THE ROLE OF LIPOPROTEIN-X 
IN THE DEVELOPMENT OF GLOMERULOSCLEROSIS 
IN FAMILIAL LCAT DEFICIENCY 

by 
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June 1997 
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ABSTRACT

Progressive glomerulosclerosis is a major complication in patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency. The absence of active LCAT in the plasma of these patients leads to the formation of an abnormal plasma lipoprotein, lipoprotein-X (Lp-X). Renal biopsies of these patients have revealed lipid deposition, macrophage infiltration, the presence of foam cells, and mesangial cell proliferation in affected glomeruli. The objective of this project was to examine the role of Lp-X in the development of glomerulosclerosis. Our results have demonstrated that Lp-X is taken up by cultured rat mesangial cells and that the lipid component of Lp-X is metabolized intracellularly. We have also investigated the role of apolipoproteins in the uptake of Lp-X. Both apo C-I and C-III inhibited Lp-X uptake while C-II (1.5 fold) and E (4 fold), as well as all four apolipoproteins combined (1.5 fold), stimulated this process. We have also observed that cell surface proteoglycans are involved in modulating the uptake of this abnormal lipoprotein. Lp-X, either alone or combined with rat peritoneal macrophages, has no effect of the proliferation of mesangial cells.
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<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CE</td>
<td>Cholesteryl ester</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CHE</td>
<td>Cholesteryl hexadecyl ether</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicron</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Free (unesterified) cholesterol</td>
</tr>
<tr>
<td>FED</td>
<td>Fish Eye Disease</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered saline solution</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin:cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>Lp-X</td>
<td>Lipoprotein-X</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL-receptor related protein</td>
</tr>
<tr>
<td>NaBr</td>
<td>Sodium bromide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>rLP-X</td>
<td>reconstituted lipoprotein-X</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>UC</td>
<td>unesterified cholesterol</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
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</table>
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To my family
1. INTRODUCTION

1.1 LIPOPROTEINS

Plasma lipoproteins are heterogeneous particles composed of lipids and proteins. They vary widely in size but almost all appear to be microemulsions (Edelstein et al., 1979). They play a crucial role in the transport and metabolism of plasma lipids.

1.1.1 The major lipoprotein classes

Lipoproteins have been traditionally divided into four main classes based on their density by sequential ultracentrifugation (Havel and Kane, 1995). Table 1 lists the major lipoprotein classes along with their corresponding densities and sizes. Table 2 summarizes the composition of the major lipoprotein classes.

The two classes (Table 1) with the largest lipoproteins, whose cores consist mainly of triglycerides (Table 2), are the chylomicrons and the very low density lipoproteins (VLDL). Chylomicrons are derived from dietary fat and its B apolipoprotein (apo) is primarily apo B-48. VLDL is synthesized and secreted by hepatocytes and contains apo B-100. The two classes with smaller lipoproteins, which mainly contain cholesteryl esters in their cores, are low density lipoproteins (LDL) and high density lipoproteins (HDL). Elevated levels of LDL and/or low concentrations of HDL are well-known risk factors of coronary artery disease (CAD) (Lavie and Milani, 1997).

The mature forms of LDL and HDL are not directly secreted by hepatocytes. Mature LDL and HDL are mainly produced by metabolic processes
Table 1. The four major classes of human plasma lipoproteins.

<table>
<thead>
<tr>
<th>Class</th>
<th>Density (g/mL)</th>
<th>Diameter (nm)</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>chylomicrons</td>
<td>0.93</td>
<td>75 - 1200</td>
<td>50 - 100 x10^6</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.93 - 1.006</td>
<td>30 - 80</td>
<td>10 - 80 x10^5</td>
</tr>
<tr>
<td>LDL</td>
<td>1.006 - 1.063</td>
<td>18 - 25</td>
<td>2,300,000</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063 - 1.210</td>
<td>5 - 12</td>
<td>175 - 360,000</td>
</tr>
</tbody>
</table>

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. Adapted from Havel and Kane (1995).

Table 2. Chemical composition of normal human plasma lipoproteins.

<table>
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<tr>
<th></th>
<th>Surface components</th>
<th>Core lipids</th>
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</thead>
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<tr>
<td></td>
<td>Cholesterol</td>
<td>PL</td>
</tr>
<tr>
<td>CM</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>VLDL</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>LDL</td>
<td>8</td>
<td>22</td>
</tr>
<tr>
<td>HDL</td>
<td>4</td>
<td>35</td>
</tr>
</tbody>
</table>

Surface components and core lipids are given as percentage of dry mass (% grams). Apo, apolipoprotein; CE, cholesteryl ester; CM, chylomicron; PL, phospholipid; TG, triglyceride. Adapted from Havel and Kane (1995).
occurring in the plasma. LDL are derived from the metabolic products of VLDL while the components of HDL are secreted with chylomicrons and VLDL. Some components of HDL are also secreted independently as HDL precursors (Hamilton et al., 1991).

1.1.2 Structure of lipoproteins

Lipoproteins are composed of two major components, lipids and proteins. A typical lipoprotein particle is spherical and consists of a polar lipid monolayer surrounding a core of nonpolar lipids. The surface monolayer consists primarily of phospholipids, unesterified cholesterol, and proteins. These proteins include albumin and apolipoproteins. Apolipoproteins solubilize the lipoprotein and enable its transport via the circulatory system. The central core of lipoprotein particles consists primarily of triglycerides and cholesteryl esters (Gotto et al., 1986).

Phospholipids are amphipathic, possessing polar and nonpolar regions. The polar or hydrophilic head of the phospholipid is exposed to the aqueous environment (plasma) and the nonpolar, hydrophobic tail faces the central core of the lipoprotein. Like the phospholipids, the protein components of the surface monolayer have amphipathic characteristics. These amphipathic properties result from regions containing both polar and nonpolar amino acid residues (Gotto et al., 1986). The fatty acyl chains of phospholipids and the nonpolar amino acid side chains of proteins are excluded from the aqueous environment by their hydrophobicity. These hydrophobic forces generate the association of polar lipids
and proteins with lipoproteins.

1.1.3 Apolipoproteins

Most of the apolipoproteins, except apolipoprotein B, are relatively soluble in an aqueous environment. These water soluble apolipoproteins are able to exchange readily between lipoprotein particles as well as with other lipid surfaces. The exchange of phospholipids and nonpolar lipids among lipoproteins requires the presence of specific transfer proteins (Fielding, 1990). Table 3 lists the apolipoproteins and their distribution in lipoproteins. From Table 3, it is clear that the major apolipoprotein associated with LDL is apo B-100 while apo E is associated primarily with HDL and to a lesser extent VLDL and LDL.

1.2 Receptor-mediated uptake of lipoproteins

1.2.1 LDL receptor

The catabolism of apolipoprotein B-containing lipoproteins occurs by receptor-mediated endocytosis (Havel and Hamilton, 1988). The LDL receptor is a transmembrane protein that recognizes the apo B-100 on lipoprotein surfaces, which includes partially catabolized VLDL and LDL. LDL receptor also binds to lipoproteins containing apo E. Once bound to the LDL receptor the lipoproteins are endocytosed via coated-pits on the plasma membrane. The lipoproteins dissociate from the receptor in the acidic environment of endosomes, allowing the LDL receptor to return to the cell surface for reuse.
Table 3. Distribution of plasma apolipoproteins in normal humans.

<table>
<thead>
<tr>
<th>Apo</th>
<th>HDL</th>
<th>LDL</th>
<th>VLDL</th>
<th>CM</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>&gt;99</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>29.0</td>
</tr>
<tr>
<td>A-II</td>
<td>&gt;99</td>
<td>-</td>
<td>-</td>
<td>&lt;1</td>
<td>17.4</td>
</tr>
<tr>
<td>B-48</td>
<td>-</td>
<td>-</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td>241.0</td>
</tr>
<tr>
<td>B-100</td>
<td>-</td>
<td>88</td>
<td>6</td>
<td>-</td>
<td>512.7</td>
</tr>
<tr>
<td>C-I</td>
<td>97</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>6.6</td>
</tr>
<tr>
<td>C-II</td>
<td>60</td>
<td>-</td>
<td>30</td>
<td>10</td>
<td>8.9</td>
</tr>
<tr>
<td>C-III</td>
<td>60</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>8.8</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19.0</td>
</tr>
<tr>
<td>E-II</td>
<td>-</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>34.1</td>
</tr>
</tbody>
</table>

apo, apolipoprotein; MW, molecular weight. Adapted from Havel and Kane (1995) and Gotto et al., 1986.
The amount of LDL receptor is regulated by the concentration of intracellular cholesterol. High levels of cholesterol lead to the down-regulation of LDL receptor while low levels of cholesterol increase LDL receptor synthesis (Brown and Goldstein, 1975). The liver is the principal site of LDL removal from the blood (Goldstein and Brown, 1987).

The apo E binding domain of nascent chylomicrons is unavailable for binding to LDL receptor (Havel and Kane, 1995). However, as chylomicrons are metabolized to chylomicron remnants, the apo E binding domain becomes available for interaction with LDL receptor. The apo B and E binding domains are also unavailable in nascent VLDL. The apo B and E binding domains gradually become exposed during lipolysis. The LDL receptor has a much lower affinity for apo B-100 than for apo E (Havel and Hamiliton, 1988).

1.2.2 LDL-receptor related protein (LRP)

Some studies have suggested that the LDL-receptor related protein (LRP) is a chylomicron remnant receptor (Kowal et al., 1989). LRP is thought to bind apo E but not apo B-48. VLDL receptor is a member of the LRP family. The VLDL receptor specifically recognizes apo E as its ligand. It has recently been shown that VLDL receptor is able to mediate chylomicron remnant uptake (Niemeir et al., 1996).

1.2.3 Scavenger receptor

Scavenger receptors take up lipoproteins whose protein components have
been modified, such as oxidized LDL. Scavenger receptor mediates endocytosis and lysosomal catabolism of modified lipoproteins (Kowal et al., 1989). The scavenger receptor is found primarily on macrophages (Beisiegel and St. Clair, 1996).

1.3 LECITHIN:CHOLESTEROL ACYLTRANSFERASE

Lecithin:cholesterol acyltransferase (LCAT) (EC 2.3.1.43) catalyzes the transfer of a fatty acid from the sn2 position of lecithin (phosphatidylcholine) to the 3-hydroxyl group of cholesterol, forming lysophosphatidylcholine and cholesteryl ester in plasma (Figure 1) (Glomset and Wright, 1964). Apo A-I is an important cofactor in this LCAT reaction (Jonas, 1991). The LCAT gene consists of 6 exons and 5 introns and has been localized to chromosome 16 (Kuivenhoven et al., 1997).

LCAT is a glycoprotein of approximately 63 kDa that is synthesized and secreted by the liver. 25% of the molecular mass of LCAT is due to carbohydrate (Marcel, 1982). LCAT circulates in the blood primarily bound to HDL and is responsible for esterifying cholesterol in plasma. Normal LCAT exhibits two activities in plasma. α-LCAT activity is specific for HDL while β-LCAT activity is specific for LDL and to a smaller extent VLDL (Carlson and Holmquist, 1985; Funke et al., 1991). The preferred substrate of LCAT is HDL.
Figure 1. The LCAT reaction. Lecithin:cholesterol acyltransferase (LCAT) catalyzes the transfer of a fatty acid from the \( sn_2 \) position of phosphatidylcholine to the 3-hydroxyl group of cholesterol. Apo A-I is an important cofactor in this reaction. This reaction produces lysophosphatidylcholine and cholesteryl ester.
Less than 10% of LCAT activity is associated with LDL (Kuivenhoven et al., 1997).

Apo A-I is the most effective cofactor in promoting LCAT activity. Apo A-II, A-IV, C-II and C-III can also, to a lesser extent, stimulate this reaction (Jonas, 1991; O and Frohlich, 1995).

Determination of LCAT activity can be approached using proteoliposomes (Dobiasova and Schutzova, 1986; O and Frohlich, 1995). LCAT activity can be measured with proteoliposomes containing unesterified cholesterol, phosphatidylcholine, and apo A-I. Assaying for either the decrease in unesterified cholesterol or the increase in free fatty acids will give a measure of the esterase and phospholipase activity of LCAT, respectively (O and Frohlich, 1995; O et al., 1993).

Reverse cholesterol transport is the system by which excess extrahepatic cholesterol is transported to the liver (Fielding, 1990). The HDL cholesteryl esters produced by the LCAT reaction are transferred to LDL and VLDL. LDL and VLDL are in turn taken up by the liver, completing the transport of excess extrahepatic cholesterol to the liver. This transfer of cholesterol esterified by LCAT from HDL to the lower density lipoproteins requires the presence of cholesteryl ester transfer protein (CETP). LCAT, HDL and CETP are believed to be vital in reverse cholesterol transport (Havel and Kane, 1995).
1.4 FAMILIAL LCAT DEFICIENCY

1.4.1 Genetic basis of LCAT deficiency

Familial LCAT deficiency is an autosomal recessive disorder resulting from various point mutations in the LCAT gene. Thirty-five mutations dispersed among the six exons of the LCAT gene have been identified (Kuivenhoven et al., 1997). Recently, a mutation in intron 4 of the LCAT gene has also been reported (Kuivenhoven et al., 1996). These mutations lead either to the expression of inactive LCAT or to the absence of LCAT protein.

Mutations in the LCAT gene that only lead to a deficiency in α-LCAT activity (associated with HDL) gives rise to Fish Eye Disease (FED), or partial LCAT deficiency. Familial LCAT deficiency results from the absence of both α-LCAT and β-LCAT activities (Kuivenhoven et al., 1997).

1.4.2 Lipoprotein-X

One unique feature of LCAT deficiency is the presence of an abnormal plasma lipoprotein, lipoprotein-X (Lp-X), in patients’ plasma. The total concentration of Lp-X in the plasma of patients is 0.3 - 1.5 mmol/L (Glomset et al., 1995). Lp-X is also present in the plasma of patients with cholestatic liver disease (Hamilton et al., 1971) and is associated with the LDL fraction (1.190 mg/mL < d < 1.063 g/mL) upon ultracentrifugation. In both of these diseases Lp-X appears to have an identical structure and chemical composition (Seidel et al., 1974).

In LCAT deficiency, Lp-X arises from the surface of chylomicron remnants
that are not further catabolized due to the absence of active LCAT (Sabesin, 1982). In cholestatic liver disease, Lp-X is thought to originate from the large amounts of bile cholesterol and phosphatidylcholine present in the plasma due to the obstruction of the bile ducts (Miller, 1990; Sabesin, 1982).

Unlike normal plasma lipoproteins, Lp-X is a bilayer vesicle and has a diameter of 30 to 70 nm (Miller, 1990; Sabesin, 1982; Seidel et al., 1974). Another unique feature of Lp-X is its high concentration of unesterified cholesterol (30% w/w) and phospholipid (60% w/w) (Miller, 1990; Sabesin, 1982; Seidel et al., 1974). Phosphatidylcholine is the major phospholipid in Lp-X while sphingomyelin comprises the minor fraction. Cholesteryl esters (2% w/w), triglycerides (2% w/w), and proteins (6% w/w) are the remaining constituents of this particle (Miller, 1990; Sabesin, 1982; Seidel et al., 1974). The major constituents of the protein fraction are albumin (40%) and the C apolipoproteins (60%). Lp-X also contains small amounts of apo E (O and Frohlich, 1995; Miller, 1990; Sabesin, 1982; Seidel et al., 1974).

The cholesteryl esters, triglycerides, and albumin form the core of Lp-X while the apolipoproteins, phospholipids, and cholesterol are on its surface. A third unique feature of Lp-X is its cathodic migration on agarose gel electrophoresis (Sabesin, 1982). Normal plasma lipoproteins have an anodic migration. Unlike the other lipoproteins that are taken up by hepatocytes, Lp-X is mainly taken up by the reticuloendothelial system (Walli and Seidel, 1984).
1.4.3 Clinical symptoms in LCAT deficiency

Clinical abnormalities in familial LCAT deficiency include corneal opacities, anemia, and proteinuria (Glomset et al., 1995).

1.4.3.1 Corneal opacities

Corneal opacities are present in all patients with LCAT deficiency since early childhood. Corneal opacities consist of grayish dots in the corneal stroma, giving the cornea a cloudy or misty appearance. While the material making up the dots has yet to be determined, ultrastructural examination of sections obtained by superficial keratectomy revealed the presence of numerous vacuoles (Bron et al., 1975; Bethell et al., 1975). Many of these vacuoles contained membranous deposits. Although the presence of excess lipids has not been verified chemically or morphologically, crystals that may be cholesterol have been visualized in the cornea. Examination of the cornea obtained from a patient revealed excess unesterified cholesterol and phospholipid (Glomset et al., 1995).

1.4.3.2 Hemolytic anemia

Hematological data and bone marrow studies have indicated that moderate hemolysis in addition to low compensatory erythropoiesis leads to the anemia seen in LCAT deficient patients (Funke et al., 1991). Studies have shown that the half-life of erythrocytes in patients is greatly reduced (16 - 17 days versus 23 - 35 days in normal subjects) (Glomset et al., 1995). Erythrocytes in LCAT deficiency have an abnormal appearance and lipid composition (Gjone et
analyses of whole erythrocytes in some patients showed nearly twice
the normal amount of unesterified cholesterol and phosphatidylcholine. Such
abnormalities may contribute to hemolytic anemia. Patients had normal amounts
of total phospholipid (Murayama et al., 1984).

1.4.3.3 Proteinuria

LCAT deficient patients frequently present with proteinuria. Proteinuria is
usually detected early in life (20 - 30 years of age) and remains moderate (0.5 to
1.5 mg protein per milliliter urine) for many years (Gjone, 1974). However,
proteinuria increases in severity as renal function deteriorates. Patients' urine
may contain protein, hyaline casts and erythrocytes. The level of albumin in
patients' serum decreases considerably with the onset of renal failure (Glomset et
al., 1995).

The majority of patients develop progressive glomerulosclerosis, a
potentially life threatening complication. Light-microscopy of renal biopsies has
revealed the presence of foam cells in glomerular tufts of affected glomeruli
(Stokke et al., 1974). Arterioles have thickened intimas and exhibit a narrowing
of the lumens (Hovig and Gjone, 1973). Deposits of lipid material have been
found in the renal arteries and arterioles. Lipid accumulated in glomeruli
consisted mainly of unesterified cholesterol and phosphatidylcholine (Table 4). In
Table 4 it can be seen that the cortex and glomeruli in the kidneys of some LCAT
deficient patients have a higher ratio (0.7 and 0.9, respectively) of unesterified
cholesterol to phosphatidylcholine relative to control (0.5 and 0.6, respectively).
Electron microscopy of renal biopsies revealed capillary lumens that were partly filled with a meshwork of membranes (Magil et al., 1982). Capillary walls appeared abnormal and endothelial cells were often missing. The basal lamina had an irregular thickness, endothelial foot processes were fused, and membrane-surrounded particles were present in both the subendothelial and subepithelial regions. Immunofluorescence has identified complement (C3) deposits in the mesangium (Imbasciata et al., 1986).

1.4.3.4 Coronary artery disease and LCAT deficiency

In the past an increased risk of coronary artery disease (CAD) was believed to be nonexistent in familial LCAT deficiency (Kuivenhoven et al., 1996; Glomset et al., 1995). While there were only a few cases of CAD in these patients, calcification in the aorta had been demonstrated and postmortem examinations had revealed atherosclerosis of the aorta and large arteries (Gjone, 1974; Stokke et al., 1974).

Renal arteries and arterioles had also shown early atherosclerotic changes. Histological examinations revealed fibrosis and hyalinization of renal arterial walls accompanied by marked narrowing of the vessel lumens (Hovig and Gjone, 1973). Studies using electron microscopy of sections from renal and iliac arteries and from the aorta showed the presence of lipid-like deposits. These deposits were present in the different layers of the vessel walls. Foam cells were found in the lipid-like deposits and there was smooth muscle cell proliferation in
Table 4. Lipid Concentrations in the kidneys of LCAT deficient patients.

<table>
<thead>
<tr>
<th>Kidneys</th>
<th>Concentration (nmol lipid/mg protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>UC</td>
</tr>
<tr>
<td>Cortex, control</td>
<td>50</td>
</tr>
<tr>
<td>Cortex, patient</td>
<td>170</td>
</tr>
<tr>
<td>Glomeruli, control</td>
<td>57</td>
</tr>
<tr>
<td>Glomeruli, patient</td>
<td>416</td>
</tr>
</tbody>
</table>

UC, unesterified cholesterol; PL, total phospholipid; TG, triglyceride. Adapted from Glomset et al., (1995).
the intimal layer (Glomset et al., 1995). Lipid analysis of an atheroma from a renal artery showed that only 35% of the total cholesterol was esterified, in contrast to 75% found in patients who died from other causes (Smith, 1965).

Recently, a case has been reported where a 53 year-old man with partial LCAT deficiency (FED) presented with premature coronary artery disease (CAD) in the absence of other risk factors (Kuivenhoven et al., 1996). FED patients lack α-LCAT activity but still retain β-LCAT activity (Carlson and Holmquist, 1985). This finding puts in doubt the belief that there is no increased risk of atherosclerosis in LCAT deficiency and FED (Kuivenhoven et al., 1996). Kuivenhoven et al. speculate that inactive or partially active LCAT increases the risk for atherosclerosis. This would support the protective role of active LCAT against CAD via its action in reverse cholesterol transport. However, no conclusive statement can be made regarding FED or LCAT deficiency and the risk of CAD until more cases are identified.

1.5 GLOMERULOSCLEROSIS

A major complication of LCAT deficiency is glomerulosclerosis, which can lead to renal failure. The mechanisms by which LCAT deficiency leads to glomerulosclerosis are still unclear. Some researchers have suggested that glomerulosclerosis and atherosclerosis may share a similar pathology (Diamond and Karnovsky, 1988). Arterial intima becomes thickened in atherosclerosis and contains vascular smooth muscle cells (SMC), macrophage foam cells, collagen fibers, and glycosaminoglycans, eventually leading to necrosis. In
glomerulosclerosis there is mesangial matrix expansion, mesangial cell proliferation, macrophage infiltration, and the presence of foam cells (Diamond and Karnovsky, 1988).

1.5.1 Glomerular mesangial cells

Glomerular mesangial cells have several functions. They provide structural support for capillary loops, modulate glomerular filtration by its smooth muscle cell activity, and generate vasoactive agents (Schlondorff, 1987). Mesangial cells are also able to synthesize and break down structural elements such as collagen, produce various growth factors and cytokines, endocytose macromolecules (including immune complexes and lipoproteins), and modulate glomerular injury through cell proliferation and production of extracellular matrix (Schlondorff, 1987).

The analogy between glomerulosclerosis and atherosclerosis is largely based on the origin and functional properties of the glomerular mesangial cell. There are two distinct functional types of mesangial cells. 5 - 10% of mesangial cells are bone-marrow derived and are responsible for the phagocytosis of macromolecules entering the mesangium. The second type of mesangial cell is smooth muscle cell-like and shares many similarities with vascular smooth muscle cells (Schlondorff, 1987).

1.5.2 Macrophages

The mechanisms by which macrophage infiltration and the presence of
foam cells may cause renal damage are as yet not fully understood. Glomerular macrophages are a locally activated mononuclear cell population which produces reactive oxygen species that may contribute to macrophage-mediated glomerular injury (Van Goor et al., 1994). Macrophages are a rich source of cytokines that modulate mesangial cell proliferation and extracellular matrix expansion (Van Goor et al., 1994).

Renal biopsies of LCAT deficient patients revealed macrophage infiltration and the presence of foam cells in affected glomeruli. Foam cells have an important pathogenic role in atherosclerosis. If glomerulosclerosis is analogous to atherosclerosis, then foam cells may also have an important role in the development of glomerulosclerosis in these patients.

1.5.3 Lipid accumulation

Observations in experimental animals and in patients with genetically determined and acquired hyperlipidemias suggest that lipids can damage the kidney and hence lead to glomerulosclerosis (Walli et al., 1993). There are strong indications that lipoproteins play a vital role in mesangial cell damage and in the development of progressive renal disease. It has been reported that lipid accumulation in perfused rat kidney was caused by the direct deposition of lipoprotein-X (O et al., 1997). Mesangial cells are able to take up LDL and apo E-containing lipoproteins (VLDL) through receptor-mediated pathways. It has been suggested that rat mesangial cells possess both LDL receptor and scavenger receptor (Coritsidis et al., 1991).
1.5.4 Extracellular matrix expansion

In the normal kidney, collagens IV, V, and laminin have been localized to the basement membrane of the glomerular tuft, Bowman's capsule, and in the mesangium (Striker et al., 1984). Fibronectin has been localized to the basement membrane region and the mesangium. Striker et al. (1984) studied the composition of the extracellular matrix in glomerulosclerosis. In this study they found that the glomerular basement membranes and mesangial areas within glomeruli principally consisted of collagen IV. They also found significant scleroses, multi-lamination of Bowman's capsule basement membranes, and periglomerular fibrosis. The sclerotic mass consisted of interstitial collagen (type III), as opposed to type IV.

Glomerulosclerosis in LCAT deficiency is histologically characterized by the deposition of lipids, especially unesterified cholesterol and phosphatidylcholine, in the glomerular membrane. It is also characterized by the expansion of extracellular matrix, infiltration of macrophages, the presence of foam cells, and mesangial cell proliferation (Diamond and Karnovsky, 1988).

1.6 RATIONALE OF THE STUDY

Patients with familial LCAT deficiency experience corneal opacities, anemia and proteinuria (Glomset et al., 1995; Frohlich and Pritchard, 1992). Proteinuria progresses dramatically as renal function deteriorates. Most of these patients develop glomerulosclerosis. The pathogenesis of glomerulosclerosis
may involve multiple factors. The deposition of lipids, foam cell formation, proliferation of mesangial cells, and extracellular matrix expansion could contribute to the development of glomerulosclerosis.

1.7 HYPOTHESES AND OBJECTIVE

Lipoprotein-X has high levels of unesterified cholesterol and phosphatidylcholine. These two lipids are also elevated in affected glomeruli. Hence, Lp-X may play a role in the accumulation of lipids in the kidneys of these patients. The objective of this project is to examine the uptake and metabolism of lipoprotein-X in mesangial cells and to study its effect on mesangial cell proliferation.

1.8 SPECIFIC AIMS

• To examine the uptake of Lp-X by mesangial cells.

• To examine the metabolic fate of Lp-X lipids.

• To determine whether Lp-X is able to induce foam cell formation.

• To examine the effect of Lp-X and macrophages on mesangial cell proliferation.
2. MATERIALS and METHODS

2.1 TISSUE CULTURE

2.1.1 Isolation and culture of rat mesangial cells

Mesangial cells were isolated from rat kidney glomeruli by a differential sieving technique and collagenase digestion (Harper et al., 1984). Sprague-Dawley rats (350 +/- 25 g) were anesthetized with halothane. The kidneys were then removed and glomeruli isolated as follows. First, any adhering tissue was removed from the kidney, followed by the removal of the cortices from the medulla. The cortical portion was minced into millimeter square pieces and passed through a series of stainless steel sieves with decreasing pore sizes (200 μ-pore, 150 μ-pore and 75 μ-pore). The resulting glomeruli remained on top of the 75 μ-pore sieve and were stripped of their capsules as well as being 100% free of tubular tissue (Kreisberg et al., 1978). Isolated glomeruli were then resuspended with 10 mL Hank’s Buffered Saline Solution (HBSS) containing 1% antibiotic-antimycotic and washed twice. Glomeruli were then incubated in 10 mL 0.2% trypsin-1% antibiotic-antimycotic in HBSS at 37°C. After twenty minutes the glomeruli were centrifuged at 1,000 rpm for one minute. The trypsin was then removed and the glomeruli resuspended in 0.1% collagenase-1% antibiotic-antimycotic in HBSS and incubated at 37°C for 40 minutes. The glomeruli were then washed and cultured in 10 mL RPMI-1640 (containing 20% heat-inactivated fetal bovine serum (FBS), 1% antibiotic-antimycotic, 5 μg/mL bovine insulin, 5 μg/mL human transferrin, and 5 ng/mL sodium selenite) at 37°C. Media was changed every two to three days. For experiments only cells between the fifth
and eleventh passages were used.

The homogeneity of the cell culture was confirmed by immunofluorescence staining (Harper et al., 1984), which was kindly performed by Dr. Alexander Magil, and by the addition of the epithelial cytotoxin puromycin to the culture medium. Immunofluorescent staining was negative for cytokeratin (rat leukocyte antigen) and positive for vimentin and smooth muscle cell specific actin. Unlike epithelial cells, mesangial cells are resistant to puromycin (100 μg/mL). Mesangial cells comprised > 99% of the cell culture. Cell viability was greater than 95% when tested with trypan blue.

2.1.2 Isolation and culture of rat peritoneal macrophages

RPMI-1640 medium, containing 10% FBS and 1% antibiotic-antimycotic, was injected (15 mL) into the peritoneal cavity of Sprague-Dawley rats (350 ± 25 g) after cervical dislocation. The abdomen was massaged for five minutes and the medium removed using a hypodermic needle. The medium was then centrifuged at 1,000 rpm for 10 minutes to pellet the cells. After centrifugation, the cells were resuspended and the cell density was determined using light microscopy and a hemocytometer. Macrophages were plated at a density of 1 - 6 x10^6 cells/35mm dish and incubated at 37°C for 2 h. Within the first 2 h of incubation macrophages are able to attach to the culture dish. Washing and changing the medium after 2 h removed any other cell types that may have been present (Adams, 1979). Fresh culture medium was then added and macrophages were used immediately for experiments.
Macrophage-conditioned medium was prepared by incubating rat peritoneal macrophages in the presence or absence of reconstituted Lp-X (rLp-X) for 24 h at 37°C. The macrophage-conditioned medium was collected and used immediately for experiments.

2.2 Preparation of plasma lipoproteins

2.2.1 Isolation of Lipoprotein-X

All procedures and methods were approved by the University Ethical Review Committee.

There is no difference between the composition and properties of lipoprotein-X isolated from familial LCAT deficiency and cholestatic liver disease (Seidel et al., 1974). Since cholestatic liver disease is more common than familial LCAT deficiency, sera were collected from patients with cholestatic liver disease (O and Frohlich, 1995). Lp-X positive sera were identified by their cathodic migration on 1% agarose gel (Figure 2).

Lp-X was prepared by ultracentrifugation and column chromatography. In brief, a fraction with a density of 1.019 - 1.063 g/mL was prepared by sequential ultracentrifugation (Sabesin, 1982; Miller, 1990). The Lp-X present in the 1.019 < d < 1.063 g/mL fraction was separated from LDL by gel filtration chromatography, using a matrix of Superose 6B beads (Pharmacia). Lp-X was eluted off the column as a distinct peak using a buffer of 0.15 M NaCl / 10 mM Tris-HCl, pH 7.4. The peak was identified by measuring absorbency at 280 nm. The purity of Lp-X was determined by the mobility of the isolated Lp-X
Figure 2. Lipoprotein electrophoresis. Samples were loaded on a 1% agarose gel and electrophoresed for 35 minutes. The lipoproteins were stained with Sudan Black. The arrows indicate the position of individual lipoproteins. Lane 1, sera from controls; Lane 2, pooled sera from obstructive jaundice patients; Lane 3, LDL isolated from normal serum (lane 3). From O and Frohlich, 1995.
on 1% agarose gel, as well as by the ratio (>95%) of total cholesterol to unesterified cholesterol. Free and total cholesterol were measured enzymatically using commercially available kits (Boehringer-Mannheim).

2.2.2 Preparation of radiolabeled Lp-X

For experiments requiring radiolabeling, Lp-X was equilibrated with $[^3]H$cholesterol- (0.05 μCi/nmol) or dipalmitoyl phosphatidyl-[Me-3H]choline (0.05 μCi/nmol)-coated filter disks at 4°C overnight. This method has been used successfully to label Lp-X and other lipoproteins by our laboratory as well as other investigators (O and Frohlich, 1995; O et al., 1993; Dobiasova and Schutzova, 1986). Various concentrations of labeled Lp-X were added directly to the culture medium.

2.3 PREPARATION OF RECONSTITUTED LIPOPROTEIN-X (rLp-X)

2.3.1 Preparation of rLp-X

rLp-X consisted of a 1:1 molar ratio of phosphatidylcholine (PC) and cholesterol, which is similar to the ratio found in Lp-X. To prepare rLp-X the lipids were dissolved and mixed in chloroform and then dried under nitrogen gas. Dried lipids were resuspended with 250 μL absolute ethanol and rapidly injected into 30 mL HBSS while vortexing. The preparation was concentrated to 4 mL with an Amicon stirred ultrafiltration cell using a YM-100 membrane. The free cholesterol content of the rLp-X preparation was determined by commercially available enzymatic kits.
2.3.2 Preparation of radiolabeled rLp-X

rLp-X was labeled with $[^3\text{H}]$cholesterol (0.05 $\mu\text{Ci/nmol}$), dipalmitoyl phosphatidyl-[Me-$[^3\text{H}]$]choline (0.05 $\mu\text{Ci/nmol}$), or $[^3\text{H}]$cholesteryl hexadecyl ether (0.3 $\mu\text{Ci/nmol}$). Radiolabeled rLp-X are referred to as $[^3\text{H}]$FC rLp-X ([$^3\text{H}$]cholesterol-labeled rLp-X), $[^3\text{H}]$PC rLp-X (dipalmitoyl phosphatidyl-[Me-$[^3\text{H}]$]choline-labeled rLp-X) and $[^3\text{H}]$CHE rLpX ([$^3\text{H}$]cholesteryl hexadecyl ether-labeled rLp-X), respectively.

2.4 UPTAKE AND METABOLISM OF LIPOPROTEIN-X (LP-X) BY MESANGIAL CELLS

2.4.1 Uptake of Lp-X and rLp-X by mesangial cells

Mesangial cells were rendered quiescent by overnight preincubation in serum-free RPMI-1640. Cells were pulse-labeled with either $[^3\text{H}]$FC- or $[^3\text{H}]$PC-labeled Lp-X or rLp-X. After incubation the cells were washed with phosphate buffered saline (PBS) containing 1% albumin, followed by two washes with PBS. The cells were lysed in 1 mL 0.2 N NaOH and then neutralized with glacial acetic acid. The uptake of Lp-X was determined by measuring the radioactivity associated with cells.

2.4.2 Metabolism of lipids by mesangial cells

The metabolism of the cholesterol and phosphatidylcholine in Lp-X in mesangial cells was also measured. Quiescent mesangial cells were incubated with $[^3\text{H}]$cholesterol-labeled Lp-X (0.05 $\mu\text{Ci/nmol}$) or dipalmitoyl phosphatidyl-[Me-
\(^3\)H]choline-labeled Lp-X (0.05 \(\mu\)Ci/nmol). After incubation, aliquots of cell lysates were used for lipid extraction. Cellular lipids were extracted by a solvent containing chloroform:methanol:water (4:2:3, v/v/v) (O et al., 1993; Wheeler et al., 1990), followed by centrifugation for ten minutes at 175 x g. After centrifugation the organic phase (lower layer) was carefully removed and then dried under nitrogen gas.

The dried lipids were resuspended in chloroform. Free cholesterol and cholesteryl ester, or phosphatidylcholine and lysophosphatidylcholine were separated by thin layer chromatography (O et al., 1997). Radioactivity associated with individual lipids was determined using liquid scintillation counting.

2.4.3 Effects of cytochalasin, suramin, and polyinosinic acid on the uptake of rLp-X

Quiescent cells were pretreated with cytochalasin D and polyinosinic acid overnight and with suramin for two hours. After pretreatment, media was removed and fresh media with the corresponding agents (cytochalasin D, polyinosinic acid, suramin) were added in addition to radiolabeled Lp-X. After incubation, the medium was removed and the cells washed with 2 mL PBS containing 1% BSA followed by two more washes with 2 mL PBS. The cells were lysed in 1 mL 0.2 N NaOH. The lysate was then neutralized with 10.5 \(\mu\)L glacial acetic acid. Radioactivity associated with cells was measured by liquid scintillation counting.
2.4.4 Effect of Lp-X and rLp-X on mesangial cell proliferation

The incorporation of \[^{3}\text{H}]\text{thymidine}\) into cellular DNA was used to determine the effect of lipoprotein-X on mesangial cell proliferation (Gupta et al., 1992). Quiescent mesangial cells were pulse-labeled with \[^{3}\text{H}]\text{thymidine}\) (1 μCi/mL) at 37°C in the presence or absence of Lp-X or rLp-X for different time periods. After incubation, the medium was removed and the cells washed three times with 2 mL PBS. The cells were lysed in 1 mL 0.2 N NaOH. The lysate was then neutralized with glacial acetic acid. The radioactivity associated with cellular DNA was measured by liquid scintillation counting.

2.5 EFFECT OF RECONSTITUTED LIPOPROTEIN-X ON MACROPHAGES

2.5.1 Lipid analyses

Rat peritoneal macrophages were incubated in the presence or absence of rLp-X for 24 h at 37°C. After incubation, the medium was removed and the cells were washed three times with 2 mL PBS. The cells were then lysed in 1 mL 0.2 N NaOH. After neutralization with acetic acid, cellular lipids were extracted as described above. Total cholesterol and free cholesterol were assayed using enzymatic kits.

2.5.2 Oil Red O (ORO) staining

Macrophages cultured on cover-slips were stained with Oil Red O (ORO) after 24 h incubation in the presence or absence of rLp-X (Luna, 1968). Cover-slips were first fixed in 10% neutral formalin for 15 minutes. They were then
rinsed in distilled water and left in 100% propylene glycol for 2 minutes followed by staining in ORO for 10 minutes. Macrophages were then treated in 60% propylene glycol for 1 minute and rinsed in distilled water. The macrophages were examined using oil immersion and light microscopy. Macrophages with 10 or more lipid droplets were identified as foam cells.

2.6 EFFECT OF RECONSTITUTED LIPOPROTEIN-X AND MACROPHAGES ON MESANGIAL CELL PROLIFERATION

The combined effect of rLp-X and macrophages on mesangial cell proliferation was also determined by measuring $[^3H]$thymidine incorporation (1 $\mu$Ci/mL). Quiescent mesangial cells were cultured in macrophage-conditioned medium in the presence or absence of rLp-X (100 - 400 nmol FC/mL). After incubation the medium was removed and the cells washed three times with 2 mL PBS. The cells were then lysed in 1 mL 0.2 N NaOH. After neutralization with acetic acid, the radioactivity associated with the cellular DNA was determined by liquid scintillation counting.
3. RESULTS

3.1 UPTAKE AND METABOLISM OF LIPOPROTEIN-X BY MESANGIAL

CELLS

3.1.1 Uptake of Lp-X

The uptake of Lp-X by rat mesangial cells was studied by incubating cells with either \[^3^H\]FC- or \[^3^H\]PC- labeled Lp-X for 10 hours. Cells were washed once with PBS containing 1% BSA followed by two additional washes with PBS before measuring the radioactivity associated with cells. As seen in Figure 3, uptake of labeled Lp-X was in a concentration dependent manner. The uptake of radiolabeled Lp-X appeared to reach a plateau at 75 - 125 nmol cholesterol/mL Lp-X.

3.1.2 Metabolism of Lp-X lipids

The metabolic fate of the lipids in Lp-X was examined in Figure 4. Mesangial cells were incubated with \[^3^H\]FC- or \[^3^H\]PC-labeled Lp-X at concentrations of 65 nmol Lp-X cholesterol/mL and 110 nmol Lp-X cholesterol/mL. It is evident from these experiments that the cholesterol in Lp-X is esterified by mesangial cells. In addition, phosphatidylcholine in Lp-X was metabolized to lysophosphatidylcholine. When treated with high levels (110 nmol/mL) of Lp-X, the cells showed a significant increase in their uptake of unesterified cholesterol and phosphatidylcholine, relative to the lower concentration (65 nmol/mL) of Lp-X. This increased uptake most likely
Figure 3. The uptake of Lp-X by rat mesangial cells. Mesangial cells were pulse-labeled with various amounts of $[^3$H]cholesterol or dipalmitoyl phosphatidyl-[Me-$^3$H]choline labeled Lp-X. After 10 h incubation the radioactivity associated with cells was determined as described in Materials and Methods. Preliminary experiments have shown that 10 h incubation is within the linear range of Lp-X uptake (data not shown). Results are expressed as the mean ± standard deviation from five separate experiments.
Figure 4. The metabolism of Lp-X by rat mesangial cells. Mesangial cells were incubated with $[^3]$Hcholesterol labeled Lp-X (65 nmol/mL or 110 nmol cholesterol/mL). After 10 h cellular lipids were extracted and the radioactivity associated with the unesterified (free) cholesterol (FC) and cholesteryl ester (CE) was determined. In separate experiments, cells were incubated with phosphatidyl-[Me-$^3$H]choline labeled Lp-X (65 nmol cholesterol/mL or 110 nmol cholesterol/mL). After 10 h, the radioactivity associated with phosphatidylcholine and lysophosphatidylcholine was determined. Results obtained from five separate experiments incubated with 65 nmol/mL Lp-X are expressed as 100 percentile and depicted as mean ± standard deviation.
contributed to the significant increase in cholesteryl ester and lysophosphatidylcholine.

3.1.3 Uptake and metabolism of rLp-X

3.1.3.1 Uptake of rLp-X

In order to demonstrate that Lp-X lipids were indeed entering the cell (and not just simply exchanging with plasma membrane lipids) rLp-X labeled with \[^{3}H\]cholesteryl hexadecyl ether (CHE) was prepared. \[^{3}H\]CHE has been shown to remain inside liposomes and does not readily exchange with membrane lipids (Bally et al., 1990). Additionally, CHE is not subject to esterase activity once it is internalized by the cell (Bally et al., 1990). Figure 5 (Panel A) shows that the uptake of \[^{3}H\]CHE rLp-X was time dependent. Uptake of \[^{3}H\]CHE rLp-X (100 nmol cholesterol/mL) began to level off after 20 h incubation. As shown in Figure 5 (Panel B), the uptake of \[^{3}H\]CHE rLp-X by mesangial cells was concentration dependent. These results suggest that the uptake of Lp-X lipids was not facilitated by a simple exchange mechanism.

The ability of mesangial cells to take up and metabolize lipids in rLp-X was compared to the uptake and metabolism of Lp-X lipids. In Figure 6 cells were treated with rLp-X, labeled with either \[^{3}H\]FC or \[^{3}H\]PC, and their uptake by mesangial cells was examined. Like Lp-X (Figure 3), the uptake of labeled rLp-X was concentration dependent. However, at the concentrations tested the uptake of rLp-X does not appear to be saturable (Figure 6).
Figure 5. The uptake of $[^3]$Hcholesteryl hexadecyl ether (CHE) rLP-X by rat mesangial cells. Cells were incubated with $[^3]$HCHE rLP-X for various time periods (100 nmol cholesterol/mL) (Panel A) or various concentrations (Panel B). After incubation, the radioactivity associated with the cells was determined. Results are expressed as DPM per mg of cellular proteins and are depicted as the as mean ± standard deviation from five separate experiments.
Figure 6. The uptake of $[^3\text{H}]$FC or $[^3\text{H}]$PC rLp-X by rat mesangial cells. Mesangial cells were incubated with various amounts of $[^3\text{H}]$cholesterol or dipalmitoyl phosphatidyl-$[\text{Me-}^3\text{H}]$choline rLp-X. After 10 h, the radioactivity associated with the cells was determined. The uptake of rLp-X is expressed as DPM per mg of cellular proteins. Each point represents the mean ± standard deviation from five separate experiments.
3.1.3.2 Metabolism of rLp-X lipids

Similar to results found in Figure 3, the cholesterol and phosphatidylcholine in rLp-X were metabolized to cholesteryl ester and lysophosphatidylcholine, respectively (Figure 7). Cells treated with 120 nmol/mL rLp-X showed a significant increase in the uptake of unesterified cholesterol and phosphatidylcholine relative to cells treated with 70 nmol/mL rLp-X. In turn, the higher levels of cholesterol and phosphatidylcholine most likely led to the significant increases in cholesteryl ester (10 nmol cholesterol/mg protein) and lysophosphatidylcholine (11 nmol cholesterol/mg protein) (Figure 7).

3.2 THE EFFECT OF APOLIPOPROTEINS ON THE UPTAKE OF LIPOPROTEIN-X

The role of apolipoproteins in Lp-X uptake was examined as shown in Figure 8. [3H]CHE rLp-X was incubated with mesangial cells in the presence or absence of individual apolipoproteins or with all four apolipoproteins combined. From Figure 8 it can be seen that both apolipoproteins C-I and C-III have a significant inhibitory effect. In contrast, apo C-II (1.5 fold) and E (4 fold) significantly stimulated the uptake of [3H]CHE rLp-X. In the presence of all four apolipoproteins the uptake of [3H]CHE rLp-X by mesangial cells was enhanced 1.5 fold.

Since the size of rLp-X may be affected by the presence of apolipoproteins, and in turn affect the rate of uptake, the diameter of each rLp-X preparation was measured by quasi-elastic light scattering analysis.
Figure 7. Metabolism of rLp-X by rat mesangial cells. Mesangial cells were incubated with $[^3]$HFC-rLp-X (70 nmol cholesterol/mL or 120 nmol cholesterol/mL). After 10 h the cellular lipids were extracted and the radioactivity associated with unesterified (free) cholesterol (FC) and cholesteryl ester (CE) was determined. In separate experiments, cells were incubated with phosphatidyl-[Me-$^3$H]choline rLp-X (70 nmol cholesterol/mL or 120 nmol cholesterol/mL) for 10 h. The radioactivity associated with phosphatidylcholine and lysophosphatidylcholine was determined. Results obtained from five separate experiments incubated with 70 nmol/mL liposomes are expressed as 100 percentile and depicted as mean ± standard deviation.
Figure 8. The effect of human apolipoproteins on the uptake of Lp-X by rat mesangial cells. An aliquot of \(^{[3]H}\)CHE rLp-X (100 nmol/mL) was mixed with individual or all apolipoproteins. The mixture (Mix) contained 100 nmol cholesterol/mL and 4-10 µg apolipoprotein/mL. This was then incubated with the mesangial cells. After 10 h the radioactivity associated with the cells was measured. Cells incubated without apolipoproteins were used as control. The results are expressed as a percentage of control and each point represents the mean ± standard deviation from five separate experiments. By student's t test * p < 0.01, where n = 5.
(Mayer et al., 1986). As seen in Table 5, the presence or absence of apolipoprotein(s) had no significant effect on particle size.

3.3 THE EFFECT OF SURAMIN, CYTOCHALASIN, AND POLYINOSINIC ACID ON THE UPTAKE OF RECONSTITUTED LIPOPROTEIN-X

Cell surface heparan sulfate proteoglycans play a role in the uptake of normal plasma lipoproteins (Hurt-Camejo et al., 1990; Vijayagopal et al., 1992; Ismail et al., 1994). Hence, the involvement of heparan sulfate proteoglycans in the uptake of Lp-X by mesangial cells was examined. Suramin is an agent that blocks the interaction between proteoglycans and lipoprotein ligands (Ismail et al., 1994). Cells treated with suramin (0.5 and 1.0 mg/mL) showed a significant 15 to 20% reduction in the uptake of rLp-X, as shown in Table 6. This result suggests that a portion of Lp-X uptake involves interactions with cell surface proteoglycans.

Cytochalasin D is an agent that blocks cytoskeleton dependent uptake (Ismail et al., 1994; Ting et al., 1995). In order to investigate the role of phagocytosis in Lp-X uptake, mesangial cells were treated with cytochalasin D. As seen in Table 6, this compound had no significant effect on the uptake of rLp-X.

By treating cells with polyinosinic acid, an inhibitor of lipoprotein uptake in smooth muscle cells (Ismail et al., 1994), the role of scavenger receptor in the uptake of rLp-X was investigated. Polyinosinic acid had no effect on the uptake
Table 5. Effect of apolipoproteins on the size of rLp-X.

<table>
<thead>
<tr>
<th></th>
<th>Mean diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLp-X</td>
<td>103</td>
</tr>
<tr>
<td>rLp-X + apo C-I</td>
<td>104</td>
</tr>
<tr>
<td>rLp-X + apo C-II</td>
<td>105</td>
</tr>
<tr>
<td>rLp-X + apo C-III</td>
<td>102</td>
</tr>
<tr>
<td>rLp-X + apo E</td>
<td>104</td>
</tr>
<tr>
<td>rLp-X + apo C-I, C-II, C-III, E</td>
<td>105</td>
</tr>
</tbody>
</table>

The size distribution of $[^{3}H]$cholesterol (FC), $[^{3}H]$phosphatidylcholine (PC) or $[^{3}H]$cholesteryl hexadecyl ether (CHE) rLp-X was determined using quasi-elastic light scattering in the absence or presence of apolipoproteins (10-15 μg/mL). The amounts of apolipoproteins were chosen to match the concentrations normally associated with Lp-X (Seidel et al., 1974). The mean diameter of particles was obtained using Gaussian analysis from two separate experiments.
Table 6. Effects of suramin, cytochalasin and polyinosinic acid on the uptake of reconstituted Lp-X.

<table>
<thead>
<tr>
<th>Total Uptake (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+ suramin 0.5 mg/mL</td>
</tr>
<tr>
<td>+ suramin 1 mg/mL</td>
</tr>
<tr>
<td>+ cytochalasin D 15 µg/mL</td>
</tr>
<tr>
<td>+ cytochalasin D 30 µg/mL</td>
</tr>
<tr>
<td>+ polyinosinic acid 25 µg/mL</td>
</tr>
<tr>
<td>+ polyinosinic acid 50 µg/mL</td>
</tr>
</tbody>
</table>

Mesangial cells were preincubated with suramin (2 h), cytochalasin D (18 h), or polyinosinic acid (18 h) in RPMI-1640 medium. [³H]CHE labeled rLp-X (100 nmol cholesterol/mL) was added to the culture medium and cells were incubated at 37 °C for 10 hours. The amounts of suramin, cytochalasin D, and polyinosinic acid used above were previously shown to be effective by other investigators (Ismail et al., 1994; Ting et al., 1995). The radioactivity associated with the cells was determined by liquid scintillation counting. The results are expressed as mean ± standard deviation from three separate experiments. The *P < 0.05 when compared with control.
of rLp-X by mesangial cells (Table 6), indicating that scavenger receptor is not involved in the uptake of Lp-X.

3.4 EFFECT OF LIPOPROTEIN-X ON MACROPHAGES

3.4.1 Lipid accumulation in macrophages

Macrophages treated with rLp-X (177 - 387 nmol FC/mL) showed a significant increase in both cellular total cholesterol (162%) and cholesteryl ester (223% to 245%) relative to control. There was a slight, but not significant, increase in free cholesterol (117%) relative to control, as shown in Figure 9.

3.4.2 Foam cell formation

Macrophages cultured on cover-slips, treated with 120 nmol FC/mL rLp-X, stained with ORO revealed the presence of foam cells, which comprised 10% of the macrophage population. Foam cells could not be detected in macrophages not treated with rLp-X. Figure 10 shows the induction of foam cell formation by rLp-X. Foam cells were identified as being any macrophage containing 10 or more lipid droplets. Figure 10 was magnified 100 X under light microscopy using oil immersion.
Figure 9. Effect of rLp-X on cellular lipids in rat peritoneal macrophages. Macrophages were plated at a density of 2 x 10^6 cells/35 mm dish and incubated for 24 h at 37°C in the presence or absence of rLp-X (177 - 387 nmol FC/mL). The results are from five separate experiments and are expressed as percentage of control and depicted as mean ± standard deviation. By student's t test, * p < 0.05 and ** p < 0.005, where n = 5.
Figure 10. Induction of foam cell formation by Lp-X. (200 X magnification) Rat peritoneal macrophages were incubated with 120 nmol cholesterol/mL rLp-X for 24 h and then stained with Oil Red O and examined under light microscopy using oil immersion. The long, thick arrow indicates a foam cell while the short, thin arrow indicates a macrophage cell identical to those seen in control treatments.
3.5 MESANGIAL CELL PROLIFERATION

3.5.1 Effect of Lp-X and rLp-X

The effect of Lp-X on mesangial cell proliferation was examined by measuring the incorporation of $[^3\text{H}]$thymidine into cellular DNA. Lp-X at low concentrations had no effect on the incorporation of radioactivity, while at higher concentrations Lp-X had a slight inhibitory, but insignificant, effect. These results are shown in Figure 11.

In order to examine the effect of the lipids in Lp-X on cell proliferation, cells were treated with rLp-X. In this study, rLp-X had no significant effect on the incorporation of $[^3\text{H}]$thymidine into cellular DNA (Figure 11). These results indicate that Lp-X and rLp-X, at the concentrations tested (5 - 200 nmol/mL), do not affect mesangial cell proliferation.

3.5.2 Combined effect of rLp-X and macrophages

Mesangial cells treated with macrophage-conditioned medium in the presence or absence of rLp-X showed no change in $[^3\text{H}]$thymidine incorporation relative to control (Figure 12).
Figure 11. The effect of Lp-X and rLp-X on the incorporation of $[^3H]$thymidine into rat mesangial cells. Mesangial cells were incubated with $[^3H]$thymidine in the presence or absence of Lp-X or liposomes. After 10 h the radioactivity associated with the cells was determined. No serum was present in the culture. Results are expressed as a percentage of control and each point represents the mean ± standard deviation from five separate experiments.
Figure 12. Combined effect of macrophage-conditioned medium and rLp-X on [³H]thymidine incorporation by rat mesangial cells. Mesangial cells were incubated in macrophage-conditioned medium in the presence of rLp-X (100 - 400 nmol FC/mL) and pulsed with [³H]thymidine for 24 h. After incubation the radioactivity associated with the cells was measured. No serum was present in the culture. The results are expressed as percentage of control and depicted as mean ± standard deviation from four separate experiments.
4. DISCUSSION

One of the major clinical features of familial LCAT deficiency is progressive glomerulosclerosis (Magil et al., 1982; O and Frohlich, 1995). A typical feature of this disorder is the presence of an abnormal plasma lipoprotein, Lp-X. Although the pathogenesis of glomerulosclerosis may involve multiple factors, the increased amounts of phosphatidylcholine and unesterified cholesterol seen in affected glomeruli may be due to the accumulation of Lp-X in the kidney. Lp-X has unusually high levels of these two lipids. Renal biopsies of LCAT deficient patients have shown mesangial cell proliferation and the presence of foam cells in addition to the accumulation of lipids in the mesangial region. It has recently been reported that lipid accumulation in perfused rat kidney was caused by a direct deposition of Lp-X (O et al., 1997). The objective of this project was to examine the uptake and metabolism of Lp-X in mesangial cells as well as its effect on mesangial cell function.

4.1 UPTAKE AND METABOLISM OF LIPOPROTEIN-X IN MESANGIAL CELLS

One of the key events in the development of glomerulosclerosis is the accumulation of lipids in the kidney (Striker and Striker, 1985; Diamond and Karnovsky, 1988). The results obtained from this study have clearly demonstrated that Lp-X is taken up in a concentration dependent manner by rat mesangial cells (Figure 3). Furthermore, the lipids in Lp-X are metabolized (Figure 4). The uptake of Lp-X by mesangial cells may contribute to the lipid
accumulation in the kidneys of LCAT deficient patients, leading to the development of glomerulosclerosis.

4.1.1 Role of apolipoproteins in Lp-X uptake

The apolipoproteins C-I, C-II, C-III, and E are associated with Lp-X. Reconstituted Lp-X was prepared in order to examine the role these apolipoproteins play in the uptake of Lp-X by mesangial cells. rLp-X contains a similar lipid content to Lp-X.

Apolipoproteins E and C-II stimulated the uptake of rLp-X while apolipoproteins C-I and C-III inhibited this process (Figure 8). These results suggest that the apolipoproteins (C-I, C-II, C-III, and E) associated with Lp-X may play a significant role in modulating its uptake. The significant effect of apolipoproteins on the uptake of Lp-X also suggests that the mechanism of Lp-X uptake may involve one of the known lipoprotein receptors.

4.1.2 The role of scavenger receptor, phagocytosis, and proteoglycans in the uptake of Lp-X

Additional experiments were performed to determine whether scavenger receptor or cytoskeleton-dependent phagocytosis are involved in the uptake of Lp-X by mesangial cells. The results indicated that scavenger receptor and non-receptor mediated phagocytosis do not play a role in Lp-X uptake (Table 6). Neither polyinosinic acid, which inhibits scavenger receptor (Coritsidis et al., 1991), nor cytochalasin D, which disrupts the machinery required for
phagocytosis, affected the uptake of Lp-X.

Furthermore, the role of cell surface heparan sulfate proteoglycans in the uptake of Lp-X by mesangial cells was examined. Heparan sulfate proteoglycans have been shown to be involved in the uptake of normal plasma lipoproteins (Hurt-Camejo et al., 1990; Vijayagopal et al., 1992; Ismail et al., 1994). Cells treated with suramin reduced Lp-X uptake by 15 to 20% (Table 6). Suramin competes with positively charged particles for sites on the negatively charged heparan sulfate proteoglycans. These results indicate that cell surface proteoglycans may play a role in the uptake of Lp-X by mesangial cells.

Abnormalities in the function of scavenger receptor, phagocytosis, or cell surface proteoglycans could lead to intracellular lipid accumulation. This would in turn lead to the accumulation of lipids in the kidney and possibly contribute to the development of glomerulosclerosis. Whether the uptake of Lp-X by mesangial cells is enhanced or attenuated by other putative participants (monocyte/macrophages, extracellular matrix expansion, and various cytokines) in the development of glomerulosclerosis still remains to be clarified.

4.1.3 Possible mechanisms for the uptake of Lp-X

Rat mesangial cells have LDL receptor on its surface, which is responsible for the uptake of LDL and VLDL (Coritsidis et al., 1991; Schlondorff, 1993). The LDL receptor recognizes its lipoprotein ligands by virtue of their associated apolipoprotein B or E (Havel and Kane, 1995; Coritsidis et al., 1991; Schlondorff, 1993). Apolipoprotein E is associated with Lp-X (Sabesin, 1982). The
stimulatory effect of apolipoprotein E on the uptake of Lp-X (Figure 8) suggests that LDL receptor may be involved in this process. If we are able to obtain sufficient Lp-X samples then competition studies will be done in order to clarify the role of LDL-R in Lp-X uptake.

Alternatively, other receptors that recognize apolipoprotein E could also be possible routes for Lp-X uptake. An example of a receptor other than LDL receptor that recognizes apo E as a ligand is LRP. LRP is thought to bind apo E (Kowal et al., 1989). Besides its role in lipoprotein metabolism, lipoprotein lipase (LPL) may also help in the association of lipoproteins with cell surfaces (Rutledge et al., 1997). LPL, possibly in conjunction with cell surface proteoglycans, could be involved in the uptake of Lp-X by mesangial cells. Complement components are sometimes present in glomerular injury (Adler et al., 1984). Complement may play a role in Lp-X uptake by an as yet unknown mechanism. However, Lp-X may just simply fuse with the cell plasma membrane. Clearly, further studies are needed to examine and identify the mechanism(s) by which Lp-X is taken up into mesangial cells.

4.2 EFFECT OF RECONSTITUTED LIPOPROTEIN-X ON MACROPHAGES

Infiltration of macrophages and the formation of foam cells have been found in the affected glomeruli of patients with LCAT deficiency. In vivo the uptake of Lp-X by infiltrated macrophages could lead to foam cell formation. Foam cells are a rich source of cytokines and growth factors. One of the growth factors secreted by macrophages is transforming growth factor β (TGF-β). TGF-
β can act as a stimulator of mesangial cell proliferation as well as induce matrix production in vitro (MacKay et al., 1989). Both mesangial cell proliferation and extracellular matrix expansion are key events in the development of glomerulosclerosis.

The process of matrix production may in turn lead to or involve the up-regulation of PDGF (platelet-derived growth factor) β-receptor in mesangial cells (Van Goor et al., 1994). Macrophages are able to secrete PDGF, a cytokine that could play an important role in the progression of glomerulosclerosis. It has been shown that PDGF selectively induces mesangial cell proliferation and extracellular matrix accumulation (Floege et al., 1993).

In addition to contributing to lipid accumulation in mesangial cells, Lp-X may also contribute to the accumulation of lipids in macrophages. The effect of rLp-X on cellular lipid levels in macrophages was examined. Macrophages cultured in the presence of rLp-X showed an increase in cellular total cholesterol and cholesteryl esters relative to control (Figure 9). rLp-X does not contain cholesteryl ester. These results suggested that the increase in cholesteryl ester was due to the uptake of unesterified cholesterol from rLp-X by macrophages. Excess cellular cholesterol is esterified and stored as lipid droplets.

The accumulation of cellular lipids in macrophages could lead to the formation of foam cells. ORO staining of macrophages treated with rLp-X revealed the presence of foam cells (Figure 10). Foam cells were not detected in untreated macrophages.

Two of the major events in glomerulosclerosis are the accumulation of
lipids and the presence of foam cells in affected glomeruli. The results obtained from these experiments indicate that rLp-X is able to cause lipid accumulation in macrophages. This could contribute to the accumulation of lipids in the kidneys of LCAT deficient patients. Results obtained from this study have shown that macrophage lipid accumulation via the uptake of Lp-X may lead to the formation of foam cells. Foam cells may contribute to the development of glomerulosclerosis by the secretion of growth factors and cytokines.

4.3 MESANGIAL CELL PROLIFERATION

Increased mesangial cell proliferation is one of the key events in the development of glomerulosclerosis. This proliferation may be due to the accumulation of Lp-X lipids by mesangial cells or to the combined effect of Lp-X and macrophages. In this study these two possible mechanisms were tested.

4.3.1 The effect of Lp-X

Considerable amounts of intracellular lipid are acquired by the mesangial cell when treated with Lp-X. This accumulation of lipid could possibly lead to enhanced cell proliferation. However, the results obtained in this study indicated that Lp-X does not induce cell proliferation, as measured by the incorporation of \(^{3}\text{H}\)thymidine (Figure 11). Increasing the time of incubation (up to 48 h) did not alter mesangial cell proliferation (data not shown). Since mesangial cell proliferation is one of the characteristics of glomerulosclerosis, it may be possible that Lp-X affects proliferation indirectly.
4.3.2 The combined effect of macrophages and Lp-X

Treatment of macrophages with rLp-X led to the formation of foam cells. Lp-X may affect mesangial cell proliferation indirectly by inducing macrophages/foam cells to secrete growth factors. To examine this possibility, mesangial cells were incubated in macrophage-conditioned medium. Macrophage-conditioned medium in the presence or absence of rLp-X had no effect on the incorporation of \(^{3}\text{H}\)thymidine by mesangial cells (Figure 12). These results suggest that other factors must be involved in mesangial cell proliferation. In addition to macrophages, further agents present in plasma in vivo may be required to stimulate mesangial cell proliferation. Whether macrophages are involved in this process is still uncertain.

4.4 CONCLUSION

Lipid accumulation, macrophage infiltration, foam cell formation, and mesangial cell proliferation are four key events in the development of glomerulosclerosis. This study has examined whether Lp-X is involved in these events and thus able to contribute to the development of glomerulosclerosis in LCAT deficient patients.

The results obtained from this study have clearly shown that Lp-X is taken up by rat mesangial cells and that the lipid components of Lp-X, phosphatidylcholine and cholesterol, are metabolized to lysophosphatidylcholine and cholesteryl ester, respectively. The uptake of Lp-X may involve cell surface
heparan sulfate proteoglycans. The accumulation of lipids in mesangial cells could contribute to the lipid accumulation seen in glomerulosclerosis. Lp-X is able to induce foam cell formation in macrophages. The uptake of Lp-X by macrophages may also contribute to the accumulation of lipids in the kidneys of these patients. However, the results obtained in this study do not suggest that Lp-X, alone or combined with macrophages, is able to induce mesangial cell proliferation. These results indicate that other factors are involved in this process.

Further studies are needed to examine the mechanisms involved in mesangial cell proliferation and to investigate the role of extracellular matrix expansion in the pathogenesis of glomerulosclerosis in familial LCAT deficiency.
5. LITERATURE CITED


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