held interpretation of the progression of atherosclerosis focuses on the hyperlipidemia and the interaction of the cell types in the artery wall (3,12-14). Poole and Florey (15) showed in 1958 that in the hypercholesterolemic rabbit the monocytes migrate under the endothelium and begin to accumulate lipid there. A number of subsequent studies have followed the condition of arteries of animals such as swine, rabbits, and monkeys during a period of high dietary fat intake, and a clear picture has started to emerge of the sequence of events leading to the development of the fatty streak.

The regular "cobblestone" appearance of endothelial cells (EC) from the arteries of control monkeys can be altered after only 12 days on an atherogenic diet. Faggiotto and Ross report the first observed change is an increased monocyte adherence (16-19). Several chemoattractants for circulating monocytes may be produced in the artery wall. These include platelet factor 4, which is released from platelet granules after adherence to the EC matrix (20); both macrophages and SMC in culture (21); and a factor from lesion-prone areas of swine arteries (22). Subsequent monocyte migration under the EC into the artery is followed by monocyte accumulation of lipid and results in a bumpy appearance in the lumen of the vessel. The continuing process of monocyte infiltration and lipid-loading has been shown to result in fatty streak formation (16-19).

Gerrity has reported that the fatty streak lesion is reversible, depending on the number of circulating monocytes that penetrate the area. Degenerating foam cells have been observed in the spleen and liver; this provides evidence for the idea that monocytes are scavenging lipid in the fatty streak and then leaving the lesion, taking the lipid away (23-26).

Escaping foam cells or their hazardous products may damage the EC, resulting in the observed disruption of the EC layer, which exposes the lipid-laden cells and the EC matrix to the bloodstream. The matrix is a site for platelet and LDL adherence (27,28). Thus the damage to the EC exposes the SMC to platelet-derived growth factor (PDGF), resulting in SMC migration and proliferation in the lesion (29-31). The proliferation of SMC narrows the lumen of the artery. PDGF-like growth factors can also be produced by the stimulated monocyte (32,33) (which also produces a growth factor for fibroblasts; 34-38), and by EC (39) and SMC (40). PDGF production by SMC is developmentally regulated (41), and in a variety of cells has been shown to accompany transformation (42). Besides stimulating SMC growth, PDGF is chemotactic for monocytes and neutrophils (43) and can also lead to their activation (44,45). PDGF is also reported to act as a vasoconstrictor (46).

The macrophage is the source of other mediators that produce potent effects in the artery wall, including interleukin-1 (IL-1) and tumor necrosis factor (TNF; 47). TNF and IL-1 share many activities (47,48) and their independent regulation results in a variety of functions. IL-1 regulates T lymphocytes and stimulates fibroblast growth and collagenase production (49,50). It induces an overall procoagulant state in endothelial cells (51-53), and has been reported to stimulate monocytes to be tumoricidal (54), and perhaps more activated for other functions.

TNF was originally discovered in the sera of BCG- (Bacille Calmette-Guerin) infected mice that had been injected with LPS (55), and the major cellular source may be activated macrophages (56). It can act as a growth factor for fibroblasts (57) and it also results in overall procoagulant activity (48,58), similar to the effects of IL-1. These changes in the artery wall could lead to progression of the plaque.

The fibrous cap that develops over the site confines the cells and their interacting products, the progressively accumulating lipids, the foam cells, and the proliferating SMC. This pathological lesion is the interaction of EC, SMC, platelets, and macrophages in close proximity, surrounded by LDL cholesterol. The complexity of influences is hard to overestimate. Each cell type present--EC, SMC, macrophage, and platelet--can secrete growth and inhibitory factors, vasoactive products of arachidonic acid metabolism, and chemotactic factors that the others may respond to by proliferation, chemotaxis, or the release of other bioactive mediators. The numerous cellular interactions in the atherosclerotic plaque have recently been reviewed by Ross (2). In addition, many studies have shown that EC, SMC, and circulating leukocytes can modify LDL for its subsequent enhanced uptake and degradation by tissue macrophages, contributing to the formation of foam cells at the fatty streak stage (59-67).

From this complex lesion, we have chosen to concentrate on two aspects of macrophage-LDL interaction. The first is the biological modification of LDL by EC, SMC, and macrophages that has been shown to accelerate macrophage foam cell formation (Chapter 3). The second aspect to be considered in this thesis is the effect of modified LDL on macrophage functions (Chapter 4).

The Fatty Streak, Monocytes, and LDL

Some quantitative assays are available to measure the amount of LDL localized in the artery wall of hyperlipidemic animals or patients. The largest proportion of LDL in the body (40%) goes immediately to the liver (68,69). In the case of acetylated LDL (Ac-LDL), 80% of the injected material appears in

the liver with a $t_{1/2}$ equal to 10 minutes. The labeled Ac-LDL is recovered mainly in the endothelial cells at 1 hour after its injection, and subsequently is located in hepatocytes (70). Smith and Staples found that in human aortas LDL achieves almost twice as high a concentration in the inner intima (next to the endothelium) as in plasma, and that the internal elastic lamina excludes LDL almost totally although allowing passage of smaller proteins, including albumin (71). However, a damaged internal elastic lamina allows LDL to build up in that region of the artery. Intact endothelium is necessary to maintain the 2-fold higher LDL concentration in the intima as compared to the plasma (72). In agreement, Minick et al find that lipid accumulates in rabbit arteries more in areas of re-endothelialization than in denuded areas (73). Smith and Staples also report that the LDL in the intima is mainly not complexed with extracellular matrix since it is freely diffusable.

Measurement of the movement of ^{125}I -labeled LDL through rabbit thoracic aorta in vivo shows that LDL passes into the artery rapidly and from both the luminal and adventitial sides. LDL accumulates less in the media, and ^{125}I -LDL decreases gradually over 67 hours (74).

Analysis of total lipids from fatty streaks shows 95% cholesterol esters, and of these 50.4 % were esterified with oleic acid. After isolation of lipid droplets from the fatty streak, the residual tissue fraction was found to have a lipid composition very similar to normal intima (75). Examination of the lipoproteins from mild and severe sclerotic plaques revealed LDL in human aortic intima in complexes with mucopolysaccharides (or glycosaminoglycans, GAGs) and sulfated polysaccharides (76). Bihara-Varga and Vegh (28) showed that GAGs selectively bind serum lipoproteins with the composition of LDL, that these GAG-LDL complexes form in the presence of calcium and that they could be isolated only from atherosclerotic aortic

intimas and not from normal aortas. In fact, increased amounts of GAGs, in particular chondroitin sulfate C, can be isolated from aortas of very early lesions, even the fatty streak (77). Dalferes et al suggest that the presence of GAGs in the intima, considering that they form insoluble complexes with LDL, could be a mechanism for lipid accumulation and sequestration in the artery wall. This mechanism could probably only operate during the fatty streak stage, since aortic chondroitin sulfates are reported to decrease with the advancement of disease (78). Lipoprotein-GAG complexes have also been isolated from rabbit aortic lesions, although those complexes are GAG-VLDL, as VLDL is the most common lipoprotein in rabbit plasma (79). The presence of an intact endothelium allows more GAG accumulation. Chondroitin sulfate A/C and heparan sulfate (but not dermatan sulfate) build up in the intima of re-endothelializing rabbit aortas (80).

Gerrity has shown in swine that the initial AS lesion, the fatty streak, consists of blood monocytes that have entered a lipid-engorged vessel of a hypercholesterolemic animal and become foam cells (23-26). Electron microscopic data form the basis of the conclusion that the lipid-laden macrophage foam cells return through the endothelium into the bloodstream. Medial SMC are not involved in the fatty streak lesion. In the aortic arch of swine the lesions do not develop beyond the fatty streak stage although fibrous lesions develop in the same animal in the abdominal aorta. Gerrity suggests that these fatty streak lesions do not progress further precisely due to the presence of a peroxidase-positive monocyte "lipid-clearance system." Perhaps, as has been suggested, the exiting foam cells might disrupt the EC integrity, resulting in the open lesions (16-19) that allow lipid and other plasma factors, including platelets, access to the SMC.

How do monocytes clear away the LDL and LDL-GAG complexes?

Macrophages have the classical LDL receptor as described in fibroblasts by Brown and Goldstein (81,82). It is a high-affinity membrane receptor that binds LDL saturably and leads to its internalization by receptor-mediated endocytosis. The LDL degradation products regulate the cholesterol metabolism of the cell. High LDL levels decrease the synthesis of LDL receptors, and suppress the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. LDL's cholesterol increases cellular cholesterol esterification (esters being the preferred storage form) by acyl-CoA:cholesterol acyltransferase (ACAT).

Macrophages also bind chemically modified LDL, i.e. acetylated, maleylated, acetoacetylated, succinylated, or malondialdehyde-treated (83-95). In human monocyte-derived macrophages the modified LDL receptor is not very active before 5 days of culture (88,89). The modifications of LDL change the epsilon amino groups of lysine to a more negatively charged form (90,91). Modified LDL might be formed in vivo by reaction with the platelet products malondialdehyde (85) or 4-hydroxynonenal (92) or by other metabolites of arachidonic acid, including 5-HPETE or leukotriene A₄ (82). LDL complexed with this array of negatively charged compounds competes with maleylated albumin (mal-BSA) and with the sulfated polysaccharides fucoidin and dextran sulfate for binding to the "modified LDL" or scavenger receptor (84,91). It has been speculated that this negative charge confers a conformational change on the apoprotein, since de-maleylated BSA still competes for binding with mal-BSA (93). It should be pointed out that monocytes express both the mal-BSA receptor and the scavenger receptor, and that the mal-BSA receptor is a chemoattractant receptor (94,95).

Uptake of acetylated LDL (Ac-LDL) or other modified forms of LDL through the macrophage scavenger receptor occurs at 20 times the rate of LDL uptake through the native LDL receptor and stimulates cholesterol esterification, resulting in enormous cholesterol ester accumulation (84-86). The scavenger receptor of mouse macrophages has 20,000-40,000 binding sites per cell and is protease sensitive. Binding is not dependent on divalent cations and reaches half-maximal at 25 μg LDL/ml at 37°C (82). The purified Ac-LDL receptor from mouse macrophage tumors has a M_r of 260,000 daltons. The receptor has a K_d of $3.1 \times 10^{-8} \text{ M}^{-1}$ (96).

Unlike the native LDL receptor which is found in coated pits in the macrophage cell membrane, the scavenger receptor is distributed more diffusely over the cell. The receptors do cluster after ligand binding but not in coated pits (97), and are reported to recycle more rapidly than the native receptors (98). The activity of the scavenger receptor can be suppressed by lymphocyte products (99,100), bacterial endotoxin (101), and a platelet secretory factor (102).

SMC are also exposed to LDL in the artery wall, and they also degrade LDL with subsequent reduction in cholesterol synthesis, but do not take up Ac-LDL avidly (103,104). However, they are subject to a variety of influences. Under hypoxic conditions SMC LDL uptake was increased, but degradation was decreased 36%. This could lead to accumulation in the artery (105). Macrophage conditioned medium increases SMC cholesterol esterification--perhaps, if Ac-LDL is present, by changing the lipoprotein lipid balance (106), or by way of a secretory product (107). Platelets are also reported to enhance cholesterol accumulation in SMC (108).

In the normal rabbit aorta, 16% of the LDL degradation is mediated by the intimal EC LDL receptor (69). Human umbilical artery and vein EC in

culture also have a high affinity LDL receptor accounting for 17% of the total LDL endocytosis at physiological LDL concentrations (109,110). Like macrophages, EC have a scavenger receptor and take up Ac-LDL more rapidly than native LDL (104,111). Additionally, in the experiments reported by Stein and Stein, confluent EC degraded more Ac-LDL than did subconfluent cultures, the opposite of the finding for LDL degradation by EC cultures. Pitas et al report liver sinusoidal EC have a K_d of $2.5 \times 10^{-9} \text{ M}^{-1}$ for modified LDL (111). Aortic EC degrade less than 1% of the Ac-LDL degraded by liver EC (112).

EC receptors, native or scavenger, are not the only mode of entry of LDL into the artery wall. The bulk of the LDL enters the cell through low affinity adsorptive endocytosis (111). LDL might also enter the artery wall by accompanying migrating monocytes, as shown by Territo et al (113).

LDL that has been exposed to EC, SMC, or macrophages in culture has been shown to be modified for enhanced degradation by a non-LDL receptor-mediated mechanism. Biologically modified LDL competes with chemically modified LDL for binding to the scavenger receptor (59-67). A similar modification of LDL is observed from LDL re-isolated from human aortas or rabbit aortic interstitial fluid (114-118). Thus, the biological modification may contribute to accelerated foam cell formation. The mechanisms postulated to underlie the cell-mediated modification of LDL include oxidation, lipolysis, and proteolysis, but in no instance has this alteration been clearly defined in molecular terms. In light of our interest in foam cell formation, we pursued the oxidative modification of LDL by a detailed study of a major oxidizing secretory product of phagocytic cells, H_2O_2 . In chapter 3, we report the susceptibility of LDL to oxidation by known concentrations of chemical oxidants, by EC, and by the diverse group of

reactive oxygen intermediates produced by monocytes. In addition, we have investigated the ability of monocytes and macrophages to respond to modified LDLs by releasing H_2O_2 .

Foam Cells and the Atherosclerotic Lesion

An analysis of atherosclerotic aortas reveals significant enrichment of catalase, acid hydrolases, and free and esterified cholesterol (119). Isolation of cells from these regions by enzymatic digestion results in cell populations that display some properties of macrophages and some of smooth muscle cells. Monocytes had previously been shown to become loaded with fat under certain culture conditions (120). Haley et al separated the total cell population into a high density and low density fraction on a metrizamide density gradient and observed that the low density fraction cells were particularly highly enriched in lysosomal hydrolases (121,122). Studies on monkeys revealed similar cell distribution and biochemical function. Interestingly, most of the accumulated lipid was found to be extracellular (123). This may be due to the compartmentalization of tissue water in the more advanced lesion (124). Subsequent electron microscopic studies by Fowler et al identified the high density cells as smooth muscle cells. The low density aortic cells were concluded to be authentic macrophages based on Fc receptors, high levels of catalase and acid hydrolases, and the ability to ingest antibody-coated particles. However, only one third of these cells had C3 receptors and they did not show the presence of lysozyme, either in intracellular or secreted form (125).

Using glass adherent cells instead of enzymatically digested cells, Schaffner et al (126) confirmed the presence of macrophage-origin cells in

the lesion. They were able to detect C3 receptors on 80-90% of the isolated glass-adherent cells. And from primate aortas they measured higher lysozyme content than in that of the rabbit. These studies conclude that the atherosclerotic lesion has two lipid-loaded populations and that in fact foam cells derive from both SMC and macrophage cell types.

Macrophage-derived foam cells from rabbit explants were shown to accumulate lipoprotein through the modified-LDL (scavenger) receptor as well as the β -VLDL receptor. Interestingly, Pitas et al also showed that explanted foam cells retained their cholesterol esters under culture conditions where in vitro loaded cells lost them (127).

Lipid analysis of macrophage foam cells has been reported by Kelley et al from an experimentally-induced granuloma in rabbit. Macrophages accumulated in rabbit aortas in response to the subcutaneous injection of the sulfated polysaccharide carrageenan. In animals pre-loaded with a high cholesterol diet for 28 days, the macrophages quickly began to accumulate lipids, particularly cholesterol esters, reaching almost 80 times the control cell cholesterol ester level by 14 days, and 178 times by 28 days. Oleate (18:1) was the most frequently found fatty acid in the cholesterol esters, comprising almost 50% of those in the high-cholesterol animals (128,129).

But the presence of foam cells is not the only difference in the advanced atherosclerotic lesion. Normal carotid artery and aortic cell populations are altered (130,131). Serial sections of the mature AS plaque tissue from human carotid artery extensively analyzed with monoclonal and polyclonal antibodies directed against specific cell lineages showed that 60% of the cells in the necrotic core of the lesion are Leu-M3 positive, representing macrophages, as are 24% of the cells in the fibrous cap. Conversely, 60% of the cells in the fibrous cap are desmin positive,

representing SMC, and only 29% of the cells in the core. Interestingly, the Leu 4 antibody marking T lymphocytes stained 20% of the cells in the fibrous cap, but almost none in the necrotic core or the surrounding areas of artery. T cells are almost absent from normal arteries. Of the T cells identified, almost 3 times as many T4+ (helper) cells are present as T8+ (suppressor) cells. SMC from plaques express HLA-DR, a class II transplantation antigen generally found only on cells of the immune system (132). This phenotypic change suggests the presence of $IFN\gamma$, a cytokine known to increase Ia on SMC and other cell types (133). This surface marker may reflect other functional changes in the SMC, and, in combination with the presence of the T4+ lymphocytes, suggests active participation of an immune reaction in the artery.

McCullagh et al have shown that the distribution of collagen types in the atherosclerotic human aorta is different from that in uninvolved areas. The SMC collagen, type I, is increased in plaques, representing a functional shift for the cells. Type I collagen forms thick bundles, resulting in more rigidity and less elasticity in that section of the artery. These features could be very important in the progression and pathology of the disease (134).

Analysis of the lipid composition of advanced plaques from human carotid and femoral arteries also revealed enormous changes from the normal artery. LDL has been immunocytochemically localized by use of antibodies to the apoprotein B. Areas of diseased human aortas (but not normal aortas) were shown to have extracellular LDL in the necrotic core of an advanced plaque, lining the internal elastic lamina and surrounding the SMC. All macrophages were negative for apo B, even though some foam cells were surrounded by it (135). Yomantas et al showed that highly lesion-prone areas also reflect intracellular apo B, not seen in less severe lesions

(136). Over 50% of the cholesterol in the advanced lesion was in a free, unesterified form. In normal artery, the fatty streak lesion, or a xanthoma, only 25% of the total cholesterol was unesterified. The plaque's esterified cholesterol had a ratio of 1.7 of linoleic/oleic acid as the fatty acid esterified, compared to a ratio of 0.36 (linoleic/oleic) in xanthomas. These differences are important because they allow conclusions about the location of cholesterol esterification. The plasma enzyme LCAT uses predominantly linoleic acid, and ACAT, the macrophage esterifying enzyme, uses predominantly oleic. The increase in ratio of linoleic/oleic from 0.36 to 1.7, combined with the high level of free cholesterol, implies a failure of the macrophages within the lesion to keep up with lipid metabolism. Rapp et al speculate that this "decreased metabolic efficiency" of the macrophages may enhance plaque progression (137). The reduced cholesterol ester proportion can also be observed from the predominance of isotropic forms of lipid droplets in more advanced lesions (75).

We have seen from the literature that the monocytes initially migrating into the artery at the time of fatty streak formation may meet native LDL, biologically modified forms of LDL, and LDL-GAG complexes. They have been shown to ingest and accumulate lipid, maintaining a high proportion of cholesterol esters, and using oleic acid primarily in the formation of the cholesterol esters. But in advanced lesions, the monocyte-derived foam cells seem unable to metabolize the volume of lipid, which leads to an increase in the proportion of free cholesterol, and to a change in the cholesterol ester profile from oleic to linoleic as the fatty acid esterified. Also as the fatty streak progresses to the advanced lesion, smooth muscle cells proliferate and become lipid-loaded foam cells. The plaque is very different from the normal artery wall or even the fatty streak, and a

possible contributing factor is the reduced capacity of the macrophage scavenging system. This may be due to impairment of the macrophages' metabolic functions after lipid loading.

Several investigators have reported reductions in macrophage functions as a result of interaction with lipid. Chapman and Hibbs have shown that macrophage tumoricidal activity is reversibly inhibited by incubation with LDL (or an LDL-associated component) and that an increase in cholesterol content of the plasma membrane inhibits cytotoxic activity and the ability of the cells to respond to endotoxin stimulation (138). An increase in the saturation of the plasma membrane phospholipids of the macrophage has been shown to decrease the rates of, and increase the energy requirements for, pinocytosis and phagocytosis (139). Accumulation of polyanions, like the GAGs prevalent in the artery wall, inhibits lysosome-phagosome fusion and decreases lysosomal membrane fluidity (140). Macrophage lysosomes are critical for digestion of scavenged debris or microorganisms (141).

Ligands of the scavenger receptor have been shown to trigger secretory functions of the cell (142). The chronic stimulation by modified-LDL ingestion could make the cells refractory to additional signals, or result in toxicity to the cell. Foam cells may suffer membrane lipid peroxidation or other physical changes due to the large lipid pools within them. The effect of lipid-loading on macrophage functions is an important issue which has not been addressed. We will report on the capabilities of the lipid-laden cell in Chapter 4.

Chapter 2 Materials and Methods

LDL Preparation and Modifications

Preparation of LDL. LDL was prepared as indicated from human plasma anti-coagulated with either EDTA (5 mM, final concentration) or CDP (1.61 g of sodium citrate dihydrate/ 0.19 g of anhydrous citric acid/ 0.14 g of disodium phosphate/ 63 ml of water for 450 ml blood). Isolation of LDL was as in ref. 143 with modifications as follows: 100 ml of freshly drawn blood from healthy volunteers was centrifuged at 850 \times g for 15 min at 25 °C. The separated plasma was then centrifuged at 1300 \times g for 15 min at 4 °C to remove platelets. The density of the plasma was adjusted with KBr and 11.5 ml of the solution was pumped under 27.5 ml saline in heat-sealing tubes (Beckman). The tubes were centrifuged in a VTi 50 vertical rotor at 45,000 rpm, 4 °C, for 3 hr, using a computerized slow acceleration and deceleration program (Beckman #9). LDL was harvested; its density was increased to 1.1 g/ml using KBr; and then it was centrifuged in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered saline (PD; GIBCO), at a density - 1.100 (adjusted with KBr), for 18 hr in a Beckman Ti 70 rotor at 40,000 rpm at 4 °C. This diminished the albumin contamination after the vertical rotor step. The resulting LDLs were called EDTA-LDL (LDL isolated from plasma anti-coagulated with EDTA) and CDP-LDL (LDL isolated from plasma anti-coagulated with citrate).

EDTA-LDL was dialyzed against PD that contained 0.01% (0.26 mM) EDTA (61). CDP-LDL was dialyzed against PD alone, which was continuously sparged with N_2 to prevent oxidation. Dialysis was at 4 °C in 50-100 times the sample volume, for each of three changes of buffer over 12 hr. Dialysis

Figure 2-1. Preparation of LDL from plasma. Human plasma was anti-coagulated with EDTA or CDP. 11.5 ml of density-adjusted plasma was layered under sterile saline (A) and centrifuged for 3 hr as described in methods. LDL is the yellow band in the center of the tube shown in B. LDL was harvested, its density was adjusted to 1.1 g/ml with KBr (C) and was centrifuged again for 18 hr. The final product is shown in D.

Figure 2-1

Preparation of LDL from Human Plasma

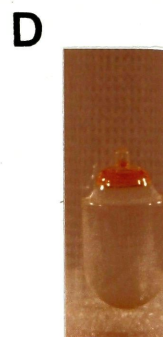


Figure 2-2

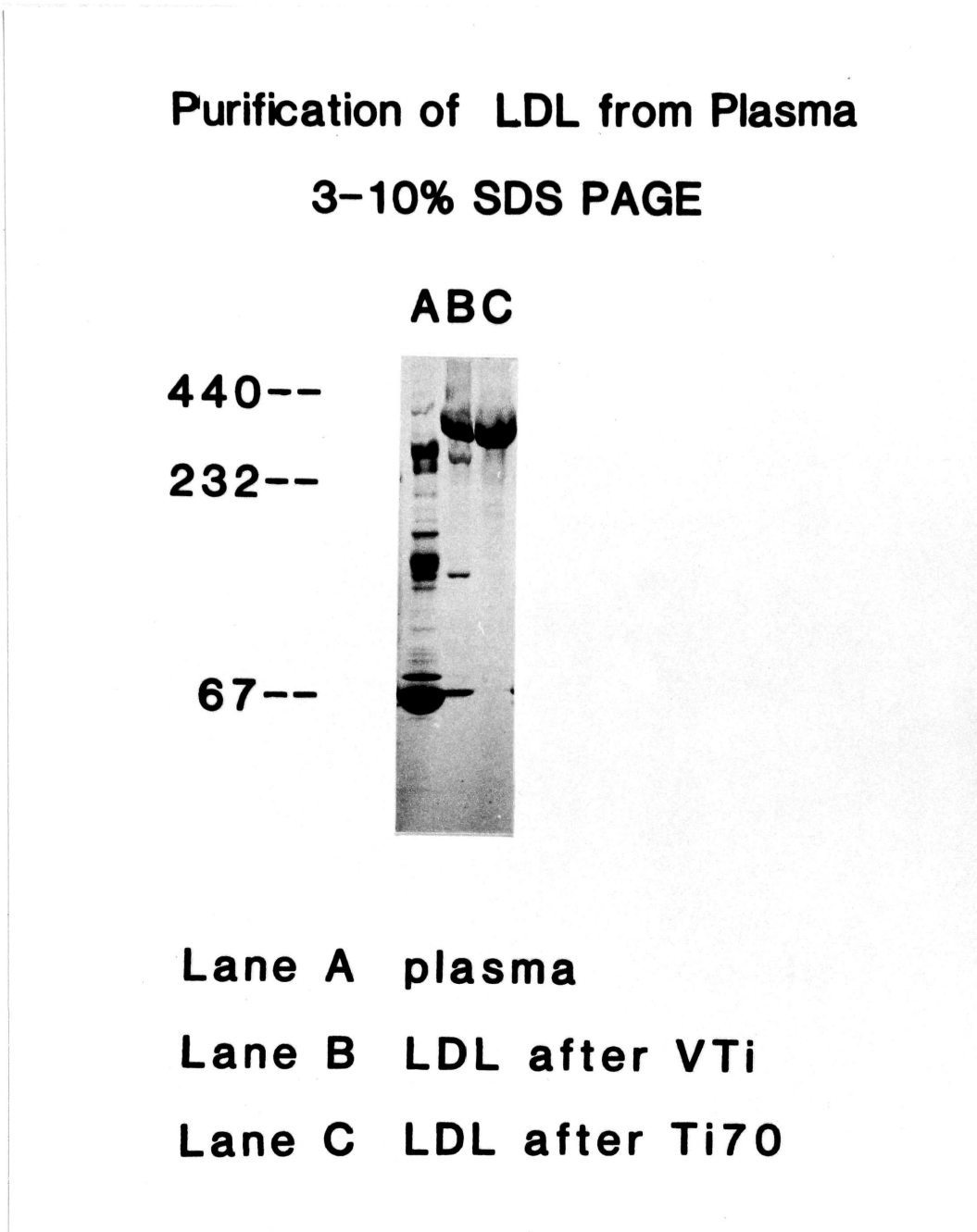


Figure 2-2. 3-10% gradient SDS Page of purification of LDL. 15 μ g of protein was loaded per lane. The gel was stained with Coomassie blue stain. Lanes are as labeled.

tubing was of 12,000-14,000 M_r cutoff (Spectrapor 2, Spectrum Medical Industries, Los Angeles). LDLs were stored in acid-washed glass tubes under N_2 . EDTA-LDL was prepared freshly every two weeks, and CDP-LDL was prepared every week. All LDLs were examined for purity by 3-10% SDS/polyacrylamide gradient gel electrophoresis (adapted from ref 144) before use. EDTA-LDL was iodinated (carrier-free $Na^{125}I$, New England Nuclear) by the method of Bilheimer et al (145) to a specific activity of 400-500 cpm/ng. Protein was determined by the method of Lowry (146).

Acetylation (Ac-LDL) and Acetoacetylation (AcAc-LDL). EDTA-LDL was modified by diketene according to the method of Weisgraber et al. (147). The extent of acetoacetylation was measured by reaction with 2,4,6-trinitrobenzenesulfonic acid (Pierce) in the spectrophotometric assay of Fields (148), as well as by 1% agarose gel electrophoresis, using a barbital buffer system at pH 8.6 (149). Each modification was accompanied by a mock-modified control.

EDTA-LDL was acetylated by repeated additions of acetic anhydride while stirring in an ice water bath, following the method of Basu et al (149). Ac-LDL was dialyzed as described for EDTA-LDL before use in experiments. Extent of acetylation was determined by agarose gel as described above.

Dextran Sulfate LDL (DS-LDL) Complexes. LDL was complexed with dextran sulfate according to the method of Kielian and Cohn (140). As described, LDL and dextran sulfate were combined in an equal weight ratio and then $CaCl_2$ was added to 0.1 M final concentration. DS-LDL was stored at 4 °C overnight and then pelleted, washed 3x with double distilled water at 4 °C, and resuspended to 1 mg/ml LDL in M-10 culture medium (see below). The mobility of DS-LDL complexes in an agarose gel is shown in Figure 2-3.

Figure 2-3

1% Agarose gel of LDL, Ac-LDL, DS-LDL

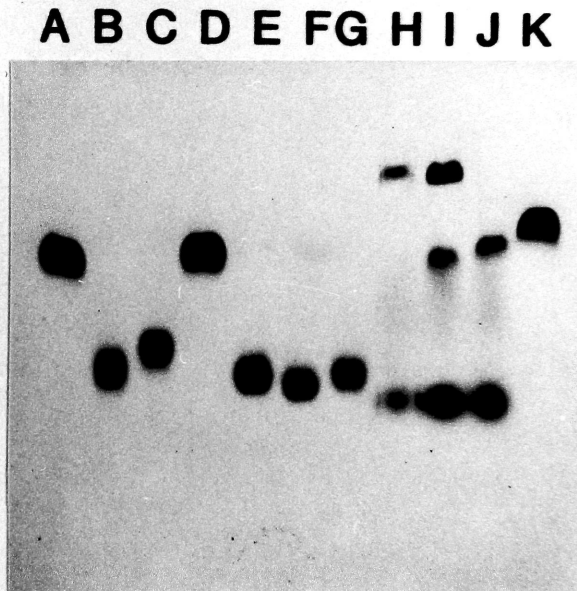


Figure 2-3. 1% Agarose gel of LDL, Ac-LDL, and DS-LDL. 15 ug of protein was loaded per lane, and the gel was stained with Coomassie blue stain. Lanes A, D, K: LDL. Lanes B, C, E, F, G: Ac-LDL. Lanes H, I, J: DS-LDL.

The variations in band patterns from different preparations may be due to size heterogeneity of the dextran sulfate.

H₂O₂ Oxidation of LDL. LDL (ca. 100 µg) was diluted to 0.5 ml in serum-free Ham's F-10 medium (Flow laboratories) and was incubated for 3 hr at 37 °C in covered glass tubes with H₂O₂ (Fisher) and other reagents as described in chapter 3. Catalase (300 U/ml) was added for 15 min at 37 °C before the samples were tested, because H₂O₂ inhibits the TBA assay by 37.5% at concentrations of 1 mM, and by 100% at 0.1 M. Samples were tested by agarose and SDS gel electrophoresis and in duplicate by the TBA assay (see below).

Endothelial Cell Modification of LDL (Ec-LDL). EC-LDL was prepared as described by Steinbrecher et al (61), using the same cell line and medium. LDL at 100 µg/ml was incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂ with confluent monolayers of EC (see below) or serum-washed cell-free flasks in serum-free Ham's F-10 supplemented with penicillin at 100 units/ml, streptomycin at 100 µg/ml, and glutamine at 300 µg/ml. Our use of 25 cm² flasks resulted in the same ratio of volume of medium to cell culture surface area as for the petri dishes used in ref. 61. After 24 hr, medium was collected, centrifuged at 300 x g for 10 min to remove cellular debris, and then tested by agarose gel electrophoresis and in duplicate in the TBA assay.

Cell Culture

Endothelial Cells (EC). A continuous line of rabbit thoracic aorta EC was obtained from V. Buonassissi (W. Alton Jones Cell Science Center, Lake Placid, NY). Cells were incubated at 37 °C in a humidified atmosphere of 95%

air/5% CO₂, in 25-cm² tissue culture flasks (Corning) in Ham's F-12 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), glutamine, penicillin, streptomycin as above. Medium was changed twice per week, and cells were passaged once per week, using 0.25% trypsin (Flow laboratories) in 1 mM EDTA. The cells were mycoplasma-free as tested by fluorescent bisbenzimidazole staining (Hoechst 33342; Aldrich).

Human Monocyte/ Macrophage Culture. Cells were isolated from human peripheral blood or buffy coat using a Ficoll/Hypaque gradient (Pharmacia) as described (150), and cultured in RPMI 1640 (KC Biologicals, Lenexa, KS) with glutamine, penicillin, and streptomycin as above, and 25% (v/v) human serum. Cells were incubated for 2 hr to allow adherence, then nonadherent cells were rinsed away and culture medium was replaced (day 0). Medium was changed again on days 1 and 4. Recombinant human γ interferon (Genentech, South San Francisco) at 100 antiviral units/ml was added on day 4. Cells were assayed for H₂O₂ release on day 7.

Mouse Peritoneal Macrophage and Foam Cell Culture. Cells were isolated by peritoneal lavage of specific pathogen-free female ICR mice (Trudeau Institute, Lake Placid, NY.) weighing 25-30 g, as previously described (151). Peritoneal cells (8×10^6) in minimal essential medium (MEM) supplemented with 10% (v/v) FBS and antibiotics and glutamine as above (M-10) were added to 35-mm plastic tissue culture dishes (Nunc, InterMed, Denmark) or at 1×10^6 to 13 mm acid-washed glass coverslips (Gold Seal 3550). Cells were incubated for 2 hr to allow adherence, nonadherent cells were rinsed away and culture medium was added (day 0). Medium with all additions was replaced daily.

Foam cells were prepared by addition of lipoproteins to M-10 as follows:

1. M-10 with 200 $\mu\text{g/ml}$ Ac-LDL, called Ac-LDL macrophages.
2. M-10 with 10 $\mu\text{g/ml}$ DS-LDL, called DS-LDL macrophages.
3. MEM with antibiotics and gentamycin and 1% FBS (v/v; M-1) with 200 $\mu\text{g/ml}$ Ac-LDL and 20 μM BSA and 100 μM oleic acid, called Low serum/Oleic (LS/OI) macrophages.

Assays

H₂O₂ assay. Freshly isolated human mononuclear leukocytes were plated at 2×10^5 cells/well and assayed for H₂O₂ release in 96-well flat-bottom polystyrene tissue culture plates (Corning). For monocyte-derived macrophages and mouse peritoneal macrophages, mononuclear cells were plated at a density of 1×10^6 cells per 13-mm glass coverslip, cultured, and assayed for H₂O₂ release in 24-well plates (Falcon, Becton Dickinson Labware, Oxnard, California). EC were plated at 1×10^4 cells per well in a 96-well plate and were assayed 1 or 2 days later. H₂O₂ was assayed by determining the H₂O₂-dependent loss of scopoletin fluorescence using a Dynatech Corporation Microfluor plate reader (152).

Thiobarbituric Acid (TBA) Assay of Lipid Peroxidation.

Samples (100–300 μl) containing 25–30 μg of LDL protein were tested as described in ref. 153. Each sample was treated with 1.5 ml of 20% (w/v) trichloroacetic acid (TCA) followed by 1.5 ml of freshly prepared 0.67% TBA (Aldrich) and heated to 95 °C for 15 min. The amount of malondialdehyde formed was determined by fluorescence measured on a Perkin-Elmer MPF-44a fluorometer (515-nm excitation, 553-nm emission wavelengths). Freshly diluted 1,1,3,3-tetramethoxy propane (Aldrich) served as standard. The assay is sensitive to 0.1 nmol of malondialdehyde.

Cholesterol Assay. Total cellular cholesterol determination was made after extraction of cellular lipids by the method of Bligh and Dyer (154). 35 mm dishes of control macrophages or foam cells were rinsed twice in PD containing 2 mg/ml BSA (PD/BSA), and twice more in PD alone before being scraped with a rubber policeman. An aliquot of the scraped cell suspension was removed for protein determination and the rest was added to 3.75 ml chloroform/methanol 1:2 (v/v) at 4 °C, covered, vortexed, and allowed to stand for 10 min. 1.25 ml of chloroform was added to each sample followed by 1.25 ml of water, both at 4 °C. The mixture was vortexed again and centrifuged at 1500 rpm at 4 °C for 10 min in a Damon/IEC CRU 5000 Centrifuge. The lower, organic phase was recovered and brought to dryness under a stream of N₂. After drying, the samples were resuspended in 200 µl chloroform/methanol and heated for 15 min at 75 °C in a heating block. Samples were again resuspended in 200 µl ethanol and 2 ml assay buffer was added to each tube before being covered and incubated in air for 30 min at 37 °C. Fluorescence was determined using the Perkin-Elmer MPF-44a fluorometer, at wavelengths of 325-nm excitation, 415-nm emission. Sigma aqueous cholesterol standard was freshly diluted for the standard curve in each assay. The assay mix was as described by Gamble et al (155), using a peroxidase-catalyzed reaction detecting H₂O₂ from enzymatic cholesterol oxidation and producing a stable fluorescent product.

Oil Red O Staining and Extraction. Coverslips were rinsed in PD/BSA and fixed in 5% formalin in PD (v/v) for 30 min. The coverslips were blotted, rinsed in PD, and stained in Oil Red O (Kodak) at 0.5% in 60% triethylphosphate (Kodak) for 15 min. The coverslips were rinsed in double distilled water, and mounted on slides with nail polish. This is a standard method (140).

For extraction and quantification of Oil Red O staining, fixed and stained coverslips were extracted in 0.8 ml dioxane, and compared to a dioxane blank at OD₅₃₀ (156). Dioxane was selected because it resulted in a higher specific OD than chloroform, chloroform/methanol 1:2, or chloroform/methanol 2:1.

Thin Layer Chromatography (TLC) of Foam Cell Lipids. One-dimensional TLC to separate lipids into individual classes was done according to the method of Falcone et al (157), using petroleum ether/ diethyl ether/ acetic acid, 97: 100: 3 (Mallinkrodt, chromair quality) as the first solvent system, and petroleum ether/ diethyl ether, 200: 6, as the second solvent system. 35 mm dishes of control macrophages and foam cells were rinsed 3x in PD and extracted in situ with 2 ml/dish of hexane/ isopropyl alcohol, 3:2 for 30 min, and then extracted again for 10 min with another 1 ml of hexane/ isopropyl alcohol, 3:2. The three ml were combined in an acid-washed tube and dried under N₂. Samples were resuspended in 100 μ l chloroform/methanol 2:1 and spotted onto pre-washed and heat-inactivated silica gel 60 precoated TLC plates (Thomas). Plates were stained with freshly prepared 0.0001% rhodamine 6G in 0.25 M dibasic potassium phosphate, and photographed under ultraviolet light.

Two dimensional TLC was performed on Redicoat (Supelco, Inc., Bellefonte, PA) heat-inactivated plates as described in ref. 158. Briefly, the first direction was chloroform/methanol/ammonium hydroxide, 65/25/5, v/v/v, and after drying the plate was turned 90 degrees and chromatographed in chloroform/acetone/methanol/acetic acid/water, 33/44/11/11/5.5, v/v/v/v/v. Lipid standards were visualized with I₂ vapor, and lipid-containing regions of the plate were scraped into scintillation vials with 1 ml water and 10 ml aquasol (National Diagnostics

Inc., Somerville, NJ) per sample before radioactivity was determined in a scintillation counter .

Phagocytosis of Immunoglobulin G-coated Sheep

Erythrocytes (ElgG). ElgG, sheep erythrocytes coated with rabbit anti-erythrocyte IgG, were kindly provided by Dr. Samuel Wright. 35 mm dishes of control macrophages and foam cells were washed 2x in MEM before 1.5 ml of MEM containing 2.0×10^7 ElgG/ml was added. After 45 min of incubation at 37 °C in a humidified atmosphere of 95% air/5% CO₂, the dishes were rinsed in PD for determination of attachment index. For phagocytosis index, the dishes were dipped 5x in distilled water for 1 second per dip and then returned quickly to PD. This procedure lysed any uningested ElgG. Cells were fixed in 2.5% gluteraldehyde in PD (v/v), and the number of attached or ingested particles was counted under phase-contrast microscopy using an inverted microscope with a 40x water-immersion objective (159).

Fibrinolysis by Macrophages and Foam Cells. Peritoneal cell suspensions were plated onto ¹²⁵I-fibrin coated 24-well plates, prepared according to an established method (160) and provided by Drs. L. Ossowski and R. Mira y Lopez. Cells were plated at 2×10^5 cells/well in MEM with 5% FBS, antibiotics and glutamine as above and 100 µg/ml soybean trypsin inhibitor to prevent fibrinolysis during culture. Nonadherent cell removal, lipoprotein additions, and daily culture medium changes were made as described for foam cells. After 48 hr of incubation, the culture medium with soybean trypsin inhibitor was removed, cells were rinsed with MEM and the fibrinolytic assay was initiated by the addition of MEM supplemented with 5% acid-treated FBS (AT-FBS). Acid treatment of FBS inactivates serum plasmin inhibitors. At the time points indicated, radioactivity of triplicate aliquots of the supernatant was determined and variation did not exceed

10%. The percent of the ^{125}I -fibrin substrate solubilized was determined after subtraction of background counts, and is expressed per 5×10^4 cells, based on nuclear cell counts (161) from the wells at the end of the fibrinolytic assay.

^{35}S -Methionine Secreted Protein Profile. 35 mm dishes of control macrophages and foam cells were washed 3x with methionine-free RPMI-1640 and labeled with ^{35}S -methionine (New England Nuclear) in methionine-free RPMI-1640 for four hr as described by Werb and Chin (162). After the incubation, the culture medium was collected, centrifuged for 3 min in an Eppendorf Microfuge, and the supernatant was transferred to a fresh tube. Before precipitation of proteins for 30 min on ice with 5% TCA, *M. lysodeikticus* at 2 mg/ml in normal saline was added as a carrier. Samples were pelleted and then washed 2x in 1 ml of 5% TCA, and finally resuspended in SDS sample buffer. After the culture medium was removed and the monolayer was rinsed 2x with PD, ^{35}S -methionine labelled cellular proteins were harvested from 35 mm dishes by adding SDS sample buffer directly to the dish. Before samples were loaded on gels, they were heated to 95 °C for 3 min and 5 μl aliquots were removed for counting with aquasol in a scintillation counter. Samples were loaded onto a 7-18% gradient SDS-PAGE, electrophoresed, and developed for autoradiography as described.

Immunoprecipitation of Apolipoprotein E. Apolipoprotein E (apo E) was labelled from control macrophages and foam cells as described above for the ^{35}S -methionine secreted protein profile. Goat anti-human apo E antiserum or control goat serum was added to the culture supernatant at 1:100 dilution in antibody buffer (150 mM NaCl, 10mM Tris-HCl, 1% powdered milk). Samples were allowed to shake on the nutator at 4 °C for 2 hr and then 50 μl of a 50% slurry of pre-cleared (with antibody buffer)

Protein A-agarose beads (Boehringer Mannheim Biochemicals) was added, and samples were rotated for an additional 1 hr. Samples were pelleted by 2 min centrifugation at highest power in a Savant High Speed Microcentrifuge. The resulting pellet was washed 5x with antibody buffer and then 5x with PD. The pellets were suspended in 50 μ l SDS sample buffer containing 60 mM dithiothreitol, heated, counted, loaded, and processed for autoradiography as above.

Release of ^3H -20:4 and 20:4 Metabolite Profile. 35 mm dishes of control macrophages and foam cells were cultured from day 1 to day 2 with the same lipoprotein additions as described and 0.5 μCi ^3H -20:4/dish. After 16 hr equilibration, monolayers were rinsed 3x with PD and 1.2 ml MEM was added to each dish, with or without 160 $\mu\text{g}/\text{ml}$ zymosan or 10 $\mu\text{g}/\text{ml}$ of the calcium ionophore, A23187, as indicated in chapter 4. Cells were incubated at 37 $^{\circ}\text{C}$ in a humidified atmosphere of 95% air/5% CO_2 for the times indicated, and aliquots of the culture supernatant were removed and radioactivity was determined in aquasol in a scintillation counter. Monolayers were lysed in 2 x 0.5 ml 0.05% triton X-100, and portions were removed for determination of radioactivity or assayed for protein.

Metabolites of 20:4 from 1 ml culture medium were extracted with 1 ml ethanol and 10 μ l 0.88% (w/v) formic acid, and then extracted twice with 1 ml chloroform. The organic phases were combined, dried under N_2 , and resuspended in the appropriate solvent for further purification by reverse-phase high-pressure liquid chromatography (HPLC) as described in ref. 163. To summarize: the 20:4 metabolite extracts were chromatographed on an Ultrasphere C-18 column, using methanol/ water/ acetic acid, 65: 34.9: 0.1 (v/v/v), pH 5.4, as solvent 1, followed by methanol/ acetic acid, 100: 0.01 (v/v) as solvent 2. The cyclooxygenase products were analyzed on a Waters

fatty acid analysis column, using water/ acetonitrile/ benzene/ acetic acid, 76.7: 23: 0.2: 0.1 (v/v/v/v) as solvent 1, followed by methanol/ acetic acid, 100: 0.01 (v/v) as solvent 2.

Interleukin 1 (IL-1) Release. IL-1 release was determined by the method of Koide et al (164,165), using conditioned medium from 48 hr control macrophage and foam cell cultures. Serial dilutions of the culture supernatant were added to wells of a 96-well plate containing $2-3 \times 10^4$ D10 G4.1 cells/well. D10 G4.1 is a T lymphocyte cell line which proliferates in response to IL-1. Proliferation was determined following a pulse of 0.5 μ Ci 3 H-thymidine (Schwartz Mann division of Becton Dickinson, Orangeburg, NY) for 16 hr. Murine recombinant IL-1 (gift of Dr. P. Lomedico, Hoffman-LaRoche) was used as a standard. At least 6 dilutions of each supernatant were assayed in duplicate and the number of IL-1 U/ml of the original supernatant was determined by linear regression analysis.

Chapter 3 Effects of Reagent and Cell-generated H₂O₂ on the Properties of LDL

Introduction

Many studies have suggested that endothelial cells, smooth muscle cells and circulating leukocytes can modify LDL, resulting in its subsequent enhanced uptake and degradation by mouse peritoneal macrophages via the scavenger receptor that recognizes chemically modified forms of LDL (59-67). This biological modification of LDL leads to enormous cholesterol ester accumulation in macrophages (59,60), and it is believed to accelerate foam cell formation *in vivo*, contributing to the formation of the fatty streak precursor lesions of atherosclerosis. While exiting to the circulation, the lipid-laden foam cells may disrupt the endothelial cells lining the artery, thereby exposing the underlying matrix to lipoproteins and platelets from the blood. This would mark the initiation of a major atherosclerotic plaque.

The mechanisms postulated to underlie the cell-mediated modification of LDL include oxidation, lipolysis, and proteolysis (61-67). The biological modification of LDL has been reported by Steinbrecher et al to coincide with the acquisition of thiobarbituric acid (TBA) reactivity, representing oxidation of the lipid portion of LDL. In no instance has this alteration been clearly defined in molecular terms. For this reason, we undertook a detailed study of the role of a major oxidizing secretory product of phagocytic cells, H₂O₂. H₂O₂ is a relatively long lived, uncharged product of the reduction of molecular oxygen that can pass across biological membranes (166,167) and oxidize substrates at both intracellular and extracellular sites. We have examined the susceptibility of LDL to oxidation by known concentrations of chemical

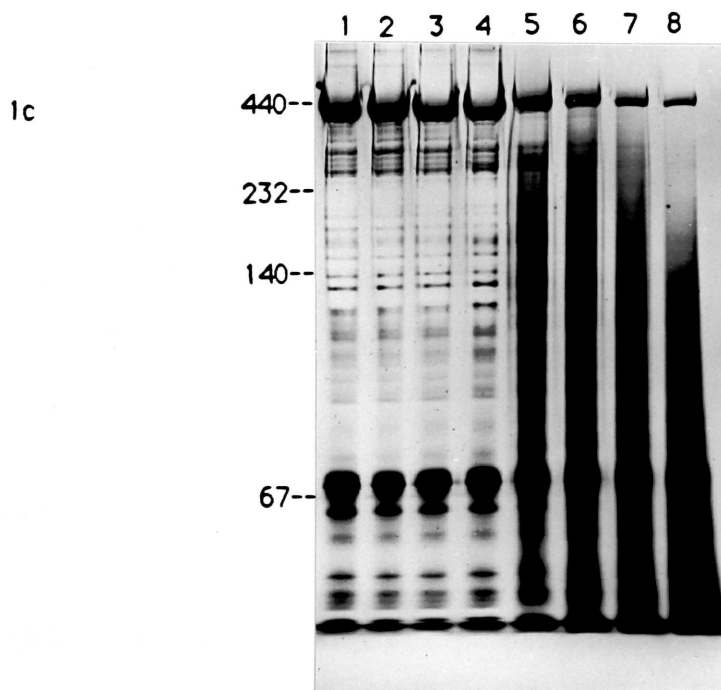
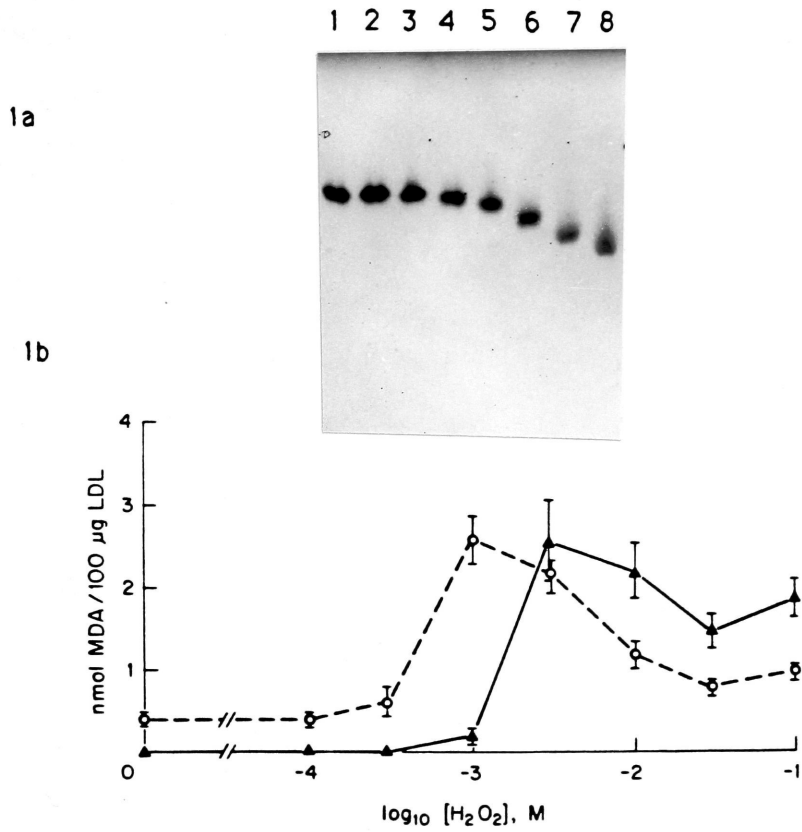
oxidants, by endothelial cells, and by the diverse group of reactive oxygen intermediates produced by monocytes. In addition, we have investigated the ability of monocytes and macrophages to respond to modified LDLs by releasing H_2O_2 . The results suggest two conclusions: first, that LDL is relatively resistant to the generation of TBA-reactive moieties by the concentrations of reactive oxygen intermediates secreted by a widely-studied rabbit aortic endothelial cell line or by human monocytes; and second, that biological modification of LDL can proceed in the absence of its detectable oxidation.

Results

Characterization of H_2O_2 -oxidized LDL. The common assay of oxidation of LDL used by Steinbrecher et al (61), by us, and by many others is the TBA assay, which measures malondialdehyde-equivalent oxidation products of conjugated unsaturated fatty acids. A fluorescent pigment is formed by the combination of two TBA molecules with such a lipid product. In LDL, sources of this material are the phospholipids and triglycerides, a small proportion of the total lipid of the particle. The quantities of defined oxidants required to generate a given level of reactivity of LDL in the TBA assay have, to our knowledge, not been reported. Accordingly, we tested the effects of various concentrations of H_2O_2 and the products of its metal-catalyzed redox reactions, using the same serum-free culture medium employed by others for cell-dependent modification of LDL. Fig 3-1 characterizes LDL that was reacted with reagent H_2O_2 and tested for oxidation of lipid in the TBA assay and for alteration of the apoprotein by 1% agarose and 3-10% gradient SDS gel electrophoresis, as described in methods.

Figure 3-1. Characterization of H₂O₂-oxidized LDL. EDTA-LDL and CDP-LDL at 100 μg/ml in Ham's F-10 medium were treated with various concentrations of H₂O₂ for 3h at 37 °C. a. Coomassie blue-stained 1% agarose gel in barbital buffer system, pH 8.6. 20 μg LDL per lane. H₂O₂ concentration: lane 1, 0; lane 2, 1x10⁻⁴ M; lane 3, 3x10⁻⁴ M; lane 4, 1x10⁻³ M; lane 5, 3x10⁻³ M; lane 6, 1x10⁻² M; lane 7, 3x10⁻² M; lane 8, 1x10⁻¹ M. b. Oxidation measured by the TBA assay of EDTA-LDL (▲—▲) and CDP-LDL (○- - -○). Data are the mean of 6 experiments ± SEM. c. Silver-stained 3-10% gradient SDS-PAGE of 20 μg LDL samples treated with H₂O₂, concentrations in each lane as in fig 1a.

Figure 3-1



Within each experiment, a single donor's plasma was used to compare EDTA-LDL and CDP-LDL. As shown in Fig 3-1B, only concentrations of H_2O_2 above 3×10^{-4} M induced TBA reactivity in EDTA-LDL, with 3×10^{-3} M H_2O_2 required for maximal reactivity. The dose required to reach half-maximal reactivity (ED_{50}) of H_2O_2 was 1.7×10^{-3} M for EDTA-LDL. However, CDP-LDL was 4-fold more sensitive to oxidation by H_2O_2 , showing TBA reactivity above background at 3×10^{-4} M, a maximum value at 1×10^{-3} M, and ED_{50} at 4.5×10^{-4} M. The decline of malondialdehyde values at supramaximal concentrations of H_2O_2 probably reflects lipid oxidation to other products not measured by this assay.

The agarose gel (Fig. 3-1A) shows the increased negative charge of EDTA-LDL reflected by its increased migration after oxidation by increasing concentrations of H_2O_2 . The plot of R_f values versus the logarithm of concentrations was linear above 1 mM H_2O_2 . At 0.3 M H_2O_2 , the highest concentration tested, the R_f value was 0.48. By extrapolation, 3 M H_2O_2 would be required to generate the R_f value of 0.51 seen for acetoacetylated LDL.

Similarly, the SDS gel (Fig. 3-1C) shows the 420 kd band of the apolipoprotein B of EDTA-LDL gradually diminished in lanes 4-8 with increasing concentrations of H_2O_2 . Above 1 mM, the protein was extensively degraded to lower M_r peptides.

Cell-derived reactive oxygen intermediates other than H_2O_2 may be involved in oxidizing LDL. Consequently, we next tested the sensitivity of LDL to oxidation by H_2O_2 in combination with cuprous or ferrous ions (Fenton's reagent, a source of O_2^- and $\text{OH}\cdot$; 168), and with lactoperoxidase and NaI (to generate hypohalous ions; Table 3-1). At 0.3 mM H_2O_2 , TBA reactivity was 1.33-fold above control when lactoperoxidase/NaI was added,

Table 3-1. Effect of Additions to Reagent H₂O₂ on Oxidation of LDL

(nmoles MDA/ 100 μg LDL)

H ₂ O ₂	no add.	LPO/NaI	FeSO ₄	CuSO ₄	SOD/Cu ⁺⁺	Mann/Cu ⁺⁺
0.3mM	0.6 ± .13	0.8 ± .16	0.8 ± .29	1.4 ± .14	0.4 ± .08	1.0 ± .22
		p<0.05	NS	p<0.01	p<0.02	NS
3.0mM	2.1 ± .26	2.5 ± .34	2.1 ± .29	2.5 ± .34	2.6 ± .44	2.5 ± .48
		p<0.01	NS	p<0.05	NS	NS
n=	8	5	4	8	4	4

CDP-LDL was incubated at 37 °C in Ham's F-10 medium for 3h as described in Materials and Methods.

Data are means ± SEM for the number of experiments indicated. Significance of difference between each addition and no addition was assessed with the two-tailed t test. NS = p>0.05. CuSO₄ was 10 μM, FeSO₄ was 50 μM, SOD was 30 U/ml, mannitol was 50 μM, LPO was 100 mU/ml, NaI was 100 μM. With no H₂O₂, CDP-LDL had 0.4 ± .05 nmol MDA/100 μg LDL, n=8, and CDP-LDL with Cu⁺⁺ had 0.8 ± 0.1, n=5. Medium alone and with all additions tested with H₂O₂ showed 0 MDA.

and 2.33-fold above control in the presence of Cu^{2+} . Addition of superoxide dismutase to the LDL incubations containing Cu^{2+} and H_2O_2 abolished the enhancement in TBA reactivity, suggesting that the effect of Cu^{2+} may be due to generation of O_2^- or a product of O_2^- and H_2O_2 , such as $\text{OH}\cdot$. Both Cu^{2+} and lactoperoxidase/NaI were able to increase slightly the effect of 3 mM H_2O_2 . The maximum observed TBA value in our experiments was 4 nmol MDA/100 μg LDL.

Coincubation of LDLs with Endothelial Cell Monolayers. We next examined the effect of the coculture of LDL with endothelial cells (EC-LDL), reproducing closely the conditions employed by Steinbrecher et al. (61). As shown earlier for H_2O_2 -oxidized LDL, we noted significant differences between EDTA-LDL and CDP-LDL (Fig. 3-2 A and B). Endothelial cells appeared to retard the formation of TBA reactivity for EDTA-LDL, since EDTA-LDL incubated in the cell-free flasks had twice the TBA value of that incubated in the presence of cells. This difference was even more pronounced when $10\ \mu\text{M}$ Cu^{2+} was present, in which case cell-free incubated EDTA-LDL was about three times as TBA-reactive as the sample incubated with endothelial cells. However, for CDP-LDL, the TBA reactivity was very similar for LDLs incubated in the absence or presence of endothelial cell monolayers, and the enhancement in oxidation due to Cu^{2+} was also less, probably because the original level was high. Thus, we were unable to demonstrate endothelial cell-dependent oxidation of LDL by means of TBA reactivity. The linear relationship between TBA oxidation and R_f values on agarose gels was maintained in these experiments (data not shown).

EC-LDL Degradation by Murine Macrophages. In confirmation of the biological modification described by Steinbrecher et al, we measured increased macrophage degradation of EC-LDL. Degradation is an indication of

Figure 3-2. Endothelial Cell Modification of LDL

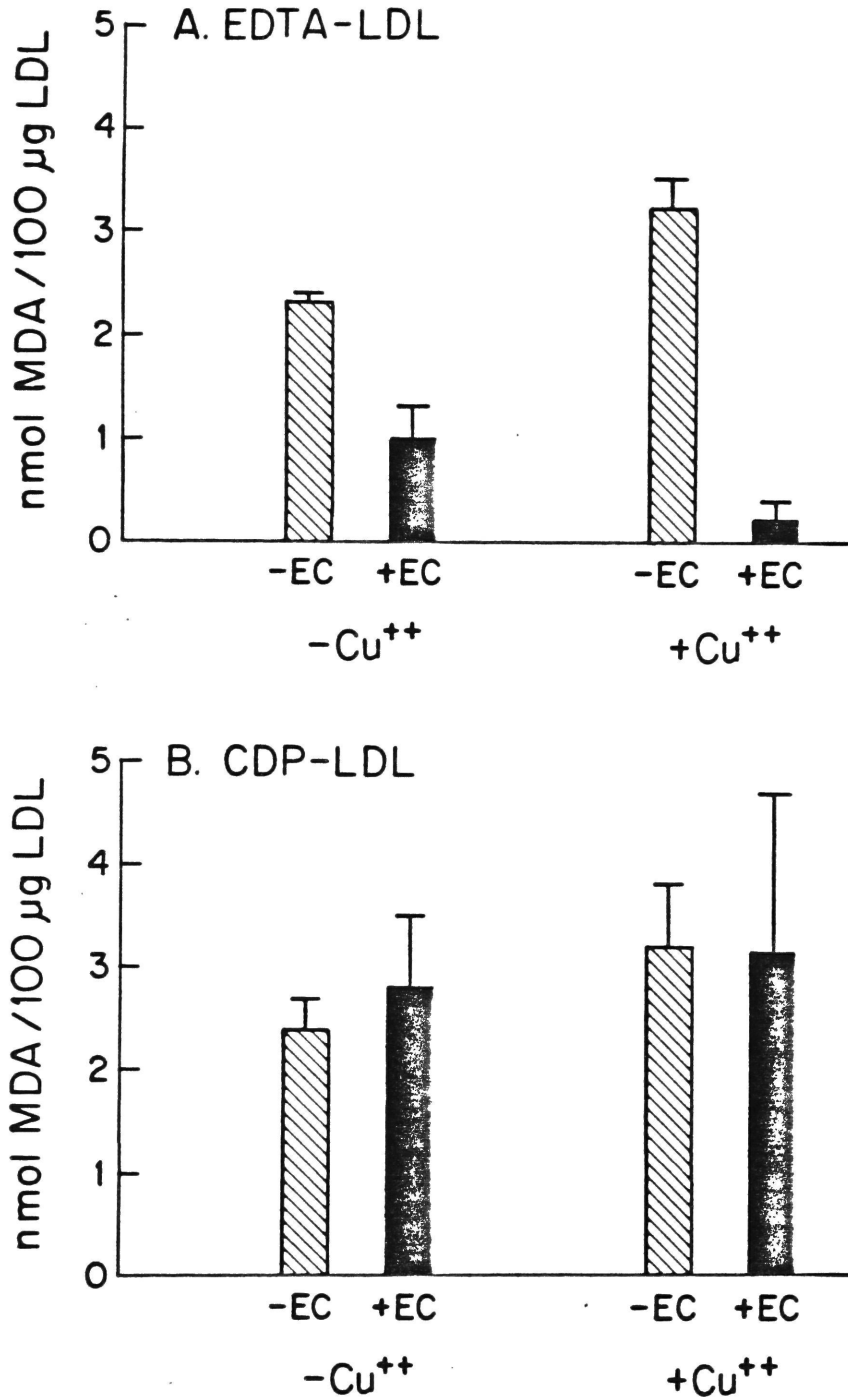


Figure 3-2. Endothelial Cell Modification of LDL. TBA assay of samples of 100 µg LDL/ml after 24 h incubations in Ham's F-10 medium in the presence (solid bars) or absence (hatched bars) of cells as indicated (+ EC). For EDTA-LDL, n=22 independent experiments without copper, n=11 with copper. For CDP-LDL, n=8 without copper, n=5 with copper. Data are means ± SEM.

Table 3-2**Mouse Macrophage Degradation of LDL**

<u>Preincubated* With</u>	<u>Degradation μg LDL/mg/5h</u>
Endothelial Cells	11.43 ± 1.14
Medium alone	3.80 ± 1.32 ^a
Not preincubated	2.45 ± 0.14 ^a

*¹²⁵I-LDL (100 μg/ml) was incubated for 24 h in serum-free Ham's F-10 medium before incubation with mouse macrophages. Data are the means ± SD for 3 experiments, a = P < 0.02 for each sample compared to preincubated with EC.

Table 3-3**Mouse Macrophage Degradation of LDL**

<u>Preincubated* With</u>	<u>Degradation μg LDL/mg/5h</u>
Endothelial Cells (EC)	4.48 ± 0.23
EC + SOD + Catalase	4.05 ± 0.80 ^a
EC + BHT + Vitamin E	3.98 ± 0.30 ^b
Medium alone	1.46 ± 0.16
Not Preincubated	1.39 ± 0.17

*¹²⁵I-LDL (100 μg/ml) was incubated for 24 h in serum-free Ham's F-10 medium before incubation with mouse macrophages. Data are the means ± SD for 3 experiments, a = not significant, b = P < 0.05 for each sample compared to preincubated with EC.

cell binding and ingestion of the LDL. Three forms of LDL were compared for the rate of degradation by cultures of resident mouse peritoneal macrophage monolayers: ^{125}I -labeled LDL incubated with endothelial cells (^{125}I -labeled EC-LDL), ^{125}I -labeled LDL incubated without endothelial cells, and not pre-incubated ^{125}I -labeled LDL. In agreement with previous reports (59-61,63-65,69), ^{125}I -labeled EC-LDL was degraded 3-5 times more rapidly by mouse macrophages than either the ^{125}I -labeled LDL incubated without endothelial cells, or the not pre-incubated ^{125}I -labeled LDL (Table 3-2). A separate set of experiments shown in Table 3-3 has different baseline values, but shows clearly that the addition of the anti-oxidants superoxide dismutase (SOD; 80 $\mu\text{g}/\text{ml}$), catalase (1000 U/ml), butylated hydroxytoluene (BHT; 20 μM), or vitamin E (100 μM) to these preincubations only slightly reduced the rate of degradation of LDL by mouse macrophages, while completely eliminating TBA reactivity (data not shown). Thus, all samples of LDL preincubated with endothelial cells showed higher rates of degradation by mouse macrophages than the LDLs preincubated without endothelial cells, with or without anti-oxidants.

Determination of Endothelial Cell H_2O_2 and O_2^- Release. To evaluate the oxidizing potential of these endothelial cells, we tested their ability to release H_2O_2 and O_2^- . Table 3-4 shows an apparent oxidation of a very small amount of the scopoletin indicator, about half of which is shown to be H_2O_2 independent by the controls omitting horseradish peroxidase and adding catalase. The addition of superoxide dismutase did not result in any increase in the 5 nmol of $\text{H}_2\text{O}_2/\text{mg}/2$ hr detected, suggesting that these endothelial cells released extremely little O_2^- .

Monocyte Oxidation of LDL. As confirmed above, endothelial cells are not reported to secrete abundant reactive oxygen intermediates (169). In

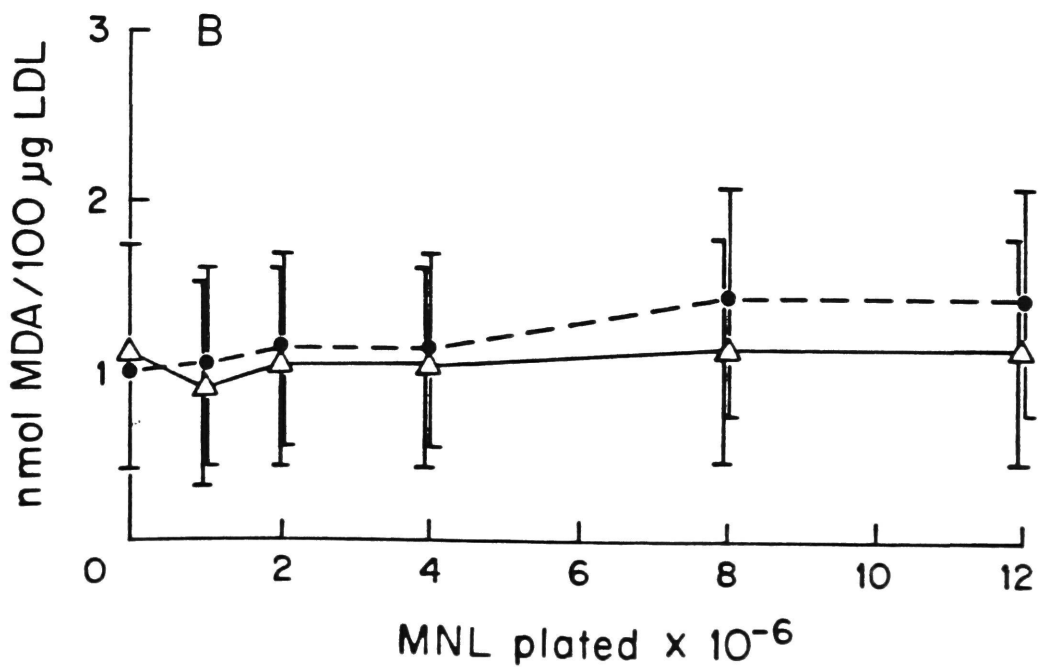
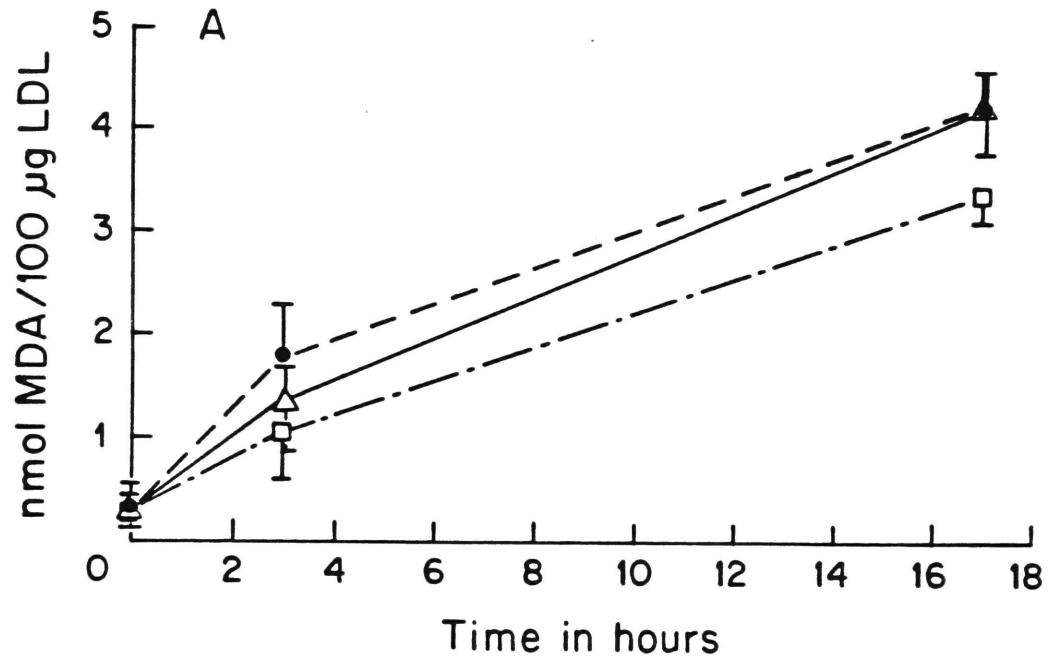
Table 3-4. Release of H₂O₂ by Endothelial Cells

Condition	-PMA	+PMA
	nmoles H ₂ O ₂ / mg protein/2h	
complete system	10.7 ± 4.9	12.0 ± 5.4
plus SOD (30 U/ml)	9.1 ± 5.3	12.9 ± 5.4
plus catalase (300 U/ml)	4.1 ± 2.2	3.4 ± 2.0
minus HPO (background)	4.8 ± 2.5	9.4 ± 4.2

Rabbit EC were trypsinized and plated in a 96-well plate for assay 2-3 days later. Phorbol myristate acetate (PMA) at 100 ng/ml was used as a potential secretagogue. Three separate experiments were done over a range of cell protein values (2-20 µg/well.) In each experiment n=9 for control, n=5 for experimental. The data are means ± standard errors of the mean. Complete system was Krebs-Ringer phosphate buffer with 5.5 mM glucose, 1 mM NaN₃, 1 purpurogallin unit/ml horseradish peroxidase (HPO), 25 µM scopoletin.

Figure 3-3. Mononuclear (MNL) Cell Oxidation of LDL. a. Time course for 8×10^6 MNL plated/16 mm well. Plates were rinsed after 2h and LDL was added at 100 $\mu\text{g/ml}$ in Ham's F-10 medium. Data are the means of four experiments \pm SEM. The three samples tested are of LDL without cells (\square ----- \square) or cells in the absence (Δ — Δ) and presence (\bullet ----- \bullet) of PMA. b. Dose curve of MNL plated as above with data taken at time = 3 h. n=3. Means \pm SEM. (\bullet ----- \bullet) with PMA; (Δ ----- Δ) no PMA.

Figure 3-3. Mononuclear Leukocyte Oxidation of LDL



contrast, human monocytes are potent sources of O_2^- , H_2O_2 , OH^\cdot , and a myeloperoxidase halogenation system (150,170). Thus, even though monocytes do not secrete enough H_2O_2 to attain the concentrations producing TBA reactivity in Fig. 3-1, their production of additional, more potent oxidants might make them effective producers of TBA reactivity in LDL. Accordingly, freshly isolated human mononuclear leukocytes were plated at 8×10^6 cells per 16-mm well, rinsed after 2 hr of incubation to remove nonadherent cells, and CDP-LDL at 100 $\mu\text{g}/\text{ml}$ was added, using the same medium as in the endothelial cell experiments, in the presence and absence of 100 ng/ml PMA to trigger the respiratory burst. In assays lasting up to 17 hours, the TBA assay did not reveal any cell-dependent oxidation of LDL (Fig. 3-3A). The TBA reactivity that is shown is approximately equal for the cell-free samples and the samples in the presence of monocytes with or without PMA. Increasing the number of cells did not increase the cell-independent TBA reactivity seen after 3 hours (Fig. 3-3B).

Monocyte/Macrophage H_2O_2 Release with LDLs as Triggers.

Ligands of the scavenger receptor have been shown to trigger macrophage secretory functions (142). A final query concerned the ability of native and modified LDLs to trigger the release of reactive oxygen intermediates-- H_2O_2 , O_2^- , OH^\cdot , and hypohalous ions--by monocytes and macrophages via this mechanism (Table 3-5). Monocytes, cultured macrophages, and macrophages treated with human recombinant IFN_γ were exposed to the lipoproteins, and their respiratory burst response was compared with that elicited by PMA, using H_2O_2 as a marker. The level of H_2O_2 release by fresh monocytes in the presence of modified LDLs was only slightly changed in comparison to the model secretagogue, PMA. The PMA-induced H_2O_2 releasing capacity declined with time in culture, but the cells could be kept near the original

Table 3-5. LDLs as Triggers for H₂O₂ Release from Human Monocytes and Macrophages

Condition	Monocytes		Macrophages			
	-	+	-	+	-	+
rIFN- γ		-		-		+
PMA	-	+	-	+	-	+
control	26 \pm 9	520 \pm 39	18 \pm 8	62 \pm 28	54 \pm 29	432 \pm 214
CDF-LDL	58 \pm 26	686 \pm 106	8 \pm 4	101 \pm 19	50 \pm 14	301 \pm 76
EDTA-LDL	54 \pm 13	623 \pm 94	57 \pm 14	124 \pm 15	56 \pm 17	343 \pm 87
AcAc-LDL	68 \pm 10	699 \pm 74	54 \pm 16	143 \pm 15	63 \pm 15	322 \pm 61
mock AcAc-LDL	75 \pm 7	648 \pm 94	26 \pm 5	117 \pm 20	67 \pm 12	269 \pm 60

LDLs were used at 100 μ g/ml in assay buffer. H₂O₂ is expressed as nmoles/mg cell protein/h for monocytes tested on day of harvest and per 3h for macrophages tested after 7 days of culture. Values are means of 4-6 experiments \pm SEM. rIFN- γ was 100 U/ml. PMA (phorbol myristate acetate) was at 100 ng/ml.

level by the addition of recombinant IFN γ . Only small changes in release were observed with native or modified LDLs at 100 $\mu\text{g}/\text{ml}$, although Oil Red O staining of replicate coverslips showed lipid accumulation.

Discussion

Incubation of EDTA-LDL with endothelial cells or smooth muscle cells in the absence of serum leads to modification resulting in more rapid degradation of the LDL when subsequently added to cultures of mouse macrophages (59-61,63-65,69). Such LDLs were markedly positive in the TBA assay, which has led to the widely embraced interpretation that a primary effect of endothelial cells and smooth muscle cells upon LDL is its oxidation. Experiments with inhibitors suggested that cell-derived H_2O_2 and O_2^- were critical to generate the modifications resulting in enhanced degradation by macrophages (61,63-65).

Human umbilical cord endothelial cells have been reported to secrete 65 nmol of H_2O_2 per mg of protein per 90 min (169) and monkey and human aortic smooth muscle cells were reported to secrete 0.3-3 nmol of O_2^- per mg of protein per min (65). However, using the rabbit aortic endothelial cell line (59), with corrections for nonspecific loss of the fluorescent indicator, we detected only 6 nmol of H_2O_2 per mg of protein per 2 hr. Our measured value of endothelial cell-derived H_2O_2 (less than 0.3% of the H_2O_2 produced by triggered fresh human monocytes, 800-1000 nmol per mg per hr; 150) can be extrapolated to 1 μM per hr per 25- cm^2 flask. This value was not increased by the addition of 90 μM L-cystine (data not shown;171). If endothelial cell H_2O_2 production is linear over 24 hr and if endothelial cell-derived H_2O_2 accumulates without any other reactions, this would represent

three orders of magnitude less H_2O_2 than was required to yield malondialdehyde values of 3 nmol per 100 μ g of LDL seen for EDTA-LDL in the same medium (Fig. 3-1). The amounts of reactive oxygen intermediates required to generate TBA-positive LDL have not previously been quantified or compared to cellular rates of secretion.

We have confirmed that rabbit endothelial cells modify LDL for enhanced uptake by macrophages but have been unable to demonstrate that this is associated with induction of TBA reactivity. Indeed, the quantities of oxidants required to yield TBA-reactive LDL (mM range) are orders of magnitude higher than those detectable under similar conditions for the same endothelial cells, or even for monocytes, potent sources of all the reactive oxygen intermediates known to be secreted by cells. A calculated value of only 0.1 mM H_2O_2 /2 hr was seen in our assay with monocytes, Fig. 3-3. These data suggest that the increase in rate of degradation of EC-LDL by macrophages was not due to oxidation by endothelial cells as manifest by TBA reactivity, but rather to some other influence of the endothelial cell monolayer.

The EDTA in the LDL used by Steinbrecher et al. (61) could be expected to suppress metal ion-dependent oxidation (172,173), and we confirmed that EDTA-LDL (0.26 mM EDTA) was 4-fold more resistant to induction of TBA reactivity than was LDL prepared without EDTA (CDP-LDL). Nonetheless, even CDP-LDL was highly resistant to generation of TBA reactivity by H_2O_2 and metal ions. Of course, biologically important oxidation not measured by the TBA assay may have occurred.

We cannot explain the marked differences in generation of TBA reactivity among apparently similar studies (61,62,174), but suspect that an ancillary factor may be involved, such as phospholipase or proteases

contaminating some preparations of LDL. Degradation of apolipoprotein B accompanying auto-oxidation of LDL has been observed by Schuh et al. (172). We observed the presence of multiple low M_r bands on SDS gels of EC-LDL samples but not of LDL incubated without endothelial cells (data not shown). This can be seen clearly also in ref. 64. We suggest that the biological modification of LDL may include proteolytic degradation.

Although neither the endothelial cells nor monocytes efficiently oxidized LDL in a fluid environment, oxidation may take place in the artery wall. The presence of both matrix-bound LDL and large numbers of monocytes in the confines of a subendothelial fatty streak (from which serum, a suppressant of cell-dependent modification of LDL, is excluded) would allow more intimate interactions of the substrate and effector cells. Additional enhancement might occur if T-cell derived IFN_γ were also present (175). LDL re-isolated from vessels has altered lipid composition and electrophoretic mobility, similar to in vitro modified LDL (115-119).

Further, we could not demonstrate triggering of H_2O_2 release from human monocytes or macrophages, with or without IFN_γ , by a variety of modified LDLs. We used both monocytes and mature macrophages, which have the scavenger receptor that has been shown to regulate macrophage secretory function (142).

Thus, the present studies lend no support to the notion that oxidation of LDL lipid to malondialdehyde-like moieties reactive with TBA is a prominent reaction caused by endothelial cells or monocytes, or that modified LDLs in turn trigger reactive oxygen intermediate release from inflammatory cells. The molecular basis of the modification of LDL by endothelial cells that leads to its enhanced uptake by macrophages remains to be defined.

Chapter 4 The Endocytic and Secretory Repertoire of the Lipid-loaded Macrophage: An in vitro Model for Foam Cells

Introduction

As described in chapter 1, a critical feature of the atherosclerotic plaque has not been examined: that is, what are the functional properties of a macrophage-foam cell? After the extensive lipid-loading, and contact with numerous cytokines and endocytic stimuli, these cells may no longer be capable of macrophage functions. We have undertaken the following studies to evaluate the endocytic and secretory capacities of the lipid-loaded macrophage. From the large number of activities associated with the macrophage, we first evaluated the phagocytic capacity of the cells by measuring Fc-mediated endocytosis of immunoglobulin-coated erythrocytes. Next we surveyed cellular and secreted proteins by metabolic labeling. This was followed by the measurement of selected secretory products that would have a large impact in the arterial environment, including apolipoprotein E, neutral proteases, H₂O₂, and the arachidonic acid metabolites. Finally, we began studies, preliminary results of which are reported below, on macrophage and lipid-laden cell response to IFN γ , and release of interleukin-1.

Cholesterol Accumulation

Normal mouse macrophages maintain a constant ratio of cholesterol to protein and have no cholesterol esters. 95% of the cholesterol is membrane-associated, mainly in the plasma and the lysosomal membranes (176).

Macrophages exchange 30% of their cholesterol with the medium per hour when cultured in 20% calf serum. A kinetic analysis of cholesterol exchange shows that macrophages have one fast-exchanging compartment representing 60-70% of the cellular cholesterol, which is the plasma membrane. The slower exchanging compartment (the remaining 30-40% of cellular cholesterol) is intracellular, probably largely made up of the lysosomal membranes (177). Excretion of cholesterol from the macrophage requires a suitable acceptor in the medium, such as serum, high density lipoprotein, or intact erythrocytes, and the rate of exchange is proportional to the concentration of acceptors in the medium (176,179-183). Cholesterol efflux and exchange occur via a trypsin-sensitive membrane component which requires protein synthesis to restore activity after proteolytic degradation (177,182).

When resident mouse peritoneal macrophages in culture are loaded with Ac-LDL, macrophages store excess cholesterol in an esterified form. After endocytosis, the LDL-derived cholesterol esters are hydrolyzed in the lysosomes by a lysosomal cholesterol esterase that has optimal activity at pH 4.0 (178-180). The free cholesterol thus generated can then be exchanged to a suitable acceptor, as described above, or re-esterified in the cytoplasm. The cholesterol esters accumulate in the cytoplasm and undergo a continuous cycle of hydrolysis to free cholesterol followed by re-esterification by ACAT. This process occurs with a half-life of 24 hours, requires ATP, and has been called the "cholesteryl ester cycle," a futile cycle (180). Two cytoplasmic cholesterol esterases that hydrolyze the cholesterol esters for efflux have been reported, one from the arterial wall and the other from macrophages. They are both regulated by cAMP-dependent protein kinase, and have neutral pH optima (184,185).

Morphology of Lipid-laden cells

Figure 4-1 shows pairs of photographs of day 2 macrophage cultures at 40x with the same field of cells seen under phase microscopy and in bright field. Mouse peritoneal macrophages were used in the foam cell experiments as a convenient source of mature tissue macrophages. The control cells (A) show a population of adherent cells--- some cells are flat and pancake-like, while others are rounded, or elongated, reflecting the normal population of macrophages from the peritoneal cavity of an unstimulated mouse. After fixation and Oil Red O staining (156), seen in bright field, control cells show almost no red color. A few cells at the lower right show some faint staining. These are the larger, pancake cells. All three lipid-loading regimens result in accumulation of Oil Red O stainable material.

The cells in B were loaded with 200 $\mu\text{g/ml}$ Ac-LDL. These are called Ac-LDL cells, and they represent cells lipid-loaded through the scavenger receptor. They act as a model for EC-LDL. The phase photograph shows a large number of inclusions in the cells which are shown in bright field to stain strongly with Oil Red O, reflecting lipid content.

The pair of pictures labeled C represent cells fed M-10 with 10 $\mu\text{g/ml}$ dextran sulfate-LDL (DS-LDL) complexes. The atherosclerotic plaque is highly enriched in sulfated glycosaminoglycans (76,77), which avidly bind LDL. DS-LDL complexes serve as a model for the extracellular matrix-bound LDL in the artery wall. Oil Red O stains these cells the most dramatically.

Serum components such as HDL are required for the release of cholesterol from the macrophages. The cells in D are loaded with MEM with a reduced serum concentration to reduce the amount of HDL and therefore

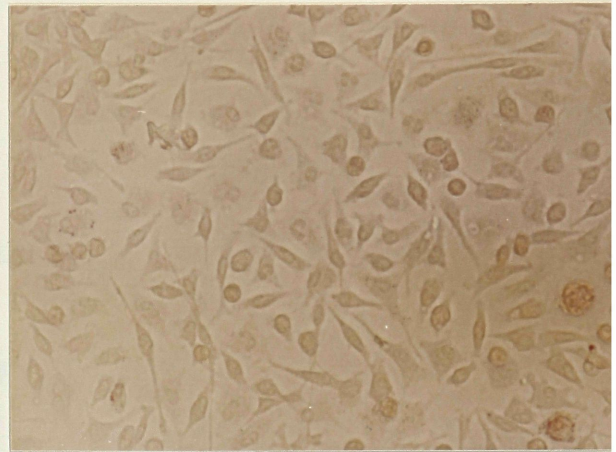
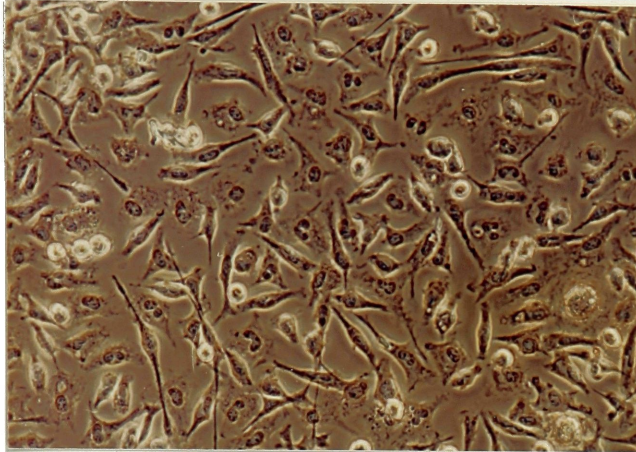
Figure 4-1. 40X photographs of Oil Red O stained mouse macrophages. Cells were cultured 2 days and then fixed in formalin and stained with Oil Red O. Panel A, control cells; Panel B, Ac-LDL cells; Panel C, DS-LDL cells; Panel D, LS/OI cells.

Figure 4-1

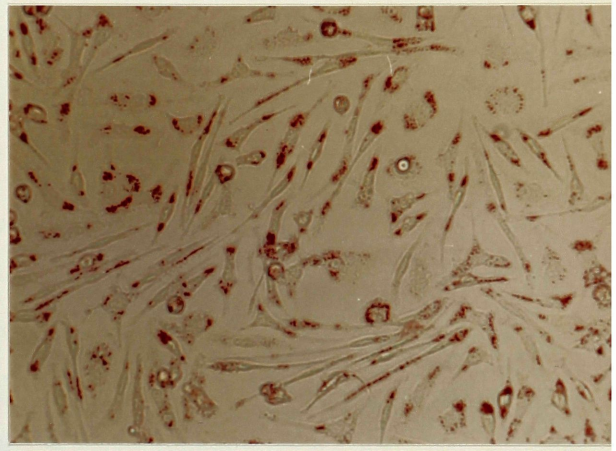
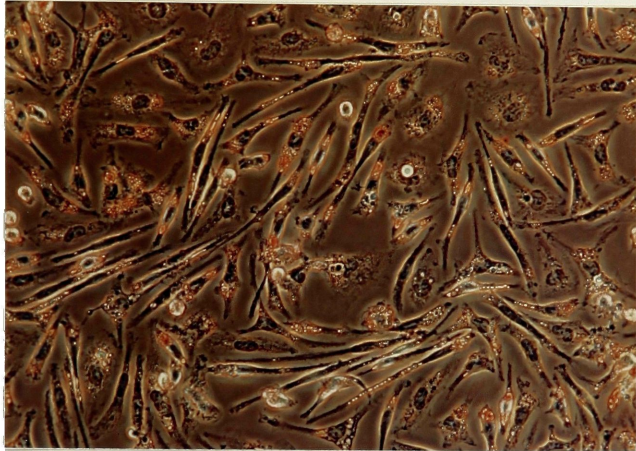
Phase

Bright Field

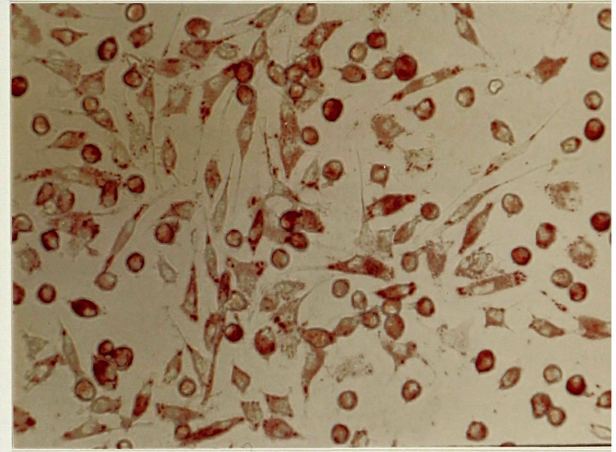
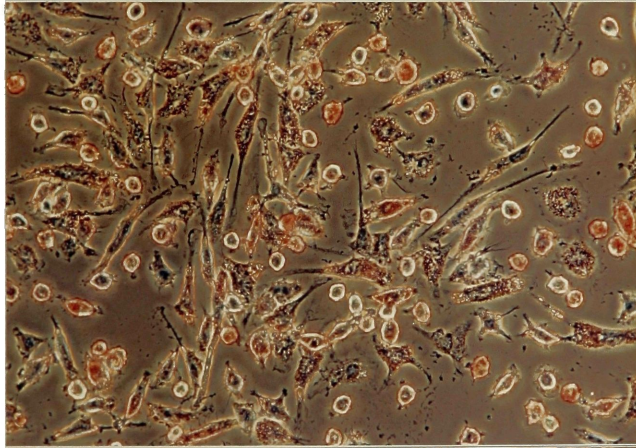
A



B



C



D

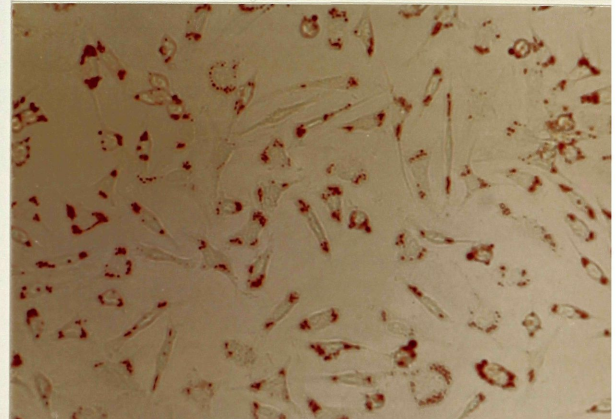
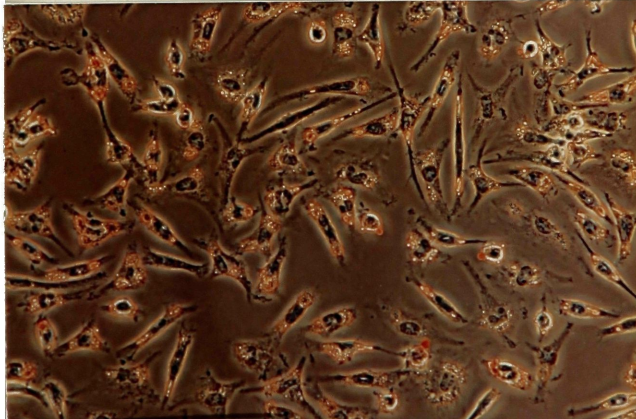


Table 4-1. Cholesterol content of foam cells

<u>Culture condition</u>	<u>Cholesterol, $\mu\text{g}/\text{mg}$ cell protein *</u>
M-10	83.9 \pm 7.7
Ac-LDL	113.3 \pm 8.9
DS-LDL	212.3 \pm 17.1
LS/O1	138.7 \pm 11.4

* Mouse macrophages were cultured as described for 2 days and then lipids were extracted by the method of Bligh and Dyer, and cholesterol was determined in the fluorescent assay of Gamble et al (see methods). Data are means \pm SEM. $P < .001$ for all lipid-fed samples compared to control. n= 21-39 separate determinations.

reduce the amount of cholesterol that could be exchanged. These cells had Ac-LDL in the same amount as above, and oleic acid to enhance macrophage cholesterol esterification. BSA in one-fourth the concentration of serum was added as a carrier of the oleic acid. These cells are called low serum/oleic cells, or LS/OI.

Cholesterol levels were determined using p-hydroxyphenylacetic acid, which detects the H_2O_2 enzymatically generated from cholesterol by the action of cholesterol oxidase and whose oxidation product is fluorescent (155). In each experiment the lipid-fed cells showed significant increases in total cholesterol (free and esterified) as compared to control M-10 fed cells (Table 4-1). Ac-LDL fed cells and LS/OI cells had between 1.4-1.7 fold more cholesterol loading than control M-10 cells. DS-LDL fed cells consistently showed the largest increase in cholesterol levels, 2.5 fold over control.

We examined the kinetics of lipid loading of resident peritoneal macrophages in vitro for up to 12 days. As shown in Figure 4-2, the macrophages fed DS-LDL quickly accumulated lipid so that after 1 day in culture they were 1.8-fold above control cells in cholesterol content. The amount of cholesterol per cell was maintained at about 270 $\mu\text{g}/\text{mg}$ cell protein after day 3, to a level that was 4.5-fold above control cells. The initially high control cell cholesterol level dropped over 3 days in culture but was maintained thereafter at about 60 $\mu\text{g}/\text{mg}$ protein. Cells that were loaded with DS-LDL for 2 days and then changed to M-10 medium for the remainder of the 12 days showed a fast initial drop in cholesterol level from 260 $\mu\text{g}/\text{mg}$ to 180 $\mu\text{g}/\text{mg}$ by day 3. However, about half of the cholesterol loaded was not lost during the 12 days. This may be due to the DS-LDL complexes. DS itself is poorly digested by macrophages (140) and may trap

Figure 4-2. Time Course of Cholesterol Accumulation by Macrophages

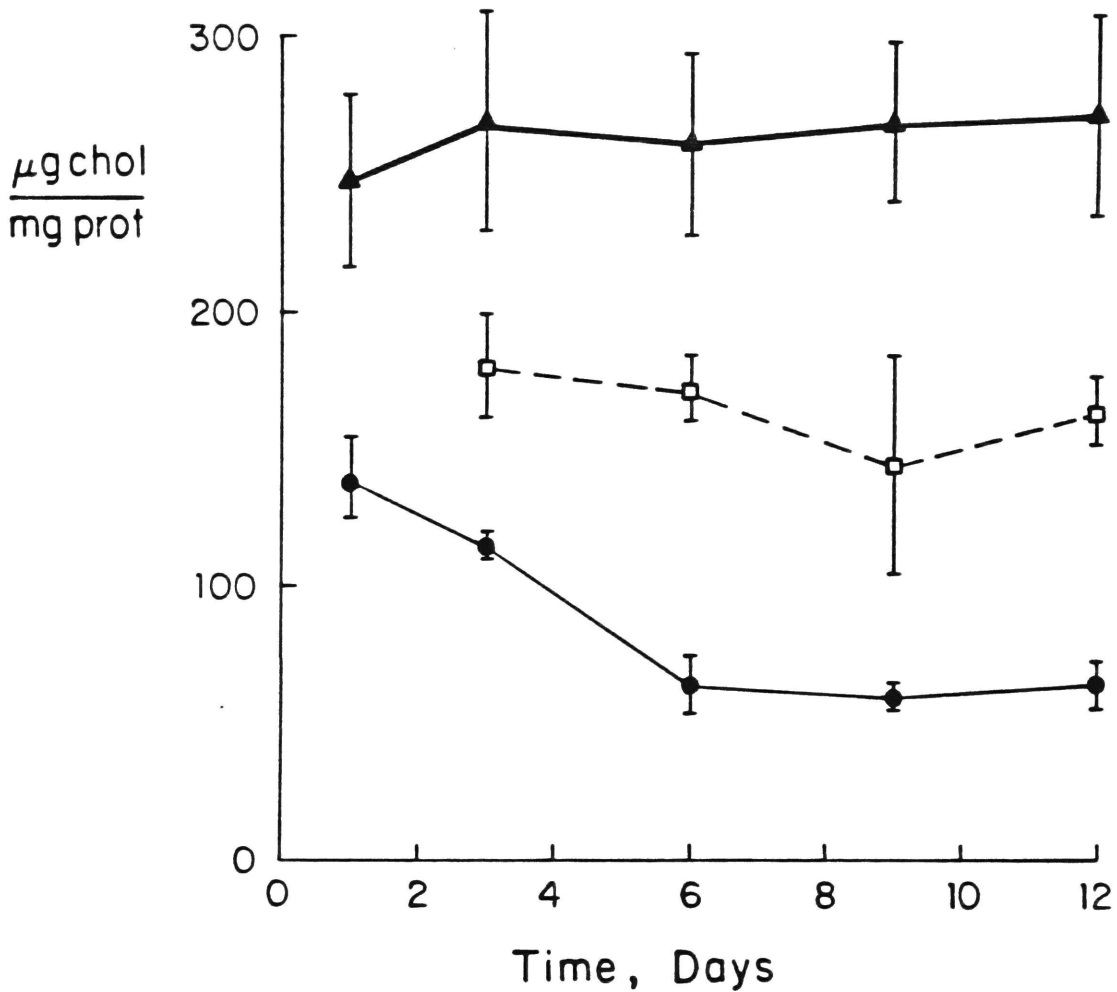


Figure 4-2. Mouse macrophages were cultured up to 12 days and assayed for cholesterol at the times shown. Solid line, control cells. Bold Line, DS-LDL cells. Dotted line, cells fed DS-LDL for 2 days, and then control medium for 10 days. Data are averages \pm SD for n=4 samples.

some cholesterol in the secondary lysosomes where it would not be available to be exchanged.

These increases in total cell cholesterol can also be measured by quantitating the Oil Red O staining in the cells (156). After 2 days of culture, coverslips were fixed and stained, and then extracted in dioxane. The optical density at 530 nm (OD_{530}) was measured against a dioxane blank. The fold over control of OD_{530} was nearly the same as that measured by total cholesterol determination. Some variability was observed between experiments. However, within any experiment the increase over control was very consistent (Table 4-2).

The lipids in these lipid-laden cells were also examined by thin layer chromatography (TLC), as shown in Figure 4-3. One dimensional TLC was performed using the 2 solvent system of Falcone et al (157). Total cellular lipids were extracted in situ with hexane, dried under N_2 , and spotted onto silica plates. After separation, the plates were stained with rhodamine and photographed under ultraviolet light. Lane A in both experiments shows the separation pattern of lipids from M-10 fed control cells, including free cholesterol and triglycerides. Almost no cholesterol ester is seen in normal macrophages. In contrast, all three lipid-loaded macrophage populations show an enormous accumulation of cholesterol ester. This is particularly prominent in lanes C and D, the DS-LDL fed and LS/OI cells. However, Ac-LDL cells also show large amounts of cholesterol ester. The lipid-loaded cells also show more free cholesterol and triglycerides than control cells. The TLC plates graphically illustrate the increases in lipid seen in the Oil Red O stained cells and quantified by Oil Red O extraction and the cholesterol assay.

Phagocytosis

Figure 4-3

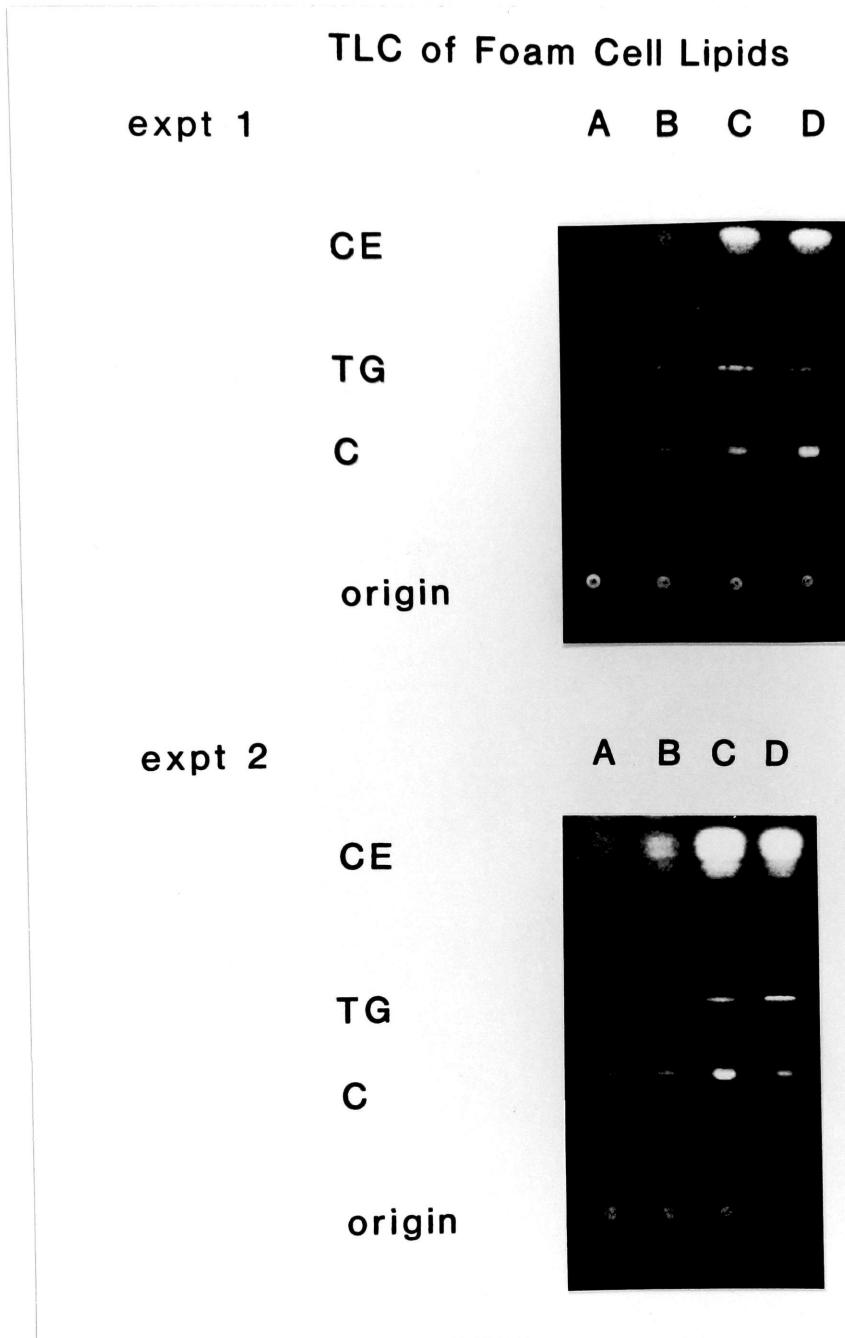


Figure 4-3. Mouse macrophages were cultured for 2 days and then lipids were extracted and chromatographed according to the method of Falcone et al (157). Plates were stained with rhodamine and photographed under UV light. Lane A, control cells; Lane B, Ac-LDL fed cells; Lane C, DS-LDL fed cells; Lane D, LS/OI fed cells.

Table 4-2. Extraction of Oil Red O from foam cells

Optical Density, 530 nm, fold over control *

<u>Culture condition</u>	<u>expt. 1</u>	<u>expt. 2</u>
M-10	1.0	1.0
Ac-LDL	1.2	2.4
DS-LDL	2.6	2.4
LS/OI	2.7	1.6

* Mouse macrophages were cultured as described, fixed and stained on day 2, and extracted in dioxane. OD of control macrophages was 0.048, n=2 per experiment, range < 10%.

Phagocytosis is a hallmark of macrophage function. Foam cells in the artery wall would be surrounded with LDL and LDL-GAG complexes, as well as tissue debris, and it is important to know if they can actively scavenge after lipid-loading. In addition, Fc-mediated uptake serves as a natural trigger for the three secretory functions that will be examined next. We measured the ability of lipid-laden cells to phagocytose sheep erythrocytes coated with immunoglobulin G (ElgG) for 45 minutes at 37 °C. Both attachment and phagocytosis of ElgG were determined. As shown in Table 4-3, nearly all the cells (94-97%) were able to bind ElgG, and approximately equal numbers of erythrocytes were bound per cell regardless of the lipid loading regimen. The number of cells with ElgG attached and the number per cell agrees well with published values (186). Similarly, a large percentage of the population was able to ingest ElgG and the number of erythrocytes per cell was almost equal in each condition. We can thus conclude that lipid-loading does not change Fc mediated endocytosis by mouse macrophages.

³⁵S-Methionine Protein Profile

Introduction. Macrophages secrete a large number of proteins with varied functions and regulate their release of these products in response to environmental signals (187). Some secreted products like lysozyme are constitutively released regardless of the state of activation. Lysozyme only acts on the β -1,4 N-acetyl glucosamine linkages of bacterial cell walls and its constitutive production is not known to be harmful (188). In contrast, the group of neutral proteases--plasminogen activator, elastase, and collagenase--can damage cellular proteins, particularly proteins of the

Table 4-3. Binding and phagocytosis of EIgG by foam cells*

<u>Culture condition</u>	<u>Attachment Index</u>		<u>Phagocytosis Index</u>	
	<u>% cells w/E</u>	<u>#E per cell</u>	<u>% cells w/E</u>	<u>#E per cell</u>
M-10	94 ± 0	8.1 ± 1.0	91 ± 4.5	4.6 ± 0.3
Ac-LDL	96 ± 2.8	7.8 ± 1.3	88 ± 6.2	4.9 ± 0.9
DS-LDL	97 ± 3.0	5.8 ± 0.4	96 ± 1.5	5.1 ± 1.7
LS/OI	96 ± 2.4	7.7 ± 2.0	93 ± 2.0	5.8 ± 0.1

* Mouse macrophages were cultured as described for 2 days. Cells were washed twice and incubated with EIgG (see methods) for 45 minutes. A brief wash with distilled water removed uningested E, to provide phagocytosis data. Attachment index and phagocytosis were determined by counting under water immersion # E/100 cells, using a Nikon 40x lens. Data are means ± SD, n=3 for all except DS-LDL, where n=2.

extracellular matrix (189). Secretion of these enzymes is very low in resident cells, but increased in inflammatory macrophages. Other proteins like the lysosomal acid hydrolases are secreted only by activated cells or in vitro in response to stimuli like immune complexes, PMA or zymosan (190).

We next observed a large number of the cellular and secretory proteins of lipid-laden macrophages by metabolic labeling with ^{35}S -methionine, shown in Figures 4-4 through 4-7. Since they illustrate such a broad number of activities, the profiles can be a revealing indicator of cellular condition.

Results. Analysis of ^{35}S -methionine-labelled cellular proteins from lipid-laden cells is shown in Figure 4-4. Lane A shows the control band pattern. Although we cannot identify very many of the numerous bands, it appears that many of the intracellular proteins are still produced after lipid loading.

SDS-PAGE of secreted proteins, however, shows several differences. In all four conditions shown in Figure 4-5, secreted proteins made up 18-23% of the total radioactivity. In the autoradiograms shown, LS/OI cells' incorporation of labeled methionine was only 45% of control, and so an additional, darker exposure of that lane is shown at the far left of the figure to facilitate comparisons. DS-LDL fed cells incorporated only 71% of control. Since BSA- and DS-fed macrophages have been shown to be more pinocytically active than control cells (191), we may assume that these cells were exposed to at least as much radiolabel as the control and Ac-LDL fed cells, and that the lower incorporation of label reflects a decrease in protein synthesis. The M-10 control cells, at the far right of the figure, show the typical pattern of secretory proteins for resident mouse peritoneal macrophages in adherent culture. There is a prominent band at 220,000 M_r ,

Figure 4-4

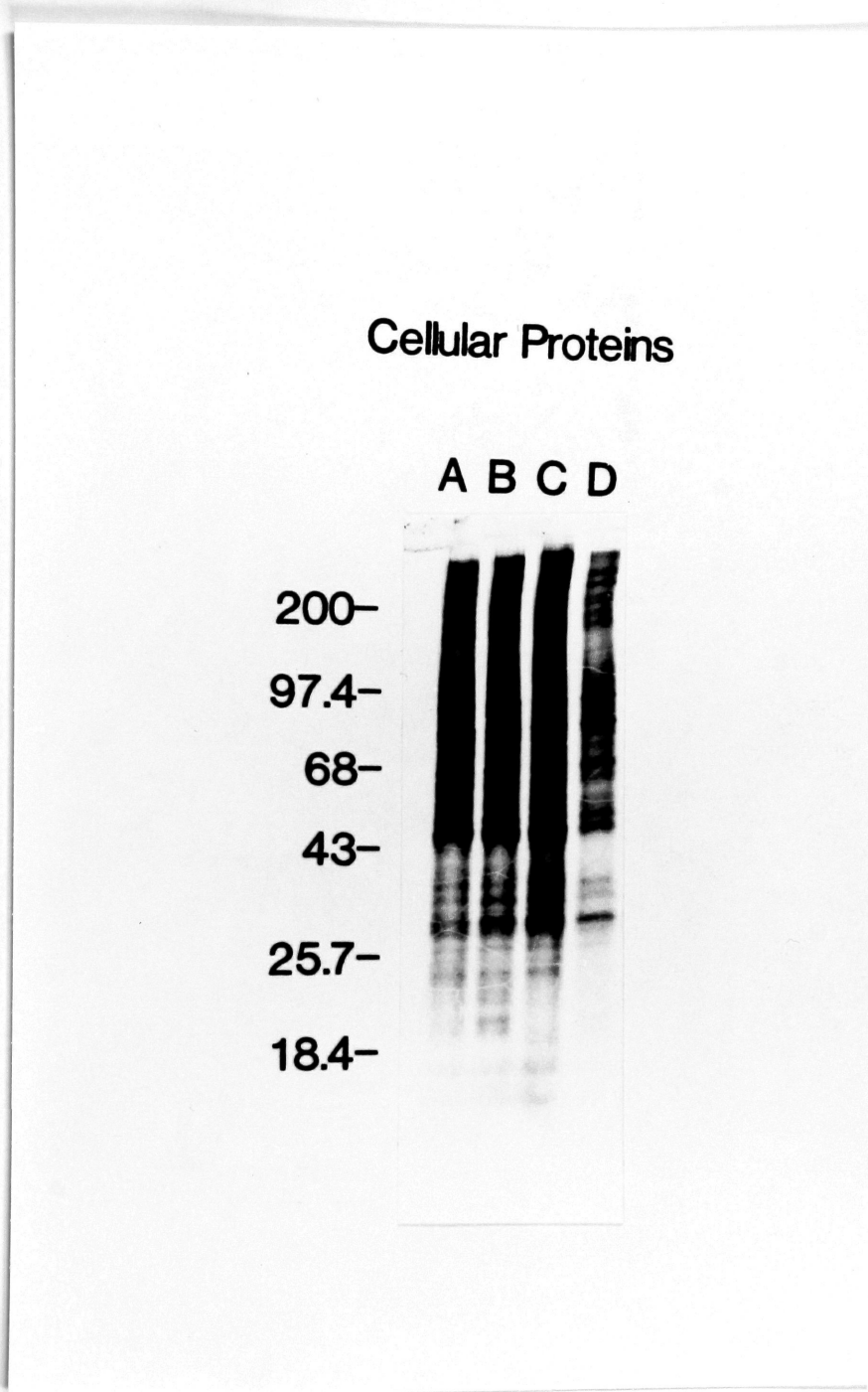


Figure 4-4. Mouse macrophages were cultured for 2 days and then labeled with ^{35}S -methionine for 4 hours. Cell monolayers were lysed in SDS sample buffer, loaded onto a 7-18% gradient SDS gel, electrophoresed and processed for autoradiography. Lane A, control; Lane B, Ac-LDL; Lane C, DS-LDL; Lane D, LS/O1.

Figure 4-5

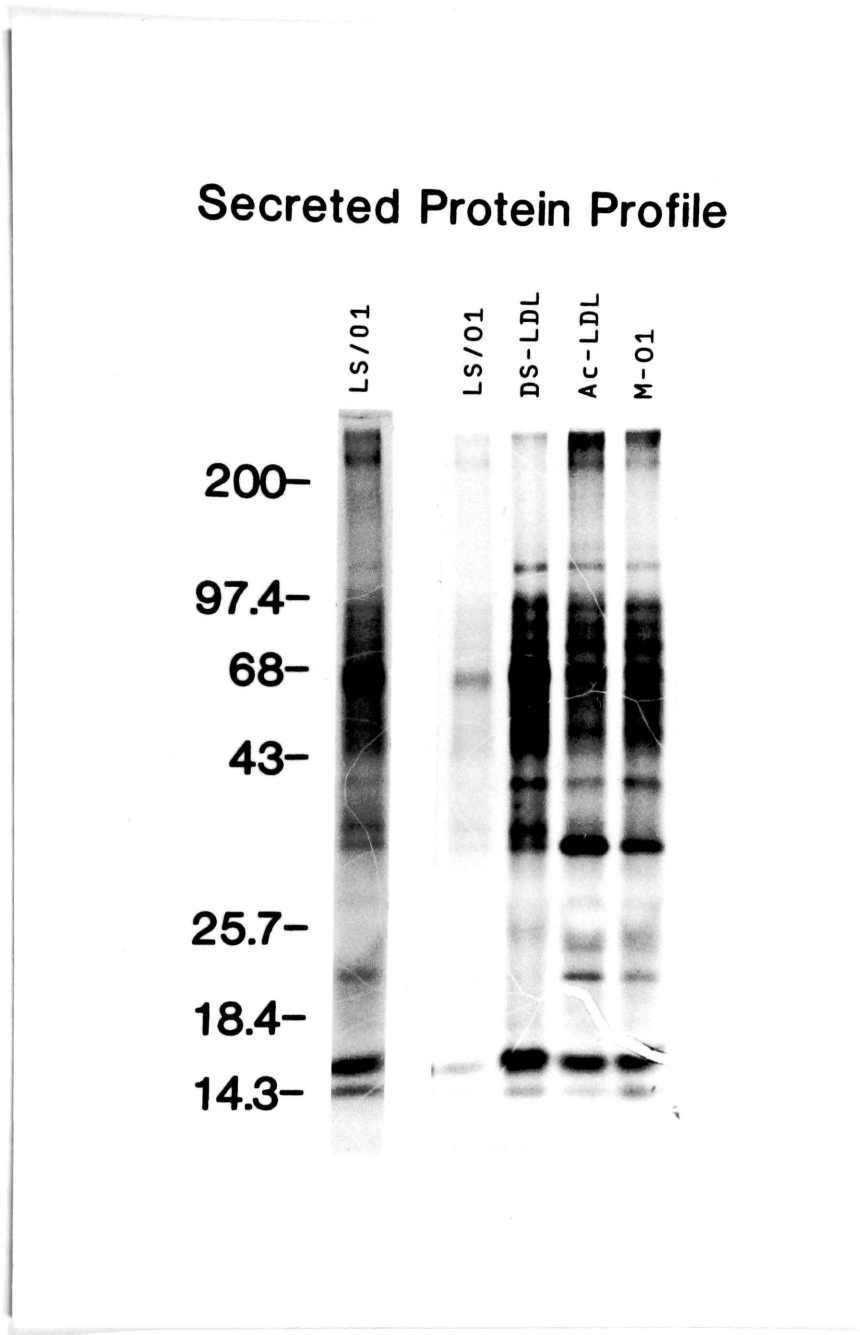


Figure 4-5. Mouse macrophages were cultured for 2 days and then labeled with ^{35}S -methionine for 4 hours. Secreted proteins were precipitated with trichloroacetic acid and resuspended in SDS sample buffer before being loaded onto a 7-18% gradient SDS gel, electrophoresed and processed for autoradiography. Samples are as marked.

which is probably fibronectin (192). This band is present in all the lanes. Similarly, lysozyme, at 14 Kd, is constitutively expressed (193).

A band at 90 Kd is increased in the DS-LDL fed cells. This may be complement factor B, which when activated can cleave and activate plasminogen (194,195). A prominent band at 68 Kd (p68) is increased in DS-LDL fed cells and LS/OI fed cells. This unidentified protein has been reported by Takemura and Werb to be elevated after DS treatment of resident cells (196). This is also true for a more dispersed band at 51-55 Kd. Both DS-LDL fed and LS/OI cells showed increases in p68 as compared to control. Endocytic signals have been shown to affect secreted proteins enormously, and this phenotype may be a result of undigested endocytosed material in secondary lysosomes. The effect of DS-LDL loading on p68 production can be seen more clearly in Figure 4-6. Here, resident cells were fed complexes of DS-LDL prepared with reduced quantities of DS and equal amounts of LDL. The ratio represents the proportion of DS to LDL used in the preparation of the DS-LDL complex. Lane A is the pattern of proteins secreted from cells loaded with DS-LDL complexes at 0.01:1, DS/LDL; the ratios for Lane B and Lane C are 0.1:1 and 1:1 respectively. The decreasing doses of DS-LDL reduce the cell's secretion of the 68 kd protein.

One of the most obvious changes in this profile is the protein at 33 Kd. This is apolipoprotein E (apo E), shown to be increased in resident cells that have been fed Ac-LDL (196,197). The secretion of apo E is highly regulated and can give information regarding the condition of the macrophage. It will be considered in detail below.

Apolipoprotein E (apo E)

Figure 4-6

Secreted Protein Profile

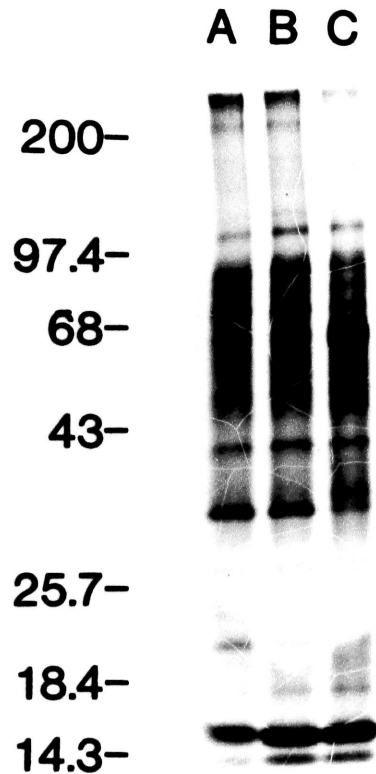


Figure 4-6. Mouse macrophages were cultured for 2 days and then labeled with ^{35}S -methionine for 4 hours. Secreted proteins were precipitated with trichloroacetic acid, resuspended in SDS sample buffer, loaded onto a 7-18% gradient SDS gel, electrophoresed and processed for autoradiography. Lane A, cells fed DS-LDL complexes prepared with a 0.01:1 ratio of DS/LDL. Lane B, cells fed DS-LDL complexes prepared with a 0.1:1 ratio of DS/LDL. Lane C, cells fed DS-LDL complexes prepared with a 1:1 ratio of DS/LDL.

Introduction. Apo E is a plasma glycoprotein that is a constituent of LDL, VLDL, and chylomicrons. It alone can account for the binding of VLDL to its receptor and the rate of degradation of VLDL (198-202). The conformation of apo E is influenced by its constitutive lipids. The purified protein tends to self-aggregate; it forms homo- and heterodimers by disulfide bridges (203).

Apo E is secreted by mouse and human macrophages. Its secretion is developmentally regulated; bone marrow-derived macrophages secrete apo E after 9 days in culture with colony stimulating factor 1 (204). Apo E secretion from macrophages is monensin sensitive, suggesting that it is secreted through the Golgi apparatus. It has been identified near the Golgi by immunofluorescence (205, 206). Apo E is released from the macrophage with phospholipids, in the form of bilayer discs resembling nascent HDL. It appears as 33,000-35,000 M_r due to sialic acid additions at the time of synthesis that are reduced by time in circulation (207). The secretion of Apo E is reported to be transcriptionally controlled (208).

Apo E discs have been shown to be recognized by the high affinity LDL receptor on fibroblasts (207). By itself, apo E secretion does not relieve the cell of excess cholesterol. As stated above, cholesterol is only excreted to specific cholesterol acceptors in the medium, like HDL or albumin. Once in the medium, however, apo E discs may expand and take in liberated cholesterol. Thus, apo E is seen as a part of the "reverse cholesterol transport" route to the liver for excess cholesterol from lipid-loaded macrophages (205). In type III hyperlipoproteinemia or familial dysbetalipoproteinemia, apo E has a defective interaction with its receptor because of point mutations in the apolipoprotein (203). When the E2

phenotype (one of three isoforms that exists in humans) is homozygously expressed, patients have elevated VLDL levels leading to pathology (209).

Apo E secretion is affected by endocytosis, presenting a complex picture of regulation depending on the substance ingested, on the prior state of activation of the cell, and on lysosomal storage. Most endocytic stimuli decrease apo E secretion; fucoidin and LPS do not increase apo E (196). When mouse macrophages are cholesterol-loaded, apo E secretion increases, and it can be up to 12% of the protein secreted by the cell. However, Takemura and Werb have shown that this is not strictly due to the cholesterol content, since EIgG feeding, which increases cholesterol, does not increase apo E secretion. It was of particular interest to us to measure the level of secreted apo E in cells that had been exposed to DS-LDL complexes. These cells have the highly elevated cholesterol content expected to increase apo E, and the presence of lysosomal DS shown to decrease apo E secretion (196).

A fraction of LDL, LDL-In, has been shown to inhibit lymphocyte functions. LDL-In is isolated by precipitation with dextran sulfate, and the suppressive concentration, 27 $\mu\text{g}/\text{ml}$, is within the physiological range. Inhibition requires membrane binding (but not internalization) to a trypsin-sensitive, saturable lymphocyte receptor. The binding is temperature-sensitive and is increased by previous incubation of the cells in lipoprotein-deficient serum and facilitated by Ca^{+2} ions. The receptor is not the LDL receptor. T8⁺ lymphocytes, the suppressor population, are the most sensitive to LDL-In suppression (210-214). Apo E from VLDL or LDL can suppress human and murine lymphocyte function. Chemically modified lipoproteins are not inhibitory, and all apo E isoforms are equally inhibitory. It has been shown to be the apo E protein, not the lipid moiety, that suppresses phaeoemagglutinin (PHA) induced increases in phospholipid turnover

(215). Similarly, the delipidated fraction of inhibitory LDL (LDL-In) is just as active as the entire particle. LDL-In and apo E may be the same product.

Results. As can be seen from figure 4-5, DS-LDL and LS/OI feeding strongly suppress secretion of a 33,000 M_r band. The band still visible at 33,000 may be a distinct protein, not apo E. In our hands the 33,000 M_r protein is dramatically increased by Ac-LDL feeding. The effect of DS-LDL feeding on apo E secretion is examined further in Figure 4-6. As described above, the cells in this experiment were loaded with DS-LDL complexes prepared with one-tenth and one-one hundredth the amount of DS and the same amount of LDL. Reducing the amount of DS in the DS-LDL complexes almost restores apo E to control levels.

This can be seen more clearly by immunoprecipitation of apo E, using a goat anti-human apo E serum, which fortunately also precipitated mouse apo E. Figure 4-7 shows that the protein is clearly induced by Ac-LDL feeding (lane B, Ac-LDL vs lane A, control). It is hardly visible from DS-LDL fed cells (lane C and lane E, identical samples), or LS/OI fed cells (Lane D). Expression of apo E returns in lanes F (DS-LDL, 0.1:1) and G (DS-LDL, 0.01:1) as the doses of DS in the DS-LDL are reduced. Takemura and Werb (196) reported the suppression of apo E secretion by endocytosis of DS. A similar mechanism may account for the decrease in apo E secretion by DS-LDL complex fed macrophages.

Fibrinolysis

Another macrophage function of great importance in the artery wall is protease secretion. Macrophages secrete several neutral proteases that are active in tissue remodeling and may be important for allowing the cell to

Figure 4-7

Immunoprecipitation of Apo E

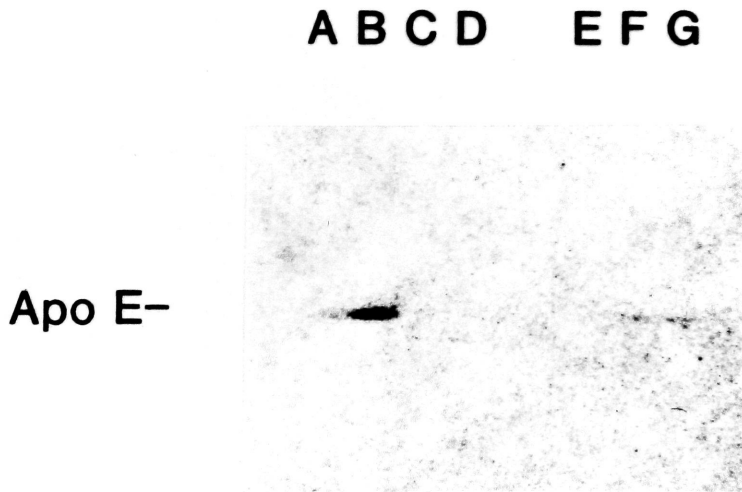


Figure 4-7. Mouse macrophages were cultured for 2 days and then labeled with ^{35}S -methionine for 4 hours. Secreted proteins were precipitated with goat anti-human apo E antiserum for 2 hours, then incubated with Protein A-agarose for an additional hour. Pellets were extensively washed and then resuspended in SDS sample buffer, loaded onto a 7-18% gradient SDS gel, electrophoresed and processed for autoradiography. Lane A, control; Lane B, Ac-LDL; Lane C and E, DS-LDL; Lane D, LS/Ol. Lane F, cells fed DS-LDL complexes prepared with a 0.01:1 ratio of DS/LDL. Lane G, cells fed DS-LDL complexes prepared with a 0.1:1 ratio of DS/LDL.

migrate. These enzymes are potentially very damaging, and their release is regulated. Johnson et al (142) have shown that ligands of the scavenger receptor trigger neutral protease release from mouse macrophages. Since foam cells are believed to migrate out of the fatty streak lesion (23-26) and plasminogen activator (PA) has an important role in cellular migration (216), we were very interested to measure PA production in lipid-loaded macrophages, to see how lipid-loading would effect the release of neutral proteases.

Plasminogen activator was first described in macrophages by Unkeless, Gordon, and Reich (160) as a 48 kd serine protease similar to the one produced by virally transformed fibroblasts. PA activity can be measured in cell lysates and in conditioned medium, or by plating the cells directly onto an ^{125}I -labeled fibrin plate. PA production can be regulated by a variety of agents, acting both in vivo and in vitro. It is decreased by glucocorticoids, inhibitors of cAMP phosphodiesterase activity, and reagents that inhibit the macrophage microtubule network, colchicine or vinblastine (216-219). Increased PA activity is seen in macrophages elicited by intraperitoneal injection of thioglycollate broth or endotoxin. Thioglycollate- or endotoxin-elicited macrophages can produce up to 100 times the PA of a resident unstimulated macrophage population (160). In vitro, PMA or Con A can act to increase cellular PA release (218).

We measured PA release by the highly sensitive fibrin plate assay (Figure 4-8; 160). Freshly isolated peritoneal cells were plated at 2×10^5 /well on ^{125}I -fibrin coated wells. After 48 hours of culture in the presence of 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor, the cells were washed and medium was replaced with MEM/10% Acid-treated FBS, serum that has had 95% of its plasmin inhibitors inactivated by treatment with 1 N HCl. In time

points measured to 22 hours, thioglycollate-elicited macrophages were shown to have potent proteolytic activity, degrading 38% of the substrate/ 5×10^4 cells (data not shown). This was enhanced about two-fold by the addition of 10 ng/ml PMA to 55.5 % substrate solubilized/ 5×10^4 cells. Control M-10 resident macrophages without PMA stimulation degraded only 8% of the fibrin layer, or 14% of the amount degraded by thioglycollate-elicited cells.

Both Ac-LDL and DS-LDL lipid-loaded macrophages were able to degrade ^{125}I -fibrin; in fact, they appeared to be more active than the M-10 cells. They degraded about 1.5 fold more ^{125}I -fibrin than control cells. This increase was seen equally with and without PMA enhancement of degradation. DS-LDL fed macrophages treated with PMA actually equaled the degradative activity of an equal number of thioglycollate-elicited cells at 22 hours. But the lipid-laden cells retain the profile of resident cells by displaying a 5-10 hour lag between PMA addition and increased PA production. Thioglycollate-elicited macrophages, as shown in Figure 4-8 and in ref. 218, respond to PMA much faster, degrading 4 times as much as control cells at the earlier 10 hr timepoint, a difference cut in half by the 22 hr determination.

These results show that lipid-laden cells have increased protease activity above control macrophages. This could enhance their mobility and migration in the vessel wall and lead to tissue degradation. Like thioglycollate-elicited macrophages, foam cells are the result of chronic ingestion of particles. If lipid-laden cells are like thioglycollate-elicited cells, we could expect to see equivalent increases in other protease activities, namely collagenase and elastase (188). In addition to allowing increased

Figure 4-8 Fibrinolytic activity of Foam Cells

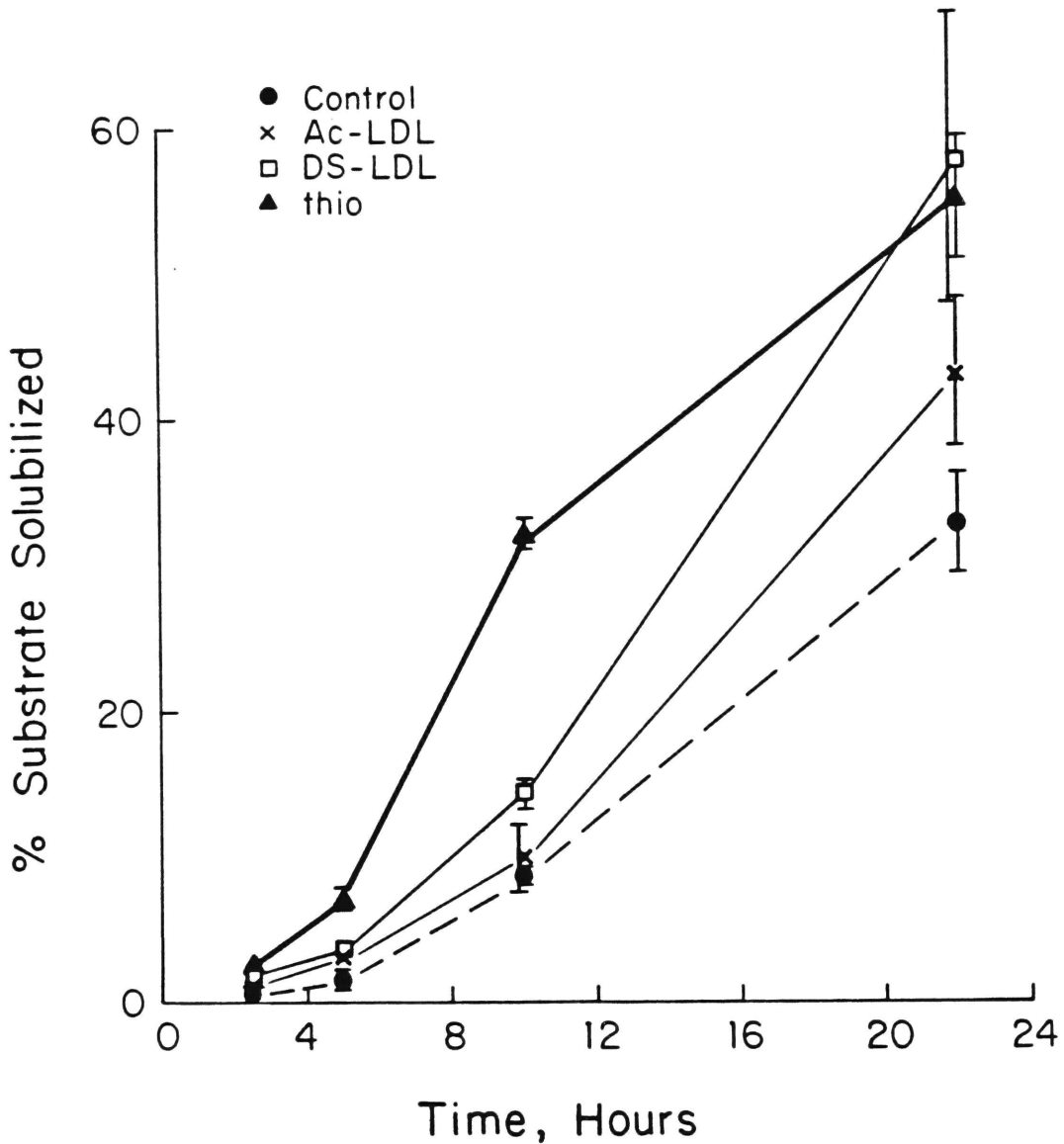


Figure 4-8. Mouse macrophages were cultured for 2 days on ^{125}I -fibrin coated wells in the presence of 100 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor. The assay was started with fresh medium containing 100 ng/ml of the triggering agent, PMA. Aliquots were taken at the times indicated and the percent of substrate solubilized was determined per 5×10^4 cells after subtraction of background counts. Samples are as indicated. Data are the means \pm SEM for 6-9 samples.

foam cell migration, active proteases in the vessel wall would certainly lead to damage to extracellular matrix proteins and increased inflammation.

The increase in PA in Ac-LDL fed cells confirms the increase in neutral proteases observed by Johnson et al (142) after maleylated-BSA feeding. However, DS-LDL had not previously been shown to do this.

H₂O₂ Release

Reactive oxygen intermediates (ROI) make up a potent part of the macrophage arsenal against invading microorganisms and tumor cells. The release of ROI can be stimulated by a variety of physiologic and non-physiologic agents (170,220,221). The macrophage, once triggered, releases ROI into its milieu, sometimes damaging "innocent bystander" cells. As discussed in chapter 3, if the macrophage in the artery wall were releasing ROI, it could inactivate protease inhibitors, damage the extracellular matrix proteins, and even lead to cell death. Endothelial cells are particularly sensitive to ROI (222-224). Previously, we showed that modified LDLs did not trigger H₂O₂ release from normal human monocytes and macrophages, with or without previous incubation with IFN γ . However, the intracellular accumulation of lipids, possibly oxidized toxic lipids, could influence ROI release from macrophages, and since H₂O₂ could accumulate, it represents a particularly hazardous macrophage product. Accordingly, we measured the release of H₂O₂ from lipid-laden cells (table 4-4).

Without PMA, all cellular incubation conditions showed very similar low levels of H₂O₂ release. The control cells (M-10) showed an 80-fold increase, as expected, with the addition of the triggering agent, PMA. The PMA-triggered increase was similar for Ac-LDL cells, 93-fold, but

substantially reduced to only a 50-fold enhancement over no PMA for LS/OI cells. In the case of DS-LDL fed cells, there was a major suppression of H₂O₂ release triggered by PMA. H₂O₂ release was only 12 fold over no PMA, and only 15% of the M-10 plus PMA value. This dramatic decrease was dose dependent, as shown in table 4-5.

Resident cells were incubated for 48 hours in the presence of DS-LDL complexes prepared as described above with 10-fold (0.1:1 DS/LDL) and 100-fold (0.01:1 DS/LDL) less DS and equal amounts of LDL. A stepwise restoration of H₂O₂ releasing capacity was observed with the decrease in DS content. Cells loaded with DS-LDL complexes released 26% of the H₂O₂ of control cells in these experiments. 0.1:1 DS/LDL complex-fed cells produced 61% of control, and 0.01:1 DS/LDL-complex fed cells produced 81% of control. Cholesterol values for the samples fed reduced DS-content complexes were 80% (0.1:1 DS/LDL) and 65% (0.01:1 DS/LDL) of the DS-LDL cholesterol value, or only 1.4- and 1.2-fold over control, compared to 2.5-fold for 1:1 DS-LDL loaded cells.

The decrease in the cells' ability to release H₂O₂ did not appear to be due to a sequestration of PMA within lipid pools. In experiments testing H₂O₂ release in response to graded doses of the triggering agent, PMA, over 4 logs, the DS-LDL fed cells showed the same pattern of release as control cells, but the magnitude at every point was dramatically reduced, reaching only 35% of control at its maximum (Figure 4-9).

Control experiments adding the H₂O₂ generating system of glucose/glucose oxidase to assays of H₂O₂ did not demonstrate scavenging of H₂O₂ by control or lipid-loaded cells, with or without PMA (data not shown).

We conclude from these experiments that lipid-loaded cells are still capable of releasing H₂O₂, although in DS-LDL cells the quantity is

Table 4-4. Release of H₂O₂ by foam cells

H₂O₂, nmol per mg of cell protein per hour*

<u>Culture condition</u>	<u>- PMA</u>	<u>+PMA</u>
M-10	6.8 ± 0.8	556 ± 47
Ac-LDL	4.3 ± 0.5	403 ± 42 ^a
DS-LDL	6.4 ± 0.5	79 ± 13 ^a
LS/O1	6.4 ± 0.8	340 ± 38 ^b

* Mouse macrophages were cultured as described and assayed for H₂O₂ on day 2 of culture. PMA at 100 ng/ml was used as a secretagogue. Data are means ± SEM for 7 experiments, n= 2-3 coverslips per condition per experiment. Statistics are for each sample compared to control, a = P<.001, b = P<.01.

Table 4-5. Effect of dose of DS-LDL on Release of H₂O₂ by foam cells

H₂O₂, nmol per mg of cell protein per hour*

<u>Culture condition</u>	<u>- PMA</u>	<u>+PMA</u>
M-10	4.6 ± 1.0	276 ± 8
DS-LDL (1:1)	5.2 ± 0.2	73 ± 7
DS-LDL (0.1:1)	3.7 ± 0.3	167 ± 2
<u>DS-LDL (0.01:1)</u>	<u>2.9 ± 0.4</u>	<u>224 ± 22</u>

* Mouse macrophages were cultured as described and assayed for H₂O₂ on day 2 of culture. The ratio in parentheses signifies the proportion of DS to LDL in the complexes. PMA at 100 ng/ml was used as a secretagogue. Data are means ± SEM. n= 3.

Figure 4-9. H₂O₂ Release in Response to Doses of PMA

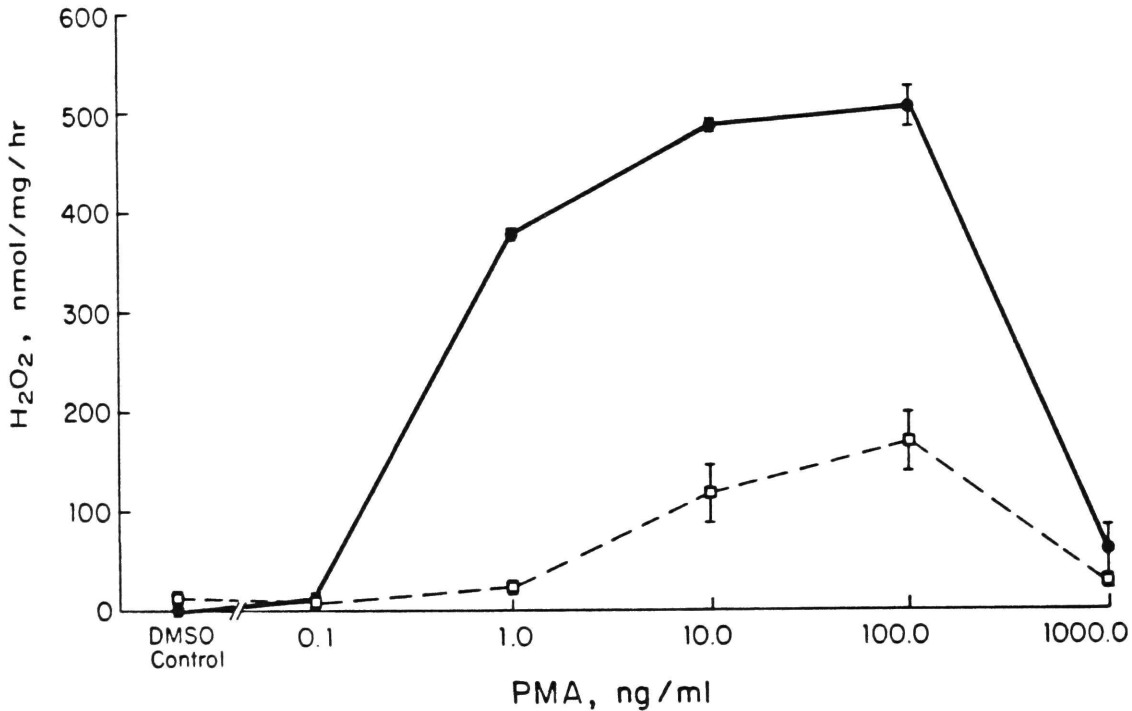


Figure 4-9. Mouse macrophages were cultured for 2 days and then H₂O₂ was determined as a function of the triggering agent, PMA. H₂O₂ assay was as described in methods. DMSO was added as a diluant control at a concentration equal to the highest concentration tested. Solid line, control cells. Dotted line, DS-LDL fed cells. Data are the means \pm SD for three samples.

significantly reduced. However, we do not believe that the increase in lipid content by itself can explain the reduction in H_2O_2 from DS-LDL fed cells, since Ac-LDL fed cells and LS/OI cells had similarly increased cholesterol levels and only slightly depressed H_2O_2 release. We suggest that the cells' H_2O_2 release may be affected as a result of endocytosis of DS. Accumulation of the highly negative DS portion of DS-LDL was reported by Kielian and Cohn to be concentrated in the secondary lysosomes and inhibit phagosome-lysosome fusion (140). DS is also known to reduce apo E secretion (196) and PA release (216). These results suggest that endocytosis of DS by the cells may suppress some cellular functions. Interestingly, thioglycollate-elicited macrophages also have a reduced capacity for H_2O_2 release, which has been attributed to ingestion of thioglycollate broth (225). Ongoing endocytosis of thioglycollate or DS-LDL complexes may compromise the macrophage's secretory abilities. These results suggested that other macrophage secretory products might be reduced in the foam cell. We next examined the bioactive lipids, the metabolites of arachidonic acid.

Arachidonic Acid Release

25% of the membrane phospholipids of macrophages are arachidonic acid (20:4). Prostaglandin E_2 (PGE_2) is the most prominent metabolite of arachidonic acid in mouse macrophages. Prostaglandins have been shown to have potent effects including vasodilation, inhibition of lymphocyte proliferation and antibody production, edema, and pain. PGE_2 in particular can increase phagocytosis and the number of Fc and Con A receptors on macrophages. The release of 20:4 from macrophages can be triggered by a variety of agents including zymosan, calcium ionophore A23187, immune

complexes, C. Parvum, LPS, colchicine, and EIgG. Different stimuli result in different magnitudes of release but a characteristic pattern has been established for resident cell populations (226). Inflammatory cells and activated cells show a different pattern of release (227). The release of PGE₂ correlates with the release by cells of acid hydrolases (190, 228).

The 20:4 release and metabolites of lipid-laden macrophages were examined by labeling the cells with ³H-20:4 for 16 hr before assaying. This time allows the label to equilibrate in the cell. The low amount of spontaneous release of ³H-20:4 into the medium was not changed by lipid-loading (Table 4-6). When zymosan was used as a trigger, control cells released 27.6% of the ³H-20:4 label when assayed at 90 minutes, as reported in ref. 229. Although Ac-LDL fed cells showed little change from control, both LS/OI and DS-LDL fed cells showed a significant reduction in total release, to 79% of control for DS-LDL fed cells and only 41% of control for LS/OI cells. We also tested the effects of the calcium ionophore A23187, thus avoiding the phagocytic stimulus for release. Although the baseline values were higher in these experiments, the same reduction of 20:4 release in both LS/OI and DS-LDL fed cells occurred in response to ionophore (Table 4-7).

We were concerned that the lipid-loaded cells, having additional sinks for lipid, might not have as much ³H-20:4 available for release as the control cells. Dvorak et al have shown the incorporation of ³H-20:4 into intracellular lipid bodies in macrophages (230). Two-dimensional TLC revealed some differences in the cells' lipid profiles, as might be expected (Table 4-8). In control, Ac-LDL fed, and DS-LDL fed cells, more than 50% of the total ³H-20:4 label was found in phosphatidyl ethanolamine and phosphatidyl choline, similar to the values Scott et al reported for resident peritoneal macrophages (158). LS/OI cells, however, have only 25% of their ³H-20:4 label in

Table 4-6. Arachidonic acid release by foam cells

Culture condition	³ H 20:4, % release at 90 min.*	
	- zym	+zym
M-10	2.6 ± 0.3	27.6 ± 0.3
Ac-LDL	3.3 ± 0.2 ^c	26.8 ± 1.4 ^c
DS-LDL	2.6 ± 0.2 ^c	21.8 ± 1.9 ^b
LS/O1	1.7 ± 0.2 ^c	11.4 ± 1.0 ^a

* Mouse macrophages were cultured as described and labeled with ³H-20:4 for 16 hours before assay for release on day 2 of culture. Zymosan was used at 160 µg/ml as a stimulant of release. Data are means ± SEM for 5 experiments, n= 2-3 samples per condition per experiment. Statistics apply to each sample compared to control, a = P<.001, b = P<.02, c = not significant.

Table 4-7. Arachidonic acid release by foam cells

³H 20:4, % release at 90 min.*

<u>Culture condition</u>	<u>- A23187</u>	<u>+A23187</u>
M-10	2.9 ± 0.1	39.8 ± 0.1
Ac-LDL	3.9 ± 0.1	36.5 ± 0.2
DS-LDL	1.8 ± 0.1	23.8 ± 0.2
<u>LS/O1</u>	<u>2.1 ± 0.4</u>	<u>19.6 ± 0.1</u>

* Mouse macrophages were cultured as described and labeled with ³H-20:4 for 16 hours before assay for release on day 2 of culture. Ionophore was used at 10 µg/ml as a stimulant of release. Data are means for n= 2 per condition.

Table 4-8. [³H] 20:4 in foam cells

³H 20:4, % located in neutral lipids*

<u>Culture condition</u>	
M-10	25
Ac-LDL	32
DS-LDL	26
<u>LS/O1</u>	<u>58</u>

* Mouse macrophages were cultured as described for 2 days and labeled with ³H-20:4 for 16 hours before use. Lipids were extracted and run on two dimensional TLC.

phospholipids. LS/OI cells have twice as much label in the neutral lipid pools. These are probably intracellularly located, and therefore less available for release. In vivo, this partitioning of 20:4 and reduced release could change cellular interactions in the artery wall.

Further analysis of the arachidonic acid profile of the lipid-loaded macrophages is shown in Table 4-9. Control macrophages released 27% of their ^3H -20:4 label as prostaglandin E_2 (PGE_2), as previously reported (229). The Ac-LDL fed cells and the DS-LDL cells hardly varied from that value. Unreacted 20:4 (control, 4%) and mono- and dihydroxy-eicosatetraenoic acid (Hetes; control, 6%) were as reported, and not significantly different in any of the lipid-loaded samples. However, LS/OI cells showed a relative shift in the cyclooxygenase pathway, resulting in an increase in PGE_2 and a decrease in prostacyclin (PGI_2) measured as its stable degradation product, 6-keto prostaglandin $\text{F}_{1\alpha}$ (6k-PGF $_1$). PGI_2 , a potent vasodilator, was only 62% of control in LS/OI cells. The relative increase in PGE_2 does not result in an absolute increase, however, since these cells have such a reduced level of total release (shown again at far left of Table 4-9).

Leukotriene C (LTC) was significantly depressed in Ac-LDL fed cells (50%) and LS/OI cells (85%). LTC, or slow-reacting substance of anaphylaxis, is a very potent vasoconstrictor, and its reduction generally correlates with markers of macrophage activation, like H_2O_2 release, or bactericidal activity (227, 231). LTC has been shown to promote endothelial cell PGI_2 production (232). Reduced production of LTC, the vasoconstrictor, could result in reduced production of PGI_2 , the vasodilator, and greatly affect vascular tone in the artery.

These studies do not include thromboxane, which is not a prominent product of mouse macrophages. However, it is the major arachidonic acid

Table 4-9. Arachidonic acid metabolites released by foam cells

[³H] 20:4, % released to zymosan at 90 min. *

Condition	(% rel)	6k-PGF _{1α}	PGE ₂	LTC	Hetes	20:4
M-10	27.6	31.4	27.0	23.2	6.2	4.2
Ac-LDL	26.8	30.9	26.2	12.1	7.6	6.6
DS-LDL	21.8	29.9	26.7	20.1	4.9	3.3
LS/O1	11.4	19.7	51.8	3.7	7.5	1.9

* Mouse macrophages were cultured as described and labeled with [³H] 20:4 for 16 hours before assay for release on day 2 of culture. Zymosan was used at 160 μg/ml as a stimulant of release. Macrophage culture medium was extracted and dried under N₂ before HPLC as described in methods. Values are expressed as the percent of the total [³H] 20:4 products formed and calculated on the basis of discernable peaks above background. n=2 for LTC, Hetes, 20:4, n=1 for PGE₂, 6k PGF_{1α}.

metabolite of fresh human monocytes, and it would be very interesting to examine the effects of lipid-loading on the human mononuclear phagocyte system (233).

Interleukin 1

To continue these studies, we evaluated another important class of proteins produced by macrophages, the growth factors. In particular, we wanted to know about the secretion of interleukin 1, a monokine that regulates T lymphocytes and stimulates fibroblast growth and collagenase production (49, 50). Il-1 also induces a 35% decrease in endothelial cell plasminogen activator production, and a 360% increase in production of a plasminogen activator inhibitor. As discussed in chapter 1, these changes reduce the fibrinolytic activity of EC and lead to an overall procoagulant state (48, 51-53). In light of these activities, and its reported ability to activate macrophages (54), Il-1 could play an important role in the progression of the plaque.

Measurements of Il-1 were made using the D-10 cell line, a T lymphocyte clone which will proliferate in response to Il-1 (164, 165). Control cells (M-10 fed) produced approximately 1 U Il-1/ml of culture supernatant collected after 24 hours. No stimulation of D-10 cell growth was seen with cell-free medium. No stimulation of Il-1 production was seen after the M-10 cells were incubated for 24 hours with 10 ng/ml PMA. But a 100-fold enhancement of Il-1 production was seen after 24 hours with 1 mg/ml LPS, as reported by Koide et al (164). In three experiments testing cell-free and cell-conditioned medium from lipid-fed macrophages, only slight variations in Il-1 production were observed comparing the lipid-laden cells

to control cells. Addition of conditioned culture medium at 10 times the highest concentration tested did not inhibit the response of the D-10 cells to recombinant Il-1, suggesting that no inhibitor of Il-1 was produced.

Response to γ Interferon

The presence of T4+ lymphocytes in the fibrous cap of the atherosclerotic lesion (130, 131) and the expression of the Ia antigen by SMC (132) suggest that the cytokine IFN γ is present in the lesion. IFN γ has pleiotropic effects on macrophages, and is called the macrophage activating factor (175). We have seen that certain macrophage activities are reduced as a result of lipid loading. It is important to know the response of the lipid-loaded macrophage to this regulatory factor. Preliminary experiments compared the effect of IFN γ on cellular accumulation of Oil Red O-stainable material, and the ability of lipid-loaded cells to respond to IFN γ by increasing expression of Ia antigens. Recombinant murine IFN γ at 100 U/ml was added to the culture medium on day 0 or day 1 of culture, and cells were assayed for total cholesterol on day 3. The effect of IFN γ on lipid-loading can be seen in Figure 4-10. The cells were fixed on day 3 and stained for Oil Red O as before. Panel A shows cells that were loaded with Ac-LDL. They show the characteristic accumulation of Oil Red O staining material. The cells in B were loaded with Ac-LDL and IFN γ . Panel C shows cells incubated for 1 day with IFN γ , and then for 2 days with IFN γ and Ac-LDL. Finally, D shows cells loaded with Ac-LDL for 1 day and then for two days with Ac-LDL and IFN γ . As can be seen from the photographs, the cellular accumulation of lipid droplets did not seem to be affected by IFN γ .

Sample _____ Figure 4-10

Ac-LDL	A
Ac-LDL + IFN γ	B
IFN γ ----> Ac-LDL + IFN γ	C
Ac-LDL ----> Ac-LDL + IFN γ	D

Lipid-laden cells were also plated in a 96-well plate with and without IFN γ and expression of the class II major histocompatibility antigen, Ia, was determined in a cell ELISA (Birkeland and Pure, manuscript in preparation). While not a quantitative assay, the cell ELISA showed an increase in Ia expression after IFN γ treatment. This increase was not affected by the order in which the cells received the cytokine and the lipid. These preliminary findings suggest that lipid-loaded cells were still able to respond to IFN γ , and did not show major differences in the lipid-laden cells' response as compared to that of control cells. We plan to continue these studies to measure the release of H₂O₂ after IFN γ treatment. Nathan et al (175) have shown that IFN γ greatly increases the macrophage production of H₂O₂. This measurement should be particularly interesting in the case of the DS-LDL cells, which we have shown have a substantially reduced release of H₂O₂.

We have seen that the three lipid-loading regimens produce Oil Red O positive cells with significantly elevated cholesterol ester levels. The secretory functions of the cells have been altered in a complex way. Modulations do not appear to be due strictly to the cellular cholesterol level. Implications for the overall condition of the lipid-laden cells will be discussed in the next chapter.

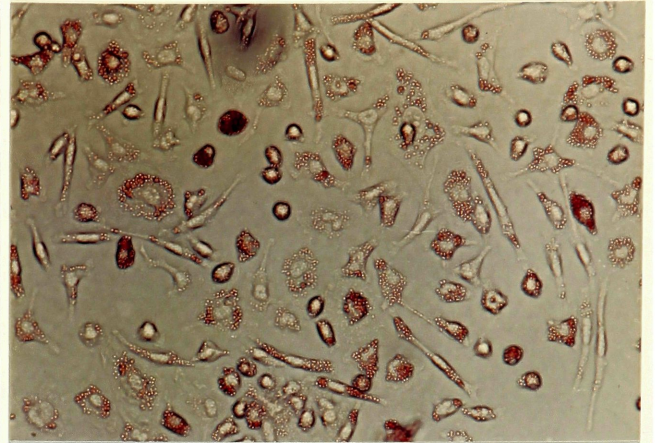
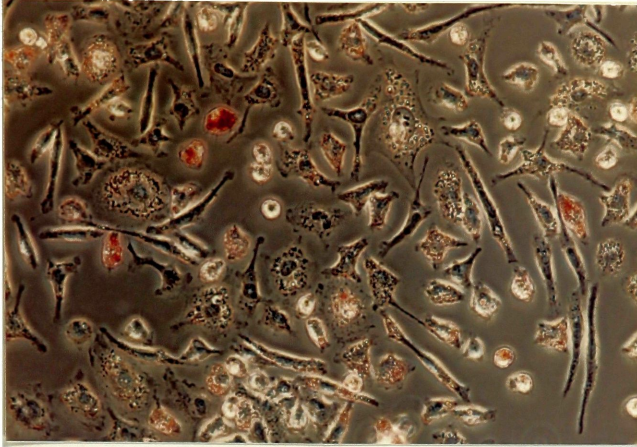
Figure 4-10. 40X photographs of Oil Red O stained mouse macrophages. Cells were cultured 3 days and then fixed in formalin and stained with Oil Red O. Panel A, Ac-LDL cells. Panel B, cells incubated with Ac-LDL and IFN γ . Panel C, cells incubated with IFN γ for 1 day and then 2 days with Ac-LDL and IFN γ . Panel D, cells incubated with Ac-LDL for 1 day and then 2 days with Ac-LDL and IFN γ .

Figure 4-10

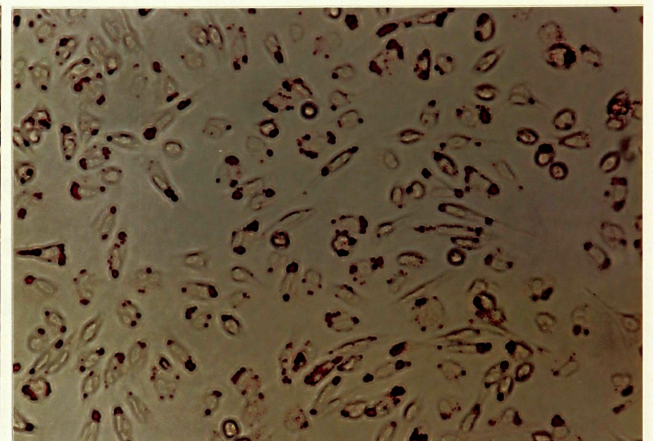
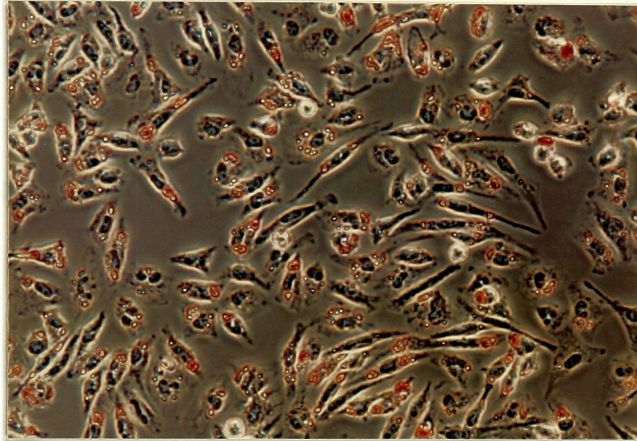
Phase

Bright Field

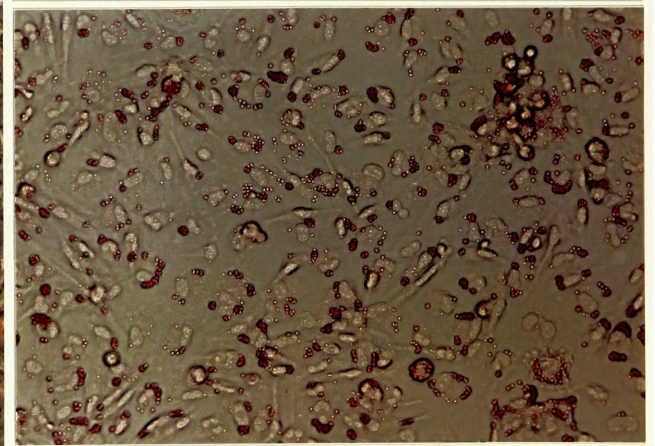
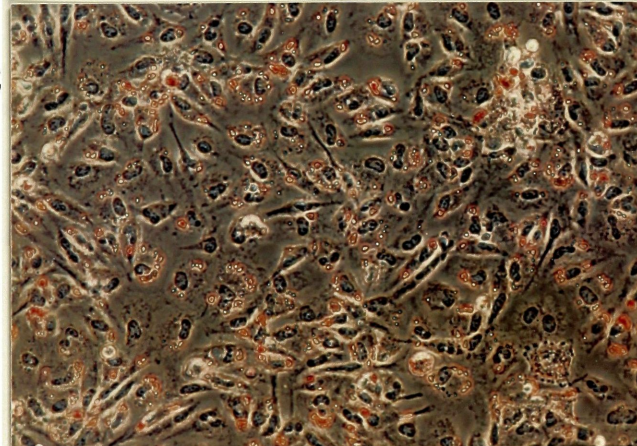
A



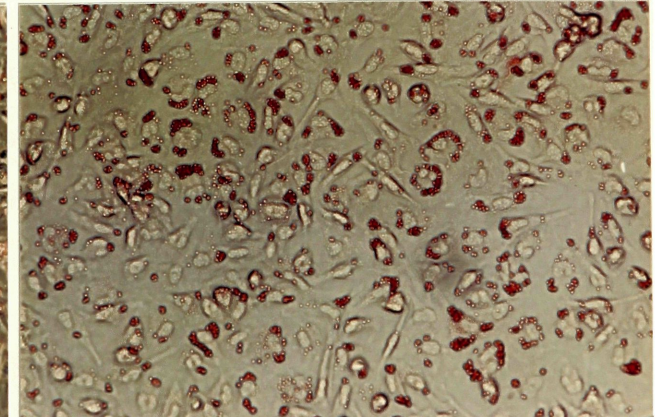
B



C



D



Chapter 5 Discussion and Conclusions

We have lipid-loaded macrophages to study the functions of foam cells. A number of reports have shown that macrophage functions are impaired as a result of interaction with lipid: cells are less cytotoxic (138), less endocytic (139), and have several of their activities depressed due to ingestion of polyanions (inhibited lysosome fusion, 140; inhibited PA release, 196; reduced Apo E secretion, 216). Macrophage-derived foam cells have been touted as a lipid clearance system (23-26), but not enough is known about the lipid-loaded cells' capabilities to evaluate whether they can help alleviate inflammation and scavenge and dispose of excess cholesterol.

To do these studies, we selected three regimens for loading cells, each of which models known conditions from the artery. The lipids of the atherosclerotic plaque have been shown to be mainly cholesterol esters derived from LDL (74), modified LDL (114-118), LDL-GAG complexes (76-79), and LDL bound to collagen and elastin (234). Lipoproteins or extracellular matrix proteins may also become modified in the artery after nonenzymatic attachment of glucose, as happens to proteins in diabetic patients (235). Diabetes is a risk factor for atherosclerotic disease. We have incubated resident mouse peritoneal macrophages with combinations of lipids that model biologically-modified LDL, LDL-GAG complexes, and low serum HDL conditions. The resulting lipid-laden cells were characterized in an attempt to define functional capacities.

Examination of the quantity and classes of the intracellular lipids of the lipid-laden cells shows that the predominant form of lipid accumulated by the cells is cholesterol ester (Figure 4-3, ref. 82, 178). Similar proportions of increase in total cholesterol levels are quantitated by a fluorescent

cholesterol assay and by direct extraction in dioxane of Oil Red O stained lipid droplets.

Despite massive increases in cholesterol esters, the cells retain many features of macrophage function. They are still capable of Fc receptor-mediated endocytosis--binding and ingestion of IgG were quantitatively undiminished from control cells. Many of the cellular proteins are still produced after lipid-loading, as shown in Figure 4-4. Secreted proteins associated with endocytosis and lipid balance are altered, but many other macrophage secretory proteins remain unchanged (Fig 4-5). Of course, we have loaded the cells for only two days, and foam cells *in vivo* may be exposed to lipid-loading for months or years.

We have shown that the lipid-laden cells are still capable of producing all the secretory products examined, including plasminogen activator, H_2O_2 , arachidonic acid and its metabolites, PGE_2 , PGI_2 , LTC, and Hetes. The quantities of products are somewhat reduced in most cases, except for the release of plasminogen activator, which appears to be increased.

The 20:4 metabolites regulate vascular tone and inflammation in the artery wall. The macrophages, EC, SMC, and platelets all produce these agents, which can then act on the neighboring cells. The large number of studies in this area reflect the complexity of these interactions in the artery. For example, leukotrienes have been shown to mediate leukocyte adhesion to endothelium (236), and to promote endothelial cell PGI_2 production (232). LDL has been reported to stimulate PGI_2 and PGE_2 production from SMC in culture (237). The macrophage product II-1 induces PGI_2 synthesis in EC and SMC (238). Macrophages make platelet activating factor, which can lead to platelet aggregation and release of 20:4 metabolites as well as PDGF (239). Such results *in vitro*, however, can only provide information about the

abilities of the cell types and the mediators, not about their interactions in vivo. At present, clarifying abilities is all we can do, since we do not have a successful in vitro model for all the aspects of cell involvement in the artery wall.

We plan to continue these studies to measure the effect of lipid-loading on macrophage production of tumor necrosis factor, TNF. Activated macrophages produce TNF (56). In addition to causing regression of some tumors, acting as a growth factor for fibroblasts, and suppressing anticoagulant activity on the endothelial cell surface (57, 58), TNF has metabolic effects on lipid balance, inhibiting lipogenic enzymes in adipocytes (240). TNF and IL-1, while having some overlapping functions, have independent regulation which results in a variety of effects (47). The lack of observed change in IL-1 production from lipid-laden cells does not predict the effects of lipid-loading on TNF release.

Our initial attempts to determine if the release of TNF was altered after lipid-loading of macrophages were unsuccessful for technical reasons. We measured cell death after incubation with conditioned medium from control and lipid-loaded cells. The results with the bioassay showed that the culture medium itself was toxic to the target cells, probably due to the high concentration of lipids. We have set up a collaboration to pursue the determination of TNF by western blot analysis.

Another factor that has a large role in the artery wall is PDGF: it is chemotactic for monocytes (43), fibroblasts and for SMC, as well as mitogenic for fibroblasts and for SMC (29-31). Macrophages make a PDGF-like factor after stimulation by various agents (32, 33). The possible effects of lipid-loading in the macrophage are unknown. We plan to measure PDGF production by lipid-laden cells. As was the case for TNF, our initial

measurements of PDGF by bioassay were inconclusive for technical reasons and we will pursue the determination of its production by lipid-loaded cells with other techniques.

To conclude: we have described an in vitro model system for foam cells and characterized the cells. We have shown that the cells maintain their capacity for all the macrophage functions examined, with some variations in quantities of products produced. We have begun studies on another very important class of proteins, the growth factors, and would like to pursue this model to evaluate possible changes in migration through intact endothelial cell monolayers, chemotaxis, and changes in function after longer periods of lipid-loading. In addition, it would be of interest to examine these questions in the human foam cell, with autologous LDL.

The macrophage that enters the artery wall has been studied extensively with respect to what products it can elaborate and under which circumstances. The effects of these agents are also under close scrutiny. The conditions in the artery dictate that the cells will become lipid-loaded. We know how much and how quickly macrophages ingest various types of lipids. We also know that certain types are not easily digested (140, 157) and remain inside the cell for long periods. We have shown that in many respects lipid-laden cells maintain macrophage functions after lipid-loading. But the decreased production of H_2O_2 and 20:4 show that inflammatory mediators are somewhat reduced. While it is difficult to predict what changes in the macrophage's large repertoire would occur in vivo, we may speculate that the foam cell is not "activated" in the defined sense (241). It may be more like an inflammatory macrophage, like the model thioglycollate-elicited macrophage. Or it may be simply a resident cell which after much abuse by

lipid-loading has become sluggish in its functions. The details of the foam cell's abilities remain to be determined.

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