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Cholesterol and lipoprotein metabolism of human promyelocytic leukemic HL-60 induced macrophages

El-Jouni, Zeinab Ezzuddine, Ph.D.

The University of Arizona, 1991
CHOLESTEROL AND LIPOPROTEIN METABOLISM OF HUMAN
PROMYELOCYTIC LEUKEMIC HL-60 INDUCED
MACROPHAGES

by

Zeinab El-Jouni

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COMMITTEE ON NUTRITIONAL SCIENCES (GRADUATE)
in Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1991
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Zeinab E. El-Jouni, entitled "CHOLESTEROL AND LIPOPROTEIN METABOLISM OF HUMAN PROMYEOLOCYTIC LEUKEMIC HL-60 INDUCED MACROPHAGES," and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of DOCTOR OF PHILOSOPHY.

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DEDICATION

This dissertation is dedicated to my parents, Ezzuddine and Montaha Jouni, in appreciation of their love, continued support and encouragement, throughout the years.

This work is also dedicated to the rest of my family for their love and generous support: Samir, Sami, Ahmed, Yussif, Naim, Rabbab, Mona and Walid.
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LIST OF ABBREVIATIONS

Ac-LDL: Acetylated-LDL
ACAT: AcylCoA:cholesterolacyltransferase
ACBP: Acyl-CoA-binding protein
Acetyl-LDL: Acetylated-LDL
ApoA: Apolipoprotein A
ApoB: Apolipoprotein B
ApoB-100: Apolipoprotein B-100
ApoB-26: Apolipoprotein B-26
ApoB-46: Apolipoprotein B-46
ApoB-74: Apolipoprotein B-74
ApoC: Apolipoprotein C
ApoE: Apolipoprotein E
Apoprotein: Apolipoproteins
ATP: Adenosine triphosphate
$B_{max}$: Maximum binding
BSA: Bovine serum albumin
Buffer B: Buffer A - BSA
Buffer A: 150 mM NaCl, 50 mM Triton X-100 and 2 mg / ml
BSA: pH 7.4
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<tr>
<td>C/PC:</td>
<td>Cholesterol/phosphatidylcholine</td>
</tr>
<tr>
<td>CHD:</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CHO:</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CO₂:</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>ºC:</td>
<td>Degrees centigrades</td>
</tr>
<tr>
<td>D₃:</td>
<td>1,25-Dihydroxyvitamin D₃</td>
</tr>
<tr>
<td>Da:</td>
<td>Dalton</td>
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<tr>
<td>DAG:</td>
<td>Diacylglycerol</td>
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<tr>
<td>DTT:</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC-LDL:</td>
<td>Endothelial cell-modified LDL</td>
</tr>
<tr>
<td>EDTA:</td>
<td>Ethylene diamine tetrasulfate</td>
</tr>
<tr>
<td>EGF:</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>GLC:</td>
<td>Gas liquid chromatography</td>
</tr>
<tr>
<td>HDL:</td>
<td>High density lipoproteins</td>
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<td>HL-60:</td>
<td>Human promyelocytic leukemic cells</td>
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<tr>
<td>HMG-CoA:</td>
<td>3-Hydroxy-3-methylglutaryl coenzyme A</td>
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<tr>
<td>HS:</td>
<td>Horse serum</td>
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<td>IDL:</td>
<td>Intermediate density lipoproteins</td>
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<td>Kₛ:</td>
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LIST OF ABBREVIATION--(Continued)

KDa: Kilodalton
LDL: Low density lipoproteins
LPDS: Lipoprotein depleted serum
LPL: Lipoprotein lipase
LRP: LDL receptor-related protein
M-CSF: Macrophage-colony stimulating factor
MDA-LDL: Malondialdehyde modified LDL
MVA: Mevalonate
NaCl: Sodium chloride
ORO: Oil red O
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PKC: Protein kinase C
RER: Rough endoplasmic reticulum
S.D.: Standard deviation
β-VLDL: Beta very low density lipoprotein
SCP₂: Sterol carrier protein
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
LIST OF ABBREVIATION--continued

SER: Smooth endoplasmic reticulum
TPA: Tetramyristic phorbol acetate
VLDL: Very low density lipoproteins
WHHL: Watanable heritable hyperlipemic
ABSTRACT

Human promyelocytic leukemic cells (HL-60) possess well regulated expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, acylCoA:cholesterol acyltransferase (ACAT), and receptor-mediated low density lipoprotein (LDL) catabolism, but lack receptor-mediated acetyl-LDL processing.

Differentiation of HL-60 cells with tetramyristic phorbol acetate (TPA) is accompanied by the loss of receptor-mediated LDL degradation and no expression of a functionally active scavenger receptor.

1,25-Dihydroxyvitamin D₃ (D₃)-induced HL-60 macrophages possess specific and saturable receptor-mediated binding for LDL, with an apparent $K_d$ of 29 $\mu$g/ml and a $B_{\text{max}}$ of 219 ng/mg. Receptor-mediated LDL degradation is specific for apoB and apoE containing lipoproteins; it is calcium dependent, and is inhibited by pronase and chloroquine.

Differentiation of HL-60 cells with D₃ for 2 days induces a 45-fold increase in acetyl-LDL degradation rate compared to undifferentiated cells. Receptor-mediated degradation of acetyl-LDL is specific for acetyl-LDL, calcium independent, inhibited by chloroquine, pronase and fucoidin treatment, and is not regulated by cellular cholesterol. Acetyl-LDL binding studies demonstrated a $K_d$ of 36 $\mu$g/ml and a $B_{\text{max}}$ of 313 ng/mg.
Delivery of cholesterol via receptor-mediated catabolism of LDL or acetyl-LDL results in significant suppression of sterol synthesis and HMG-CoA reductase activity, and significant induction of ACAT activity relative to macrophages incubated with LPDS (P<0.001). However, receptor-mediated degradation of acetyl-LDL, but not LDL, significantly increases cholesteryl ester content (P<0.001).

D₃-induced HL-60 macrophages incubated with or without LDL for 48 hr exhibited large empty vacuoles with little or no lipid stainable material. In contrast, macrophages incubated with acetyl-LDL exhibited a dramatic increase in lipid stainable material which imparted the macrophages with a foamy appearance.

In conclusion, HL-60 cells treated with D₃ for 48 hr undergo activation differentiation assuming the structural and functional characteristics of human monocyte-derived macrophages. Thus, D₃-induced HL-60 macrophages are a suitable in vitro system to study lipoproteins and cholesterol regulation as related to macrophage involvement in atherosclerosis.
CHAPTER 1

INTRODUCTION

Atherogenesis involves complex cascades of interactions among environmental and genetic factors including the endothelial and smooth muscle cells of the arterial wall; monocytes and platelets; and plasma lipoproteins (Schwartz et al. 1989). In man, the atherosclerotic lesions are characterized by the accumulation of lipids in and around the intima cells; and is associated with cellular and fibrous proliferation resulting in a subsequent narrowing of the vessel lumen (Gerrity 1981). Lipid-laden foam cells are a major component of early atherosclerotic plaques. These cells are laden with lipids, mainly cholesteryl esters. There has been considerable controversy as to the exact origin of these cells. Evidence from animal and human model diseases indicates that monocyte-derived macrophages and smooth muscle cells of the arteries may be the progenitors of foam cells (Ross 1986).

Monocyte-macrophage adhesion to and infiltration into the arterial intima is the earliest process of atherosclerotic lesions (Watanable et al. 1989). The phenomenon of mononuclear cell adhesion and transendothelial passage has been observed not only in the aorta but also in coronary arteries (Scott et al. 1986) and carotids (Vos et al. 1983). Once blood monocytes have entered the arterial intima
by a process of transendothelial migration, they undergo activation differentiation, then they assume the structural and functional characteristics of foam cells after uptake of modified LDL. Specific interest has been centered on the regulation of lipoprotein uptake by macrophages and their involvement in the initiation and propagation processes of atherosclerosis. Numerous studies have demonstrated that macrophages in culture can be induced to accumulate massive amounts of lipids and to take on the appearance of foam cells. The primary model systems for studies of the major biochemical and histological characteristics of foam cells have been mouse peritoneal macrophages (Goldstein et al. 1979; Brown et al. 1980), human blood monocyte-derived macrophages (Soutar and Knight 1984; Patel and Knight 1985), and macrophage-like cell lines both of human and animal origin (Via et al. 1985a; Drevon et al. 1981). Each of these model systems, however, has limitations due to the technical difficulties in obtaining and culturing blood monocytes or due to species related differences in the types and expression of membrane lipoprotein receptors. For example, studies have shown that rat fibroblasts have high receptor affinity for certain rat lipoproteins yet bind human LDL ineffectively (Innerarity et al. 1980). In addition, mouse peritoneal macrophages have an apo B/E receptor but they bind human LDL with low affinity (Knight and Soutar 1986).

The purpose of the studies presented here was to develop a readily available model for in vitro analysis of the interactions between human plasma lipoproteins and human macrophages, as related to foam cell development. To serve as a valid
model system of macrophage-lipoprotein interactions, the cell line should meet the following criteria:

1. Easy to obtain and culture.
2. Have a regulated native LDL receptor.
3. Exhibit an unregulated scavenger receptor pathway.
4. Able to accumulate cholesteryl esters when incubated with chemically modified LDL.
5. Take on the appearance of foam cells when loaded with lipids.

This literature review includes current knowledge regarding characterizations, functions and regulations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, acyl-coenzyme A: cholesterol acyltransferase (ACAT), and native and scavenger receptors of LDL; all of which play important roles in the regulation of cellular cholesterol and lipoprotein metabolism. In addition, recent advances in understanding differentiation and lipid metabolism in HL-60 cells and chemically-induced HL-60 macrophages are included.
CHAPTER 2

LITERATURE REVIEW

CELLULAR CHOLESTEROL REGULATION

All mammalian cells require cholesterol for basic functions of growth, replication, and maintenance. The free cholesterol in the cell is determined by a balance between input and output. There are two principal sources of cholesterol input: 1) endogenous synthesis in situ regulated by HMG-CoA reductase, the rate limiting enzyme in the biosynthesis of cholesterol, and 2) receptor-mediated uptake of cholesterol-rich lipoproteins from the extracellular environment (Brown and Goldstein 1986). Both of these pathways are regulated by the cellular free cholesterol concentration and, therefore, are under the influence of end-product repression (Goldstein and Brown 1984). A third, but less significant source of cholesterol to the cells is via non-receptor-mediated uptake of extracellular lipoproteins. Cellular cholesterol output governs all of the processes that remove cholesterol from the cell including: 1) secretion of free cholesterol to extracellular sterol acceptors, and 2) cholesterol utilization for synthesis of membranes and catabolism to steroid hormones and bile acids.
There are two important enzymes which regulate cellular endogenous cholesterol synthesis and storage of cholesterol as cholesteryl esters, HMG-CoA reductase and ACAT respectively.

**3-HYDROXY-3-METHYLGLUTARYL-COENZYMEO REDUCTASE**

**Characteristics and Structure**

HMG-CoA reductase catalyzes the first committed step in the synthesis of cholesterol by reducing the six-carbon intermediate HMG-CoA to mevalonate in the presence of NADPH (Brown and Goldstein 1980). The enzyme exhibits a diurnal rhythm in nocturnal animals with nadir at noon (McNamara and Rodwell 1972) and an optimal catalytic activity at pH 7.0 (Goldstein and Brown 1984).

HMG-CoA reductase is an integral membrane glycoprotein present in the smooth endoplasmic reticulum (SER) (Chin et al. 1984). The nucleotide sequence of a full length cDNA for the human HMG-CoA reductase codes for a 888 amino acids which share a high degree of homology with the hamster enzyme (Luskey and Steven 1985). The enzyme isolated from hamster cells by an immunoprecipitation technique has an apparent molecular weight of 90-94 KDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The complete amino acid sequence of the hamster enzyme has been deduced from a full length cDNA (Chin et al. 1984). Based on a computer analysis of the amino acid sequence, Liscum et al. (1985) have postulated that the structure of HMG-CoA reductase is as follows:
1) There are seven membrane spanning regions, each with 23-30 amino acids in alpha-helical configuration, a highly conserved region among species.

2) The loop between the sixth and the seventh membrane-spanning region projects into the lumen and contains the potential N-linked glycosylated sites of the enzyme.

3) The C-terminus projects into the cytoplasm, whereas the N-terminus projects into the SER lumen.

4) The C-terminal cytoplasmic projection contains two cysteine-rich extended beta-structure that contribute to the active sites.

The reductase gene has been characterized in detail by Reynold et al. (1984a). The hamster reductase gene is 25 kb with 20 exons and 19 introns. It has 16 different 5' untranslated regions, unusual features which probably explain the existence of multiple reductase mRNAs of different sizes (Reynold et al. 1985).

Regulation of HMG-CoA Reductase

Regulation of HMG-CoA reductase has received great attention due to its complexity. The enzyme is subject to multivalent regulatory mechanisms where feedback inhibition by cholesterol and nonsterol metabolites of mevalonate play important roles. The enzyme activity is transcribed at a relatively high level when cellular sterols are depleted and is repressed when sterols accumulate. Within the total mass of cellular free cholesterol there resides a "regulatory pool" of membrane cholesterol. This pool monitors the concentration of cellular free cholesterol and
mediates signals to sites that control HMG-CoA reductase activity and the LDL receptor pathway. The sterol-mediated regulation of reductase activity is exerted at several molecular levels including transcription, translation, protein turnover, and catalytic efficiency of the enzyme (Reynolds et al. 1984b; Tanaka et al. 1983; Von Gunten 1989).

The molecular basis of induction and/or suppression of HMG-CoA reductase by sterols have been intensively studied by Osborne and his coworkers. Osborne et al. (1987), using hamster liver cells, have shown that sterol-regulated expression of the HMG-CoA reductase gene requires the binding of transcription factors to specific sequences in the promotör region which consists of an octanucleotide sequence. Interestingly, seven out of the eight nucleotides match with a sequence in the regulatory region of the gene for LDL receptor (Osborne et al. 1988). This could explain the basis for the reciprocal responses of the activities of HMG-CoA reductase and LDL receptor to changes in cellular sterol content.

Delivery of cholesterol to cells via a lipoprotein receptor-mediated process is followed by suppression of reductase activity. When cultured human fibroblasts are transferred from a medium containing LDL to a medium containing lipoprotein depleted serum, HMG-CoA reductase activity increases more than 20-fold (Brown et al. 1974). Thus, LDL-cholesterol inhibits enzyme activity and Lusky et al. (1983) have demonstrated that this inhibition is mediated at the transcriptional level.

Kandutsch et al. (1978, 1982) have suggested that the intracellular sterol
directly responsible for HMG-CoA reductase regulation is not cholesterol itself, but an oxysterol related metabolically to cholesterol. The presence of an oxysterol pool has been confirmed by many investigators (Brown and Goldstein 1974; Kreiger et al. 1978; Kandutsch et al. 1978 & 1982; Gibbons 1983). That a greater suppression of HMG-CoA reductase activity is obtained when Chinese hamster ovary cells (CHO) are incubated in the presence of 25-hydroxycholesterol, compared to cells incubated with LDL, is consistent with oxysterol being a more potent inhibitor of the enzyme.

Regulation by mevalonate was studied in detail using Mev-1, a mutant cell line from CHO cells that lacks HMG-CoA synthase which catalyzes the synthesis of HMG-CoA. This cell line exhibits an absolute need for mevalonate for normal growth, and has a very high reductase activity compared to the wild type (Schintzer-Polokoff et al. 1982). When these cells are incubated with cholesterol or 25-hydroxycholesterol, reductase activity is not completely suppressed. However, the addition of mevalonate to the incubation media results in suppression of enzyme activity. This provides evidence that nonsterol metabolites of mevalonate are able to suppress HMG-CoA reductase activity.

Regulation of HMG-CoA reductase at the level of enzyme protein synthesis and degradation (long-term regulation) has been characterized under a variety of conditions. Using radioimmune precipitation techniques it has been shown that induction of enzyme synthesis is obtained when rats are fed mevinolin (competitive inhibitor of the enzyme) or cholestyramine (a resin that binds bile acids), and when
rat hepatocytes are incubated in the presence of mevinolin (Edwards et al. 1983).

Changes in the catalytic activity of enzyme molecules, without changing the number, have been demonstrated in a variety of tissues including developing brain, human fibroblasts, liver and intestine. These changes, referred to as short-term regulation, are brought about by isosteric and allosteric effectors and by covalent modification of the enzyme (phosphorylation/ dephosphorylation of the enzyme) (Beg and Brewer 1981, Beg et al. 1978, 1979, 1980). A bicyclic system has been proposed for the modulation of reductase activity. HMG-CoA reductase is inactivated when phosphorylated by the action of an ATP-dependent protein kinase (reductase kinase). However, the enzyme is reactivated by a fluoride sensitive phosphatase. The major phosphorylation site on the enzyme is the linker region outside the membrane-spanning region (Parker et al. 1989). Reductase kinase, which catalyzes the phosphorylation of reductase, itself is regulated by phosphorylation/ dephosphorylation by cAMP-independent reductase kinase kinase/ reductase kinase Phosphatase. Although endogenous feedback control exists, cholesterol synthesis is never completely suppressed (Reichl and Miller 1989).
ACYL-CoA:CHOLESTEROL ACYLTRANSFERASE (ACAT)

Function and Structure

ACAT catalyzes the transfer of long chain fatty acid, preferentially oleate, from acyl-CoA to the beta-hydroxyl group of cholesterol forming cholesteryl esters. Enzyme activity has been demonstrated in many cells and tissues including liver (Balasubramanian et al. 1978), intestine (Helgerud et al. 1981), artery wall (Bell 1984), fibroblasts and macrophages (Brown et al. 1980). Recently the enzyme has been purified from human leukocytes and porcine liver (Schmitz and Beuck 1990). It is an integral membrane enzyme associated with the rough ER (Balasubramanian et al. 1978). The enzyme has an apparent molecular weight of 58 kDa (Schmitz and Beuck 1990).

The major role of ACAT is to act in conjunction with the LDL receptor and HMG-CoA reductase to minimize fluctuations in cellular free cholesterol concentrations. When free cholesterol flux into cells increases, reductase activity decreases, LDL receptor expression decreases, and ACAT activity increases. ACAT activity can be induced some 500-fold when cholesterol deprived cells are incubated with LDL (Brown and Goldstein 1983a). These coordinated changes take place to maintain cholesterol homeostasis, as well as to protect the cells by preventing deterioration of membranes from the potential cytotoxicity of free cholesterol (Schmitz and Beck 1990).
Regulation of ACAT

The available data indicate the existence of at least four regulatory factors affecting ACAT activity:

1) Substrate supply (cholesterol, acyl-CoA's): Cytoplasmic cholesteryl ester accumulates when mouse peritoneal macrophages are incubated with acetyl-LDL, and it disappears when the source of incoming cholesterol is removed from the media (Brown and Goldstein 1980). The findings that a very small increase in microsomal free cholesterol content leads to a tremendous increase in ACAT activity is in part explained on the basis of a substrate activation theory (Hashimoto et al. 1974).

2) Quality of phospholipid synthesis: Doolittle and Chan (1982) have demonstrated that when ACAT is solubilized by treating microsomes with detergent, its catalytic activity is not expressed unless the solubilized enzyme is incorporated into phospholipid-cholesterol vesicles. Recently, Schmitz and Beuck (1990) have shown that microsomes of mouse peritoneal macrophages preincubated with synthetic free cholesterol/phosphatidylcholine (C/PC) liposomes exhibited a higher ACAT activity compared to microsomes incubated with C/sphingomyelin. The authors also proposed that when cholesterol is associated with PC, they formed drop-like structure of C/PC liposomes are better substrates for ACAT than other synthetic liposomes.

3) Lipid carrier proteins: Acyl-CoA-binding protein (ACBP), sterol carrier protein (SCP₂), etc. can regulate ACAT activity in both directions, suppression and
induction, depending on their degree of preloading with oleoyl-CoA (Schmitz and Beuck 1990). When partially purified ACAT was incubated with ACBP preloaded with [14C]-oleoyl-CoA, the incorporation rates of [14C]-oleate into cholesteryl ester increases linearly with increasing amounts of ACBP preloaded with oleate. However, when increasing concentration of ACBP not loaded with oleoyl-CoA were incubated with the enzyme, ACAT activity was inhibited.

4) Phosphorylation/dephosphorylation regulation: Catalytic activity of the ACAT enzyme can be modulated reversibly by phosphorylation (Suckling et al. 1983). It has been proposed that the enzyme's activity increases when phosphorylated, however, covalent addition of the phosphate group to the enzyme has not been demonstrated. The evidence that the enzyme is active when phosphorylated is based on observation that ACAT activity in isolated microsomes increases under conditions in which phosphorylation is favored (Skrzypczak and Higgins 1985). A conclusive test of the hypothesis that ACAT activity can be modulated by phosphorylation/dephosphorylation will only be possible when the enzyme is completely purified and characterized.

LIPOPROTEIN METABOLISM

Clinical interest in lipoproteins arises from their association with coronary heart disease (CHD), the number one killer in the United States. Plasma lipoproteins are metabolically diverse particles formed from the non-covalent
association of lipids and one or more apolipoproteins (apolipoprotein: apoA, apoB, apoC, etc.). The apoprotein moiety of the lipoproteins represent a unique group of proteins that are able to bind, solubilize, and transport blood lipids in stable forms. Besides binding, solubilization and transport of lipids, other functions for apoproteins have been identified including activation of enzymes involved in lipid and lipoprotein metabolism, and recognition of ligands for cellular lipoprotein receptors (Pownall et al. 1987).

Plasma lipoproteins are separated into various classes based on the density at which they float during ultracentrifugation. They are further classified on the basis of particle size, electrophoretic mobility, or affinity chromatography. There are six major classes of lipoproteins in normal plasma: chylomicrons, chylomicron remnants, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL); all of which differ in composition, density, electrophoretic mobility, and size.

Chylomicrons, the largest lipoproteins, are synthesized by the intestine to transport dietary cholesterol and triglycerides from the site of absorption in the intestinal epithelium to various cells of the body. The main apoproteins of the particles are apoB-48 and apoE. Chylomicron triglycerides are hydrolyzed by lipoprotein lipase (LPL), an enzyme bound to the luminal surface of the endothelium of blood capillaries. LPL requires apoC-II for activation and is enhanced by apoA-I. The fatty acids liberated during hydrolysis are utilized as an energy source by various
tissues and excess fatty acids are taken up by adipocytes for storage as triglycerides. The lipoprotein particles generated by the action of LPL are referred to as chylomicron remnants. These small, apoB-48, apoE, and cholesterol-rich remnants are cleared rapidly by the liver via the apo E and apo B/E receptors (Salter and Brindley 1988).

VLDL are assembled in and secreted by the intestine and liver into the circulation. Hepatic VLDL are rich in apoB-100 and apoE, whereas, intestinal VLDL are high in apoA-I and apoA-IV, both forms of VLDL acquire their apoC from HDL (Kane 1983). Once the VLDL particles enter the circulation, they are acted upon by LPL. About 90% of the VLDL-triglycerides are hydrolyzed, releasing fatty acids and 2-monoglycerides (Nelson and Ackerman 1988). However, most of VLDL’s apoC and some apoE are transferred to HDL (Alexander et al. 1976). The net result from the action of LPL is the formation of IDL and VLDL remnants; relatively smaller particles, with a high cholesteryl ester: triglyceride ratio, and a low apoC: apoE ratio. In man, the remnant particles are either taken up by the liver and other tissues via the apo B/E receptors or are converted into LDL (50-90%). Little is known about this conversion. One theory postulates that the continuing action of LPL on VLDL remnant may result in the formation of LDL (Deckelbaum et al. 1979). Another theory points to the possibility that VLDL conversion to LDL is brought about by the action of heparin-releasable hepatic lipase. This enzyme is attached to the luminal surface of the liver sinusoids, and catalyzes the catabolism
of VLDL-triglycerides (Goldberg et al. 1982).

Most of the IDL formed contain apoB-100, apoC and apoE and are rapidly cleared from circulation via the apo B/E receptors. A portion of IDL loses most of its apoE and apoC to HDL, giving rise to LDL (Brewer et al. 1988). In man, the LDL particles formed are solely from the conversion of VLDL particles (Oloffson et al. 1987a). However, in animals it is believed that the liver contribute to LDL production directly (Eisenberg 1984). Eventually all of the LDL formed are cleared from circulation by either receptor-mediated endocytosis via the apo B/E receptor or by non-receptor mediated uptake. The regulation of LDL uptake will be discussed later.

HDL are secreted by liver and intestine as non-spherical particles resembling disks, known as nascent HDL, containing free cholesterol (Eisenberg 1984). Intestinal HDL are high in apoA-I, and hepatic HDL are high in apoE. Besides hepatic and intestinal sources of HDL, the particles are assembled within the plasma during lipolytic processing of chylomicrons and VLDL (Jaons et al. 1988). The HDL acquire their apoproteins and phospholipids from different potential sources including nascent HDL, and the hydrolysis of chylomicrons and/or VLDL. Phospholipid transfer protein transfers phospholipids from apoB containing lipoproteins to HDL and has been identified in man (Albert et al. 1988). The sources of free cholesterol in the HDL particles are nascent HDL, the surface coat of lipolyzed triglyceride-rich particles, and cell membranes (Jaons et al. 1988).
Lecithin-cholesterol acyl transferase (LCAT), activated by apoA-I and apoA-IV, catalyzes the formation of cholesteryl ester which moves to the core of HDL. The synthesized cholesteryl esters are transferred to other lipoproteins by lipid transfer or exchange proteins. Thus, HDL particles play an important role in reverse cholesterol transport, and as the site of cholesterol esterification by LCAT.

HDL particles can become smaller or larger in size when, and if, protein and lipids are depleted or added to the particles. HDL exists as two major subfractions, HDL₂ and HDL₃ (apoE-free HDL) (Weis 1988). HDL₂ are larger and lighter than HDL₃, which are rich in protein and more dense. Evidence indicates an inverse correlation between plasma HDL levels and incidence of atherosclerosis, suggesting that HDL may protect against atherogenesis. This protective role is believed to be related to "reverse cholesterol transport", a process by which cholesterol is transported from peripheral tissues to liver for excretion.

LOW DENSITY LIPOPROTEINS

Composition and Physical Characteristics

Epidemiological studies have demonstrated a positive relationship between total plasma cholesterol and LDL cholesterol levels and CHD incidence. Human LDL is the major cholesterol-containing particle in the plasma, has beta mobility on electrophoresis on agarose gel, a density range of 1.019-1.063 g/ml, and floatation rates of Sₙ 0-12. Based on sedimentation velocity or sedimentation equilibrium LDL
has a molecular weight of $2.0-2.5 \times 10^6$, and a diameter of 190-250 Å based on electron microscopy. Based on chemical analysis, small-angle X-ray scattering, and electron microscopy, the mature LDL particle has been shown to be a sphere comprised of a polar shell of phospholipid, free cholesterol, and protein (mainly apoB-100), and a core of non-polar lipids including esterified cholesterol and triglycerides (Decklbaum et al. 1977). Dry weight percent composition of the LDL particle averages 20-25 % protein, 7-10 % triglyceride, 15-20 % phospholipid, 7-10 % free cholesterol, and 35-40 % esterified cholesterol (Chen et al. 1981).

There are two or more subclasses of LDL particles with different chemical, physical and immunological properties, and different metabolic behavior. This heterogeneity is probably related to differences in the apoproteins present on the particle or related to differences in the expressed immunoreactive sites. Although apoB-100 is the major apoprotein present on LDL, it has been shown that the LDL particle carries other apoproteins including apoC-III and apoE (Lee and Alaupovic 1986).

**Apolipoprotein B-100**

ApoB-100 plays an essential role in the metabolism of LDL; it is synthesized in the liver and is an essential constituent of VLDL, IDL and LDL where each has one copy of the apoprotein. ApoB-100 is essentially the only apoprotein B present in normal LDL, however, apoB-74 and apoB-26 have also been isolated from some normal individuals' LDL (Kane et al. 1980).
The complete amino acid sequence of apoB-100 has recently been deduced from its cDNA sequence (Chen et al. 1986; Cladaras et al. 1986). It is a large monomeric glycoprotein of 4536 amino acid residues, insoluble in aqueous media when delipidated, and with a molecular weight of 513 KDa (Scott 1989). It contains 20 potential glycocylated sites present as N-linked high-mannose oligosaccharide units and N-linked complex oligosaccharides. There are 25 cysteine residues, 12 out of the 25 are located in the amino terminal region, and crosslinked by disulfide bonds that provide a potential complex globular structure at the amino terminus of the apoprotein (Chen et al. 1986).

The protein has a 40% alpha-helical structure and about 20% beta-sheets which give apoB-100 its distinguished features among other apoproteins (Gotto et al. 1968). Based on the hydrophobicity profile, measured by plotting hydrophobicity as a function of amino acid residues (Kyte and Doolittle 1982), it has been demonstrated that many regions of hydrophobic sequences alternate with hydrophilic sequences. This phenomenon lead Knott et al. (1985) to the conclusion that apo B-100 is woven in and out of the LDL particle at irregular intervals, and thus explains why apoB-100 is not exchangeable among lipoprotein particles. However, the apoprotein can bind to the LDL receptor (Brown and Goldstein 1986), and interacts with glycosaminoglycans including heparin (Weisgraber and Rall 1987).

ApoB-100 is present as a single gene on chromosome 2, with 43 Kb, containing 29 exons (Higuchi et al. 1988). Evidence indicates that apoB-48, the major
apolipoprotein of chylomicrons, and apoB-100 are encoded in the same gene. Changes in the sequence of apoB-100 have been shown to affect function. The first mutation, leading to overproduction of apoB-100 and an elevated cholesterol levels, could come about from changes in the promoter region. Secondly, a mutation of the region coding for the amino acids that bind to the LDL receptor, could result in lower binding affinity, and therefore, could lead to an increase in plasma cholesterol levels (Talmud et al. 1990).

THE LDL RECEPTOR (APO B/E RECEPTOR)

Prospective

Since the discovery of the LDL receptor in cultured mammalian cells by Goldstein and Brown in 1974, intensive studies have been carried out to elucidate the role of the receptor in the regulation of cellular and total body cholesterol metabolism. The fundamental importance of this receptor in the regulation of plasma LDL cholesterol levels is already shown by studies in patients with familial hypercholesterolemia, an autosomal dominant disorder characterized by elevated plasma cholesterol levels, development of premature atherosclerosis, and complete or partial absence of LDL receptor activity (Brown and Goldstein 1986).

Location and Composition

The gene for the LDL receptor is found on chromosome 19 (Lindgren et al. 1985). It is made up of about 45 kb; is divided into 18 exons and 17 introns, with a
lack of introns in the untranslated 5'flanking region (Sudhof et al. 1985). The LDL receptor, a transmembrane glycoprotein with a single polypeptide chain, was first purified to homogeneity from bovine adrenal cortex (Schneider et al. 1982). Complete cDNA sequence of the human LDL receptor revealed the presence of 860 amino acids. Twelve amino acid residues are cleaved co-translationally, leaving a protein of 839 amino acids (Yamamoto et al. 1984).

The apparent molecular weight of the receptor determined in human fibroblasts using immunoblotting techniques, with the antibody IgG-C7 as a probe, is about 164 KDa (Beisigel et al. 1982). The receptor exhibits 18 O-linked oligosaccharide chains attached to serine or threonine and 2 complex N-linked oligosaccharides attached to asparagine residues (Cummings et al. 1983).

**Biosynthesis and Orientation of the LDL Receptor**

The LDL receptor is synthesized in the rough ER, where the N-linked high mannose are added co-translationally. Then the receptor moves to smooth ER, where GalNAc transferase catalyzes the addition of GalNAc to serine and threonine residues on the O-linkages. This process is followed by the modification of the N-linked units and the addition of the galactose and sialic acid residues to the O-linked core sugar in the Golgi complex. The fully mature receptor is transferred to plasma membranes in coated vesicles where it clusters in coated pits to begin its endocytic cyclic pathway (Pathak et al. 1988).

Deletion techniques by spontaneous or in vitro mutations of the cDNA
suggested that the receptor is divided into five domains (Brown and Goldstein 1986). The first domain is the ligand binding domain and consists of 292 amino acid residues forming the N-terminal. This domain is divided into seven imperfect repeats, each of which is made up of 40 amino acids, six of which are cysteine that appear to be involved in disulfide bonds, providing the receptor with some stability (Lehrman et al. 1987a). At the C-terminal of each repeat resides a cluster of negatively charged residues. These residues are partially complementary to the positively-charged receptor-recognition sites on apoB-100 and apoE proteins, providing the receptor binding sites with the lipoprotein ligands.

The second domain of the receptor is made up of 400 amino acids. The amino acid sequence of this domain exhibits considerable homology to the mouse epidermal growth factor (EGF) precursor and to the LDL receptor-related protein (LRP), a cell surface protein that binds apoE containing lipoproteins but not LDL, referred to as the apo E receptor (Beisiegel et al. 1989). This domain is required for normal binding of LDL to the receptor. It has been shown that in the presence of acidic pH, similar to lysosomal pH, conformational changes of this domain take place causing release of the bound ligand and movement of the receptor to the plasma membrane, and thus, subsequent involvement of the receptor in another endocytic pathway (Davis et al. 1987).

The third domain of the receptor is made up of 58 amino acids including 18 serine or threonine residues. It is believed that this O-linked region of the domain
gives the receptor stability in vivo (Kingsley et al. 1986). The fourth domain of the receptor is a membrane spanning sequence of 22 hydrophobic amino acids. This region helps to anchor the receptor in the membrane in the right orientation, with the C-terminal on the cytoplasmic face and the N-terminal on the opposite face (Lehrman et al. 1987b).

The fifth domain is a 50 amino acids cytoplasmic C-terminal tail. This region is highly conserved, and exhibits high homology to the cytoplasmic domain of the bovine LDL receptor (Russell et al. 1984). The domain is made up of tyrosine rich short sequences of amino acids, which direct the clustering of the receptor to coated pits (Herz et al. 1988). This short sequence with its aromatic amino acids is not a unique feature of the LDL receptor, it has been shown that other transmembrane proteins, including LRP, have similar sequences and are clustered in coated pits of plasma membranes (Herz et al. 1988).

Lipoprotein-Receptor Interactions

There are several criteria that characterize ligand-receptor interactions of regulated receptors; 1) specificity; 2) high affinity; 3) saturation; and 4) ligand feedback control (Innerarity et al. 1986; Brown and Goldstein 1986). The interaction between LDL and its receptor has been shown to adhere to these criteria. Binding of $^{125}$I-LDL is inhibited competitively by the addition of unlabeled LDL to fibroblasts in culture. It is specific for apoB and apoE containing lipoproteins, with a $K_d$ of $2.8 \times 10^9$ M and saturation point of about 25 $\mu$g/ml for $^{125}$I-LDL. Furthermore,
cholesterol-derived LDL results in a feedback suppression of the mRNA encoding the LDL receptor (Dawson et al. 1988).

The interaction between the LDL receptor and its ligands (apoB-100 and apoE containing lipoproteins) has been further characterized by Mahley et al. (1984). This interaction is ionic in nature. It takes place between the positively charged clusters of amino acid residues of the ligands and the negatively charged amino acids of the LDL receptor binding region. Selective modification of the positive charges of the lysine residues on apoB-100 or apoE with acetoacetylation, maleylation, carbamylation, reductive methylation and acetylation (Lopes-Virella 1988; Weisgraber et al. 1987; Harberland and Fogelman 1987), or of arginine residues with 1,2-cyclohexanedicione, have been shown to abolish their ability to bind to the LDL receptor.

Binding of apoB-100 and apoE containing lipoproteins to the LDL receptor (apo B/E) requires the presence of a divalent cation, preferentially calcium. This interaction could be abolished by the presence of the chelating agent ethylenediamine tetraacetic acid (Goldstein and Brown 1977). Recently it has been demonstrated that repeat 1 of the binding domain of the LDL receptor binds calcium. That the binding of LDL to its receptor is not inhibited by deleting this repeat suggest that all of the repeats bind calcium (Van Driel et al. 1987a).

Although apoB-100 and apoE containing lipoproteins are able to bind to the LDL receptor, their receptor's binding affinities are different. ApoE containing
lipoproteins exhibit 15-fold higher binding affinity compared to apoB containing lipoproteins (Pitas et al. 1979). Recent studies of the LDL receptor have shown that the receptor can exist as dimers, trimers, or higher multimers in the cell membrane (Van Driel 1987b). One LDL molecule with its single apoB-100 binds to only one receptor in the clustered region. However, apoE containing lipoproteins (chylomicron remnants and VLDL), each with more than one copy of the apoE, bind to clusters of the LDL receptor (Van Driel et al. 1987b). This observation correlates with the very rapid clearance of apoE containing lipoproteins from the plasma. Moreover, binding of LDL to its receptor is temperature dependent, LDL receptor exhibits higher binding affinity for LDL at 4°C relative to 37°C. This observation is explained on the basis that the ligands are internalized at higher temperature (Brown and Goldstein 1975).

**Internalization and Degradation**

The pioneering work of Goldstein and Brown and coworkers delineated the sequence of events by which LDL binds to its receptor on extrahepatic cells and the subsequent internalization and degradation (Goldstein and Brown 1977). ApoB-100 of the LDL molecule acts as recognition and binding site for the LDL receptor. The ionic interaction takes place in the coated pits of the cell membrane which is associated with a protein, clathrin, of apparent molecular weight of 180 kDa and other proteins with molecular weights of approximately 100 kDa, 50-55 Kda, 36 kDa, and 33 kDa (Pearse 1976; 1988). It has been postulated that LDL receptors bind to
the 100/50 kDa proteins which act as adaptors that bind the receptor to coated pits and allow the receptors to bind their ligand (Pearse 1988).

The binding of the LDL molecule to its receptor in coated pits is followed by invagination of the coated pits into the cytoplasm and the formation of non-coated vesicles termed endocytic vesicles, via a process known as endocytosis (Goldstein and Brown 1977). The invaginated vesicles fuse together and are progressively acidified by an adenosine triphosphate (ATP)-driven proton pump, leading to the dissociation of the lipoprotein from its receptors. The dissociated receptors return to the cell surface to be involved in another endocytic cycle. The apoB-100 rich endosomes fuse with primary lysosomes. There, the protein moiety of LDL is exposed to a variety of hydrolases. The protein is hydrolyzed into amino acids, and the cholesteryl esters are acted upon by an acid cholesteryl ester hydrolase releasing free cholesterol (Goldstein et al. 1975). Outside the lysosomal department, released free cholesterol acts upon three metabolic regulatory pathways:

1) It reduces the synthesis of mRNA for HMG-CoA reductase, thus ensuring that the cell will utilize the cholesterol derived from LDL and not over synthesize endogenous cholesterol (Osborne et al. 1988).

2) It decreases the number of LDL receptors by suppressing the synthesis of mRNA encoding the receptors (Goldstein and Brown 1984).

3) Finally, it induces ACAT activity, which catalyzes the esterification of cholesterol into cholesteryl ester, and thus lowers cellular free cholesterol levels.
(Brown and Goldstein 1985).

All of these responses minimize fluctuations in the amount of cellular free cholesterol to within very narrow concentrations.

**Regulation of The LDL Receptor**

Synthesis of the LDL receptor has been shown to be subject to feedback inhibition by cellular cholesterol (Goldstein and Brown 1984). Induction of the LDL receptor in fibroblasts, by incubation with lipoprotein depleted serum, has been demonstrated by many investigators. However, when the induced fibroblasts are incubated in the presence of LDL, a progressive and concentration dependent decrease in the number of receptors is achieved, as measured by binding at 4°C (Brown and Goldstein 1975). The same results are obtained when 25-hydroxycholesterol is added to the incubation media. Furthermore, when LDL and 25-hydroxycholesterol are removed from the media, LDL receptor number is increased. These data clearly demonstrate that the LDL receptor is under finely tuned feedback regulation by cholesterol. That changes in the receptor number, and not affinity, is obtained when fibroblasts are incubated with LDL suggests that the regulation of the LDL receptor by LDL is mediated by controlling the rate of receptor synthesis.

Advances in understanding the regulation of the LDL receptor gene have indicated the involvement of second messengers in the regulation process (Auwerx et al. 1989a). When the human monocytic leukemia cell line THP-1 is treated with
TPA, a transient increase in transcription of the LDL receptor and HMG-CoA reductase are demonstrated. This effect is believed to be mediated by protein kinase C (PKC) (Auwrex et al. 1989b). The increase in mRNA levels of the LDL receptor is not secondary to the differentiation of the THP-1 cells into macrophages, because similar increases of mRNA levels encoding the receptor are obtained when a natural activator of PKC and a non-differentiation inducer is used. Although the mechanisms by which PKC induces LDL receptor gene transcription are not completely elucidated, it has been suggested that PKC-mediated induction of the LDL receptor and HMG-CoA reductase involves a post-transcriptional modification such as phosphorylation of one or more trans-acting factors (Lee et al. 1987).

**MODIFIED LDL RECEPTOR (SCAVENGER)**

**Modified LDL**

Histochemical methods, electron microscopy and surface markers of human lesions have implicated macrophages in naturally occurring atherosclerosis in man (Mitchinson et al. 1987). The observation that foam cells, whose cytoplasm is loaded with lipid droplets and which occur in fatty streaks and at the edge of plaques, are macrophages helps to explain some aspects of the disease. Particularly important is the interaction with and uptake of lipoproteins by macrophages (Klurfield 1985). However, in vitro and genetic studies indicate that the LDL receptor is not required and may not be involved in atherosclerosis. Thus the two most likely mechanisms
by which macrophages accumulate lipids, specifically cholesteryl esters, are either by LDL uptake via a low affinity, non-receptor-mediated process related to LDL concentration, or by uptake of modified LDL via a high-affinity, scavenger receptor-mediated pathway.

Macrophages possess a wide variety of receptors that can recognize various lipoproteins:

1) Acetyl-LDL receptors that bind acetyl-LDL, succinyl-LDL, and malondialdehyde modified LDL (MDA-LDL) (Goldstein et al. 1979).
2) Beta-VLDL receptors (Goldstein et al. 1980).
3) Receptors for dextran sulfate which may interact with LDL-glycosaminoglycan complexes (Lyons 1987).
6) Receptors for oxidized LDL (Steinberg et al. 1988).
7) HDL receptors (Schmitz et al. 1988).

Intensive work has been carried out to identify the naturally occurring ligands for these receptors, yet not all of them have been classified. Brown and Goldstein (1983b) have confirmed the presence of MDA in human blood platletls and macrophages during arachidonic acid metabolism. When human monocyte-derived macrophages are incubated with MDA-LDL they bind and degrade the ligand via the
acetyl-LDL receptors resulting in massive accumulation of cholesteryl esters. However, Schecter et al. (1981) have indicated that the little concentration of MDA in blood can not account for the modification of LDL, so that it is not longer recognized by the apo B/E receptor.

Beta-VLDL have been isolated from plasma of animals with severe hypercholesterolemia (Mahley et al. 1980). When mouse peritoneal macrophages are incubated with beta-VLDL, they take up the ligand via a high affinity receptor-mediated pathway, which is distinct from LDL and acetyl-LDL receptors (Brown and Goldstein 1983b). The strongest evidence that beta-VLDL are taken up by beta-VLDL receptors is the fact that Watanable heritable hyperlipemic (WHHL) rabbits, which are defective in LDL receptor activity, express beta-VLDL receptors (Van Lenten et al. 1983, 1985). In contrast to these reports, Ellsworth et al. (1986) have demonstrated that specific polyclonal antibodies directed against the estrogen-induced LDL receptor of rat liver compete for the uptake of beta-VLDL in J774 cells and mouse peritoneal macrophages. This observation indicates that beta-VLDL are taken up, at least partially, by an LDL receptor pathway.

The presence of glycosylated LDL in plasma of patients with diabetes has been illustrated by Schleicher et al. (1981). In fibroblasts the recognition of glycosylated LDL by the apo B/E receptor decreases as the glycosylation of the LDL molecule increases (Lopes-Virella 1988).
LDL undergoes several modifications when incubated with vascular endothelial cells or aortic smooth-muscle cells. When macrophages are incubated with endothelial cell-modified LDL (EC-LDL), they accumulate cholesteryl esters (Henriksen et al. 1983). It is believed that the generation of superoxide anions (free radicals) by endothelial and smooth-muscle cells, in addition to macrophages, leads to the peroxidation of LDL lipids and the extensive breakdown of apoB-100 (Steinberg et al. 1989). Moreover, this modified LDL acts as a chemotactic agent for monocytes in vitro (Quinn et al. 1987).

Oxidatively modified cytotoxic LDL functioning as a monocyte chemoattractant is of considerable significance, particularly in view of the potential role that oxidized LDL play in the pathogenesis of foam cells and atherosclerotic plaques (Steinberg et al. 1988). Kita et al. (1987) have demonstrated the potential in vivo importance of oxidized LDL with a dramatic inhibition of the progression of arterial lesions in atherosclerosis-prone WHHL rabbits treated with the antioxidant drug probucol. In vitro studies have suggested that probucol prevents lipid storage in macrophages by both preventing the oxidative modification of LDL and suppressing the uptake of modified LDL by the cells (Yamamoto et al. 1988).

Brown and Goldstein (1983b) have reviewed the disappearance of intravenous injection of chemically modified LDL from the animals' plasma in minutes after their administration. These studies included:

1) Acetoacetyl-LDL and beta-VLDL in dogs.
2) Acetyl-LDL in mice and rats.

3) Succinylated LDL in rats.

4) Oxidized LDL in guinea pigs.

The fact that chemically modified LDL are rapidly cleared from plasma in animal studies supports the hypothesis that the formation of modified lipoproteins in vivo represents a natural process. However, under normal conditions it is difficult to detect the appearance of the modified lipoproteins in blood, due to their rapid disappearance from the circulation. Whether the modifications occur naturally or are chemically introduced, they produce LDL particles that interact with macrophage receptors, resulting in a massive increase of cholesteryl esters content, and leading to the morphological transformation of macrophages to foam cells (Mahley et al. 1980; Brown and Goldstein 1983b).

As mentioned above, a wide variety of chemically modified LDL exist, however, acetyl-LDL are the most commonly used ligand for acetyl-LDL receptors. Acetylation of LDL by acetic anhydride blocks the epsilon amino group on the lysine residue of the LDL apoB molecule (Basu et al. 1976). This type of modification increases the net negative charge on the LDL particle, thus increasing its electrophoretic mobility relative to native LDL. Blocking the lysine residues of LDL is not sufficient to convert LDL into ligands for the acetyl-LDL receptor because reductively methylated LDL, in which the lysine residues are blocked but the net charge is not changed, cannot bind to acetyl-LDL receptors (Mahley and Innerrarity
In addition, LDL attached to albumin cannot bind to the acetyl-LDL receptors, presumably because the effect on the net charge of LDL is not large enough. Therefore, negative charges play an important role for recognition of chemically modified LDL by acetyl-LDL receptors. Acetyl-LDL receptors have been shown to bind other chemically modified LDL including acetoacetyl-LDL, succinyl-LDL, MAD-LDL and maleyl-LDL. All of these modifications increase the net negative charge of the apoB particles.

**Characteristics of the Scavenger Receptors**

The scavenger receptor for chemically modified LDL was first described in mouse peritoneal macrophages by Brown et al. (1983a) and Goldstein et al. (1979). Similar to the binding of LDL to its receptor, surface binding of acetyl-LDL to scavenger receptors is followed by internalization, adsorptive endocytosis, and delivery of lipoproteins to lysosomes (Brown and Goldstein 1983b). Within the lysosome, lipoprotein components (lipid and protein) are hydrolyzed by the action of a variety of acidic lysosomal enzymes. The released free cholesterol enhances the catalytic activity of ACAT, which in turn catalyzes the esterification of cholesterol to cholesteryl esters. The newly synthesized esterified cholesterol enters the cytoplasmic pool of the macrophage where it participates in a cycle of hydrolysis and re-esterification (Brown et al. 1979b; 1980). Unlike the native LDL receptor, the scavenger receptor is not down-regulated by the increase of cellular free cholesterol. Thus macrophages continue to take up modified LDL acquiring more lipids,
ultimately resulting in the formation of foam cells, the major cell types found in atherosclerotic plaques.

Scavenger receptors for acetyl-LDL are expressed by macrophages from all species examined to date. They are present on peritoneal macrophages, Kupffer cells, mouse macrophages of the J774 line, monocyte-derived macrophages, and by endothelial cells of rat liver, and parenchymal cells in vivo and in vitro (Nagelkerki et al. 1983). In general these macrophages express acetyl-LDL receptors and few or no native LDL receptors. An interesting exception is the human blood monocyte-derived macrophages that express both native and scavenger receptors, in about equal numbers, during stages of their maturation (Fogelman et al. 1981; Knight and Soutar 1982).

Several studies suggest the existence of two scavenger receptors: one receptor specifically binds MDA-LDL and methyl-albumin; and the other receptor recognizes both acetyl-LDL and MDA-LDL, referred to as acetyl-LDL receptor (Via et al. 1985b; Harberland et al. 1986). Although the two receptors have been studied intensively, the acetyl-LDL receptor has received more attention.

In vitro studies using mouse peritoneal macrophages demonstrated that surface binding of acetyl-LDL is saturable, with the half-saturation point at approximately 25 µg of protein/ml at 37°C. The receptor binds 20,000-40,000 particles of acetyl-LDL per cell at 4°C. The binding does not require calcium, is inhibited by chloroquine (inhibitor of lysosomal enzymes), and is specific for acetyl-LDL, MDA-
LDL, LDL incubated in the presence of endothelial cells, and polyanionic compounds including fucoidin, polyvinyl sulfate and dextran sulfate. In addition, the receptor does not bind or recognize native LDL (Brown and Goldstein 1983b). All of the criteria of LDL-apo B receptor interactions: specificity, saturation and high affinity, but not ligand feedback control, are observed for the acetyl-LDL. Incubating macrophages with acetyl-LDL does not result in the down-regulation of receptor number. Thus, the regulatory pool of free cholesterol, which is able to suppress LDL receptor number does not affect acetyl-LDL receptor expression. However, excess free cholesterol in macrophages does down-regulate HMG-CoA reductase and up-regulate ACAT activities (Brown et al. 1980).

Via et al. (1985b) have reported the partial isolation and purification of a mouse macrophage protein of 283 KDa that recognizes and binds acetyl-LDL. Further characterization of the protein by ligand-blotting techniques revealed that the receptor has an apparent molecular weight of 260 KDa (Via et al 1985b).

Recently progress has been made toward deducing the primary structure of the scavenger receptor from bovine macrophages (Kodama et al. 1990). This receptor is a trimeric membrane protein of 220 KDa molecular weight. Partial amino acid sequence of the protein, as a starting point for cloning and expression in COS cells, revealed the presence of two complementary cDNAs with their products as scavenger receptors type I and type II. Both receptors are integral membrane proteins. There are six domains in the type I receptor:
1) N-terminal cytoplasmic domain.
2) Transmembrane domain.
3) Spacer domain.
4) Alpha-helical domain
5) Collagenous domain.
6) C-terminal cysteine-rich domain.

All of these domains are present in type II receptors except for the C-terminal domain. Analysis of the type II form of the receptor indicates that the specificity of the receptors resides in the fourth and fifth domains (Kodama et al. 1990).

Matsumoto et al. (1990) have also identified two cDNAs for the human macrophage scavenger receptors cloned from a cDNA library derived from the TPA-treated human THP-1 cells. Comparison of the human and bovine cDNAs sequences indicated that most of the structural characteristics of the bovine receptor are conserved in the human receptor, except for domain VI of type II receptor. Moreover, immunohistochemical studies using an anti-peptide antibody which recognizes human scavenger receptors indicated the presence of the scavenger receptors in the macrophages of lipid-rich atherosclerotic lesions, suggesting the involvement of the scavenger receptors in the formation of foam cells and in the development of atherosclerotic lesions in human subjects. The scavenger receptor gene is located on chromosome 8 which is in agreement with an independent mapping of the murine receptor (Freeman et al. 1990).
The molecular cloning of the human scavenger receptor cDNAs is a breakthrough for further studies concerning their physiological role, pathological involvement, genetic polymorphism, and relationship to atherosclerotic lesions all of which are of importance to understand the pathogenesis of atherosclerosis.

HUMAN PROMYELOCYTIC LEUKEMIA CELLS (HL-60)

Characteristics of HL-60 cells

The human promyelocytic leukemia cell line HL-60 was derived from a patient with acute promyelocytic leukemia (Wathne et al. 1989). The cells have normal regulatory responses for both HMG-CoA reductase and receptor-mediated LDL degradation (Via et al. 1985; Muller et al. 1987). The saturable binding of LDL to the apo B/E receptor has a dissociation constant of $3.2 \times 10^{-9}$ M, there are 2700 receptors per cell (Wathne et al. 1989), and exogenous LDL cholesterol up regulates ACAT activity in HL-60 cells (Wathne et al. 1989). Thus, HL-60 cells exhibit metabolic and regulatory patterns that are similar to those reported for monocyte-derived macrophages (Via et al. 1985; Collins et al. 1978; Murao et al. 1983).

HL-60 cells are unusual among other human myeloid leukemias in that they continuously proliferate in suspension (Collins 1987). It has been postulated that the presence of abnormalities in cellular oncogenes play a role in the unusual proliferating capacity. Of great interest is the ability of the cells to differentiate in vitro to a variety of phenotypic myelomonocytic lineage: 1) eosinophils, 2)
granulocytes, 3) monocytes, 4) macrophage-like cells. These phenotypic changes can be brought about by different inducers. 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1,25-dihydroxyvitamin D₃ are the primary macrophage inducers (Collins 1987; Via et al. 1985). Induction of differentiation of these cells into macrophages is of primary importance to the aims of this project. Therefore, only the characteristics of HL-60 cells induced to macrophages will be discussed.

**TPA-Induced HL-60 Macrophages**

Phosphorylation of proteins is a key role in regulating cellular functions (Hunter and Cooper 1985). The kinases and phosphatases governing such phosphorylations are themselves targets for the action of growth factors, hormones, and other extrinsic factors participating in the control of cellular events (Hunter and Cooper 1985). Protein kinase C (PKC), a component of the transduction pathway, is a serine- and threonine-specific enzyme that depends upon calcium and phospholipid for activity (Takai et al. 1979). At physiological calcium concentrations diacylglycerol is required for activity of PKC (Kishimoto et al. 1980). From studies of PKC in vitro, it has become apparent that phorbol esters capable of tumor promotion can mimic the effect of diacylglycerol in enzyme activation (Castanga et al. 1982).

HL-60 cells possess specific receptors for TPA, and evidence indicate that the receptor is protein kinase C itself (Rovera et al. 1979, Niedel et al. 1983; Vanderbark et al. 1984). Differentiation can occur within 20 minutes after incubating cells with
TPA (Rovera et al. 1979) and TPA induction of HL-60 cells is associated with a rapid profound loss in proliferative capacity. The macrophages markedly adhere to plastic with prominent pseudopodia formation and also adhere to each other (Rovera et al. 1979a, b).

Minutes after exposure of HL-60 cells to TPA, PKC is activated which in turn phosphorylates at least 14 proteins, most important are two cytosolic proteins with 17 and 27 kd in size, believed to be involved in differentiation (Feurstein and Cooper 1983). However, activation of PKC cannot by itself account for the differentiation of HL-60 cells. When HL-60 cells are treated with the synthetic diacylglycerol 1-oleoyo-2-acetylglycerol, PKC is activated, several proteins are phosphorylated, yet no differentiation is observed (Kreutter et al. 1985).

It appears that the level of differentiation of HL-60 cells and the pattern of expression of oncogenes are closely related (Cayre et al. 1987). Recent advances on the mechanism of action of TPA on HL-60 cells, as measured by a modified polymerase-chain reaction (PCR) method, indicate that differentiation of the cells is associated with transcription of c-fms (Wu et al. 1990). The c-fms gene encodes the cell surface receptor for the macrophage-colony stimulating factor (M-CSF) which is involved in macrophage growth and differentiation (Chen et al. 1984). Although treatment of HL-60 cells with TPA or D₃ results in an increase of the mRNA levels for c-fms, Wu et al. (1990) have demonstrated that differentiation by the two inducers follow two different pathways. After treatment with a c-fms anti-
sense oligomer, TPA-induced differentiation of HL-60 macrophage is inhibited whereas D₃-induced macrophage differentiation is not affected. Thus, during differentiation, TPA initiates a process that involves c-fms expression, whereas with D₃, differentiation occurs without c-fms expression (Wu et al. 1990). The exact mechanism by which TPA induces differentiation requires more research.

**TPA Macrophage-Lipoprotein Interaction**

The interaction between lipoproteins and TPA-induced macrophages have been intensively studied in many cell types including THP-1, monocyte-derived macrophages, U-937, and others. The effect of TPA treatment on the expression on acetyl-LDL receptor varies from study to study depending on the type of cells being tested. Addition of TPA to mouse peritoneal macrophages is associated with a reduction of acetyl-LDL receptor activity (Leake et al. 1989; Rouis et al. 1984a). In contrast, TPA treatment of HEL cells (Papayannopoulou et al. 1983), THP-1 cells (Hara et al. 1987), and rabbit fibroblasts and smooth muscle cells (Pitas 1990) results in increased expression of acetyl-LDL receptor. Moreover, when macrophages are incubated with acetyl-LDL they bind and degrade the modified lipoprotein leading to massive accumulation of cytoplasmic lipid resulting in foam cell formation (Hara et al. 1987). TPA-treated macrophages have also been reported to secrete apoE (Kayden et al. 1985). That apoE promotes reverse cholesterol transport and facilitates the interaction of VLDL with lipoprotein receptors on cells suggests a central role for apoE in the regulation of lipid metabolism in these cells (Innerarity
et al. 1986). Furthermore, macrophages (Mahoney et al. 1986) and macrophage-like cell lines (Tajima et al. 1985) have been shown to secrete lipoprotein lipase (LPL) which plays an important role in the uptake of triglyceride-rich lipoproteins. In addition, TPA exerts a reduction (Maziere et al. 1986; Rouis et al. 1984a; Papayannopopoulo et al. 1983) or complete loss (Hara et al. 1987; Maziere et al. 1986) of LDL receptor activity depending on the type of cell examined.

The interaction between TPA-induced HL-60 macrophages and lipoproteins has received little attention. From what has been reported, it appears that TPA-induced differentiation of HL-60 cells is associated with many characteristics observed in monocyte-derived macrophages. When HL-60 cells are treated with TPA, the apoE gene is induced, however, the differentiation of HL-60 cells is not associated with LPL gene induction (Auwerx et al. 1988). This observation is also demonstrated in HEL cells that possess unregulated acetyl-LDL receptors and are able to form foam cells in response to acetyl-LDL uptake (Auwerx et al. 1988). Yachnin et al. (1984) have demonstrated that differentiation of HL-60 cells by TPA is associated with a dose-dependent increase in cellular HMG-CoA reductase activity. In addition, TPA induces an increase in [14C]-mevalonate conversion to cholesterol and its precursors, suggesting an enhancement of post-HMG-CoA reductase events in cholesterol biosynthesis. Although Yachnin et al. (1984) did not investigate the effect of TPA on LDL receptor activity, TPA-induced HL-60 macrophages have been shown not to express LDL and acetyl-LDL receptor activities (Via et al. 1985a).
Thus, it appears that the increase of HMG-CoA reductase activity in TPA treated HL-60 cells is due to the loss of receptor-mediated endocytosis of LDL cholesterol resulting in the induction of endogenous cholesterol synthesis.

The mechanism whereby the tumor-promoting phorbol esters inhibit LDL uptake remains unknown. However, the fact that mezerein, another PKC activator, mimics the effect of TPA on LDL receptor activity in fibroblasts, suggests that PKC is involved in the inhibition phenomenon. Rouis et al. (1984a) suggested that TPA could induce phosphorylation of the receptor and the formation of a cryptic pool, by inhibiting receptor recycling. The generation of a cryptic receptor pool involves many potential mechanisms: a) ligand-induced clustering and internalization of the ligand-receptor complex (Carpenter and Cohen 1976); b) a change in membrane lipids results in receptor masking; supported by the fact that phorbol esters are known to influence membrane lipid composition and metabolism (Sutton and Martin 1982); c) covalent modification of the receptor or a component of the receptor attachment mechanism such that membrane association in intact cells is inhibited (May et al. 1984); d) alteration in the ionic milieu secondary to changes in pH or calcium ion activity (Fallen and Schwartz 1986); and/or e) receptor redistribution and increased net internalization (Fallen and Schwartz 1986).

Although maturation of macrophages by TPA is associated with partial or complete loss of LDL receptor activity, treatment of THP-1 cells with TPA results early (within 3 hr) in a transient increase in LDL receptor and HMG-CoA reductase
mRNAs. The mechanism by which TPA-mediated transcriptional activation of THP-1 cells has been postulated to occur by blocking the synthesis of a negative regulatory protein or by modifying this protein (possibly by phosphorylation by PKC) to a form unable to bind the positive transcription factor (Auwerx et al. 1989b).

In summary, the mechanism through which TPA mediates its differentiation and inhibitory effects on the LDL receptor activity has not been precisely determined and requires more attention.

**D₃-Induced HL-60 Macrophages**

Myeloid leukemic cells differentiate into macrophages when challenged with the active form of vitamin D₃ (Miyaura et al. 1981; Abe et al. 1981). This observation, coupled with a wide variety of nontraditional target cells for D₃, suggests that D₃ may play a more fundamental role in cell differentiation than previously appreciated. 1,25-Dihydroxyvitamin D₃ is the only active metabolite of vitamin D that causes significant morphological and functional changes in HL-60 cells (Mangelsdorf et al. 1984). D₃-mediated differentiation of HL-60 cells to monocytes/macrophages occurs via a receptor-mediated process. Mangelsdorf et al. (1984) have demonstrated that HL-60 contains about 4,000 D₃ receptors per cell. Immunoprecipitation technique using a monoclonal antibody (IVG8C11) raised against porcine intestinal receptor for D₃ revealed that the size of the D₃ receptor of HL-60 cells is 53 kDa. The level of D₃ receptors of HL-60 cells undergoes up-regulation, followed by down-regulation upon continuous exposure to D₃ (Lee et al.
HL-60 cells exhibit significant functional changes as early as 1 day, after treatment with D₃. Differentiation becomes more pronounced the longer the hormone remains in the culture medium. The presence of D₃ is required and must be maintained over a long period of time relative to temporal events occurring at the molecular level (Mangelsdorf et al. 1984). Growth of D₃-induced HL-60 macrophages exhibits a time-dependent biphasic dose response to D₃. Significant inhibition of growth is obtained when HL-60 cells are treated with 10⁻⁸ M D₃. However, significant stimulation in growth above undifferentiated cells occurs as HL-60 cells are cultured with 10⁻⁹ M D₃ (Mangelsdorf et al. 1984).

In general, differentiation into macrophages is associated with some functional and morphological changes. D₃-induced HL-60 macrophages are larger in size compared to undifferentiated cells. The macrophage is 13-15 μm in diameter; its nuclei is large and its grayish cytoplasm is abundant and contains few granules (Mangelsdorf et al. 1984).

Recent studies indicate that the mechanism of cell differentiation by D₃ is similar to other steroid hormones (Evans 1988). Steroid hormones bind to high affinity intracellular receptors that share similar structural organization with distinct ligand and DNA-binding domains. Ligand-bound receptors interact with specific enhancer/suppressor DNA elements resulting in the modulation of gene expression (Evans 1988). It has been shown that D₃ modulates the expression of a number of
proteins, few of which have been studied at the transcriptional level.

The similarities of action of TPA and D₃ on HL-60 cells suggest a role of PKC in mediating cell differentiation. However, there are conflicting reports regarding the involvement of PKC in the differentiation process when D₃ is used as the inducer. Most of this controversy could be attributed to the different conditions of the experiments as well as to the type of cells used (Mezzetti et al. 1987). Mezzetti et al. (1987) have demonstrated that differentiation of U-937 cells to macrophages by D₃ and TPA takes place via two different mechanisms. TPA significantly and rapidly affects PKC, whereas D₃ exerts no effect on PKC (Obeid et al. 1990). On the other hand, Zylber-Katz and Glazer (1985) have reported that D₃ induces a progressive increase in calcium and phosphatidylserine-dependent protein phosphorylation in HL-60 cells. Moreover, D₃ induces an increase in the phorbol ester receptor number of D₃-induced HL-60 macrophages without altering receptor affinity (Martell et al. 1988). Interestingly, D₃-induction of phorbol ester receptors of HL-60 cells involves transcriptional regulation of PKC (Obeid et al. 1990). The effect of D₃ on phorbol ester receptors is not unique to HL-60 cells. Ways et al. (1987) have reported that phorbol ester receptors of U-937 cells are also induced in response to D₃, and that the enhancement of TPA-induced differentiation of U-937 cells by D₃ is concentration dependent. It appears that PKC is involved somehow in the differentiation process, yet the precise role of this enzyme in HL-60 cell differentiation remains to be elucidated.
Vitamin D and Lipoprotein Metabolism

An in vivo atherogenic role of dietary vitamin D has been postulated. Vitamin D induces aortic calcification and atherosclerosis lesions in several animals and cells in culture (Hines et al. 1985; Schenk et al. 1965). In humans, dietary vitamin D inconsistently affects serum cholesterol leading to either a hypercholesterolemia (Huang et al. 1977; Morrisson et al. 1972), no effect (Hines et al. 1985), or hypocholesterolemia (Jurgens et al. 1971). However, a consistent finding in animal studies indicates that accumulation of cholesterol in aortas and liver is associated with a diet high in cholesterol and vitamin D (Hines et al. 1985; Huang et al. 1977; Schenk et al. 1965), suggesting that the primary action of vitamin D is on the cellular cholesterol metabolism rather than on plasma levels of cholesterol.

The effect of D₃ on cholesterol and lipoprotein metabolism in HL-60 cells has not been investigated and the effect of vitamin D on lipoprotein metabolism in other macrophages has received little attention. D₃ has been shown to alter lipid metabolism in human monocyte-macrophages. When cultured for six days in the presence of D₃, human monocyte-macrophages accumulate significantly more triglycerides than untreated cells (Johnson et al. 1977). In mouse alveolar macrophages, D₃ enhances the synthesis of triglycerides in part by activating diacylglycerol-acyltransferase (Miyaura et al. 1987). Furthermore, in human monocyte-derived macrophages, D₃ induces cholesterol esterification, leading to accumulation of intracellular cholesteryl esters (Roullet et al. 1989); however, this
phenomenon is obtained only in the presence of acetyl-LDL. The increase in total cholesterol content observed when monocyte-macrophages are incubated with acetyl-LDL suggests that more acetyl-LDL are internalized, perhaps related to an increase in scavenger receptor number (Roullet et al. 1989). The authors of this study postulated that the stimulation of cholesteryl ester storage may reflect a differentiating effect of D₃ on the scavenger receptor. However, other mechanisms may be evoked such as, a) D₃ mediated activation of ACAT similar to diacylglycerol acyltransferase activation of mouse alveolar macrophages (Miyaura et al. 1987), with no effect on the receptor, and b) D₃ inhibition of cholesteryl ester hydrolysis, leading to accumulation of cholesteryl ester. Roullet et al. (1989) have reported the only study that proposed an enhanced expression of acetyl-LDL receptors by macrophages in response to D₃ treatment. However, the authors failed to prove the validity of their hypothesis.

**SUMMARY**

Clearly, atherosclerosis represents an extremely complex disease and different pathological processes. Its onset and progression are very subtle, slow and silent. Today, in spite of a tremendous quantity of accumulated information, we can provide only a partial explanation as to why atherosclerosis is so common, why macrophages are involved in this disease, why macrophages cannot control the amount of cholesterol uptake, why the scavenger receptor is not regulated, etc. Moreover, the
cellular aspects of human atherosclerosis remain the least understood. Most of the information on this problem has been gained by histological analysis of autopsy and biopsy material, which do not provide sufficient quantitative data for reliable assessment of cellular dynamics in disease vessels. In addition, the systematic experimental work with autopsy material is complex and difficult. Of late, the methods of quantitative morphology and cell culture were effectively applied to exploration of atherosclerosis.

At present, cell culture is the only way to study cellular manifestations of human atherosclerosis. Despite the fact that cell culture is a convenient object of study, the existing systems suffer many problems. First, is the non-human origin of the cell culture, where most of these cells are of animal origin. Secondly, is the cross-specie studies which introduces another variable. In most studies, if not all, lipoproteins are usually obtained from human, then are used to study the lipoprotein-animal macrophage interactions. Finally, monocyte-derived macrophages obtained from human blood besides being difficult to obtain, lack the consistency of results due to individual variation. Therefore, the need for a human cell line that can provide accessibility to macrophages where the major cellular manifestations of atherosclerosis can be reproduce under simple chemical conditions, is clear.
CHAPTER 3

LIPOPROTEIN RECEPTORS OF HL-60 MACROPHAGES: EFFECT OF DIFFERENTIATION WITH TETRAMYRISTIC PHORBOL ACETATE AND 1,25-DIHYDROXYVITAMIN D₃
SUMMARY

The human promyelocytic leukemic cell line HL-60 is a unique model for studies of the effects of macrophage differentiation on expression and regulation of lipoprotein receptors. Undifferentiated HL-60 cells express a regulated LDL receptor and lack the acetyl-LDL scavenger receptor. HL-60 macrophages differentiated with tetramyristic phorbol acetate failed to degrade LDL and acetyl-LDL via receptor-mediated processes. Differentiation with D_3 induced macrophages exhibiting specific, saturable receptors for LDL and acetyl-LDL. The LDL receptor of D_3-induced macrophages exhibits specificity for apoB and apoE containing lipoproteins, is calcium dependent, and inhibited by pronase and chloroquine. Maximal degradation of acetyl-LDL was achieved within two days of D_3 treatment and was specific for acetyl-LDL, calcium independent, inhibited by chloroquine, and sensitive to pronase and fucoidin treatment. Incubation of D_3-induced macrophages with LDL or acetyl-LDL resulted in reductions in sterol synthesis and receptor-mediated degradation of LDL; the scavenger receptor pathway was unaltered. These results demonstrate that D_3-induced HL-60 macrophages exhibit patterns of sterol and lipoprotein metabolism and regulation which make them a useful system for in vitro studies of lipoprotein-macrophage interactions as related to foam cell development and atherogenesis.
INTRODUCTION

Mammalian cells acquire cholesterol through either de novo synthesis or receptor-mediated endocytosis of lipoprotein cholesterol via specific, saturable, high affinity lipoprotein receptors. Most of these receptor pathways are regulated by the cellular cholesterol and when cellular cholesterol levels increase, three metabolic responses occur to maintain cholesterol homeostasis: a) down-regulation of LDL receptors at the transcriptional level; b) suppression of HMG-CoA reductase; the rate limiting enzyme of cholesterol biosynthesis; and c) induction of ACAT activity; the enzyme responsible for cholesterol esterification (Brown et al. 1979a, Mahley and Innerarity 1983, Fogelman et al. 1981, Patel and Knight 1985). While these regulatory responses are able to maintain cellular cholesterol homeostasis under most conditions, unregulated uptake of modified lipoproteins can result in cholesteryl ester accumulation and foam cell development (Fogelman et al. 1981, Goldstein et al. 1979, Brown et al. 1979b, Brown et al. 1980).

Histochemical analyses, electron microscopy and surface markers of human lesions have implicated tissue macrophages in the development of foam cells which occur in the process of atherosclerosis in man (Mitchinson and Ball 1987). In vitro and genetic studies indicate that lipoprotein uptake via the LDL receptor is probably not a major factor in foam cell development and the two most likely mechanisms by which macrophages accumulate cholesteryl ester are either by LDL uptake via a low-affinity, non-receptor-mediated pathway, or by uptake of modified LDL via a high-
affinity, scavenger receptor-mediated pathway. Macrophages, major cellular component of atherosclerotic plaques, possess unique and specific characteristics. In vitro differentiation of blood monocytes to macrophages is associated with a decrease in LDL receptor activity, enhanced scavenger receptor activity, and secretion of lipoprotein lipase and apoE (Auwerx et al. 1988).

The human promyelocytic leukemia cell line HL-60 has been shown to differentiate into macrophages in response to TPA and D₃. HL-60 cells exhibit classic regulatory responses for both HMG-CoA reductase activity (Wathne et al. 1989) and receptor-mediated LDL degradation (Via et al. 1985a). TPA induced differentiation of HL-60 cells has been reported to block receptor-mediated LDL degradation (Via et al. 1985) and to increase sterol synthesis rates and HMG-CoA reductase activity (Yachnin et al. 1984). Differentiation of HL-60 cells with TPA is also accompanied by induction of the apoE gene (Auwerx et al. 1988). In contrast to the data available regarding the effects of TPA induced macrophage differentiation of HL-60 cells, little is known regarding the effects of D₃-induced differentiation on lipid and lipoprotein metabolism by these cells. In many aspects, D₃-induced HL-60 macrophages exhibit metabolic patterns similar to those reported for human monocyte-derived macrophages (Via et al. 1985a, Collins et al. 1978, Murao et al. 1983).

In an attempt to develop a model system for in vitro analysis of the interactions between human plasma lipoproteins and human macrophages, studies
were carried out to characterize the lipoprotein receptors of the human promyelocytic leukemia cell line HL-60 prior to and following induction of macrophage differentiation by TPA or D3. Our results demonstrate that HL-60 cells exhibit a specific, saturable, and regulated LDL receptor with no expression of a scavenger receptor for chemically modified LDL. Macrophage differentiation of HL-60 cells by TPA resulted in the loss of receptor-mediated LDL degradation and no measurable degradation of acetyl-LDL. In contrast, D3-induced HL-60 macrophages express specific and saturable receptors for both LDL and acetyl-LDL. These data suggest that D3-induced HL-60 macrophages can be a valuable model system for in vitro studies of human lipoprotein-macrophage interaction and its regulation as related to foam cell development and atherogenesis.
METHODOLOGY

Materials: HL-60 cells were obtained from American Type Culture Collection (ATCC) Rockville, MD; Na$^{125}$I was purchased from Amersham, Arlington Heights, IL; RPMI-1640, glutamine, penicillin/streptomycin, horse serum (HS) and fetal calf serum (FCS) were purchased from Flow Labs, McLean, VA; TPA (dissolved in acetone and stored at -80°C), chloroquine, pronase (103 PUK/mg), bovine serum albumin (BSA), mezerien (dissolved in ethanol and stored at -20°C), and non-specific acid esterase from Sigma, St. Louis, MO; 1,25-dihydroxyvitamin D$_3$ (stored in small aliquots in ethanol at -20°C) was the gift of Dr. Milan R. Uskokovic, Hoffman-LaRoche, Inc, Nutley, NJ; mevinolin was a gift from Dr. AL Alberts, Merck, Sharp & Dohme, Nutley, NJ. [1,2-$^3$H]-cholesterol (60 Ci/mmol) and [2-$^{14}$C]-acetate (55 mCi/mmol) were obtained from New England Nuclear Corp. Boston, MA; heparin sepharose affinity columns were purchased from Pharmacia, Piscataway, N.J.; and neutral alumina AG 7 (100-200 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA.

HL-60 Cell Culture: Human promyelocytic leukemic HL-60 cells were grown in RPMI-1640 supplemented with 15% heat inactivated horse serum, 24 mM sodium bicarbonate, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, in a humidified incubator of 5% CO$_2$ at 37°C. Cells were maintained by passage of 1.5 x 10$^6$ cells/ml every 3 days. Cell viability was determined by trypan blue exclusion.
**Macrophage Differentiation:** HL-60 cells (1.0 x 10^6 cells/ml) maintained for 24 hr in fresh RPMI-1640 medium containing 15% HS were centrifuged 10 min at 400 g at 4°C and resuspended at a density of 1.0 x 10^6 cells/ml in RPMI 1640 medium containing 15% heat inactivated lipoprotein deficient fetal calf serum (LPDS) (Mangelsdorf et al. 1984). To induce macrophage differentiation, 5.0 x 10^{-8} M D_3 or 1.5 x 10^{-6} M TPA were added to the cells. After a 48 hr incubation at 37°C the suspended cells were discarded; adhered cells were washed twice with phosphate buffered saline (PBS), detached with PBS/1 mM ethylenediamine tetraacetic acid (EDTA) buffer, scraped with a rubber policeman, centrifuged at 400 g for 10 min at 4°C, washed twice with PBS, resuspended in PBS to a density of 1.5 - 2.0 x 10^6 cells/ml and used in the various experiments.

**Assessment of Cell Growth:** To examine the effect of D_3 on cell growth, 5 ml of cells at 10^5 cells/ml were incubated in RPMI-1640 supplemented with 15% HS and 5.0 x 10^{-8} M D_3. Control cultures contained the ethanol vehicle at 0.1% (v/v). 100 μl of each cell culture were removed every 24 hr for 4 days and cells were counted with a hemacytometer, using trypan blue exclusion to determine cell viability.

**Lipoprotein Isolations:** Plasma from normolipidemic volunteers recruited from staff and students at the University of Arizona was used to isolate VLDL (d < 1.006 g/ml), LDL (d 1.02 - 1.063 g/ml) and HDL (d 1.063 - 1.21 g/ml) by sequential ultracentrifugation at 15°C (Havel et al. 1955). Lipoproteins were dialyzed at 4°C against a buffer of 0.15 M NaCl, 3 mM EDTA (pH 7.4) for 24 hr prior to use. To
isolate apo E-free HDL, density fractionated HDL was subjected to heparin-sepharose affinity chromatography (Weisgraber and Mahley 1980). LPDS was prepared from the d > 1.23 g/ml fraction according to the method of Mills et al. (1984).

**Acetylation and Radiolabeling of LDL:** LDL was acetylated using acetic anhydride (Basu et al. 1976) and dialyzed against NaCl-EDTA buffer for 24 hr at 4°C. Electrophoresis of the modified LDL on agarose gels at pH 8.6 demonstrated enhanced mobility toward the cathode as compared to native LDL. $^{125}$I-labeling of LDL and acetyl-LDL were carried out according to the method of McFarlene (1958) as modified by Goldstein et al. (1983). Radiolabeled lipoproteins (350-500 cpm/ng) were stored at 4°C in 3% human albumin to minimize autoradiation denaturation and preparations were used within three weeks.

**Analysis of LDL and Acetyl-LDL Degradation Rates:** Rates of lipoprotein degradation by HL-60 cells and D$_3$- or TPA-induced HL-60 macrophages were determined by the method of Chait et al. (1982). In brief, 10 $\mu$g/ml $^{125}$I-LDL or $^{125}$I-acetyl-LDL were incubated with 0.4 - 0.5 x 10$^6$ cells/ml in the presence of 50 mM CaCl$_2$ and 15% LPDS at 37°C with (non-specific degradation) or without (total degradation) a 20-fold excess of unlabeled native or acetyl-LDL. Reactions were terminated after 4 hr by addition of 0.5 ml of 50% (w/v) trichloroacetic acid (TCA) and microfuged for one min. Following centrifugation, 0.25 ml of 10% (w/v) silver nitrate was added to the supernatant to precipitate free $^{125}$I (Henricksen et al. 1981).
TCA soluble radioactivity was determined using a gamma counter. Receptor-mediated specific degradation was calculated as the difference between non-specific and total degradation.

**Competition Assays:** Competition studies of $^{125}$I-LDL degradation (10 \( \mu \text{g/ml} \)) were performed in the presence of 0, 50, 100, and 200 \( \mu \text{g/ml} \) protein of VLDL, LDL, acetyl-LDL, total HDL and apo E-free HDL.

**Measurement of Sterol Synthesis Rates:** For analysis of sterol synthesis rates, HL-60 and D$_3$-induced HL-60 macrophages were cultured for 48 hr in 15\% LPDS. During the last 24 hr of incubation, either 100 \( \mu \text{g/ml} \) LDL or acetyl-LDL was added to the culture media. Cells were collected, washed three times with PBS and resuspended in PBS. 1.0 x 10$^6$ cells were incubated with 2.5 mM (5 dpm/pmole) [2-$^{14}$C]-acetate for 4 hr at 37\(^\circ\)C (McNamara et al. 1985). One ml of 50\% KOH was added to terminate the reaction followed by addition of [$^3$H]-cholesterol (4,0000 dpm) as an internal recovery standard and the samples were saponified at 70\(^\circ\)C for 1 hr. Sterols were extracted with petroleum ether, passed through aluminum columns AG 7 (100-200 mesh) and eluted with acetone - diethylether (1:1) as previously described (McNamara et al. 1985). The incorporation rate of [$^{14}$C]-acetate into sterols was determined by scintillation counting and are expressed as nmoles acetate incorporated per mg cell protein per 4 hr incubation.

**Other Assays:** Calcium dependence of LDL and acetyl-LDL degradation was determined in the absence or presence of 2 mM calcium after washing the cells three
times with calcium-free or calcium-containing PBS respectively. To ensure cellular calcium depletion, degradation assays were performed using an incubation mixture containing 1 mM of the chelating agent EDTA. For pronase sensitivity analysis, cells were pretreated with 3 μg/ml pronase for 20 min and washed three times with PBS. Chloroquine sensitivity was determined by pretreating cells with 50 mM chloroquine for 1 hr, and measurement of 125I-lipoprotein degradation in the presence of 50 mM chloroquine. Competition studies with fucoidin (10μg/ml) were determined for acetyl-LDL degradation in D3-induced HL-60 macrophages (Adelman and Clair 1988a). For studies of the effect of mezerein, HL-60 cells were incubated with 7.0 x 10^-8 M mezerein for 48 hr followed by measurements of LDL degradation rates.

Non-specific esterase was determined using α-naphthylacetate as a substrate and Fast RR blue as the chromogen (Yam et al. 1971). Protein concentrations were determined using a modified Lowry procedure (Markwell et al. 1978) calibrated against bovine serum albumin.

Statistical Analysis: One way analysis of variance (ANOVA) was used to determine significant difference between treatments (Glantz 1981). Data are presented as mean ± standard deviation (S.D.) for the number of assays shown.
RESULTS

PRELIMINARY DATA

Effect of D₃ on Cell Growth: HL-60 cells incubated with 15% HS (control) continued to grow and multiply with a doubling time of about 24 hr (Fig. 1). Total number of cells (macrophages and suspended) treated with $5.0 \times 10^{-8}$ M D₃ exhibited a slower rate of growth. A significant decrease of growth rates was observed at the fourth day of induction with D₃ ($P < 0.001$). However, the number of macrophages, as confirmed by acid esterase and adherence to flask, increased with increased exposure to D₃, by the fourth day, more than 50% of cells were macrophages compared to the first day where only 12% were macrophages. The data indicated that longer exposure to D₃ is required to obtain more macrophages.

Effect of Differentiation on Cellular Protein: Protein values for HL-60 cells incubated with 15% HS (control) remained constant up to 4 days in culture (Fig. 2). Induction of differentiation by D₃ resulted in macrophages that possessed significantly higher protein content compared to controls. The increase in protein content of D₃-induced HL-60 macrophages became more pronounced the longer the exposure to the inducer. These results suggest that the presence of the inducer is required and must be maintained over a long period of time.
FIGURE 1: Effect of D₃ on Growth of HL-60 Cells: HL-60 cells were incubated with 15% HS with (macrophages (MQ) and suspended (SUS)) or without (control) D₃ for 4 days. Cells were counted with a hemacytometer. Cell viability was determined with trypan blue exclusion. Values represent mean ± S.D. for 3-4 measurements.
FIGURE 2: Effect of Induction of Differentiation by D3 on Cellular Protein Concentrations. HL-60 cells at $10^5$ cells/ml were incubated with 15% HS for the indicated days in the presence or absence (control) of D3. Every 24 hr treated and untreated cells were counted using hemacytometer and protein concentrations were determined. The values are mean ± S.D. for 3-4 measurements. Using one way ANOVA, the values are significantly different at $P<0.001$ (*).
Receptor-mediated LDL and acetyl-LDL degradation rates in D₃-induced HL-60 macrophages exhibited a triphasic response to cell passage (Fig. 3). LDL degradation rates increased sharply with increasing passage of cells from 27 then reached a plateau between 54 and 120, and decreased slowly after that (Fig. 3A). The majority of this increase was attributed to increase in receptor-mediated degradation. Receptor-mediated acetyl-LDL degradation exhibited the same response to cell passage as LDL degradation (Fig. 3B). However, higher passage of 40 were required to achieve significant increase in degradation rates of acetyl-LDL relative to LDL degradation rates. Maximum induction of acetyl-LDL degradation was obtained at a passage number 59, and remained constant up to passage number 120. The loss of acetyl-LDL receptor activity in response to passage number was more rapid compared to LDL receptor activity. Therefore, to obtain consistency in results only cells between the passages 65 and 110 were used.

**Macrophage Differentiation:** Induction of differentiation of HL-60 cells to macrophages by either TPA or D₃ was confirmed by positive acid esterase activity. TPA-treated HL-60 cells had prominent pseudopodia and exhibited intense adherence to plastic dishes as well as to each other. In contrast, D₃-induced HL-60 macrophages adhered to plastic dishes without adhering to each other. Both TPA- and D₃-induced HL-60 macrophages lost their proliferation capacity.
FIGURE 3: Effect of Cell Passage on Degradation Rates of LDL and Acetyl-LDL.
Top panel: LDL degradation rates by HL-60 cells and D3-induced HL-60 macrophages. Bottom panel B: Acetyl-LDL degradation rates by D3-induced HL-60 macrophages. HL-60 cells at different passages were incubated with 15% LPDS containing 5.0 x 10^{-8} M D_3 for 48 hr prior to determination of degradation rates. Receptor-mediated degradations were calculated as the difference between total degradations and non-receptor-mediated degradations. The values represent mean ± S.D. for 3-10 measurements.
**LDL and Acetyl-LDL Degradation Rates:** HL-60 cells incubated in 15% LPDS for 48 hr exhibited specific, saturable receptor-mediated degradation of LDL (Fig. 4A). Kinetic analysis of the saturation curve indicated that the apparent saturation point was approximately 15 μg/ml of LDL. High-affinity receptor-mediated LDL degradation accounted for over 85% of the total LDL degradation rate of HL-60 cells. Regulation of LDL degradation rates was demonstrated by incubating cells with 100 μg/ml LDL for 24 hr which resulted in a significant 86% reduction in the rate of LDL degradation. D₃-induced HL-60 macrophages also exhibited saturable, specific LDL receptor-mediated degradation (Fig. 4B). The maximum degradation rate was observed at 27 μg/ml LDL. At a concentration of 10 μg/ml, 75% of the degradation was via a receptor-mediated process.

Induction of macrophage differentiation with D₃ resulted in rates of LDL degradation which were virtually identical to rates of undifferentiated HL-60 cells. In contrast, macrophage induction of HL-60 cells with TPA resulted in an almost complete loss of receptor-mediated LDL degradation (Fig. 5). These data demonstrate that while HL-60 cells could be induced to differentiate into macrophages by both TPA and D₃, the inducers produce distinctly different effects on expression of the LDL receptor and receptor-mediated LDL degradation rates.
FIGURE 4: Saturation Kinetics of LDL Degradation by HL-60 Cells and D3-Induced HL-60 Macrophages. Panel A: LDL degradation rates in HL-60 cells. Panel B: LDL degradation rates in D3-induced HL-60 macrophages. HL-60 cells and D3-induced HL-60 macrophages were incubated with 15% LPDS for 48 hr prior to determination of LDL degradation rates. Total degradation (○) was measured at the specified concentrations of 125I-LDL. Non-receptor-mediated degradation (▲) was determined in the presence of a 20-fold excess of unlabeled LDL. Receptor-mediated LDL degradation (●) was calculated as the difference between total degradation and non-receptor-mediated degradations. The values represent mean ± S.D. for 6 measurements.
FIGURE 5: LDL Degradation Rates of HL-60 Cells and D₃-Induced and TPA-Induced HL-60 Macrophages. Cells were incubated with 15% LPDS in the presence or absence of 5.0 x 10⁻⁸ M D₃ or 1.5 x 10⁻⁶ M TPA for 48 hr and LDL degradation rates were determined at a concentration of 10µg/ml ¹²⁵I-LDL with (non-receptor-mediated) or without (total) a 20-fold excess of unlabeled LDL. Values represent mean ± S.D. for 6 measurements.
Analysis of $^{125}\text{I}$-acetyl-LDL degradation by HL-60 cells and TPA- and D$_3$-induced HL-60 macrophages demonstrated that both HL-60 cells and TPA-induced macrophages failed to degrade acetyl-LDL (Fig. 6). In striking contrast, maturation of HL-60 cells with D$_3$ induced acetyl-LDL receptor expression and, after 48 hr treatment with D$_3$, 88% of the total rate of acetyl-LDL degradation was receptor mediated (Fig. 6).

To investigate possible mechanisms involved in the loss of apo B/E receptor-mediated LDL degradation following incubation with TPA, two experiments were conducted using either HL-60 cells or D$_3$-induced HL-60 macrophages, both of which degrade LDL via receptor-mediated endocytosis. HL-60 cells (1.0 x 10$^6$ cells/ml), either in the presence or absence of D$_3$, were incubated with LPDS for 46 hr followed by a 2 hr incubation with 1.5 x 10$^{-6}$ M TPA and determination of $^{125}\text{I}$-LDL degradation rates (Fig. 7). Analysis of LDL degradation rates indicated that TPA exerted the same effect on both HL-60 cells and D$_3$-induced macrophages with an 80% reduction of receptor-mediated LDL degradation with TPA treatment; non-specific LDL degradation was not affected by TPA treatment.

Absence of acetyl-LDL degradation by TPA-induced HL-60 macrophages could be due to either a failure of TPA to induce expression of the acetyl-LDL receptor or an inhibitory effect of TPA on receptor activity, as seen for the LDL receptor. To determine whether TPA blocked the activity of the acetyl-LDL
FIGURE 6: Acetyl-LDL Degradation Rates of HL-60 Cells and D3-Induced and TPA-Induced HL-60 Macrophages. Cells were incubated with 15% LPDS in the presence or absence of 5.0 x 10^{-3} M or 1.5 x 10^{-6} M TPA for 48 hr followed by determination of acetyl-LDL degradation rates at a concentration of 10\mu g/ml {^{125}}I-acetyl-LDL with (non-receptor-mediated) or without (total) a 20-fold excess of unlabeled acetyl-LDL. Values represent mean ± S.D. for 6 measurements.
FIGURE 7: Receptor-Mediated LDL and Acetyl-LDL Degradation Rates of HL-60 Cells and D3-Induced HL-60 Macrophages Following Treatment with TPA for 2 Hrs. HL-60 cells and D3-induced HL-60 macrophages were incubated with 15% LPDS for 48 hr (control) or 46 hr plus a 2 hr treatment with $1.5 \times 10^{-6}$ M TPA. Receptor-mediated LDL degradation in control and TPA-treated HL-60 cells (left) and D3-induced HL-60 macrophages (center) at 10$\mu$g/ml $^{125}$I-LDL. Receptor-mediated acetyl-LDL degradation in control and TPA-treated D3-induced HL-60 macrophages (right) at 10$\mu$g/ml $^{125}$I-acetyl-LDL. Values represent mean ± S.D. for 6 measurements.
receptor, D3-induced HL-60 macrophages, which express acetyl-LDL receptors, were treated with 1.5 x 10^{-6} M TPA for zero or 2 hr prior to measurement of acetyl-LDL degradation (Fig. 7). Incubation with TPA had no effect on non-receptor-mediated acetyl-LDL degradation; however, receptor-mediated acetyl-LDL degradation was reduced by 85% with TPA treatment, whether added at zero time or after a 2 hr pretreatment. The data indicate that TPA directly blocks receptor-mediated degradation of both LDL and acetyl-LDL by D3-induced HL-60 macrophages.

To determine whether the action of TPA on LDL receptor activity was concentration dependent, HL-60 cells were treated with varying concentrations of TPA for 2 or 48 hr followed by determination of LDL degradation rates (Fig. 8). Cells incubated with TPA for 2 hr exhibited a concentration dependent reduction in the rate of LDL degradation. At TPA concentrations of 1.5 x 10^{-9} M and 1.5 x 10^{-7} M, LDL receptor-mediated degradation rates were 64% and 29% of that found for untreated HL-60 cells. Incubation of HL-60 cells with TPA for 48 hr resulted in a significant 85% reduction (P<0.001) of receptor-mediated LDL degradation at all concentrations tested.
FIGURE 8: Dose Response to TPA of LDL Receptor-Mediated Degradation by HL-60 Cells. Receptor-mediated LDL degradation rates of HL-60 cells were determined for cells incubated with 15% LPDS for 2 hr (solid bars) or 48 hr (hatched) in the absence (control) or with varying concentrations of TPA. LDL degradation rates were determined at a concentration of 10 μg/ml LDL protein. Values represent mean ± S.D. for 3-6 measurements; all values were significantly different from control (one-way ANOVA, P<0.001).
To examine whether the reduction of LDL receptor mediated degradation was unique to TPA, the effect of mezerein, a protein kinase C (PKC) activator and inducer of macrophage differentiation of HL-60 cells, was used to determine effects on LDL degradation. Cells incubated with $7 \times 10^{-8}$ M mezerein for 48 hr exhibited a 90% reduction in LDL receptor-mediated degradation relative to D3-induced HL-60 macrophages (Appendix A1). These results suggest involvement of PKC in the TPA and mezerein mediated inhibitory effects on LDL receptor-mediated degradation by HL-60 cells.

Since only D3-induced HL-60 macrophages exhibited both native and modified LDL receptors, subsequent studies to characterize the regulatory responses of these receptors were carried out in D3-treated HL-60 cells.

**Time Courses of LDL and Acetyl-LDL Degradations:** Analysis of the time courses of expression of LDL and acetyl-LDL degradation in D3-induced HL-60 macrophages are presented in Table I. Macrophages treated with D3 for 2 days degraded LDL to the same degree as untreated HL-60 cells. Prolonged exposure of macrophages to D3 resulted in a 46% reduction in LDL degradation rates compared to HL-60 cells. However, D3 treatment resulted in a 45-fold increase in the rate of acetyl-LDL degradation compared to undifferentiated HL-60 cells. The scavenger receptor, which was fully expressed after 2 days of treatment with D3, remained constant throughout the six days of maturation.
### TABLE I: TIME COURSE OF LIPOPROTEIN DEGRADATION RATES IN D₃-INDUCED HL-60 MACROPHAGES

<table>
<thead>
<tr>
<th>Lipoprotein Degradation</th>
<th>Incubation Period (Days)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ng/mg-4hr</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td></td>
</tr>
<tr>
<td>-Total</td>
<td>261 ± 32</td>
</tr>
<tr>
<td>-Nonspec</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>-R-Mediated</td>
<td>235 ± 29</td>
</tr>
<tr>
<td>Ac-LDL</td>
<td></td>
</tr>
<tr>
<td>-Total</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>-Nonspec</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>-R-Mediated</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Cells were incubated in 15% LPDS containing D₃ for the indicated treatment period. ¹²⁵I-labeled LDL or acetyl-LDL degradation rates were measured at a concentration of 10 μg/ml in the absence (total) and presence (nonspecific) of a 20-fold excess of unlabeled autologous lipoprotein. Values are presented as the mean ± S.D. for 3-6 determinations. Nonspec (Nonspecific), R (Receptor), Ac-LDL (Acetyl-LDL).
**Receptor Specificity:** To investigate the specificity of receptor-mediated LDL degradation by D₃-induced HL-60 macrophages, competition studies were carried out using various lipoproteins as competitors (Fig. 9). Both LDL and VLDL, which contain apoB-100, effectively competed with ¹²⁵I-LDL for receptor mediated degradation. At a concentration of 200 μg/ml protein, ¹²⁵I-LDL degradation was reduced by 91% with LDL and 85% with VLDL. Total HDL was also recognized by the receptor and, at a concentration of 200 μg HDL protein/ml, the rate of LDL degradation was decreased by 33%. Neither acetyl-LDL nor apoE-free HDL competed with ¹²⁵I-LDL. These data demonstrate the presence of specific apo B/E receptors on D₃-induced HL-60 macrophages which recognize and are involved in the degradation of both apoB- and apoE-containing lipoproteins.

The specificity of the scavenger receptor of D₃-induced HL-60 macrophages was also determined by competition studies (Fig. 10). ¹²⁵I-acetyl-LDL degradation was slightly reduced by excess LDL (29%), apoE-free HDL (15%), total HDL (13%), and VLDL (20%). In contrast, excess acetyl-LDL effectively competed with ¹²⁵I-acetyl-LDL for scavenger receptor sites reducing the degradation rate by 75%. The data demonstrate that D₃-induced HL-60 macrophages possess a specific scavenger receptor which recognizes chemically modified acetyl-LDL.
FIGURE 9: Lipoprotein Competition Studies for $^{125}$I-LDL Degradation by D$_3$-Induced HL-60 Macrophages. D$_3$-induced HL-60 macrophages were incubated with 15% LPDS for 48 hr followed by determination of $^{125}$I-LDL degradation at 10μg/ml (100%) and in the presence of the indicated concentrations of LDL (○), VLDL (●), total HDL (□), apo E-free HDL (□), and acetyl-LDL (△). Values represent mean ± S.D. of 6 measurements.
FIGURE 10: Lipoprotein Competition Studies for $^{125}$I-acetyl-LDL Degradation by D$_3$-Induced HL-60 macrophages. D$_3$-induced HL-60 macrophages were incubated with 15% LPDS for 48 hr followed by determination of $^{125}$I-acetyl-LDL degradation at 10μg/ml (100%) with the indicated concentrations of acetyl-LDL (◇), LDL (○), VLDL (●), total HDL (□), and apoE-free HDL (■). Values represent mean ± S.D. for 6 measurements.
Regulation Studies: To further characterize the regulation of native and scavenger LDL receptors of D3-induced HL-60 macrophages, LDL and acetyl-LDL degradation rates were determined in the presence of calcium (control), absence of calcium with added EDTA, and in the presence of calcium plus pronase, chloroquine (lysosomal enzyme inhibitor) or fucoidin (negatively charged compound which competes with acetyl-LDL for receptor sites) (Fig. 11). Receptor mediated LDL degradation by D3-induced HL-60 macrophages was reduced 95% with EDTA in the absence of calcium as compared to control macrophages. Chloroquine treatment, which inhibits lysosomal degradation, resulted in a 70% reduction in LDL receptor-mediated degradation. Incubating the macrophages with the proteolytic enzyme pronase resulted in an 80% reduction in LDL degradation as compared to control cells. In contrast, receptor-mediated acetyl-LDL degradation by D3-induced HL-60 macrophages exhibited no dependency for calcium (Fig. 11). The inhibitory effect of chloroquine was observed for both receptor- and non-receptor-mediated acetyl-LDL degradations. The receptor was partially sensitive to pronase and fucoidin with acetyl-LDL degradation rates being significantly reduced 56% in the presence of pronase ($P < 0.005$) and 77% in the presence of fucoidin ($P < 0.02$) as compared to control values.
FIGURE 11: Effect of Calcium (Control), EDTA, Chloroquine, Pronase and Fucoidin on Receptor-Mediated LDL and Acetyl-LDL Degradation by D3-Induced HL-60 Macrophages. HL-60 cells were incubated with D3 (5.0 x 10^{-8} M) and 15% LPDS for 48 hr prior to determination of receptor-mediated degradations of LDL and acetyl-LDL. Degradation rates were determined at 10\mu g/ml of ^{125}I-LDL or ^{125}I-acetyl-LDL in the presence of calcium (control) or calcium-free media containing EDTA (1 mM) and in the presence of either chloroquine (50 mM), pronase (3\mu g/ml) and fucoidin (10\mu g/ml), [refer to Methodology section]. Values represent mean ± S.D. of 3-6 determinations. Using one way ANOVA, the values are significantly lower than control at P<0.005(*) or P<0.02(**).
Exogenous cholesterol delivered to macrophages by preincubation with either 100 µg/ml LDL or acetyl-LDL for 24 hr suppressed \(^{125}\text{I}-\text{LDL}\) receptor-mediated degradation to the same extent, indicative of the presence of both native and scavenger LDL receptors on the same cell (Fig. 12A). Incubation of macrophages with LPDS significantly increased receptor-mediated LDL degradation (4-fold, \(P < 0.001\)) compared to macrophages incubated with LDL. Measurements of \(^{125}\text{I}-\text{LDL}\) degradation rates by \(\Delta_3\)-induced HL-60 macrophages incubated with 100 µg/ml LDL plus mevinolin, a competitive inhibitor of HMG-CoA reductase, demonstrated a significant (\(P < 0.001\)) 2-fold increase in LDL degradation rates as compared to control macrophages incubated with LDL alone. A maximum induction of 6.5-fold of apo B/E receptor-mediated LDL degradation was obtained for cells incubated in LPDS and mevinolin. In contrast to the well regulated LDL receptor, \(\Delta_3\)-induced HL-60 macrophages incubated with LDL or acetyl-LDL did not decrease scavenger receptor-mediated degradation of acetyl-LDL compared to macrophages incubated with LPDS, consistent with the lack of regulation of the scavenger receptor resulting from delivery of exogenous cholesterol to macrophages (Fig. 12B).
FIGURE 12: Regulation of Receptor-Mediated LDL and Acetyl-LDL Degradation in D3-Induced HL-60 Macrophages by LPDS, LDL (Control), Acetyl-LDL, and Mevinolinin the Presence of Either LPDS or LDL. HL-60 cells were incubated with D3 and 15% LPDS for 24 hr. Incubation continued for another 24 hr with D3 and either LPDS, 100 µg/ml LDL, 100 µg/ml acetyl-LDL, or 1µM mevinolin in either LPDS or LDL. Degradation rates of LDL (A) and acetyl-LDL (B) were determined (refer to Methodology section for details). Values represent mean ± S.D. for 3-6 determinations. Significantly different from control (+ LDL) at P < 0.001 (*).
Sterol Synthesis: Differentiation of HL-60 cells with D₃ resulted in a 2.6-fold increase in the incorporation rate of [2-¹⁴C]-acetate into sterols as compared to undifferentiated cells (Table II). Addition of 100 μg/ml LDL to the incubation media resulted in reductions of 54% and 88% in sterol synthesis rates of HL-60 cells and D₃-induced macrophages respectively. Incubation with 100 μg/ml acetyl-LDL had no effect on the rate of sterol synthesis by HL-60 cells, which lack an acetyl-LDL receptor, and caused a 68% reduction in sterol synthesis rates of D₃-induced HL-60 macrophages (Table II). The data demonstrate that the delivery of exogenous cholesterol to macrophages via the degradation of either LDL or acetyl-LDL is capable of suppressing the rate of endogenous sterol synthesis.
Table II: REGULATION OF STEROL SYNTHESIS IN HL-60 CELLS AND D₃-
INDUCED MACROPHAGES

<table>
<thead>
<tr>
<th>Addition</th>
<th>HL-60 Cells (n=3)</th>
<th>D₃ Macrophages (n=6)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>3.6 ± 0.4ᵃ</td>
<td>9.3 ± 1.1ᵃ</td>
</tr>
<tr>
<td>LDL</td>
<td>1.7 ± 0.2ᵇ</td>
<td>1.1 ± 0.4ᵇ</td>
</tr>
<tr>
<td>Acetyl-LDL</td>
<td>3.7 ± 0.4ᵃ</td>
<td>3.0 ± 0.7ᶜ</td>
</tr>
</tbody>
</table>

Cells were incubated in LPDS for 24 hr followed by addition of either 100 μg/ml LDL or acetyl-LDL for 24 hr and measurement of the incorporation of [¹⁴C]acetate into sterols. Data presented as mean ± S.D. for the number of assays shown in parentheses. Values in the same column with different superscripts are significantly different (P < 0.001).
DISCUSSION

HL-60 cells have been shown to be a valuable model for studying chemically induced macrophage or granulocyte differentiation in vitro (Collins et al. 1978, Mangelsdorf et al. 1984, Rovera et al. 1979a, Breitman et al. 1980, Elias et al. 1980). This cell line, with its bipotential induction characteristics, can be induced to differentiate into granulocytes by dimethyl sulfoxide or retinoic acid, and into macrophages in response to TPA and the active forms of vitamin D₃ specifically 1,25-dihydroxyvitamin D₃. In this report, we have shown that induction of macrophage differentiation of HL-60 cells by TPA and D₃ exerted distinctly different effects on the activities of receptors for native and modified LDL.

**Lipoprotein Receptors of HL-60 Cells:** Undifferentiated HL-60 cells exhibited specific, saturable receptor-mediated LDL degradation. Analysis of LDL degradation rates in cells incubated in media containing 100 μg/ml LDL demonstrated low levels of receptor-mediated LDL degradation which could be induced 4-fold by incubating the cells in LPDS. In contrast to the findings of an active and regulated LDL receptor, HL-60 cells failed to degrade acetyl-LDL indicating the absence of the scavenger receptor. These results confirm the reports of Wathne et al. (1989) and Via et al. (1985a) which indicated that HL-60 cells possess LDL receptors but lack acetyl-LDL receptors. In a similar manner, the human monocytic leukemia cell line THP-1 and the human histiocytic lymphoma cell line U-937 have been shown to have native LDL but not modified LDL receptors (Hara et al. 1987, Rouis et al. 1984a).
In general, modified LDL receptors are not expressed by non-macrophage cells (Brown and Goldstein 1983b).

**Effect of TPA on HL-60 Cells:** Treating HL-60 cells with TPA induced differentiation to a macrophage-like cell; however, the macrophages exhibited only 5% of the receptor mediated LDL degradation rate of HL-60 cells and no detectable receptor-mediated acetyl-LDL degradation. Similar findings regarding expression of LDL and acetyl-LDL receptors by TPA-induced HL-60 macrophages have been reported by Via et al. (1985a). Comparable inhibitory effects by TPA on LDL receptor activity have also been reported for other cell lines including MRC5 human fibroblasts (Maziere et al. 1986), U-937 cells (Rouis et al. 1984a), THP-1 cells (Hara et al. 1987) and HEL cells (Papayannopoulou et al. 1983). Studies by Rouis et al. (1984a) and Maziere et al. (1986) suggest that the TPA-mediated reduction in LDL degradation results from a decrease in receptor number and not a change in receptor affinity for LDL. While there appears to be a consistency of reports that TPA treatment reduces receptor-mediated LDL degradation in macrophage-like cells, Auwerx et al. (1989b) reported an induction of LDL receptor mRNA during early macrophage differentiation of THP-1 cells by TPA. These authors provided evidence that the TPA effect was mediated by PKC and that PKC exerts its effect by inactivating directly or indirectly a labile negative protein, rendering it unable to bind to a positive transcription factor and to form an inactive complex. This is consistent with the finding that other PKC activators induce LDL receptor mRNA while PKC
inhibitors block the induction (Auwerx et al. 1989b). It would appear, however, that activation of PKC has two effects, induction of LDL receptor mRNA and a decrease in receptor mediated LDL degradation. Treatment of MRC5 fibroblasts (Maziere et al. 1986) and D3-induced HL-60 macrophages with mezerein, a PKC activator (Miyake et al. 1984), results in a similar loss of receptor-mediated LDL degradation.

The inhibitory effect of TPA on receptor-mediated LDL uptake is not unique to the LDL receptor in that the binding of other ligands including insulin (Grunberger and Gorder 1982, Thomopoulos et al. 1982), insulin-like peptides (Rouis et al. 1984b), transferrin (Pellicci et al. 1984), and epidermal growth factor (King and Cutrecasas 1982) are blocked by TPA. It has been hypothesized that TPA treatment alters the activities of a variety of cell surface receptors by receptor masking due to either induction of cryptic receptors (Jaken et al. 1983), covalent modification of receptors (May et al. 1984), alterations in the ionic milieu secondary to changes in pH or calcium ion activity (Fallon and Schwartz 1986), or receptor redistribution and increased net internalization (Fallen and Schwartz 1986). A TPA-mediated reduction in LDL uptake would in part explain the observed increases in rates of sterol synthesis and HMG-CoA reductase activity of TPA induced HL-60 macrophages (Yachnin et al. 1984) and the increased levels of LDL receptor and HMG-CoA reductase mRNA of TPA treated THP-1 cells (Auwerx et al. 1989a, 1989b) if the feed-back suppression of these genes by LDL cholesterol is released due to decreased uptake of LDL.
The effect of TPA treatment on expression of the acetyl-LDL receptor appears to be highly variable in that some studies report decreased scavenger receptor expression while others report an induction of the receptor. The finding reported here that TPA treatment does not induce expression of the acetyl-LDL receptor in HL-60 cells has been reported by others (Via et al. 1985a). Consistent with the findings that TPA decreases receptor-mediated degradation of acetyl-LDL by D3-induced HL-60 macrophages, Leake et al. (1989) and Rouis et al. (1984a) reported that addition of TPA to mouse peritoneal macrophages decreased acetyl-LDL uptake and degradation. In contrast, TPA treatment of HEL cells (Papayannopoulos et al. 1983), THP-1 cells (Hara et al. 1987, Via et al. 1989), and rabbit fibroblasts and smooth muscle cells (Pitas 1990) results in increased expression of the scavenger receptor. Why there should be such pronounced differences between different cell lines in terms of expression of the scavenger receptor following TPA treatment is unclear and may relate to different responses to PKC activation and receptor distribution. At present, it is not clear whether TPA-induced HL-60 macrophages express an acetyl-LDL receptor, as found for D3-induced HL-60 macrophages, which TPA subsequently blocked, or whether TPA mediated macrophage differentiation failed to induce expression of the scavenger receptor.

The finding that mezerein, another PKC activator, mimics the effect of TPA suggests that PKC may be involved in the observed inhibitory effect of TPA on LDL degradation rates of HL-60 cells and macrophages. Macrophage differentiation of
HL-60 cells treatment with TPA and D₃ involves, in part, the activation of PKC which causes the phosphorylation of a number of protein substrates resulting in long term cellular responses (e.g. differentiation). It has been shown that PKC activates the transcription of LDL receptor mRNA levels during the early differentiation stages of THP-1 cells (Auwerx et al. 1989b). However, it is conceivable that the effect of these two inducers on LDL and acetyl-LDL receptors is via different mechanisms, although both inducers have similar effects on PKC and membrane lipids (Auwerx et al. 1989b, Obeid et al. 1990, Wali et al. 1990). TPA, a homolog of diacylglycerol (DAG), activates PKC directly and influences membrane lipids causing receptor masking, whereas D₃ induces the transcription of several genes including PKC independent of phospholipid hydrolysis and DAG generation (Obeid et al. 1990). These differential responses to the two inducers of macrophage differentiation may in part explain the findings of this study.

**LDL Receptor of D₃-Induced HL-60 Macrophages:** In this report we have demonstrated that D₃-induced HL-60 macrophages possess both native and modified LDL receptors. Induction of maturation with D₃ for 48 hr resulted in LDL degradation rates identical to untreated HL-60 cells. Increasing the time of exposure to D₃ resulted in a 46% decrease in LDL degradation which remained constant after 4 days in culture. Similar results have been reported for human monocyte-derived macrophages where the LDL receptor activity either remains constant or increases during the first week in culture, then decreases during the second week (Fogelman
et al. 1981, Knight and Soutar 1986). Specificity of the apo B/E receptor-mediated LDL degradation by D₃-induced macrophages, as measured by competition studies, demonstrated that LDL manifested the greatest competition among lipoproteins tested. Selective chemical modifications (acetylation) of lysyl residues on LDL apo-B abolished the ability of LDL to be recognized by the apo B/E receptor as reported by other investigations (Mahley and Innerarity 1983, Brown et al. 1981).

The LDL receptor of D₃-induced HL-60 macrophages was further characterized to demonstrate that degradation was calcium dependent and that the receptor was protein in nature, as demonstrated by the inactivation of receptor-mediated degradation with pronase treatment. Similar results have been reported by Goldstein and Brown (1974) for the fibroblast apo B/E receptor. Following LDL uptake, degradation occurs by lysosomal enzymes as evident from the inhibition of degradation by the lysomotropic compound chloroquine which decreased both specific and non-specific degradations. A 4-fold induction of the LDL receptor was obtained by preincubating D₃-induced HL-60 macrophages with LPDS which is similar to the induction reported for human monocyte-derived macrophages (Fogelman et al. 1981, Patel and Knight 1985) and fibroblasts (Knight and Soutar 1986, Goldstein and Brown 1974). A maximum induction of 6.5-fold for receptor-mediated LDL degradation rates was observed in the presence of LPDS and 1μM mevinolin. Mevinolin, which inhibits endogenous cholesterol synthesis, induces LDL receptor synthesis and consequently LDL degradation (Wathne et al. 1989). Wathne
et al. (1989) reported a similar increase of 2-fold in LDL degradation rates for HL-60 cells treated with mevinolin. No significant difference was obtained in the rate of receptor-mediated LDL degradation in macrophages incubated with either LDL or acetyl-LDL. Availability of cholesterol delivered to macrophages through LDL or acetyl-LDL degradation caused the same degree of down regulation of the LDL receptor. Taken together, these data demonstrate that D$_3$-induced HL-60 macrophages exhibit the classical apo B/E receptor and that expression of the receptor is under feed back regulation.

**Scavenger Receptor of D$_3$-Induced HL-60 Macrophages:** Characterization of the scavenger receptor of D$_3$-induced HL-60 macrophages indicated that the receptor was Ca$^{+2}$ independent, chloroquine sensitive, and partially sensitive to pronase and fucoidin. To establish that acetyl-LDL was entering cells via a specific acetyl-LDL receptor pathway, competition studies demonstrated that the scavenger receptor exclusively recognized and degraded acetyl-LDL. Moreover, the scavenger receptor pathway was neither down-regulated by pretreating the cells with acetyl-LDL for 24 hr nor induced by pre-incubating the macrophages in LPDS. In addition, the expression of the acetyl-LDL receptor activity was fully induced within 48 hr of treatment with D$_3$ and it remained relatively constant up to 6 days in culture. Similar results are reported for TPA-treated THP-1 macrophages (Hara et al. 1987) as well as for human monocyte-derived macrophages (Via et al 1985a).
D₃-induced HL-60 macrophages incubated in LPDS exhibited a 2.6-fold increase in the incorporation rate of [¹⁴C]-acetate into sterols compared to undifferentiated cells. The increased sterol synthesis rates may be due to the need of macrophages to expand their cellular membrane during the course of differentiation. D₃ treatment of HL-60 cells was accompanied by an increase in cell volume and consequently by an increase of cellular membrane as reported by Mangelsdorf et al. (1984). In the presence of either acetyl-LDL or LDL, D₃-induced HL-60 macrophages exhibited a reduction in sterol synthesis indicative of the presence of both native and modified LDL receptors. In addition, a reduction of 88% and 68% in sterol synthesis in the presence of LDL and acetyl-LDL respectively demonstrated that the amount of cholesterol delivered to the macrophages from LDL and acetyl-LDL regulated cholesterol synthesis.

Vitamin D₃ induces in HL-60 cells a phenotype which resembles that of monocytes-macrophages (Murao et al. 1983). D₃-induced HL-60 macrophages are the only chemically induced human macrophage system reported to date that possesses functionally active native and modified LDL receptors. Compared to D₃-induced HL-60 macrophages and human monocyte-derived macrophages, human leukemic cell lines (THP-1 and HEL) (Hara et al. 1987, Papayannopoulou et al. 1983, Auwerx et al. 1989b) and murine cell lines (Balb/c and P388D1) treated with TPA (Via et al. 1985a), and mouse peritoneal macrophages (Goldstein et al. 1979, Brown et al. 1979b, Brown et al. 1980) express acetyl-LDL receptor with little or no
LDL receptor activities.

In conclusion, our findings demonstrate that induction of HL-60 cell differentiation with D$_3$ results in macrophages exhibiting both a regulated apo B/E receptor and a scavenger receptor whereas induction with phorbol, and non-phorbol, PKC activators results in an immediate dose dependent inhibition of LDL degradation and a long-term inhibitory effect on apo B/E receptor mediated degradation of both LDL and acetyl-LDL. D$_3$-treated HL-60 cells represent a potentially valuable model of human macrophages as a homogenous and readily available cultured cell system which can be utilized to investigate regulatory mechanisms involved in macrophage-lipoprotein metabolism and the factors involved in macrophage cholesterol flux and foam cell development.
CHAPTER 4

D₃-INDUCED HL-60 MACROPHAGES: REGULATORY RESPONSES TO CHOLESTEROL AND LDL METABOLISM
SUMMARY

Differentiation of human promyelocytic leukemic HL-60 cells with D₃ results in macrophages expressing surface receptors for both native and modified LDL. To further characterize macrophage-lipoprotein interactions, the three pathways controlling cellular sterol homeostasis namely receptor-mediated lipoproteins binding; HMG-CoA reductase and ACAT were determined. D₃-induced HL-60 macrophages exhibited specific and saturable receptor-mediated processing for both native and modified LDL. Analysis of binding kinetics revealed that the macrophages bind LDL and acetyl-LDL with same affinities, yet, possess significantly different number of receptors (219 ng/mg and 313 ng/mg respectively).

Delivery of cholesterol to macrophages via receptor-mediated processing of LDL or acetyl-LDL results in significant suppression of conversion of HMG-CoA to mevalonate compared to macrophages incubated with LPDS. ACAT activity was regulated in concert with LDL receptor and HMG-CoA reductase. An increase of 1.6- to 2.5- fold in the incorporation rate of [¹⁴C]-oleate into cholesteryl ester was achieved by increased availability of cholesterol to macrophages via receptor-mediated lipoprotein pathway. Maximum increase of ACAT activity was obtained in macrophages incubated with 25-hydroxycholesterol in the presence of LDL or acetyl-LDL, suggesting that the presence of an oxysterol is a more powerful activator of the enzyme than substrate availability. The increase in ACAT activity in macrophages incubated with acetyl-LDL for 48 hr paralleled the increase in cellular
total cholesterol content and the increase of oil red O lipid stainable material, providing the macrophages with their foamy appearance. The data suggest that D3-induced HL-60 macrophages provide a suitable model of human macrophages with the characteristics required to study the interaction between macrophages and lipoproteins.
INTRODUCTION

Substantial evidence implicates modified lipoproteins, specifically LDL, in the initiation and propagation of atherosclerosis (Harberland and Fogelman 1987). Scavenger receptors which recognize modified LDL (acetyl-LDL) are expressed by all macrophages examined to date. They are present on Kupffer cells, mouse peritoneal macrophages and the cell line J774, monocyte-derived macrophages and \( \Delta_3 \)-induced HL-60 macrophages (Fogelman et al. 1981, Ellsworth et al. 1986, Jouni and McNamara 1991, Brown and Goldstein 1983b). Similar to the binding of LDL to the native LDL receptor, surface binding of acetyl-LDL to its receptor is followed by internalization via adsorptive endocytosis and delivery of the lipoproteins to lysosomes (Goldstein et al. 1983). Within the lysosome, lysosomal enzymes hydrolyze lipoprotein components (lipid and protein) releasing free cholesterol which acts on three regulatory responses controlling cholesterol homeostasis. One of these responses is the induction of ACAT activity, an intracellular enzyme that catalyzes the transfer of fatty acid from acyl-CoA to the \( \beta \)-hydroxyl group of cholesterol (Brown et al. 1979b). Newly formed esterified cholesterol enters the cytoplasmic cholesteryl ester pool in macrophage where it participates in a cycle of hydrolysis and re-esterification (Brown et al. 1980). A second regulatory response, due to an increase in the pool of cellular cholesterol, is product feedback transcriptional suppression of HMG-CoA reductase (Lusky et al. 1983), the rate limiting enzyme in the biosynthesis of cholesterol. In addition, free cholesterol induces reductase
degradation (Gil et al. 1985), thus accounting for a reduction in endogenous cholesterol synthesis. The third regulatory response is the down-regulation of the native LDL (apo B/E) receptor at the transcriptional level to decrease delivery of extracellular lipoprotein cholesterol to the macrophage (Russel et al. 1983). In contrast to the down regulation of the LDL receptor by free cholesterol, the cytoplasmic pool of cellular cholesterol does not suppress scavenger receptor expression; hence, uptake and hydrolysis of acetyl-LDL by macrophages does not result in the suppression of acetyl-LDL receptors. Therefore, when macrophages are incubated with acetyl-LDL, they continue to take up the modified LDL, releasing free cholesterol to the intracellular pool which stimulates ACAT activity, leading to cholesteryl ester accumulation and subsequent foam cell formation (Goldstein et al. 1979).

Studies already presented have shown that D₃-induced HL-60 macrophages express specific and saturable receptors for both native and modified LDL, with the former receptor being regulated and the latter being unregulated (Jouni and McNamara 1991). In an attempt to further characterize lipoprotein-D₃-induced HL-60 macrophage interaction, cellular regulatory responses, specifically HMG-CoA reductase and ACAT activities, and the ability of macrophages to accumulate cholesteryl esters and to form foam cells, were investigated. Results indicated that when D₃-induced HL-60 macrophages were incubated with acetyl-LDL, HMG-CoA reductase activity was significantly suppressed, thus decreasing cholesterol synthesis.
In addition, ACAT activity was induced leading to cholesteryl ester accumulation that imparted the cells with a lipid rich foamy appearance.
METHODOLOGY

Materials: HL-60 cells were obtained from ATCC Rockville, MD; Na\textsuperscript{125}I was purchased from Amersham, Arlington Heights, IL; RPMI-1640, glutamine, penicillin/streptomycin, FCS were purchased from Flow Labs, McLean, VA; BSA, alpha-naphthyl acetate esterase kit with Fast RR Blue Salt, Mayer's Hematoxylin, Oil Red O, DL-3-hydroxy-3-methylglutaryl coenzyme A, mevalonolactone, glucose-6-phosphate dehydrogenase and NADP from Sigma, St. Louis, MO; [1-\textsuperscript{14}C]-oleate (57 mCi/mmol), [1,2-\textsuperscript{3}H]-cholesteryl ester (60 Ci/mmol), and DL- [5-\textsuperscript{3}H]-mevalonic acid (48.6 mCi/mmol), DL-hydroxy-[3-\textsuperscript{14}C]-methylglutaryl coenzyme A (51.6 mCi/mmol) were from New England Nuclear, Boston, Mass. 1,25-dihydroxyvitamin D\textsubscript{3} (stored in small aliquots in ethanol at \(-20^\circ\)C) was the gift of Dr. Milan R. Uskokovic, Hoffman-LaRoche, Inc, Nutley, NJ.

HL-60 Cell Culture: Human promyelocytic leukemic HL-60 cells were grown in RPMI-1640 supplemented with 15% heat inactivated FCS, 24 mM sodium bicarbonate, 2 mM L-glutamine, 100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin, in a humidified incubator of 5\% CO\textsubscript{2} at 37\(^\circ\)C. Cells were seeded at a density of 1.5 x 10\textsuperscript{6} cells/ml. Cell viability was determined by trypan blue exclusion.

Macrophage Differentiation: HL-60 cells (1.0 x 10\textsuperscript{6} cells/ml) maintained for 24 hr in fresh RPMI-1640 medium containing 15\% FCS, were centrifuged at 400 g for 10 min at 4\(^\circ\)C and resuspended at a density of 1.0 x 10\textsuperscript{6} cells/ml in RPMI-1640 medium supplemented with 15\% heat inactivated LPDS. To induce differentiation along the
macrophage pathway, cells were incubated with $5.0 \times 10^{-8}$ M $D_3$ (Mangelsdorf et al. 1984). After a 48 hr incubation at 37°C, adhered cells were collected and resuspended in PBS to a density of $1.5 - 2.0 \times 10^6$ cells/ml for use in the various experiments.

**Lipoprotein Isolation**: Plasma from normolipidemic volunteers was used to isolate LDL ($d \ 1.02 - 1.063$ g/ml) by sequential density ultracentrifugation at 15°C (Havel et al. 1955). Isolated lipoproteins were dialyzed at 4°C against a buffer of 0.15 M NaCl, 3 mM EDTA (pH 7.4) for 24 hr, filter sterilized and used within three weeks. LPDS was prepared from the $d > 1.23$ g/ml fraction according to the method of Mills et al. (1984).

**Acetylation and Radiolabeling of LDL**: LDL was acetylated using acetic anhydride (Basu et al. 1976) and dialyzed against the NaCl-EDTA buffer for 24 hr at 4°C. Electrophoresis of the modified LDL on agarose gels at pH 8.6 demonstrated enhanced mobility toward the cathode as compared to native LDL. $^{125}$I-labeling of LDL and acetyl-LDL were carried out according to the method of McFarlene (1958) as modified by Goldstein et al. (1983). Radiolabeled lipoproteins (350-500 cpm/ng) were stored at 4°C in 3% human albumin to minimize autoradiation denaturation and all preparations were used within three weeks.

**Binding Studies**: LDL and acetyl-LDL binding by $D_3$-induced HI-60 macrophages were determined following the method of Chait et al. (1982). Varying concentrations of $^{125}$I-LDL or acetyl-LDL were incubated with $0.5 - 0.7 \times 10^6$ cells/ml of $D_3$-induced
HL-60 macrophages in the presence of 15% LPDS at 4°C with (non-receptor-mediated binding) or without (total binding) a 40-fold excess of unlabeled lipoprotein. After 2 hr, the reaction was terminated by washing the cells twice with ice-cold PBS containing 0.2% BSA (wt/v), microfuge centrifugation for 1.5 min, followed by another wash with PBS. The cells were resuspended with 200 μl PBS and transferred to a 400 μl microfuge tube containing 200 μl of a mixture of dibutylphthalate: dinonylphthalate (2:1) (v/v) and microfuged for 1 minute. The supernatant was aspirated and the tube sliced above the pellet and counted for radioactivity in a gamma counter. All values were corrected for blank values obtained from cell-free incubations. Receptor-mediated binding was calculated as the difference between non-receptor-mediated and total binding. The data are presented as nanograms 125I-lipoproteins bound per mg cell protein. Specific binding kinetics were analyzed using Woolf plot, and dissociation constants (Kd) and total binding capacities (Bmax) obtained by best-fit linear regression.

**HMG-CoA reductase Assay:** The rate of conversion of [14C]-HMG-CoA to mevalonate was used to determine HMG-CoA reductase activity (Yachnin et al. 1984). HL-60 cells or D3-induced HL-60 macrophages were incubated with 15% LPDS for 24 hr. Cells were then incubated with LPDS or LPDS plus 100 μg/ml of LDL or acetyl-LDL protein for an additional 24 hr. Cells were collected as described above and washed twice with PBS. 1.5 x 10⁶ cells/ml were incubated with the lysing solution [0.05 M K₂HPO₄, 0.1 M EDTA, 70 mM Triton X-100 (pH 7.5) and
0.5 M dithiothreitol] for 10 min at 37°C and centrifuged at 5,000 rpm for 5 min at room temperature. The supernatant was incubated in a final volume of 200 μl with 50 μl of incubation cofactor [3 mM NADP, 22 mM glucose-6-phosphatase, 14 mM Triton X-100 (pH 7.5), 5 mM DTT, 0.3 IU glucose-6-phosphate dehydrogenase, and 50 μM DL-[14C]-HMG-CoA (7 cpm/pmole)]. After 2 hr the reaction was terminated by the addition of 25 μl 10 N HCL containing 60 mM mevalonate (MVA) and [3H]-MVA (5,000 dpm as an internal standard). The mixture was incubated for 20 min at 37°C and the non-lipid soluble material were precipitated by centrifugation for 1 min. 100 μl of the supernatant were spotted on activated (1 hr at 100°C) silica gel G sheets (Eastman) and developed with benzene:acetone (1:1, v/v) (Shapiro et al. 1974). The regions that corresponded to MVA were scraped and counted for radioactivity using a scintillation counter. Recovery of the internal standard averaged 80%. The amount of [14C]-HMG-CoA converted to [14C]-MVA are reported as nmole MVA/mg cell protein per min.

ACAT Assay: The rate of incorporation of [14C]-oleate into cholesteryl ester was used to determine ACAT activity as described by Goldstein et al. (1983). HL-60 cells or D3-induced HL-60 macrophages were incubated with LPDS for 24 hr followed by the addition of 100 μl LDL or acetyl-LDL with or without 25-hydroxycholesterol for an additional 24 hr. Cells were collected as described above by centrifugation at 1,000 rpm for 10 min at 4°C. 1.0 X 10⁶ cells/ml were incubated in the same media supplemented with 20 μl of sodium [14C]-oleate-bovine serum
albumin complex (10 mM \[^{14}\text{C}\]-oleate - 1.2 mg/ml albumin, 10,000 dpm/nmol of \[^{14}\text{C}\]-oleate) at 37°C in 5% CO\(_2\), in a humidified incubator. After 18 hr, the cells were washed twice with 2 ml of buffer A (150 mM NaCl, 50 mM Triton X-100, and 2 mg/ml BSA at pH 7.4), and once with 2 ml buffer B (same as buffer A with no BSA). An internal standard containing \[^{3}\text{H}\]-cholesteryl oleate (1.6 x 10^5 dpm) was added, and the cells were delipidated using hexane:isopropanol (3:2, v/v). The solvent was dried under nitrogen and the lipids were re-dissolved with petroleum ether:ether (98:2, v/v) and the labeled cholesteryl esters isolated by silica gel column as described by Chautan et al. (1988). Cholesteryl esters were eluted with 6 ml of the same solvent, dried overnight and radioactivity was counted by scintillation counter. All values were corrected for procedural error by subtracting the values from counts obtained from cell-free incubations. Recovery of the internal standard averaged 85%. The data are presented as nmole of \[^{14}\text{C}\]-oleate incorporated into cholesteryl ester per mg cell protein per 18 hr.

Oil Red O Cytochemistry: Cells were stained with Oil Red O (ORO) following the method of Winzerling et al. (1991). D\(_3\)-induced HL-60 macrophages were incubated with 15% LPDS for 24 hr followed by an additional 48 or 72 hr in the presence of 100 \(\mu\text{g/ml}\) LDL or acetyl-LDL. Cells were collected as described above and resuspended in PBS. 0.1 - 0.2 X 10^6 cells were cytospun at 150 g for 5 min, and slides were fixed in 60% isopropanol for 5 min. Slides were stained with ORO, counterstained in Mayer's hematoxylin, and wet-mounted with Aqua-mount.
were made using a Zeiss 35M camera mounted on a Zeiss microscope.

**Cholesterol Content:** Cholesterol content of D₃-induced HL-60 macrophages was determined by GLC using coprostanol as an internal standard (McNamara et al. 1985). D₃-induced macrophages were incubated with 15% LPDS for 24 hr; one set of cells continued in LPDS, another set incubated in LPDS supplemented with either 100 μg/ml LDL or acetyl-LDL for another 24 hr. Adhered cells were washed with PBS and reconstituted in PBS to a density of 10 X 10⁶ cells/ml. To measure total cholesterol content, 4.5 ml KOH:methyl alcohol (20:70) and 25 μl coprostanol (3.5 mg/ml) were added and samples were saponified at 70°C for 2 hr. Lipids were extracted with isopropanol:hexane (1:2, v/v). The organic layer evaporated under nitrogen, 200 μl TMS added, and aliquots analyzed by gas chromatography for cholesterol content. Free cholesterol was measured in the absence of KOH:methanol mixture and saponification. Cholesterol esters were calculated as the difference between free and total cholesterol.

**Other Assays:** Protein concentrations were determined using a modified Lowry procedure (Markwell et al. 1978) calibrated against bovine serum albumin.

**Statistical Analysis:** One way analysis of variance was used to determine significant difference between treatments (Glantz 1981). Data are presented as mean ± standard deviation for the number of assays shown.
RESULTS

**Macrophage Differentiation:** HL-60 cells incubated with D$_3$ for 48 hr induced differentiation along the macrophage pathway as confirmed by positive acid esterase activity and adherence to petri-dishes as described previously (Jouni and McNamara).

**Lipoprotein Binding Assays:** To assess the effects of differentiation on LDL receptor expression of D$_3$-induced HL-60 macrophages, LDL binding was determined in induced macrophages following incubation in media containing LPDS. Macrophages exhibited specific and saturable receptor-mediated LDL binding with apparent saturation point at 30 µg/ml (Fig. 13). When binding data were analyzed by a Woolf plot (Fig. 13 insert), a single linear relationship was obtained, indicative of a single receptor. Receptor affinity for the ligand ($K_a$), was 29 µg/ml, and the maximum binding, which reflects the number of receptors ($B_{\text{max}}$), was 219 ng/mg cell protein.

Figure 14 represents kinetic analysis of receptor-mediated binding of acetyl-LDL to D$_3$-induced HL-60 macrophages. Receptor-mediated binding reached a plateau at about 30 µg/ml. The Woolf plot indicated a single class of receptors with $B_{\text{max}}$ of 313 ng/mg cell protein and a $K_a$ of 36 µg/ml (Fig. 14 insert). These binding studies indicate that D$_3$-induced HL-60 macrophages possess specific and saturable receptors for both native and modified LDL.
FIGURE 13: Saturation Kinetics of LDL Binding by D₃-Induced HL-60 Macrophages. HL-60 cells incubated with 15% LPDS, were differentiated into macrophages by treatment with D₃ (5.0 x 10⁻⁸ M) for 48 hr. Binding studies were determined by incubating macrophages with indicated concentrations of ¹²⁵I-LDL in the presence (non-receptor-mediated) or absence (total) of 40-fold excess of unlabeled LDL. Receptor-mediated binding of ¹²⁵I-LDL was calculated as the difference between total binding and non-receptor-mediated binding. Woolf Plot transformations are presented in the insert. The values represent mean ± S.D. for 3-6 measurements.
FIGURE 14: Saturation Kinetics of Acetyl-LDL Binding by D3-Induced HL-60 Macrophages. D3-induced HL-60 macrophages were incubated with RPMI-1640 containing 15% LPDS for 48 hr. Binding studies were determined by incubating the macrophages with indicated concentrations of $^{125}$I-acetyl-LDL with (non-receptor-mediated) or without (total) 40-fold unlabeled acetyl-LDL. Receptor-mediated binding of $^{125}$I-acetyl-LDL was calculated as the difference between total binding and non-receptor-mediated binding. $K_d$ and $B_{max}$ were determined by Woolf Plot transformations (insert). Each value represents mean ± S.D. for 3-6 measurements.
**HMG-CoA Reductase Activity:** To determine the ability of HL-60 cells and D₃-induced HL-60 macrophages to suppress HMG-CoA reductase activity in response to cholesterol preloading, cells were incubated with LPDS in the presence of 100 µg/ml LDL or acetyl-LDL and conversion of [¹⁴C]-HMG-CoA to mevalonate was investigated (Fig. 15). HL-60 cells incubated with 100 µg/ml LDL for 24 hr exhibited only 46% of the activity of HMG-CoA reductase of macrophages incubated with LPDS. HMG-CoA reductase activity for cells incubated with acetyl-LDL was virtually identical to cells incubated with LPDS. From these results it is concluded that HL-60 cells lack cellular surface receptors for acetyl-LDL; however, they can down regulate HMG-CoA reductase in response to LDL derived cholesterol. D₃-induced HL-60 macrophages incubated with LPDS for 48 hr demonstrated the highest HMG-CoA reductase activity of 31.0 pmole/min-mg relative to macrophages incubated in either 100 µg/ml LDL (14.2 pmole/min-mg) or acetyl-LDL (21.2 pmole/min-ml) (Fig. 15). Loading the macrophages with cholesterol by incubation with LDL or acetyl-LDL significantly reduced the conversion of HMG-CoA to mevalonate by 54% (P<0.001) and 32% respectively (P<0.001). Thus, delivery of cholesterol to macrophages via a receptor-mediated process results in suppression of reductase activity via a product feedback mechanism.
FIGURE 15: Regulation of HMG-CoA Reductase of HL-60 Cells and D₃-Induced. HL-60 Macrophages Incubated with LPDS, LDL or Acetyl-LDL. HL-60 cells and D₃-induced HL-60 macrophages were preincubated with RPMI-1640 containing 15% LPDS for 24 hr. A set of cells was continued in LPDS for another 24 hr, and to the other sets 100 μg/ml LDL or 100 μg/ml acetyl-LDL were added for another 24 hr. Activity of HMG-CoA reductase was determined according to the method of Yachnin et al. (1984). The amount of [¹⁴C]-HMG-CoA converted to [¹⁴C]-MVA are reported as n mole MVA/ mg cell protein/ min. The values represent mean ± S.D. for 3-4 measurements. Significantly different from each other and from HL-60 cells at P<0.001(*).
ACAT Activity: Figure 16 represents the up regulation of ACAT activity in HL-60 cells and D3-induced HL-60 macrophages by exogenous sources of cholesterol. HL-60 cells incubated with 100 μg/ml LDL for 24 hr exhibited a significant 1.8-fold increase in the incorporation rate of [14C]-oleate into cholesteryl ester compared to cells incubated with media containing LPDS (control) (P<0.001). Cells incubated with acetyl-LDL for 24 hr demonstrated the same ACAT activity as control cells, indicative of the absence of modified LDL receptor for acetyl-LDL on HL-60 cells. Incubating cells with LDL in the presence of 25-hydroxycholesterol (an ACAT activator) resulted in a significant 4-fold increase in ACAT activity compared to control cells (P<0.001).

The incorporation rates of [14C]-oleate into cholesteryl ester by D3-induced HL-60 macrophages incubated with LPDS or LDL for 24 hr were virtually identical to rates in undifferentiated cells (Fig. 17). However, macrophages incubated with acetyl-LDL for 24 hr exhibited a significant (2.3-fold) increase of ACAT activity compared to macrophages incubated with LPDS (control) (P<0.001). Thus, cholesterol delivered to macrophages through receptor-mediated uptake and degradation of acetyl-LDL is capable of affecting the regulatory pool of cellular cholesterol resulting in induction of ACAT activity. Maximum increase of ACAT activity was obtained by macrophages incubated with 25-hydroxycholesterol in the presence of LDL (18.3 ± 1.9 nmol/mg-18 hr) or acetyl-LDL (20.8 ± 2 nmol/mg-18 hr). These results indicate that the presence of an exogenous source of cholesterol
FIGURE 16: Regulation of ACAT Activity of HL-60 cells. HL-60 cells were preincubated with RPMI-1640 containing 15% LPDS for 24 hr followed by the addition of 100 µg/ml LDL or acetyl-LDL for another 24 hr. The incorporation rate of [14C]-oleate into cholesteryl ester was determined following the method described by Goldstein et al. (1983). The data are presented as nmol of [14C]-oleate incorporated into cholesteryl ester per mg cell protein per 18 hr. The values represent mean ± S.D. for 3-4 measurements. Using one way ANOVA, the values are significantly different from LPDS at P < 0.001 (*), and from LDL and LPDS at P < 0.001 (**).
FIGURE 17: Regulation of ACAT Activity of D₃-Induced HL-60 Macrophages. HL-60 cells were preincubated with RPMI-1640 containing 15% LPDS and 5.0 x 10⁻⁸ M D₃ for 24 hr. Some cells were continued in LPDS, to the others 100 μg/ml LDL or acetyl-LDL with or 25-hydroxycholesterol were added. ACAT activity was determined according to the method of Goldstein et al. (1983). The incorporation rate of oleate into cholesteryl ester is presented as nmol of [¹⁴C]-oleate incorporated into cholesteryl ester per mg cell protein/18 hr. The values represent mean ± S.D. for 3-4 measurements. Using one way ANOVA values are significantly different from LPDS at P<0.001 (*).
along with an oxysterol in the incubation media can induce the incorporation rate of
$[^{14}C]$-oleate into cholesteryl ester beyond the rate observed for lipoprotein derived
cholesterol alone.

**Cholesterol Content:** To investigate whether the enhancement in cholesteryl ester
synthesis by D$_3$-induced HL-60 macrophages exposed to LDL or acetyl-LDL was
paralleled by intracellular accumulation of cholesteryl esters, HL-60 cells in LPDS
were treated with D$_3$ for 24 hr followed by the addition of LDL or acetyl-LDL for
an additional 24 hr and the cholesterol content (free and esterified) was determined
(Table III). Cholesterol content of HL-60 cells treated with LPDS for 48 hr was used
as a base line. HL-60 cells incubated with LPDS exhibited significantly lower total
cholesterol (12.2 ± 1.3 µg/mg) compared to D$_3$-induced HL-60 macrophages
incubated with LPDS (control) (17.7 ± 2.9 µg/mg) (P < 0.001). Similar to D$_3$-induced
HL-60 macrophages, the majority of cholesterol in HL-60 cells was free cholesterol.

D$_3$-induced HL-60 macrophages treated with 100 µg/ml LDL exhibited a
significant increase (1.5-fold) in cholesterol content compared to control cells
(P < 0.001) (Table III). Free cholesterol and cholesteryl ester concentrations in D$_3$-
induced HL-60 macrophages incubated with LDL were significantly higher than
control (P < 0.001). Addition of 100 µg/ml of acetyl-LDL to D$_3$-induced HL-60
macrophages resulted in a significant increase of cholesterol content compared to
control cells and macrophages incubated with LDL. This increase was attributed to
<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>TOTAL</th>
<th>FREE</th>
<th>ESTERIFIED</th>
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<tr>
<td>HL-60 CELLS</td>
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<tr>
<td>LPDS</td>
<td>12.2 ± 0.8</td>
<td>9.0 ± 0.3</td>
<td>3.2 ± 0.4b</td>
</tr>
<tr>
<td>D₃-MQ</td>
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<td></td>
</tr>
<tr>
<td>LPDS</td>
<td>17.7 ± 2.9</td>
<td>14.1 ± 3.8</td>
<td>3.4 ± 1.7b</td>
</tr>
<tr>
<td>LDL</td>
<td>26.3 ± 0.7</td>
<td>18.4 ± 4.1a</td>
<td>7.9 ± 3.6</td>
</tr>
<tr>
<td>ACETYL-LDL</td>
<td>35.4 ± 2.4</td>
<td>20.4 ± 3.3a</td>
<td>15.0 ± 2.4</td>
</tr>
</tbody>
</table>

HL-60 cells preincubated with LPDS for 48 hr and cholesterol content were determined as a base line for comparison. D₃-induced HL-60 macrophages were preincubated with LPDS, LDL or acetyl-LDL for 24 hr. Cholesterol content was determined in the presence (total) or absence (free) of KOH:methyl alcohol, using coprostanol as an internal standard. Esterified cholesterol was calculated as the difference between total and free cholesterol. Data are presented as mean ± S.D. for 6-7 measurements. Values in same column with same superscripts are not significantly different from each other. Values in same column with no superscripts are significantly different (p<0.001).
significant increase in both free (36%) and esterified cholesterol (66%) relative to control cells (P < 0.001). However, esterified cholesterol and not free cholesterol was significantly higher than in macrophages incubated with media containing LDL (P < 0.001).

**Foam Cell Formation:** Figures 18, 19 and 20 represent the extent of lipid accumulation as measured by ORO in D₃-induced HL-60 macrophages incubated for 48 and 72 hr in media containing either LPDS, LDL or acetyl-LDL respectively. HL-60 cells treated with D₃ and LPDS up to three days possessed large empty vacuoles with no ORO lipid stainable materials (Fig. 18). However, macrophages incubated with 100 μg/ml LDL for two or three days, accumulated little lipid as observed with the few red stains in the vacuoles, but the majority of the macrophages did not accumulate any lipid, reflecting the regulation of the LDL receptor by LDL derived cholesterol (Fig. 19). In contrast, macrophages incubated with 100 μg/ml acetyl-LDL accumulated substantial lipid stainable ORO material relative to macrophages incubated with LPDS or LDL (Fig. 20). This increase in lipid accumulation imparted the macrophages their foamy appearance. Progressively increasing the time of exposure to acetyl-LDL resulted in macrophages with higher amounts of lipid stainable materials, thus, indicating that cholesterol delivery via un-regulated scavenger pathway is capable of causing massive accumulation of lipid.
FIGURE 18: Lipid Stainable ORO Material of D3-Induced HL-60 Macrophages Incubated with LPDS. D3-induced HL-60 macrophages were preincubated with LPDS for 48 hr and foam cell formation was determined as the ability of macrophages to accumulate ORO stainable material.
FIGURE 19: Lipid Stainable ORO Material of D3-Induced HL-60
Macrophages Incubated with LDL. D3-induced HL-60 macrophages were preincubated with 100 μg/ml LDL for 48 or 72 hr and foam cell formation was determined as the ability of macrophages to accumulate ORO stainable material. [A] macrophages preincubated with LDL for 48 hr, [B] macrophages preincubated with LDL 72 hr. Magnification 500 X.
FIGURE 20: Lipid Stainable ORO Material of D3-Induced HL-60 Macrophages Incubated with Acetyl-LDL. D3-induced HL-60 macrophages were preincubated with 100 µg/ml acetyl-LDL for 48 or 72 hr and foam cell formation was determined as the ability of macrophages to accumulate ORO stainable material. [A] macrophages preincubated with acetyl-LDL for 48 hr, [B] macrophages preincubated with acetyl-LDL 72 hr. Magnification 500 X.
DISCUSSION

Deposition of lipid in the arterial intima plays a central role in the initiation and propagation of atherosclerosis (Gerrity 1981). In this respect, macrophage foam cells are considered to play a significant role in the accumulation of cholesteryl ester; a major lipid component of atherosclerotic lesion (Schwartz et al. 1989). D₃-induced HL-60 macrophages, like monocyte-derived macrophages, express unregulated, high-affinity scavenger receptors for chemically modified LDL that are able to take up and degrade acetyl-LDL without feedback suppression of scavenger receptor activity (Fogelman et al. 1981). In theory, intracellular cholesterol derived from receptor-mediated uptake of LDL acts at three regulatory sites that maintain cholesterol homeostasis. These pathways involve decrease of endogenous cholesterol synthesis by transcriptional suppression of HMG-CoA reductase (Lusky et al. 1983) and induction of reductase degradation (Gil et al. 1985), down-regulation of the LDL receptor (Russel et al. 1983), and induction of the ACAT activity (Goldstein et al. 1974). In the present study, these three regulatory responses were investigated in HL-60 cells and in D₃-induced HL-60 macrophages.

HL-60 cells have been reported to differentiate into macrophages in response to treatment with 1,25-dihydroxyvitamin D₃ (Mangelsdorf et al. 1984) and studies have demonstrated that macrophage maturation of this cell line is associated with the induction of expression of the modified LDL receptor (acetyl-LDL), without the loss of the LDL receptor activity (Jouni and McNamara 1991). LDL receptors of D₃-
induced HL-60 macrophages exhibit feedback regulation, are calcium dependent, and inhibited by treatment with pronase or with chloroquine. It has also been found that the acetyl-LDL receptor is not regulated by intracellular cholesterol, demonstrated no calcium dependency, but is inhibited by pronase, chloroquine and fucoidin (Jouni and McNamara 1991).

**LDL and Acetyl-LDL Receptors of D₃-Induced HL-60 macrophages:** Binding of LDL and acetyl-LDL to D₃-induced HL-60 macrophages exhibited typical saturation kinetics as a function of ligand concentrations. Analysis of the binding data of LDL and acetyl-LDL indicated that macrophages possess a single class of binding sites for each ligand with the same apparent binding affinities but have significantly different numbers of receptors per mg cell protein (p<0.001). The observation that macrophages exhibited similar degradation rates of LDL and acetyl-LDL (Jouni and McNamara 1991) but different number of receptors, with the acetyl-LDL receptor number being 1.4-fold higher than that of LDL receptor number, suggests differences in the rates of endocytosis for LDL and acetyl-LDL receptors. These differential degradation rates could be explained by two possible mechanisms: the first mechanism could arise from an accelerated rate of the exteriorization of the LDL receptor relative to the acetyl-LDL receptor. The second mechanism could involve a reduced internalization rate at which acetyl-LDL receptor is endocytosed.

**Regulation of HMG-CoA Reductase:** Activities of the LDL receptor and HMG-CoA reductase responded similarly to changes in the amount of cytoplasmic free cellular
cholesterol. HMG-CoA reductase activity of D3-induced HL-60 macrophages incubated in LPDS averaged 31 pmole/min/mg, a value 1.5 times higher than that observed for HL-60 cells. Addition of LDL (100 μg/ml) to LPDS culture media for 24 hr resulted in a 54% suppression of HMG-CoA reductase activity of both HL-60 cells and D3-induced HL-60 macrophages. Nevertheless, the absolute activity of the enzyme was found to be 1.5 times higher in D3-induced HL-60 macrophages relative to HL-60 cells. This difference in activity is attributed to the need of macrophages to incorporate more cholesterol in cellular membrane during the course of differentiation-induced increase in cell volume (Mangelsdorf et al. 1984). The finding that D3-induced HL-60 macrophages exhibited identical LDL degradation rate as undifferentiated cells (Jouni and McNamara 1991) but significantly higher HMG-CoA reductase activity, suggests that the need for macrophages to incorporate more cholesterol during the expansion of cellular membrane depends primarily on endogenous sources. This observation is supported by the fact that sterol synthesis in D3-induced HL-60 macrophages, as measured by the incorporation of [14C]-acetate into sterol, is 2.6-fold higher than in HL-60 cells under identical conditions (Jouni and McNamara 1991). Moreover, these results are also in agreement with the report of Patel et al. (1984) where human monocyte-derived macrophages obtain about 50% of the cholesterol that they need for growth from endogenous synthesis.

Addition of LDL at a concentration of 20 μg/ml, which approximates the saturation concentration for the LDL receptor of D3-induced HL-60 macrophages,
did not significantly suppress HMG-CoA reductase activity (see appendix B1). This finding is in agreement with the report of Knight et al. (1983) where growing monocyte-derived macrophages maintain induced activity of HMG-CoA reductase in the presence of LDL at concentrations sufficient to saturate the LDL receptor. Patel and Knight (1985) suggested that the requirement of large quantities of a non-sterol product of mevalonate metabolism not available from the medium require macrophages to maintain high levels of reductase activity when incubated with LDL concentrations lower than or equal to the saturation level of LDL for the receptor.

The lack of expression of acetyl-LDL receptor by undifferentiated HL-60 cells was reflected in their ability to maintain high HMG-CoA reductase activity when cells were incubated with acetyl-LDL. In contrast, addition of acetyl-LDL to D₃-induced HL-60 macrophages resulted in a 32% reduction in enzyme activity compared to macrophages in LPDS demonstrating that reductase activity is regulated by cholesterol derived via receptor-mediated uptake of acetyl-LDL.

**Regulation of ACAT:** ACAT is regulated in concert with LDL receptor and HMG-CoA reductase to ensure cholesterol homeostasis by minimizing fluctuations of cellular free cholesterol concentrations (Tsuada et al. 1986). When the concentration of cholesterol in the cell is altered, subsequent changes in the rate of esterification of intracellular cholesterol follows due to changes in ACAT activity. Although little is known about ACAT regulation, it is postulated that cholesterol delivery to ACAT is probably a major determinant of the enzyme activity (Hashimoto et al. 1974).
Studies in fibroblasts suggested that an increased availability of cholesterol stimulates ACAT activity by two mechanisms: it provides additional substrate and it increases enzyme catalytic activity (Brown and Goldstein 1980). In theory, an increase in ACAT activity results from either enzyme activation by substrate supply (short-term activation) (Hashimoto et al. 1974) or by a non-substrate modulation of enzyme activity (long-term activation) (Hashimoto et al. 1983).

In this study, availability of substrate, free cholesterol, via receptor-mediated uptake of lipoprotein in HL-60 cells and D3-induced HL-60 macrophages resulted in a 1.6- to 2.5- fold induction in the incorporation rate of [14C]-oleate into cholesteryl ester. The finding that ACAT activity in undifferentiated and differentiated cells was induced by 5.1- and 4.9- fold respectively upon incubating the cells with LDL in the presence of 25-hydroxycholesterol, suggests that substrate availability does not solely account for the induction of ACAT to its maximum capacity, and that the presence of an oxysterol in the incubation medium is a more powerful activator of the enzyme. When D3-induced HL-60 macrophages were incubated with acetyl-LDL and 25-hydroxycholesterol, ACAT activity was 2.5- and 5.8-fold higher than macrophages incubated only with acetyl-LDL or LPDS respectively. The mechanism by which the oxysterol induces ACAT activity is not well elucidated; however, it has been shown that 25-hydroxycholesterol induces reductase degradation by a short half-life protein (Chang et al. 1981). The fact that ACAT and reductase activities are reciprocally regulated by 25-hydroxycholesterol, suggests that the same protein might affect the
two enzymes differently. Moreover, changes in cellular membrane fluidity by 25-hydroxycholesterol could play an important role in maximally inducing ACAT activity. It is relevant to add that the intrinsic changes in ACAT activity in response to alterations in membrane fluidity is manifested by changes in the amount of sterol and oxysterol in a domain of the membrane adjacent to the enzyme (Suckling et al. 1982, Doolittle and Chan 1982).

**Cholesterol Content and Foam Cell Formation**: The increase in ACAT activity of D3-induced HL-60 macrophages incubated with LDL or acetyl-LDL paralleled the increase in cellular total cholesterol content. When the cholesteryl ester mass was expressed as the percentage of total cholesterol, cholesteryl ester accounted for a significantly larger percentage following incubation with either LDL or acetyl-LDL relative to macrophages in LPDS. However, macrophages incubated with acetyl-LDL exhibited the highest cholesteryl ester content indicative of enhanced uptake of acetyl-LDL via the non-regulated receptor. These findings are in agreement with other macrophage systems, including THP-1 cells and monocyte-derived macrophages (Hara et al. 1987).

Morphological observations verify the results reached from the biochemical experiments. Macrophages exhibited an acceleration in the synthesis of cholesteryl ester by exposure to acetyl-LDL for 48 hr and were shown to have a dramatic increase in ORO stainable material which gave these macrophages their foamy appearance. Consistent with this observation is the massive accumulation of
esterified cholesterol in LDL receptor-negative homozygotes familial hypercholesterolemia, much of which must have derived from an existing mechanism for the unregulated uptake of a modified LDL by macrophages in vivo (Goldstein and Brown 1983). In contrast, macrophages pre-incubated with LDL exhibited very little ORO stainable material with numerous empty vacuoles, demonstrating that cholesterol delivered to the macrophages via regulated, receptor-mediated pathway does not lead to massive accumulation of cholesteryl ester. Macrophages incubated in LPDS, with no exogenous sources of cholesterol, contained large empty vacuoles with no lipid droplets. These morphological findings are in agreement with the amount of lipid accumulated in the macrophages as well as with the ACAT activity, in which macrophages loaded with cholesterol by incubation with acetyl-LDL exhibited the highest amount of cholesteryl ester compared to macrophages incubated with LDL or LPDS.

These studies indicate that D₃-induced HL-60 macrophages provide a suitable model of human macrophages with all the characteristics required to study the interaction between macrophages and lipoproteins. In addition, the macrophages can form foam cells that are essential component of atherosclerotic lesions.
CHAPTER 5

SUMMARY AND CONCLUSIONS

The long-range goal of this dissertation research project was to develop a model system for in vitro analysis of the interactions between human macrophages and plasma lipoproteins. Undifferentiated HL-60 cells exhibited a specific and saturable receptor-mediated LDL catabolic pathway, yet the cells failed to process acetyl-LDL indicating the absence of the scavenger receptor, a characteristic of macrophages. This cell line, with its bipotential induction characteristics, can be induced to differentiate into macrophages in response to TPA or D₃. The present studies were based on the hypothesis that both TPA and D₃ induce structural and functional characteristics of macrophages in HL-60 cells. Further, these macrophages could be used as an in vitro human model system to study regulation of cholesterol and lipoprotein metabolism as related to foam cell formation.

Differences in structural characteristics between TPA- and D₃-treated HL-60 macrophages were observed. TPA-induced HL-60 macrophages had prominent pseudopodia and exhibited intense adherence to plastic dishes and to each other. In contrast, D₃-induced HL-60 macrophages adhered loosely to plastic dishes only, and were larger in size compared to TPA-induced and undifferentiated HL-60 cells.
Treating HL-60 cells with TPA was associated with complete loss of receptor-mediated LDL degradation with no induction of scavenger receptor activity. The mechanism by which TPA exerts its effect on native and scavenger receptor activities for LDL and modified LDL is not known at present. However, the fact that mezerine, an activator of PKC, mimics the effect of TPA on HL-60 cells suggests that TPA exerts its effect via PKC. It has been established that TPA activates PKC by substituting for diacylglycerol, a product of phosphatidylinositol pathway and is an important intracellular regulator of PKC. The ability of diacylglycerol derivatives to mimic certain cellular responses exerted by phorbol esters suggests that activation of PKC may serve as a mechanism mediating these phorbol esters effects. It is also conceivable that TPA influences membrane lipids thus causing receptor masking. At present it is difficult to draw conclusions about the exact effect of TPA on scavenger receptor activity in TPA-induced HL-60 cells. Perhaps TPA-induced macrophages express an acetyl-LDL receptor which TPA subsequently blocked or, TPA-induced macrophages fail to induce expression of scavenger receptor activity. To answer these questions, further studies are needed to determine the effect of TPA on mRNA levels of the scavenger receptor. Nevertheless, the data clearly demonstrate that HL-60 cells induced with TPA to macrophages are not a suitable model system to study lipoprotein-macrophage interactions.

D3-induced HL-60 macrophages possessed both native and modified LDL receptors with apparent $K_d$ values of 29 $\mu$g/ml and 36 $\mu$g/ml and $B_{max}$ levels of 219.
ng/mg and 313 ng/mg respectively. The LDL receptor of D₃-induced HL-60 macrophages exhibited all the characteristics of the classic apo B/E receptor. The receptor-mediated pathway of LDL catabolism was calcium dependent, EDTA sensitive, and chloroquine and pronase inhibited. Studies also demonstrated that the receptor was under feedback regulation by cellular cholesterol. Increasing the amount of free cholesterol by incubating macrophages with media containing LDL or acetyl-LDL resulted in a significant suppression of receptor activity. A 4-fold increase of receptor-mediated LDL degradation was obtained when macrophages were incubated with LPDS for 24 hr. Maximum induction of receptor activity was obtained when macrophages were treated with mevinolin and LPDS. Thus, a decrease of cellular cholesterol, whether achieved by incubating macrophages with LPDS or mevinolin, induced LDL receptor expression and consequently LDL degradation.

The scavenger receptor of D₃-induced HL-60 macrophages was calcium independent, chloroquine sensitive, and partially sensitive to pronase and fucoidin. The scavenger receptor pathway was neither down-regulated by pretreating macrophages with LDL or acetyl-LDL, nor was induced by preincubating macrophages with LPDS.

D₃-induced HL-60 macrophages exhibited a 2.6-fold increase in sterol synthesis compared to undifferentiated HL-60 cells. This increase could be in response to increased cholesterol needs for the synthesis of cellular membranes during the course
of differentiation which is accompanied by an increase in cell volume.

As expected, HMG-CoA reductase responded reciprocally to changes in cytoplasmic free cholesterol. Addition of LDL or acetyl-LDL to D₃-induced HL-60 macrophages resulted in 54% and 32% suppression of HMG-CoA reductase activity respectively compared to macrophages incubated with LPDS.

ACAT activity was also regulated in association with LDL receptor and HMG-CoA reductase activities, all of which function to minimize intracellular cholesterol fluctuations and ensure sterol homeostasis. Availability of free cholesterol, via receptor-mediated uptake of lipoproteins, resulted in a 1.6- to 2.5-fold increase in ACAT activity compared to macrophages incubated with LPDS. Maximum induction of ACAT activity was obtained in macrophages incubated with acetyl-LDL and 25-hydroxycholesterol. The mechanism by which ACAT activity is maximally induced is proposed to be in response to membrane fluidity manifested by changes in the amount of sterol and oxysterol present.

D₃-induced HL-60 macrophages incubated with LDL or acetyl-LDL exhibited significantly higher cholesterol content relative to macrophages incubated with LPDS. However, macrophages incubated with acetyl-LDL exhibited a maximum increase in cholesterol content with the majority of this increase being in the form of cholesteryl esters. Further, incubating macrophages with acetyl-LDL resulted in a dramatic increase in ORO stainable material, which imparted to macrophages a foamy appearance. In contrast, macrophages incubated with LDL exhibited very little ORO
stainable material with numerous empty vacuoles, demonstrating that delivery of cholesterol via regulated, receptor-mediated pathway does not lead to massive accumulation of cholesteryl ester.

In summary, TPA and D$_3$ exerted distinctly different effects on structural and functional characteristics of macrophages, despite the fact that both inducers transmit their action via signal transduction. However, D$_3$- and not TPA-treated HL-60 macrophages exhibited functional receptors for native and modified LDL. Given the role of macrophages in foam cell formation, and, perhaps, more generally in cholesterol scavenging, these findings raise the interesting possibility that D$_3$-induced HL-60 macrophages can be used as a human model system to study cholesterol and lipoprotein metabolism as related to foam cell formation and atherogenesis.

Regulation of sterol and lipoprotein metabolism in D$_3$-induced HL-60 macrophages are in agreement with that of human monocyte-derived macrophages and peritoneal macrophages, the only human systems that possess both native and modified LDL receptor activities. The three systems have well regulated apo B/E receptors while the scavenger receptors are not regulated by cellular cholesterol feedback mechanisms. LDL and acetyl-LDL binding studies in monocyte-derived macrophages exhibit a wide range of $K_d$ and $B_{max}$ depending on incubation conditions, age of macrophages, length of incubation, etc. Binding studies in D$_3$-induced HL-60 macrophages demonstrated a $K_d$ value and a $B_{max}$ level within the observed ranges of monocyte-derived macrophages. Similar to monocyte-derived macrophages,
loading D_3-induced HL-60 macrophages with cholesterol by incubation with acetyl-LDL induces a 3- to 4-fold increase in amount of cholesteryl esters relative to macrophages incubated with LPDS. Thus, D_3-induced HL-60 macrophages exhibit patterns of lipoprotein and sterol metabolism similar to those reported for human monocyte-derived macrophages.

D_3-induced HL-60 macrophages could be used in future studies that may help us to gain not only a better insight into atherosclerotic development, but also the means to combat the disease. D_3-induced HL-60 macrophages could be used to answer several interesting questions including a) the exact mechanisms through which TPA and D_3 induce macrophage differentiation of HL-60 cells, b) whether TPA induces expression of the acetyl-LDL receptor, c) mechanisms of cholesterol efflux and influx in macrophages, d) possible pharmacological studies to regulate scavenger receptor activity and thus reduce the ability of macrophages to accumulate cholesterol esters and form foam cells.

Although D_3-induced HL-60 macrophages possess all the characteristics required to study lipoprotein-macrophage interaction, the system is limited by its sensitivity to environmental changes that may alter functional capabilities. Further, incubating macrophages under specific conditions, a single facet of plaque etiology, does not mimic reality of atherosclerosis which involves a complex cascades of interaction among environmental and genetic factors including endothelial and smooth muscle cells of the arterial wall, monocytes, platelets and plasma lipoproteins,
all of which are not considered when studying lipoprotein-macrophage interactions in vitro. Nevertheless, the use of a highly homogenous population becomes quite obvious if we are to progress in our understanding of the interaction between macrophages and atherosclerosis.
FIGURE A1: Receptor-Mediated-Regulation of LDL and Acetyl-LDL by D₃-Induced HL-60 Macrophages. D₃-induced HL-60 macrophages were pre-incubated with 15% LPDS with or without 100 μg/ml LDL or acetyl-LDL for 24 hr. LDL and acetyl-LDL degradation rates were determined at 10 μg/ml ¹²⁵I-lipoproteins with (non-receptor-mediated) or without (total) a 20-fold excess of unlabeled lipoproteins. Values represent mean ± S.D. for 6 measurements.
FIGURE A2. Effect of Mezerine on LDL Receptor-Mediated Degradation by D$_3$- or TPA-Induced HL-60 Macrophages. Cells were incubated with 15% LPDS in the presence or absence of D$_3$ or TPA for 48 hr and degradation rates were determined at 10 µg/ml $^{125}$I-LDL with (non-receptor-mediated) or without (total) a 20-fold excess of unlabeled LDL. Values represent mean ± S.D. for 3-6 measurements.
**TABLE B: HMG-CoA REDUCTASE ACTIVITY OF D3-INDUCED HL-60 MACROPHAGES**

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<tr>
<th>TREATMENT</th>
<th>pmol/mg-min</th>
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<tr>
<td>LPDS</td>
<td>31.0 ± 2.1</td>
</tr>
<tr>
<td>LDL (20μg/ml)</td>
<td>28.1 ± 1.8</td>
</tr>
<tr>
<td>LDL (100μg/ml)</td>
<td>14.2 ± 0.9*</td>
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</table>

D3-induced HL-60 macrophages were pre-incubated with RPMI-1640 containing either LPDS or LDL at LDL receptor saturation of 20 μg/ml, or 100 μg/ml). HMG-CoA reductase activity was determined as the conversion rate of $[^{14}\text{C}]-\text{HMG-CoA}$ to $[^{14}\text{C}]-\text{mevalonate}$. Values represent mean ± S.D. for 3-4 measurements. Using one way ANOVA, the values are significantly different from other values at $P<0.001(*)$. 
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