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MAJ ATCHLEY DANIEL H

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OXIDIZED LDL DENSITY LIPOPROTEIN ANTIBODIES AND OXIDIZED LOW DENSITY LIPOPROTEIN-CONTAINING IMMUNE COMPLEXES: CHARACTERISTICS AND PATHOGENIC SIGNIFICANCE IN DIABETES

by

Daniel H. Atchley

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies.

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Abstract

Mounting evidence suggests that two major vascular complications of diabetes mellitus, atherosclerosis and nephropathy, are pathogenically analogous processes, with atherosclerosis occurring primarily in the macrovasculature and nephropathy involving the microvasculature. Additionally, several lines of evidence point to oxidized low-density lipoprotein (oxLDL) as a major participant in both processes. OxLDL is found deposited in both types of vascular lesions, and appears to influence several events. It causes influx of macrophages, which ingest the oxLDL and are transformed into foam cells – a key component of atherosclerosis and glomerulosclerosis. OxLDL also appears to promote the proliferation of smooth muscle cells in atherosclerosis and mesangial cells in glomerulosclerosis; and expansion of the extracellular matrix in both lesions. Previous work in our laboratory has focused on the immunogenicity of oxLDL, and we have shown that heterogeneous LDL-containing immune complexes (LDL-IC), made with human LDL and rabbit LDL-hyperimmune antisera, are unmatched in their ability to drive the transformation of human monocyte-derived macrophages into foam cells. Recently, we undertook a three-part endeavor to better understand the pathogenic role of LDL-IC in microvascular and macrovascular complications of diabetes. Our goals were to 1) further characterize oxLDL antibodies that we isolated from serum and immune complexes; 2) considering the similarities between nephropathy and atherosclerosis, we wished to determine if LDL-IC are associated with diabetic nephropathy, as is observed in macrovascular disease; 3) determine if artificially

prepared homogeneous oxLDL-IC, made with oxLDL and human oxLDL antibodies, produce similar results in macrophages to those observed with the use of heterogeneous LDL-IC.

Our characterization of oxLDL antibodies revealed that free and complexed antibodies, purified from serum and IC were predominately of the IgG isotype, with IgG1 and IgG2 subclasses predominating. Interestingly, we found that the avidity of oxLDL antibodies isolated from ICs was significantly higher than that of free oxLDL antibodies. These findings suggest that oxLDL-ICs form stable circulating complexes and are composed of IgG subclasses that may render them pro-inflammatory. To investigate the potential association of LDL-IC with diabetic nephropathy, we compared the concentrations of free oxLDL antibodies, avidities of free and IC-bound oxLD antibodies, and concentrations of surrogate markers of LDL-ICs (IC-cholesterol and IC-apolipoprotein B) in polyethylene glycol-precipitated ICs in three nephropathy classes of type 1 diabetic subjects. Subjects were classified according to their levels of urinary albuminuria, with the normoalbuminurics demonstrating a urinary albumin excretion (UAE) of <30mg/day; microalbuminurics had a UAE of 30-300mg/day; and macroalbuminurics had a UAE of greater than 300mg/day. We observed no significant difference in serum oxLDL antibody levels between groups, but identified a significant increase in IC-cholesterol and IC-apoliprotein B in the microalbuminuric and macroalbuminuric groups when compared to the normoalbuminuric group. Also, avidity levels of oxLDL isolated from immune complexes appeared to increase as nephropathy worsened. We interpreted

these data to indicate a positive association between concentrations of LDL-ICs, and diabetic nephropathy, and that stable immune complexes formed with antibodies of higher affinity may play a role in the pathogenesis of renal disease in type 1 diabetes. Since these studies, in conjunction with our previous studies of heterogeneous LDL-IC-induced foam cell formation in human macrophages implicated LDL-IC as a potential participant in atherosclerosis and glomerulosclerosis, but required validation with homogeneous LDL-IC. To meet this objective, we purified human oxLDL antibodies and synthesized oxLDL-IC by mixing these antibodies with oxLDL. We then precipitated the oxLDL-IC using 4% polyethylene glycol, and incubated the oxLDL-IC with THP-1 macrophages (a human monocyte-derived macrophage cell line). After 48 hours incubation, we extracted the cholesterol from these cells, and measured intracellular cholesterol ester accumulation. We observed intracellular cholesterol ester accumulation in cells incubated with oxLDL-IC that exceeded that of cells incubated with oxLDL alone, and these levels approached that observed in cells incubated with heterogeneous LDL-IC. These findings validate the continued use of heterogeneous LDL-IC in studies of foam cell formation. In conclusion, these findings demonstrate a positive association of diabetic microvascular disease and LDL-ICs that is reminiscent of that observed between LDL-IC and macrovascular disease. We have shown that homogeneous oxLDL-IC causes foam cell formation in macrophages, therefore are likely participants in microvascular (nephropathy) and macrovacular (atherosclerosis) diseases

processes. Lastly, we have validated the continued use of heterogeneous LDL-ICs in studies of macrophage: LDL-IC interactions.

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List of Abbreviations

Ab	antibody
ACAT	acyl CoA:cholesteryol acyltransferase
AER	albumin excretion rate
AGE	advanced glycated end products
ALE	advanced lipoxidation end products
ApoB	apolipoprotein B
ATTC	American Type Culture Collection
BBS	borate buffered saline
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CE	cholesteryl esters
CFA	complete Freund's Adjuvant
CHOL	cholesterol
CML	carboxymethyllysine
CURL	compartment of uncoupling of receptor and ligand
DCCT	Diabetes Control and Complications Trial
DM	diabetes mellitus
EDIC	Epidemiology of Diabtetes Interventions and Complications
EDTA	ethylenediaminetetraacetic acid
ESRD	end-stage renal disease
FC	foam cell

FcγR	Fc gamma receptor
FCS	fetal calf serum
Fc-γR	Fc gamma receptor
HAGG	heat aggregated gamma globulin
HbA1C	hemoglobin A1C
HDL	high density lipoprotein
HMG CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNE	hydroxynonenal
IC	immune complexe
IFA	incomplete Freund's Adjuvant
IgG	immunoglobulin gamma
IL-1	interleukin-1
IMDM	Iscove's modified Dulbecco's medium
Kd	dissociation constant
LDL	low density lipoprotein
LDL-IC	low density lipoprotein immune complexes
LDL-R	low density lipoprotein receptor
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
NLDL	native low density lipoprotein
OxLDL	oxidized low density lipoprotein
OxLDL-IC	oxidized low density lipoprotein immune complex
PBS	phosphate buffered saline

PDGF-AA	platelet-derived growth factor AA
PEG	polyethylene glycol
PMA	phorbol 12-myristate 13-acetate
RID	radial immunodiffusion
ROS	reactive oxygen species
SFM	serum free medium
TGL	triglyceride
TNF-α	tissue necrosis factor alpha

Chapter I

Introduction

Diabetes mellitus (DM-Attachment 1) has reached epidemic proportions in the developed nations of the world. This notoriously chronic debilitating disease is characterized by hyperglycemia and afflicts approximately 16 million people in the United States (ADA, 1998). All long-term diabetic complications are essentially the manifestation of diabetic vascular injury, encompassing both microvascular disease (nephropathy, retinopathy, and neuropathy) and macrovascular disease (atherosclerosis, ischaemic heart disease, cerebrovascular disease, and peripheral vascular disease) (Breyer, 1992; Cooper et al., 1997; Kamanna et al., 1997; Lloyd et al., 1996; Lopes-Virella and Virella, 1998; Maser et al., 1991; Nathan, 1993; Orchard et al., 1990; Raptis and Viberti, 2001); with accelerated and aggressive atherosclerosis being the leading cause of morbidity and mortality in people with diabetes (Table 1).

Although the pathogenesis of diabetes-related complications is poorly understood, the relationship between the development of diabetic complications and poorly controlled hyperglycemia is well established, and is associated with non-enzymatic glycosylation of proteins, especially collagen, hemoglobin, and lipoproteins (Brownlee et al., 1984; Nathan, 1996).

Table 1. Complications of diabetes in the United States (CDC, 1999; NCHS/CDC, 2000)

Deaths

- Diabetes is the direct cause of an estimated 65,000 deaths among Americans and is a contributing cause in another 95,000 deaths annually.

Heart Disease and Stroke

- People with diabetes have a two to four-fold increase in heart disease and are two to six times more likely to have a stroke than people who do not have diabetes.

Kidney Disease

- Diabetes is the single most common cause of end-stage renal disease (ESRD).

- Thirty to forty percent of those with diabetes develop ESRD.

- Currently over one-third of all patients with ESRD have diabetes and this number is expected to reach 50% if the current rate of increase continues.

Blindness

- Diabetes is the leading cause of blindness among adults 20 to 74 years of age.

Amputations

- Diabetes is the underlying cause of the majority of lower limb amputations.

Irrefutable evidence supporting this contention came from the nine-year Diabetes Control and Complications Trial (DCCT) reported in 1993. In this large multi-center study, rigid maintenance of near-normal glucose blood levels reduced the vascular complications of diabetes by more than 50% in 1441 subjects with type 1 diabetes (DCCT/Research/Group, 1993).

The Oxidant Stress Hypothesis

Although compelling evidence points to glycation as a significant participant in the development of many complications of diabetes, the majority of researchers and clinicians in the field have well-founded doubts that it alone is directly responsible for diabetic complications because, quite simply, the structural changes observed in glycated tissues do not adequately explain all of the clinical observations (Wolff, 1987). It is widely accepted that the "missing link," in diabetes-related pathology is answered by a well-supported hypothesis that looks beyond glycation to one of its known effects: the oxidant stress hypothesis (Baynes, 1991; Hunt et al., 1993; Hunt and Wolff, 1991; Miyata et al., 1999; Salahudeen et al., 1997; Wolff, 1987). This hypothesis may well be the missing piece of the diabetes puzzle, helping explain the diversity and severity of vascular complications. At the core of this hypothesis, glycation has been identified as the primary trigger for chronic complications of diabetes. Hyperglycemia leads to increased glycation of tissues, and a concomitant increased production of free radical reactive oxygen species (ROS). Being

chemically unstable moieties, free radicals are extremely reactive, interacting directly and indirectly with numerous macromolecules and tissues, causing oxidant stress-related pathogenesis (Baynes, 1991; Hunt et al., 1993; Hunt and Wolff, 1991; Salahudeen et al., 1997; Wolff, 1987).

The proposed mechanism by which this takes place is as follows: when protein is exposed to glucose in vitro, glucose, like other alpha-hydroxyaldehydes, can enolize. Trace amounts of transition metals, iron and copper, then catalyze the conversion of the endiol to an endiol radical anion, which is oxidized to a ketoaldehyde. This "autoxidation" of glucose produces a steady flow of ROS that contribute to the oxidant stress insult. These free radicals are capable of causing significant structural and physiological damage by mediating the reaction of various glucose reactive products (Amadori adduct, etc.) with membrane proteins, as well as by oxidatively modifying numerous proteins (Baynes, 1991; Hunt et al., 1993; Hunt and Wolff, 1991; Salahudeen et al., 1997; Wolff, 1987) (Figure 1).

Diabetic Vascular Complications and Low Density Lipoprotein (LDL)

In diabetes research, LDL has received considerable, and well deserved, attention during the past decade. Not only is it increased in diabetes, but it is susceptible to glycation and oxidative modification; and its oxidized analog, oxidized LDL (oxLDL), has been convincingly linked to both microvascular and

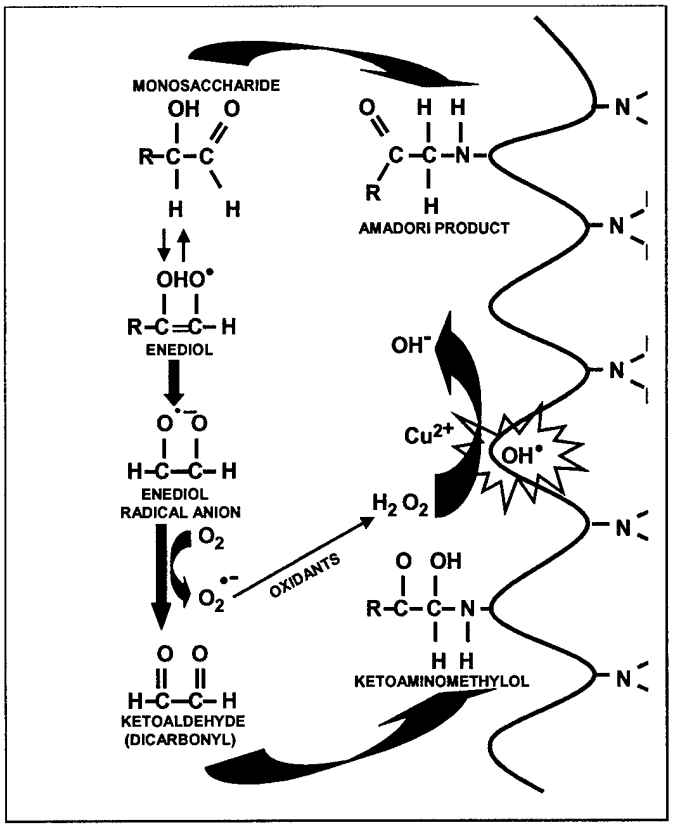

Figure 1. Substrate oxidation-derived damage. Substrates that can autoxidize, such as glucose, lead to the production of dicarbonyls and oxidants. Glucose oxidation is catalyzed by transition metals, such as copper, which are either free in solution or sequestered by protein. Copper attached to the protein may participate in glucose oxidation, and is involved in oxidative protein alterations involving hydrogen peroxide. The attachment of glucose to protein via the Amadori pathway is also shown (Hunt et al., 1993; Hunt and Wolff, 1991).

macrovascular disease (Esterbauer et al., 1992; Kreisberg, 1998; Steinberg, 1993; Steinberg et al., 1989). However, before discussing the potential pathogenic role of oxLDL in diabetes, it seems pertinent to first summarize some relevant properties of unmodified, or native, LDL (nLDL).

By definition, human nLDL (Figure 2) is the population of lipoproteins that can be isolated by density-gradient ultracentrifugation within a range of 1.019 to 1.063 g/mL. LDL functions in the blood as the primary supplier/carrier of free and esterified cholesterol to the peripheral tissues of the body (Olson, 1998). Its molecules are spherical particles of variable size, measuring 19-25 nM, with molecular masses of 1.8 to 2.8 million. Each LDL molecule consists of a hydrophobic lipid core containing 90% cholesteryl esters (CE) and 10% triglycerides, surrounded by a phospholipid monolayer which contains a massive protein, apolipoprotein B (apoB) embedded in the outer layer (Myant, 1990).

The biochemical composition of LDL is given in table 2. If one uses 2.5 million as the mean molecular weight of LDL, each sphere is calculated to contain approximately 1600 molecules of CE and 170 molecules of triglycerides in the lipophilic core. Surrounding the oily core is a monolayer of approximately 700 phospholipid molecules (primarily phosphatidylcholine with minute amounts of sphingomyelin and lysophosphatidylcholine) and 600 molecules of free cholesterol.

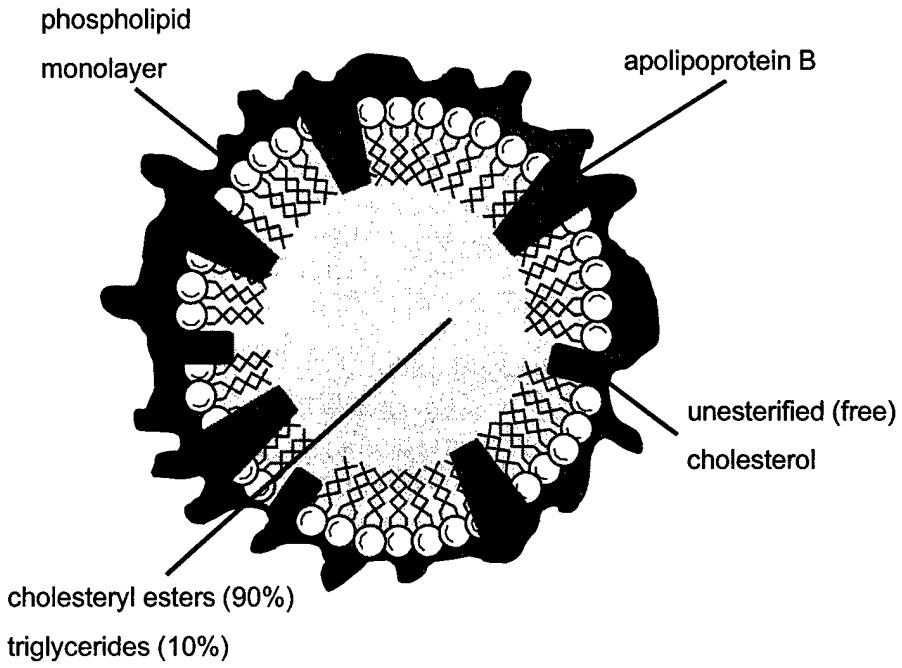

Figure 2. Schematic of a LDL particle. A hydrophobic lipid core consisting of relatively large quantities of cholesteryl esters (CE) and small quantities of triglycerides is surrounded a hydrophilic phospholipid monolayer, with a large protein moiety (apolipoprotein B) embedded.

Table 2. Chemical composition of human LDL (Esterbauer et al., 1992).

Component	mean (SD)
Phospholipid (μmol/mg protein)	22.3 (3.0)
Triglyceride (mg/mg protein)	5.6 (2.7)
Free cholesterol (mg/mg protein)	9.6 (0.7)
Cholesteryl ester (mg/mg protein)	42.2 (3.8)
Protein (%)	22.0 (1.9)

As expected, the polar heads of the phospholipids, which are positioned along the outermost periphery of the LDL particle, interface with water and enhance the solubility of LDL in the blood. Also, embedded in the phospholipid monolayer of LDL is apoB, the largest known single-chain protein, weighing an impressive 550 kDa. Functions of apoB are multifaceted, with it enhancing aqueous solubility, and appearing to be involved in receptor-mediated endocytosis of LDL into cells. Structurally, it is unlikely that apoB sits on the LDL molecule like a hat; rather experimental data suggest it embraces a large area of the phospholipid surface of LDL in an octopus-like manner (Esterbauer et al., 1992).

Extending the discussion of LDL beyond structure to include function, a brief review of normal intracellular cholesterol metabolism as described by Brown and Goldstein (Brown and Goldstein, 1984; Brown and Goldstein, 1986) is needed to help frame the questions addressed in this thesis. As illustrated in figure 3, intracellular cholesterol is generally derived from two sources: the exogenous (major) or LDL receptor (LDL-R) pathway, and the endogenous (minor) or 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase pathway. Under normal circumstances, intracellular levels of unesterified (free) cholesterol are under tight control and maintained within a very narrow range. As intracellular levels of free cholesterol increase, the following four effects serve as components of a negative feedback loop: 1) cholesterol is incorporated into the

cell membranes; 2) synthesis and expression of new LDL receptors is inhibited;

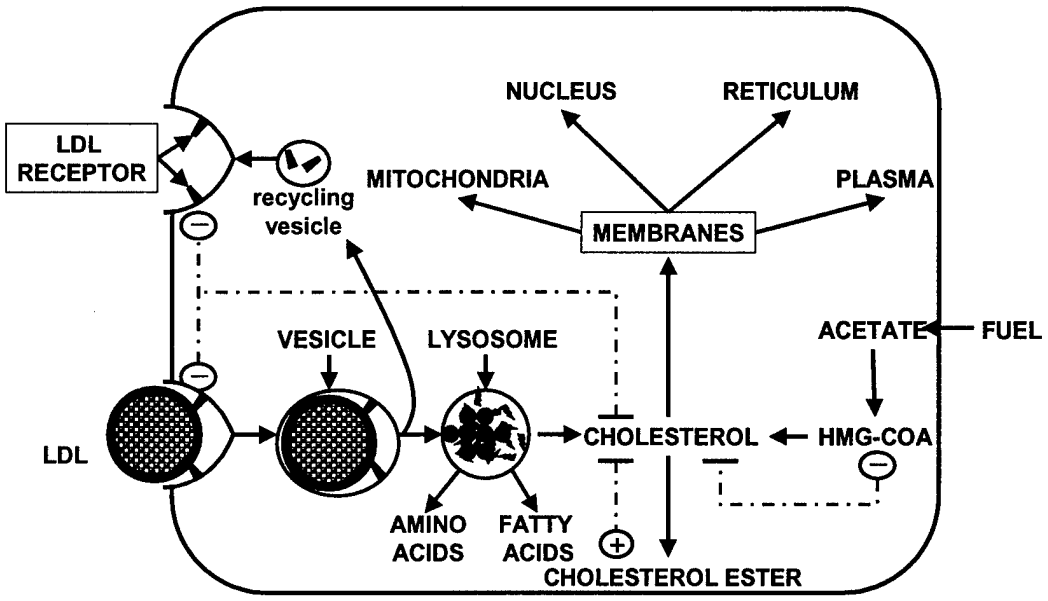

Figure 3. Normal cholesterol homeostasis in mammalian cells. LDL binds to its receptor and is ingested by receptor-mediated endocytosis, followed by internalization and vesicle formation. The vesicle then fuses with a compartment of uncoupling of receptor and ligand (CURL), where the LDL receptors are recycled to the cell's surface and LDL is delivered to a lysosome for degradation of apoB to its constituent amino acids along with cleavage of the esterified cholesterol to unesterified (free) cholesterol and fatty acids. Intracellular levels of unesterified cholesterol are normally tightly regulated. Abnormally high levels of intracellular cholesterol trigger intracellular metabolic events indicated by + (enhancing) or – (inhibiting). The bold outer lines of LDL indicate the protein moiety (apoB); the inner spheres indicate lipid. Modified from the description of Brown and Goldstein, published in 1979 (Brown and Goldstein, 1979).

3) cholesterol synthesis is curtailed by reducing the synthesis of HMG CoA reductase; and 4) the activity of acyl CoA:cholesterol acyltransferase (ACAT) is increased, driving the synthesis of cholesterol esters for storage in the cytoplasm. These regulatory events are mediated by sterol regulatory element binding protein, which monitors the free cholesterol concentration in the cell and adjusts the expression of cholesterol regulatory genes (Chin and Chang, 1981; Mazzone et al., 1988; Wang et al., 1994). The inhibition of LDL receptor expression and HMG-CoA reductase, in conjunction with enhanced ACAT activity, effectively curtails internalization of LDL and de novo synthesis of cholesterol, while enhancing storage of cholesterol esters.

Oxidation, LDL, and Diabetes

Knowledge regarding the mechanisms of oxidation of LDL *in vivo* is incomplete; however, what follows is a summary of current thought regarding this process, and a description of some of the known products of LDL oxidation (Esterbauer et al., 1991; Miyata et al., 1999; Steinbrecher, 1987). Current dogma contends that oxidation of LDL requires a microenvironment within the arterial wall where LDL is trapped and sequestered from circulating antioxidants and where the levels of redox-active metal ions are increased (Lopes-Virella and Virella, 1994b). Modification may occur directly, as a result of the generation of ROS, followed by oxidation of amino acids contained within apoB, or indirectly by reactive carbonyl compounds generated by the autoxidation of carbohydrates, lipids, or amino

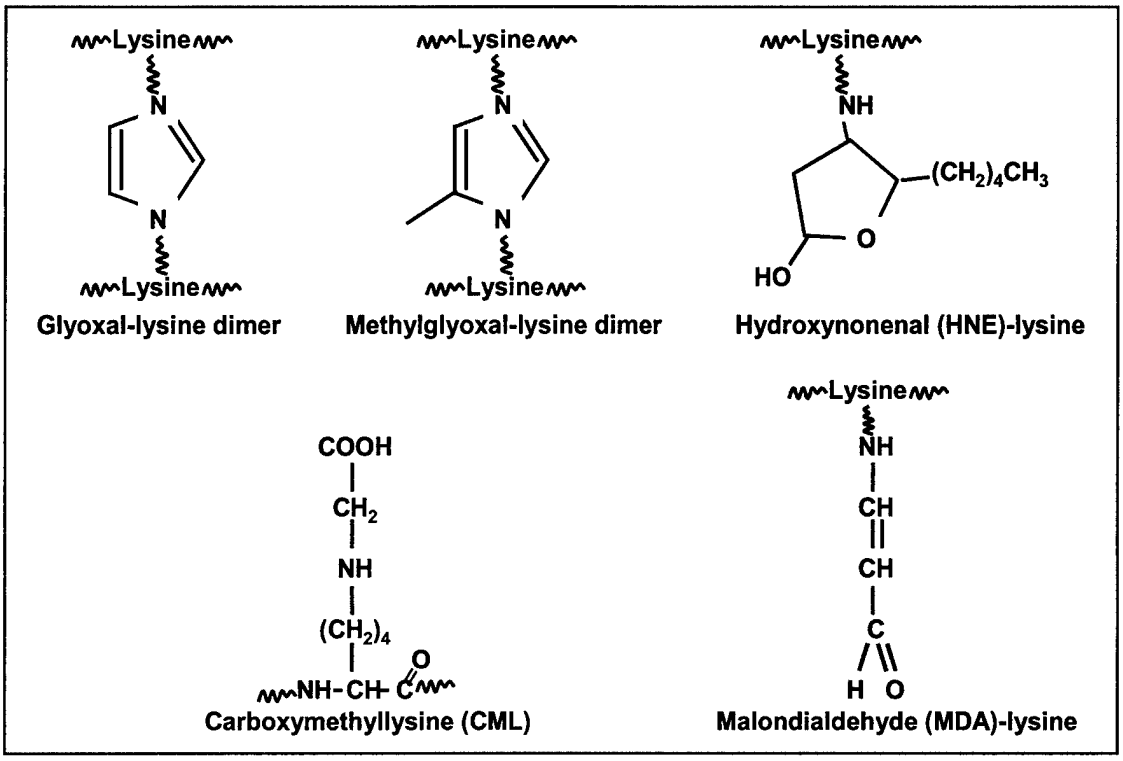

Figure 4. Oxidation produces neoepitopes. Autoxidation of carbohydrates produces glyoxal and methylglyoxal-lysine dimmers; Lipid peroxidation produces malondialdehyde (MDA) lysine and hydroxynonenal (HNE)-lysine; oxidation of the hydroxyaminoacid, serine produces carboxymethyllysine (CML) – adapted from Miyata *et al.* (Miyata et al., 1999).

acids (Miyata et al., 1999) (Figure 4). Autoxidation of carbohydrates produces reactive carbonyl compounds, such as glyoxal (Wells-Knecht et al., 1995) and methylglyoxal (Ahmed et al., 1997). Lipid peroxidation of polyunsaturated fatty acids residing in phospholipids and CE in the lipoprotein core are prone to form highly reactive degradation products, of which, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)-modified lysine, tyrosine, arginine, and histidine are best described (Chen et al., 1992; Esterbauer et al., 1991; Palinski et al., 1989).

Oxidation of the hydroxyamino acid, L-serine produces a carbonyl compound highly reactive with proteins, leading to formation of carboxymethyllysine (CML) (Anderson et al., 1997). Each of the reactive fragments described above may, in turn attach covalently to lysine and histidine residues of apoB, and in the case of MDA/4-HNE, form advanced lipoxidation end products (ALEs). In addition to the generation of LDL oxidation-induced adducts, free radical-induced damage of the protein component of LDL has been shown to cause fragmentation of apoB, with the fragments subsequently forming high molecular weight aggregates (Bellamy et al., 1989) *in vitro*. In toto, a large variety of LDL neoepitopes are likely to be generated by these oxidation-induced processes. As noted earlier in this chapter, an overwhelming body of evidence exists suggesting that diabetes is associated with increased oxidant stress and enhanced lipid peroxidation (Baynes, 1991; Hunt et al., 1993; Hunt and Wolff, 1991; Mooradian, 1991; Rabini et al., 1994).

Following this line of evidence, oxidation of LDL can occur via one or more of several mechanisms, including: 1) Autoxidation of glucose or Amadori compounds generates ROS capable of oxidizing LDL (Azevedo et al., 1988; Gillery et al., 1988; Hunt et al., 1993; Hunt et al., 1990; Hunt and Wolff, 1991; Kawamura et al., 1994; Mullarkey et al., 1990; Wolff, 1987); 2) glycation of apoB may generate free radicals, thus initiating oxidation of the LDL particle (Wolff, 1987); 3) nonenzymatic glycation may prolong the half-life of LDL circulating in the blood, which, in combination with trapping of glycated LDL in the vessel wall (due to covalent cross-linking to glycated structural proteins) increases the probability of oxidation (Brownlee et al., 1984; Kortland et al., 1992); 4) small, dense LDL particles are found in increased concentrations in poorly controlled diabetes, and are more easily oxidized than larger, less dense LDL particles found in non-diabetic control subjects and subjects with tightly controlled diabetes (de Graaf et al., 1991; James and Pometta, 1990; James and Pometta, 1991; Lahdenpera et al., 1994; Tomkin and Owens, 1994); 5) increased plasma triglyceride levels, which are commonly observed in poorly controlled diabetes, are significantly correlated with increased susceptibility of LDL to oxidation in patients with atherosclerosis (Gin et al., 2000; Kamanna et al., 1997; Kreisberg, 1998; Lopes-Virella et al., 1983; Regenstrom et al., 1991).

Interestingly, discovery of oxLDL, along with a rapidly growing appreciation of the major role post-translationally modified LDL seems to play in atherogenesis,

occurred during investigations of an apparent paradox. In a series of sentinel experiments aimed at characterizing the LDL receptor, Brown and Goldstein (Brown and Goldstein, 1986; Brown and Goldstein, 1990) made an unexpected discovery. Their work demonstrated, for the first time, that virtually all cholesterol in a cell is derived from LDL, and that endocytosis of LDL is receptor-mediated. As they continued their investigations, they discovered that people who lack functional LDL receptors (familial hypercholesterolemia) develop atherosclerotic lesions that are indistinguishable from those found in people with fully functional LDL receptors. In searching for answers to this apparent paradox, their elegant studies demonstrated that the risk of cardiovascular disease was not directly due to native LDL as many believed. Indeed, it is common knowledge that all cells require cholesterol supplied by LDL for a variety of functions to include steroid hormone production and membrane synthesis. However, the abnormal accumulation of LDL cholesterol in atherosclerotic plaques appeared to be heavily influenced by post-translational modification of LDL. They discovered that the accumulation of native LDL in the cytoplasm of cells is a tightly regulated process, with increasing levels of intracellular cholesterol triggering decreased expression of LDL receptors. However, post-translational modification (acetylation and oxidation, for example) of LDL appeared to negate the stringent control exerted by the cells' surface LDL receptors, promoting unchecked entry via scavenger receptors. They observed that when monocyte-derived macrophages ingested native LDL, lipid-induced negative feedback caused

down regulation of LDL receptors, and prevented over-accumulation of LDL in the macrophages. However, when macrophages ingested modified LDL (i.e. oxLDL), negative regulation appeared to be bypassed, causing unregulated uptake of LDL and the resultant transformation of these macrophages into lipid-laden foam cells (FCs) (Brown and Goldstein, 1990). Adding to this paradox, Lopes-Virella *et al.* discovered that human monocyte-derived macrophages incubated with oxLDL or LDL-containing immune complexes (LDL-IC) exhibited an unexpected up regulation of LDL receptors (Lopes-Virella et al., 1991). Although these cells had extremely elevated levels of cholesterol, inexplicably they continued ingesting more lipoprotein than they could excrete, causing cholesterol to be stored in their cytoplasm in the form of cholesteryl ester droplets, ultimately transforming them into foam cells and causing cell death. Interestingly, during these experiments, observations were reported that are very germane to this dissertation, and will be discussed in more detail shortly: LDL-containing immune complexes appeared to enhance foam cell formation more efficiently than modified LDL alone (Gisinger and Lopes-Virella, 1992; Griffith et al., 1988; Lopes-Virella et al., 1997a; Lopes-Virella et al., 1991; Lopes-Virella et al., 1997b; Lopes-Virella and Virella, 1992).

Table 3. Characteristics of OxLDL (Lopes-Virella and Virella, 1996; Schwartz et al., 1991; Steinberg, 1993)

- Chemotactic for monocytes, T lymphocytes
- Expands extracellular matrix
- Phagocytized by macrophages, causes foam cell transformation
- Cytotoxic
- Enhances leukocyte adhesion to the endothelium

Modified LDL and LDL-IC are Involved in Complications of Diabetes

Research during the past decade has produced compelling evidence that two major complications of diabetes, atherosclerosis (primarily a macrovascular disease) and glomerulosclerosis (primarily a microvascular disease), share some pathogenic elements; and that oxLDL (Table 3) plays a significant role in both processes (Diamond, 1991; Hunt et al., 1993; Hunt and Wolff, 1991; Kamanna et al., 1997; Magil, 1999; Moorhead et al., 1997; Salahudeen et al., 1997; Witztum, 1994a).

Although atherosclerosis is generally considered a macrovascular disease and glomerulosclerosis is mostly a microvascular disease, there are striking similarities between the two disease processes, leading one researcher to describe glomerulosclerosis as, "glomerular atherosclerosis" (Diamond, 1991; Moorhead et al., 1997). It is believed that both processes are initiated by endothelial insult (due to mechanical, chemical, bacterial, or viral injury), followed by trapping and oxidation of LDL, monocyte recruitment and activation, smooth muscle (mesangial) cell proliferation, and fibrous tissue formation (Colwell et al., 2001; Lopes-Virella and Virella, 1998; Raptis and Viberti, 2001; Ross, 1999).

Multiple lines of evidence converge on oxLDL, implicating it as a key pathogenic factor in these vascular diseases. First, oxLDL is present in both atherosclerotic and glomerulosclerotic lesions, as demonstrated by immunofluorescent staining

of human histological sections of samples obtained from both types of lesions with monoclonal antibodies to oxLDL (Itabe et al., 1994; Lee and Kim, 1998; Magil, 1999). Second, oxLDL appears to have chemotactic properties, contributing to the accumulation of monocyte-derived macrophages in both atherosclerotic plaques and glomerulosclerotic lesions (Berliner et al., 1995; Berliner et al., 1990; Kamanna et al., 1997; Kamanna and Kirschenbaum, 1997; Kamanna et al., 1999; Quinn et al., 1987; Quinn et al., 1985). Third, oxLDL has been demonstrated to induce proliferation of smooth muscle cells in atherosclerosis and smooth muscle cell-like mesangial cells in glomerulosclerosis, and cause the expansion of the extracellular matrix in both types of lesions (Diamond, 1991; Kamanna et al., 1997; Moorhead et al., 1997). Fourth, oxLDL has been shown to stimulate the transformation of macrophages into lipid-laden foam cells, and is considered cytotoxic (Ghosh et al., 1996; Itabe et al., 1994; Magil, 1999; Salahudeen et al., 1997; Steinberg, 1993; Steinbrecher et al., 1990). The accumulation of foam cells in lesion sites not only represents a hallmark of both atherosclerosis and glomerulosclerosis, but mounting evidence points to the central importance of these monocyte-derived macrophages in the pathogenesis of both lesions (Diamond, 1991; Itabe et al., 1994; Magil, 1999; Mitchinson, 1987; Ross, 1999; Witztum, 1994a)

LDL-IC and CE Accumulation in Macrophages

One property of oxLDL, and other modified analogs of LDL, that has received

considerable attention from our research group, and several others, is the immunogenicity of modified LDL. Even very minor modifications of LDL are sufficient to make it antigenic, as reflected by the common presence of oxLDL autoantibodies in humans (Lopes-Virella and Virella, 1996; Steinberg and Witztum, 1999; Steinbrecher et al., 1984a; Yla-Herttuala, 1998). Since it is reasonable to assume that that these antibodies will bind to their respective antigens, provided the antigen is present, an area of growing interest is the potential role of oxLDL and oxLDL-containing immune complexes (IC) in foam cell formation, atherogenesis, and glomerulosclerosis (Diamond, 1991; Lopes-Virella and Virella, 1998; Lopes-Virella et al., 1999; Magil, 1999). Exploration of the role of these IC in atherosclerosis has led to the observation that oxLDL and antibodies reacting with modified lipoprotein particles are present in atherosclerotic plaques, each having been identified and purified from such lesions (Palinski and Witztum, 2000; Steinberg et al., 1989).

In glomerulosclerosis, antibodies to oxLDL have not yet been separated from glomerulosclerotic lesions, and the potential role of oxLDL-IC in diabetic glomerulonephritis is unknown. There is, however, evidence suggesting that IC play a role in diabetic nephropathy that comes from the common observation of IC in the glomeruli of patients with diabetic nephropathy, as indicated by immunohistochemical staining and the presence of subendothelial high elecron density deposits (Ainsworth et al., 1982; Mazzucco et al., 2002; Osterby et al.,

2002; Severgina et al., 1994; Yoshikawa et al., 1990). In streptozotocin-induced diabetic rats, the presence of circulating IC was increased and associated with IgG deposition in the glomerular mesangium (Abrass, 1984). Additionally, diabetic glomerulosclerotic lesions have been reported to be exudative, contain foam cells, oxLDL, immunoglobulins, complement, fibrinogen, and albumin (Fioretto and Mauer, 1997, Magil, 1999 #113; Miller and Michael, 1976; Suzuki et al., 2001; Velosa et al., 1976).

Focusing on atherosclerosis, a connection between lesion formation and oxLDL-containing immune complexes has gained substantial recognition during the last decade, along with a growing body of evidence suggesting an active participation of LDL-IC in this process (Klimov et al., 1988; Lopes-Virella and Virella, 1994a; Lopes-Virella and Virella, 1998; Mironova et al., 1997; Salonen et al., 1992; Tertov et al., 1990a; Tertov et al., 1990b; Yla-Herttuala et al., 1994). For an excellent review of the involvement of LDL-IC and atherosclerosis in diabetes, the reader is referred to the 1997 *Diabetes Review* article by Lopes-Virella et al. (Lopes-Virella et al., 1997b). LDL-IC are believed to be important participants in the development of atherosclerosis via several avenues (Figure 5). Macrophages ingest LDL-IC more avidly than uncomplexed LDL via the Fcγ receptor I (FcγRI), thus contributing to foam cell formation. The cross-linking of Fcγ receptor by LDL-IC, in-turn, induce the release of pro-inflammatory cytokines (Lopes-Virella et al., 1997a; Virella et al., 1995).

Our research group pioneered this area of research, being first to examine the effects of presentation of LDL-containing IC on human monocyte-derived macrophages, subsequently engaging in extensive investigations of this phenomenon (Gisinger and Lopes-Virella, 1992; Gisinger et al., 1991; Griffith et al., 1988; Lopes-Virella et al., 1997a; Lopes-Virella et al., 1991; Lopes-Virella et al., 1997b; Lopes-Virella and Virella, 1996; Lopes-Virella and Virella, 1998). Research from our group demonstrated that the incubation of monocyte-derived macrophages with insoluble LDL-IC prepared with heterologous (rabbit) anti-LDL antibodies and human LDL led to massive (approximately 13-fold) intracellular accumulation of cholesterol and CE, causing foam cell formation in human monocyte-derived macrophages (Lopes-Virella et al., 1991). This set of experiments further indicated that the accumulation of cholesterol and CE resulted from a combination of increased uptake and impaired intracellular metabolism of the LDL ingested as part of an LDL-containing IC. The amount of LDL in human monocyte-derived macrophages after 5 hours incubation with heterologous LDL-IC was 7-fold higher than that observed in control macrophages incubated with equal amounts of native LDL. A totally unexpected finding was the lack of down regulation of the LDL receptor in macrophages with very high levels of intracellular cholesterol (Griffith et al., 1988), thus allowing unregulated uptake of LDL-IC into these lipid-engorged cells via two currently recognized receptor-mediated avenues: FcγRI, the primary ingestion pathway,

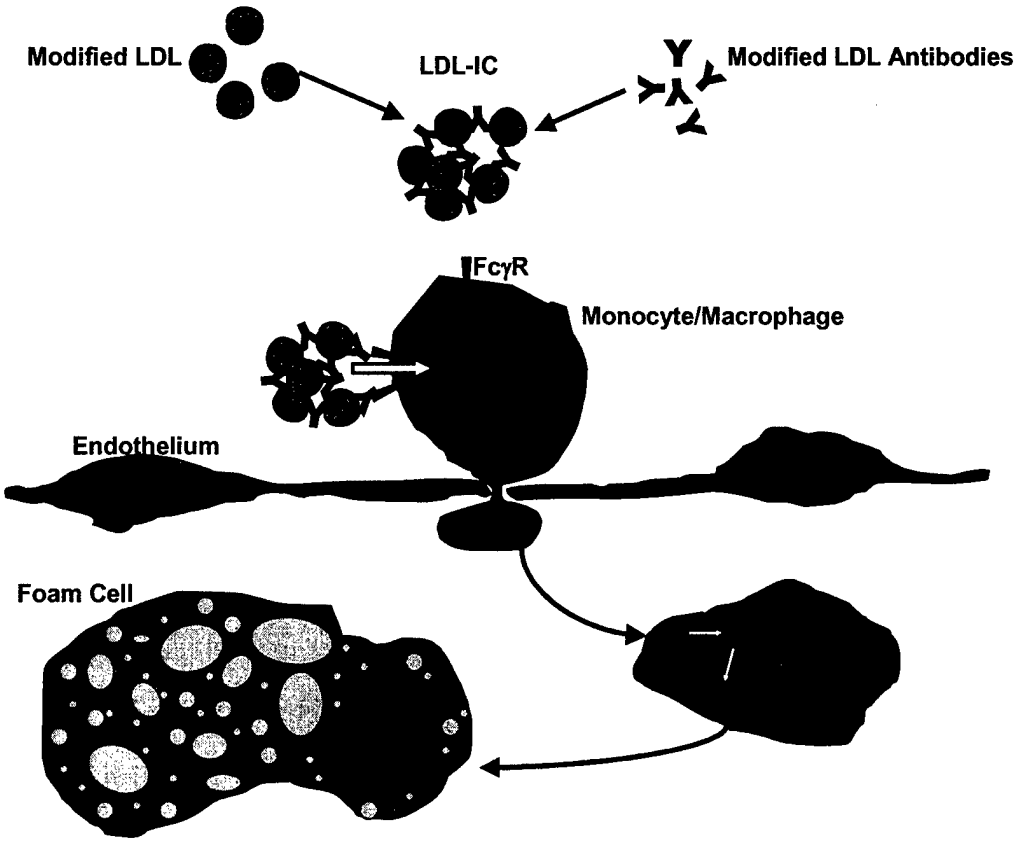

Figure 5. LDL-IC and foam cell formation (adapted from (Shunk, 1993). Antibodies are formed against modified LDL (e.g. oxLDL), and complex with their antigen, forming LDL-IC. Monocyte/macrophages from the circulation internalize the LDL-IC primarily through their FcγRI (Lopes-Virella et al., 1997a). As a consequence, the cells become activated, release cytokines that activate endothelial cells, adhere to the endothelium and undergo diapedesis. The CE from the lipoprotein core of the LDL component of the LDL-IC is hydrolyzed lysosomally and subsequently re-esterified by ACAT. When these lipid-engorged macrophages contain >50% of their total cholesterol as CE, they are, by definition, foam cells. Foam cells, or their immediate precursors, may contribute to the progression of the lesion by continuing to secrete various cytokines which, for example, promote smooth muscle cell proliferation (Ross, 1999), cause endothelial cells to express leukocyte adhesion molecules (Bevilacqua et al., 1985; Bevilacqua et al., 1989; Cavender et al., 1987), or are cytotoxic (Debets et al., 1990; Lopes-Virella and Virella, 1998; Nakagawara et al., 1981; Nathan, 1987). Monocyte/macrophages may also produce oxygen radicals (Nakagawara et al., 1981; Nathan, 1987), matrix metalloproteinases (Huang et al., 2000; Musson et al., 1980), and collagenases (Werb et al., 1980), which could further exacerbate lesion formation by promoting continued lipoprotein oxidation, damage to the subendothelial extracellular matrix, and damage to other cells in the intima.

and, to a much lesser extent, the LDL receptor (Griffith et al., 1988; Lopes-Virella et al., 1997a). Our research group also showed that the degradation of LDL bound to IgG was significantly impaired, barely surpassing the degradation of native LDL (1.09-fold higher), and led to a 200-fold increase in intracellular CE accumulation (Lopes-Virella et al., 1991). The characterization of the *in vitro* effects of heterologous LDL-IC was complemented by the demonstration that LDL-IC isolated from human sera had similar properties. Klimov *et al.* (Klimov et al., 1985) demonstrated excessive intracellular cholesterol and CE accumulation in mouse peritoneal macrophages exposed to LDL-IC isolated from human serum compared to that of macrophages incubated with LDL alone (Klimov et al., 1985). Later, the same investigators reported that LDL-IC isolated from the blood of patients with atherosclerosis by immobilized staphylococcal A affinity chromatography caused intracellular accumulation of CE in mouse pericardial macrophages, and morphological transformation of these cells into foam cells (Klimov et al., 1988). Mironova et al. (Mironova et al., 1997), working in our laboratory, observed significant CE accumulation in human monocyte-derived macrophages incubated with human IC isolated from human sera by PEG-precipitation.

LDL-IC and Scavenger Receptor Expression

As previously mentioned, the experiments with heterologous LDL-IC led us to the discovery of a paradoxical upregulation of LDL receptor in macrophages

exposed to LDL-IC containing large amounts of intracellular CE. Our studies of the binding kinetics of nLDL and macrophages stimulated with LDL-IC showed a 20-fold increase in LDL binding and that this increase in binding was due to an increase in LDL receptor number (Griffith et al., 1988). This observation was confirmed by inhibition of native LDL binding when the receptors were blocked using monoclonal antibodies against LDL receptors. Furthermore, up regulation of LDL-receptors was an LDL-IC specific phenomenon. When IC were prepared with other lipoproteins, including VLDL and HDL, and presented to human monocyte-derived macrophages, LDL receptor up regulation was not observed. These data are interpreted as suggesting that the increased expression of LDL receptors may significantly increase the influx of cholesterol-rich LDL into a cell that cannot adequately process accumulated cholesterol and that has lost the ability to down regulate LDL receptor expression.

In a separate series of investigations, Kiener *et al.* (Kiener et al., 1995) used a similar experimental design to implicated scavenger receptors in a similar function. Using phorbol-myristate acetate (PMA) treated THP-1 cells as a human monocyte-derived macrophage model, they investigated the effect of LDL-IC and acetylated LDL on these cells. After presenting the THP-1 cells with LDL-IC, they exposed the cells to acetylated LDL and were able to show an increase in intracellular accumulation of acetylated LDL. Following this, they discovered that the increase was due to a marked increase in scavenger

receptors, and not due to up regulation of LDL receptors. These observations, when conjoined with ours, suggest that LDL-IC transform macrophages into cells with exaggerated avidity for LDL, rapidly and actively taking up both native and modified forms of this lipoprotein, via LDL receptors and scavenger receptors. As a consequence, the cells accumulate cholesterol that they are unable to process. In summary, LDL-IC prepared with human LDL and rabbit anti-LDL antibodies appear to be the most efficient mechanism for the transformation of human monocyte-derived macrophages into foam cells (Griffith et al., 1988; Lopes-Virella et al., 1991). These observations needed to be confirmed in experiments where homologous LDL-IC made with modified human LDL and human anti-LDL antibodies would be incubated with human macrophages.

LDL-IC and Cytokine Release

Not only do LDL-IC cause the up regulation of macrophage LDL receptors and scavenger receptors, but their ingestion also results in the activation of a variety of pro-inflammatory macrophage functions. Researchers in our group have observed that LDL-IC are capable of inducing the synthesis and release of cytokines (TNF-α and IL-1), and stimulating respiratory burst more efficiently than any other IC (Virella et al., 1995).

Cytokine release, which is a common event observed with IC-activated macrophages, is a key step in the inflammation pathway, and has special

significance in atherogenesis. For example, in endothelial cells, IL-1 has been shown to stimulate the synthesis and surface expression of procoagulant activity and platelet activating factor (Bevilacqua et al., 1984; Breviario et al., 1988). Platelet activating factor can interact with granulocytes and directly or indirectly cause increased vascular permeability (Martin et al., 1988), and induce a positive feedback mechanism (Warner et al., 1987) causing even more release of IL-1 from endothelial cells, and induce expression of platelet-derived growth factor AA (PDGF-AA). PDGF-AA can then trigger fibroblast and smooth muscle cell proliferation through an autocrine growth-regulating mechanism (Raines et al., 1989). In addition to the effects of IL-1, TNF-α is known to induce similar responses, such as cell membrane expression of procoagulant activity and production of IL-1 by endothelial cells (Hansson et al., 1989; Nawroth et al., 1986). Both TNF-α and IL-1 can increase endothelial expression of VCAMs, promoting the adherence of monocytes/macrophages (Beekhuizen and van Furth, 1993; Cybulsky and Gimbrone, 1991; Pohlman et al., 1986). Added to this, foam cells, or their macrophage precursors, may contribute to the progression of the lesion by secreting various cytokines which act in a variety of ways, to include promoting smooth muscle cell proliferation (Ross et al., 1990), causing endothelial cells to express adhesion molecules (Bevilacqua et al., 1985; Bevilacqua et al., 1989; Cavender et al., 1987), or be cytotoxic (Debets et al., 1990; Lopes-Virella and Virella, 1998; Nakagawara et al., 1981).

In glomerulosclerosis, the accumulation of foam cells is also associated with disease progression (Klahr et al., 1988; Magil, 1999; Rovin and Schreiner, 1991). Amongst the pathogenetic alterations observed are glomerular remodeling caused by the release of growth factors (Lovett et al., 1983; Nathan, 1987), and glomerular functional changes via the elaboration of cytokines (Schreiner and Kohan, 1990). Additionally, there is a growing body of evidence implicating monocyte chemoattractant protein-1 (MCP-1), a specific chemoattractant for monocytes, in recruiting and activating monocytes/macrophages to the glomerulus in proliferative glomerular diseases (Banba et al., 2000; Murphy, 1994; Oppenheim et al., 1991; Rovin et al., 1992; Santriano et al., 1993; Yoshimura and Leonard, 1990).

LDL-IC and Endothelial Cell Damage

Since endothelial insult is considered to be an initiating factor in atherogenesis and glomerulosclerosis, appreciable research emphasis has focused on identifying factors that may be involved in it. Though not in the scope of this thesis, modified lipoproteins have long been implicated in vascular endothelial cell dysfuntion (Kamanna et al., 1997; Kamanna et al., 1999; Lopes-Virella and Virella, 1996; Ross et al., 1984). However, in addition to the direct effects of modified LDL on the endothelium, it is suspected that the activation of macrophages by insoluble LDL-IC may exert an indirect pathogenic effect on endothelial cells by generating reactive oxygen species (Virella et al., 1995) and

causing free radical damage, while promoting the oxidation of LDL. Yet another indirect pathogenic action of LDL-IC is through the formation of foam cells. These lipid-laden monocyte-derived macrophages may add further insult to the endothelium through the release of matrix metalloproteinases (Huang et al., 2000; Nathan, 1987). Additionally, a significant proportion of the damage induced by these cells is widely attributed to the release of superoxide radicals, and other reactive oxygen species which can cause increased oxidation of cell membrane lipids, among other effects (Baynes, 1991; Berliner et al., 1995; Boyce et al., 1989; Colwell et al., 2001; Hunt et al., 1993; Hunt and Wolff, 1991; Virella et al., 1995; Witztum, 1994a).

LDL-IC and Fc-γ Receptors

Having shown that LDL-IC can induce foam cell transformation in human monocyte-derived macrophages and cause these cells to express proinflammatory cytokines, two issues remain unanswered. First, how is it that LDL-IC interact with the macrophage; and second, how do these complexes become associated with damaged endothelium?

Regarding the interaction of monocyte-derived macrophages and LDL-IC, there is convincing evidence suggesting that LDL-IC is taken up Fc-γ receptors (Fc-γR). The first report of this came from the studies of Gisinger *et al.* (Gisinger et al., 1991), where they demonstrated that competing with heat aggregated gamma globulin (HAGG) effectively blocked CE accumulation in macrophages

incubated with LDL-IC. Blocking with native LDL and acetylated LDL had minimal effect on macrophage CE accumulation, ruling out the significant participation of LDL receptors and scavenger receptors, respectively. Subsequent studies have identified FcγRI and FcγRII as primary participants in the ingestion of LDL-IC by human monocyte-derived macrophages (Lopes-Virella et al., 1997a; Morganelli et al., 1995). The role of FcγR in the receptor-mediated uptake of LDL-IC was recently validated, when Shaw *et al.* reported that oxLDL-IC prepared with IgG-Fab fragments, which are not recognized by Fcγ receptors, were not ingested by macrophages (Shaw et al., 2001).

Endothelial cells would be an ideal platform for immobilization of circulating LDL-IC. Unfortunately, endothelial cells do not normally express FcγR, therefore the mechanism by which circulating LDL-IC interact with endothelial cells is unknown (Ryan, 1986). One possibility that fits well with the "response to injury" hypothesis of atherosclerosis and glomerulosclerosis is that infected or damaged endothelial cells have been reported to express FcγR (Bengualid et al., 1990; Bruggeman and van Dam-Mieras, 1991; Cines et al., 1982; Friedman, 1984; Hajjar, 1991; MacCormac and Grundy, 1996; Ryan, 1986). Since many of these agents have been associated with atherosclerosis, and have been identified in atherosclerotic lesions, it is not unreasonable to suggest that their presence could up regulate FcγR in these cells, and thus provide an anchor for soluble LDL-IC. Alternatively, IgG/IgG-containing IC might bind to cytoskeleton filaments

exposed on the surface of damaged endothelial cells (Bevilacqua et al., 1986). Regardless of the mechanism, once immobilized, an environment would be created where IgG-containing LDL-IC could interact with, and activate, macrophages and exacerbate lesion formation (Huang et al., 2000; Virella et al., 1995).

Thesis Purpose Statement

The main objective of the research reported in this dissertation was the verification of the role of human LDL-IC in diabetic nephropathy and foam cell formation. This investigation was suggested by three main observations. First, people with diabetes are at increased risk for macrovascular disease (atherosclerosis), microvascular disease (glomerulosclerosis), and dyslipidemia. Interestingly, atherosclerosis and glomerulosclerosis are somewhat analogous processes, sharing several pathogenic characteristics, including the presence of lipid-engorged monocyte-derived macrophages (foam cells). Second, foam cells are highly suspect participants, not innocent bystanders, in both processes. Third, macrophages are transformed into foam cells two ways: endocytosis of modified LDL (oxLDL, for example); but most efficiently when they ingest LDL-IC.

Based on a review of the literature and research regarding these observations, three primary goals were established for this dissertation.

1) Isolate and characterize oxLDL antibodies that are both free in circulation (unbound) and associated with LDL, forming soluble LDL-IC in serum.

2) Determine if LDL-IC are associated with diabetic nephropathy/glomerulosclerosis. It has already been shown that these complexes have macrovascular (atherosclerosis) connections; we tested the hypothesis that their presence was also linked to microvascular complications (e.g. glomerulosclerosis).

3) Determine if LDL-IC made from human oxLDL and purified human oxLDL antibodies stimulate foam cell formation. This goal was essential to validate the results obtained using LDL-IC prepared from human LDL and rabbit antibodies to human LDL in previous investigations of the pathogenic significance of LDL-IC.

CHAPTER 2

MATERIALS AND METHODS

Subjects and Samples

This study used blood samples collected from 1,068 subjects with type 1 diabetes enrolled in the Epidemiology of Diabetes Intervention and Complications (EDIC) study between May 1997 and May 2000. Blood was collected by venipuncture at the EDIC clinics, clotted at room temperature for 1 hour and centrifuged to separate serum. EDTA (1 mg/mL) was added to the serum to prevent LDL oxidation, serum aliquots were immediately stored at -70°C, and they were shipped monthly to the Medical University of South Carolina on dry ice. Informed consent was obtained from all the subjects included in the study. The patients were grouped following American Diabetes Association criteria (ADA, 2000) based on the average urinary albumin excretion (UAE) values measured in two determinations performed in a two year period, one prior to the collection of blood samples, and the other after. Patients excreting <30 mg/24 hr of albumin in the urine were considered normoalbuminuric; those excreting 30 to 299 mg/24 hr were classified as microalbuminuric, and those excreting ≥300 mg/24 hr were considered macroalbuminuric. Thirty-three

macroalbuminuric subjects and 29 microalbuminuric were selected for isolation of circulating IC based on their matching with 43 patients with normal albuminuria for gender, age (within 4 years), and HbA_1c levels (within 1%, except in three cases where the HbA_1c was greater than 9.5% and the differences exceeded 1%). A subgroup of 47 patients (22 with normal albuminuria, 11 with microalbuminuria, and 14 with macroalbuminuria) was randomly selected from the larger group of 105 patients for detailed characterization of soluble immune complexes.

Assay of OxLDL Antibodies

The concentration of antibodies to oxLDL was determined by the competitive enzyme immunoassay previously described (Virella et al., 1993). The assay is based on determining the difference in immunoglobulins binding to immobilized oxLDL between two aliquots of the same serum, one of which has been absorbed with oxLDL [400 μg/mL phosphate buffered saline, pH 7.4, containing 1% (w/v) bovine serum albumin (PBS-BSA)], while the other has been incubated with PBS-BSA alone (unabsorbed aliquot). The enzyme-conjugated antibody utilized in the assay was a polyvalent anti-IgG antibody that reacts not only with γ heavy chains, but also with κ and λ light chains, thus measuring antibodies of all isotypes. The assays were calibrated with a series of dilutions of a human serum calibrator with known concentrations of oxLDL antibodies (Koskinen et al., 1998). The antibody concentrations were calculated from the difference in O.D.

(414 nm) between unabsorbed and absorbed aliquots of the tested sera and were expressed in μg/mL.

Isolation of Circulating OxLDL Antibodies

OxLDL antibodies were isolated using a previously described affinity chromatography protocol (Mironova et al., 1996; Virella et al., 2000). In brief, freshly isolated LDL was dialyzed against a coupling buffer containing 0.1 mol/L of $NaHCO_3$ and 0.5 mol/L of NaCl (pH 8.3) and incubated overnight on a rocker at 4°C with CNBr-activated Sepharose 4B (Pharmacia Biotech) prepared in accordance with the manufacturer's instructions. At the end of this incubation, free binding sites were blocked with 0.2 mol/L glycine and, after extensive washing and degassing, the gel suspension was transferred to a chromatography column and washed thoroughly with PBS, pH 7.4. The column was then equilibrated with PBS containing 10 μmol $CuCl_2$, and the oxidation was allowed to proceed for 18 hr at 37°C. The oxidation reaction was stopped by washing the column extensively with PBS containing 300 μmol of EDTA and 200 μmol of butylated hydroxytoluene (BHT) per liter followed by five washes, alternating coupling buffer and 0.5 mol/L sodium acetate + 0.5 M NaCl, pH 4. Finally, the column was equilibrated with 0.01 mol/L $NaHCO_3$ buffer, pH 8.3. To isolate anti-oxLDL antibodies from serum samples, 2 mL of serum was diluted in 8 mL of 0.01 mol/L $NaHCO_3$ buffer, pH 8.3 and allowed to diffuse into the column. After loading, the column was incubated overnight at 4°C, and

unbound proteins were washed off with the equilibrating buffer. The bound antibodies were eluted with 0.1-mol/L $NaHCO_3$ (pH 8.3) buffer containing 0.5 mol/L NaCl. The purified antibodies were dialyzed against PBS, pH 7.4, and stored at -20°C until analyzed.

Immunoglobulin Isotypes in Purified OxLDL Antibodies

The distribution of immunoglobulin isotypes in the fractions eluted from the oxLDL column was determined by quantitating IgG, IgM, and IgA by radial immunodiffusion (RID) using low level RID kits purchased from The Binding Site Limited, San Diego, CA. The distribution of IgG subclasses in the oxLDL antibody elution was also determined by radial immunodiffusion using low level RID kits also purchased from The Binding Site.

Characterization of Soluble IC Precipitated with PEG

A modification of the technique described by Chenais et al. (Chenais et al., 1977) was used to precipitate immune complexes from sera. In brief, a 3 mL aliquot of serum was gently mixed under constant and gentle stirring with an equal volume of a freshly prepared and sterilized 8% polyethylene glycol (PEG) solution in borate buffered saline (BBS), pH 8.4, that had been added drop by drop. The samples, containing a final PEG concentration of 4%, were incubated overnight at 4°C and then centrifuged at 3000 rpm for 20 min. The supernatant was discarded and the precipitate was washed once with 14 mL of chilled 4%

PEG in BBS, centrifuged again and gently resuspended at 37°C in 3 mL PBS containing 0.01% EDTA, pH 7.4. Most studies were carried out with freshly isolated IC. When necessary, aliquots were stored at –20°C until further testing.

The protein content of PEG precipitates was measured by a modified Lowry assay (Griffith et al., 1988). The cholesterol content of the precipitated IC was determined by gas chromatography after extraction of the PEG precipitates with chloroform:methanol (2:1, v/v) (Hara and Radin, 1978). The apolipoprotein B content of IC was determined by quantitative immunoelectrophoresis (Lopes-Virella et al., 1982).

Isolation of IgG from PEG-Precipitated Immune Complexes

Aliquots of purified IC stored at –20°C were thawed and warmed to 37°C for 20 minutes and resuspended by gentle vortexing. The resuspended IC samples were loaded onto a protein G column (Pharmacia) and the column was washed with 0.1 M $NaHCO_3$ + 0.5 M NaCl buffer, pH 8.3. This buffer was used because its ionic strength is sufficient to elute human oxLDL antibodies from an immobilized oxLDL column, (Mironova et al., 1996; Virella et al., 2000) and therefore, was expected to dissociate any oxLDL-IgG containing IC, allowing the elution of oxLDL while retaining IgG due to the high avidity interaction between IgG and protein G. After washing, IgG was eluted using 0.1M Glycine-HCl-buffer, pH 2.7; and the fractions were immediately neutralized with 1M Tris-HCl-

buffer, pH 9.0. Neutralized eluates were then dialyzed against PBS overnight, aliquoted, and stored at –20°C until tested.

IgG Subclass Distribution in OxLDL Antibodies Purified from PEG-Precipitated Immune Complexes

IgG fractions isolated from PEG-precipitated immune complexes, prepared as previously described, were thawed, diluted to a total volume of 5 mLs with 0.01 mol/L $NaHCO_3$ buffer, pH 8.3 and allowed to diffuse into the oxLDL column. This column was then incubated overnight at 4°C, and unbound proteins were washed away with the 0.01mol/L $NaHCO_3$ (pH 8.3) equilibrating buffer. Following this step the bound antibodies were eluted with 0.1-mol/L $NaHCO_3$ (pH 8.3) buffer containing 0.5 mol/L NaCl. The purified antibodies were stored at -20°C until further testing. The distribution of IgG subclasses in the fractions eluted from the oxLDL column was determined by quantitating IgG1, IgG2, IgG3, and IgG4 by radial immunodiffusion (RID) using low level RID kits purchased from The Binding Site Limited, Birmingham, England. Unfortunately, the initial step of separating IgG from the immune complexes precluded isolation and quantitation of IgM and IgA antibodies specific for oxLDL in circulating soluble immune complexes.

Determination of the Dissociation Constants (Kd) of Purified Antibodies

An estimate of the avidity of each purified antibody eluate was obtained through

the measurement of K_d (a measure that is inversely proportional to avidity) by competitive enzyme immunoassay, using a modification of Friguet's method (Friguet et al., 1985), as adapted to the characterization of oxLDL antibodies by Mironova et al. (Mironova et al., 1996). For this purpose, we coated flat-bottomed Immulon type I plates with 7.5 μg of oxLDL per well. Purified oxLDL antibody was used at a final concentration of 100 μg/mL. A series of antibody aliquots was absorbed using concentrations of the apoB component of oxLDL ranging from 7.36×10^{-7} to 1.15×10^{-8} mol/L, calculated using an estimated molecular weight of 545kD for apoB.

Absorbed and unabsorbed samples were incubated in oxLDL-coated plates overnight at 4°C, and after this step the samples were processed as described for the anti-oxLDL antibody assay. The concentrations of antigen along with the absorbance values measured in unabsorbed and absorbed samples were used to construct a plot of v/a vs. v where v corresponds to bound antibody and v/a to bound antibody/free antigen at equilibrium (Friguet et al., 1985). The slope of the plot was used to calculate the K_d for each sample tested.

LDL Isolation

Blood was collected in EDTA (1 mg/mL) from normal volunteers after 12 hours of fasting, and plasma was collected after centrifugation. The LDL fraction was isolated from whole plasma by sequential ultra centrifugation through a KBr

discontinuous gradient and collected as the fraction floating at a density of 1.019 to 1.063 g/mL using a Beckman L5-50, type 50 rotor, washed by ultra centrifugation, dialyzed against a 0.15 M NaCl solution containing 1mM EDTA, pH 7.4 and stored under nitrogen in the dark (Lopes-Virella et al., 1982). LDL preparations were then passed through an Acrodisc filter (0.2 μm pore size) in order to remove aggregates, and a small aliquot taken for determination of protein concentration by the method of Lowry (Lowry et al., 1951).

LDL Oxidation

LDL was oxidized using the method described by Steinbrecher (Steinbrecher, 1987). To remove residual KBr and EDTA, the freshly isolated LDL was passed through a PD-10 column (Pharmacia Biotech, Uppsala, Sweden). Phosphate-buffered saline (PBS), pH 7.4, was oxygenated at 2 liters/min for 10 minutes, and $CuCl_2$ was added to a final concentration of 10 μmol/L for every 300 μg of LDL. The LDL final concentration was adjusted not to exceed 1.5 mg/mL, and the highest concentration of $CuCl_2$ used was 40μmol/L when the concentration of LDL was between 1.2 and 1.5 mg/mL. Oxidation was carried out at 37°C, and to continuously monitor the degree of oxidation, a 100 μg aliquot of the $CuCl_2$ treated LDL was diluted in 2 mL of PBS and continuously monitored at 37° spectrophotometrically using a wavelength of 360 nm for excitation and a wavelength of 430 nm to measure fluorescence emission (Cominacini et al., 1991). Four to six hours after fluorescence reached its peak (approximately 18

hours total time) EDTA and butyl-hydroxytoluene (BHT) was added to the incubation mixture at final concentrations of 300 and 200 μmol/L, respectively to stop the oxidation process. Copper and BHT were removed from the oxLDL preparations after termination of the oxidation reaction by overnight dialysis against 4 liters of a 0.15 mol/L concentration of NaCl containing 300 μmol/L of EDTA, pH 8.0. After dialysis, oxLDL was filtered through a sterile 0.22-μm-pore-size filter to sterilize and remove aggregates and stored at 4°C. The final protein concentration in each preparation was determined by a Lowry assay (Lowry et al., 1951).

Rabbit Immunization and LDL-hyperimmune antibody isolation

For immunization, a narrow density cut (1.030-1.050 g/mL) of unmodified human LDL was isolated. Each New Zealand White rabbit received a series of 3 immunizations over a six-week period, and were boosted as needed. For the primary immunization, 1 mg of unmodified LDL was mixed with Complete Freund's Adjuvant (CFA) to a final concentration of 1mg/mL, and the rabbit was injected intramuscularly with 0.5 mL LDL/CFA at two bilateral sites. Two weeks later, 1 mg of LDL was mixed with incomplete Freund's Adjuvant (IFA) to a final concentration of 1mg/mL, and the rabbit was injected intramuscularly with 0.5 mL LDL/IFA at two bilateral sites in different quadrants than the primary immunization. Two weeks later, 1mg of LDL was mixed with sterile PBS and the rabbit received a series of ten-100μL intradermal immunizations. Blood was

collected, and the hyperimmune serum was separated and frozen 10-14 days after the last immunization. Purified IgG was obtained from human LDL hyperimmune rabbit serum using Protein G Sepharose (Pharmacia Biotech) following the manufacturer's instructions.

Preparation of Heterologous Insoluble LDL-Immune Complexes

Insoluble immune complexes were prepared by incubating unmodified human LDL and rabbit anti-LDL antiserum at a 1:8 dilution in sterile PBS overnight, at 4°C. The concentrations of LDL and anti-LDL antibody used to prepare the immune complexes were determined by a precipitin curve, constructed by incubating one milliliter of diluted rabbit anti-LDL antiserum with varying amounts of LDL (50-500 μg) at 4°C overnight. The following day, the protein content of the insoluble LDL-IC was determined after washing the precipitate three times with sterile PBS by Lowry assay (Lowry et al., 1951) calibrated with serial dilutions of heavily heat-aggregated IgG containing known amounts of IgG. Using the results of precipitin curves, the ratio of antigen:antibody giving the greatest amount of precipitate was 200 μg LDL and 1 mL of a 1:8 dilution of our rabbit anti-LDL antiserum, with the typical yield being 750 μg immune complexes per mL of diluted rabbit anti-LDL antiserum. Insoluble LDL-IC preparations were sterilized by gamma irradiation.

Preparation of Human OxLDL-IC [oxLDL-IC(hu)]

Human oxLDL-IC were prepared using human oxLDL and purified human oxLDL antibodies. To establish the optimal concentrations for the formation of oxLDL-IC(hu), we performed a precipitin curve experiment in which 400 mg of purified human oxLDL antibodies was gently mixed with varying amounts of oxLDL (50-500 μg/mL) in a fixed volume (1 mL) and incubated overnight at 4°C. The following day human oxLDL-IC were precipitated using a modification of the technique described by Chenais et al. (Chenais et al., 1977). In brief, an equal volume of freshly prepared and sterilized 8% polyethylene glycol (PEG) solution in borate buffered saline (BBS), pH 8.4, was added drop by drop under constant and gentle stirring. The samples, containing the mixtures of antigen and antibody and a final PEG concentration of 4%, were incubated overnight at 4°C and then centrifuged at 3000 rpm for 20 min. The supernatant was discarded and the precipitate was gently resuspended in sterile 0.5 mL PBS containing 0.01% EDTA, pH 7.4. The protein content of these precipitates was determined by the method of Lowry (Lowry et al., 1951). Using the results of precipitin curves, the ratio of antigen:antibody giving the greatest yield of precipitate was 150 μg oxLDL per 1 mL purified oxLDL antibodies (400 μg/mL), with the typical yield being 250 μg oxLDL-IC. All studies were carried out with freshly isolated oxLDL-IC, stored no longer than 24 hours at 4°C and sterilized by gamma irradiation.

Cell Culture

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). THP-1 cells are well recognized for their ability to be transformed into macrophage-like cells (Auwerx, 1991). Compared to other human myeloid cell lines, such as HL-60, U937, KG-1, or HEL (Figure 6) cell lines, THP-1 cells are more closely related to monocytes. Additionally, differentiated THP-1 cells have been shown to mimic monocyte-derived macrophages morphologically and physiologically, making them a model of choice for studies of monocyte-derived macrophages (Auwerx, 1991). The cells were cultured at 37° C in a 5% CO_2 atmosphere in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) (Sigma) and antibiotics (penicillin @ 100 U/mL, streptomycin @ 100 μg/mL, amphotericin B @ 0.25 μg/mL). Transformation of the THP-1 cells into macrophages was performed in the medium described above with the addition of 160 nmol/L phorbol 12-myristate 13-acetate (PMA) for 3 days. After PMA treatment the medium was aspirated, the adherent cells were washed 3 times with sterile PBS, and the medium was replaced with a specially formulated serum free medium (SFM) (Stevenson et al., 1984) for stimulation with varying concentrations of oxLDL and oxLDL-IC. The SFM was composed of IMDM with the addition of bovine serum albumin (fatty acid free, 0.4%), human transferrin

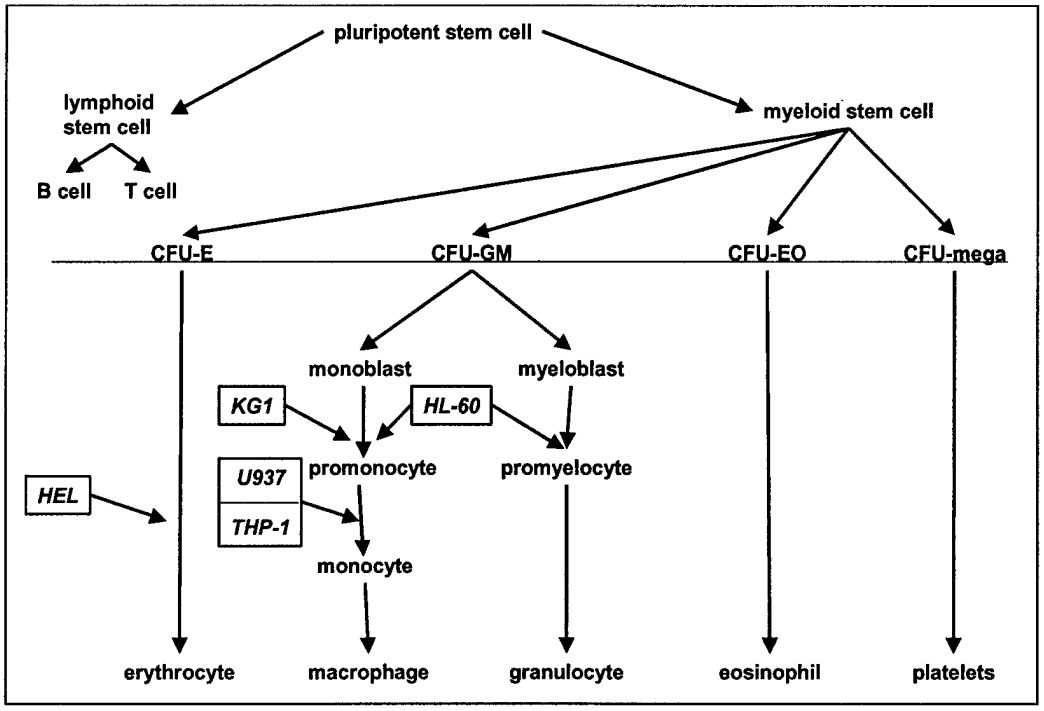

Figure 6. Hematopoietic cell differentiation: outline of the differentiation of hematopoietic stem cells. Some of the more frequently used myeloid leukemia cell lines are boxed. CFU=colony forming unit; E=erythrocyte; GM=granulocyte/monocyte; EO=eosinophil; and mega=megakaryocyte (Auwerx, 1991).

(1μg/mL), and L-phosphatidyl choline (1μg/mL) all from Sigma Chemical Company (St. Louis, MO); human insulin (0.128 U/mL, Eli Lilly Co., Indianapolis, IN); and ferrous chloride (0.07 nM, Fisher Scientific, Fairlawn, NJ).

Measurement of Free and Esterified Cholesterol Content

PMA-activated THP-1 cells (1 X 10^6cells) were incubated for 48 hr with human oxLDL-IC while oxidized low-density lipoprotein (oxLDL), insoluble oxLDL-IC, and SFM were used as controls. After incubation, the medium was removed, the cells were extensively washed with PBS, and the cell monolayer was extracted with hexane/isopropanol (3:2) (v/v) as previously described (Hara and Radin, 1978). Free and total cholesterol were assayed on a gas chromatograph equipped with a hydrogen flame ionization detector. A fused silica capillary column packed with DB17 was used for chromatographic separation and its temperature was maintained at 250°C during the separation. Nitrogen was used as the gas carrier.

To assay total cholesterol, the cellular extracts were evaporated to dryness and the residue was hydrolyzed by Ishikawa's method (Ishikawa et al., 1974) as previously described (Lopes-Virella et al., 1982). Cholesteryl ester levels were calculated by subtracting free cholesterol from total cholesterol levels. β-stigmasterol was used as an internal standard. After the lipid extraction, the cell

pellet was solubilized with 1 M NaOH, and the protein content determined (Lowry et al., 1951).

Statistical Analysis

Results are expressed as mean ± standard error, unless stated differently. All data were analyzed using InStat (GraphPad, San Diego, California) and SAS (SAS Institute, Inc., version 8, Cary, N.C.) software. ANOVA, with Bonferroni correction, was used to compare the results of different tests in the three UAE groups, using the data from patients with normal albuminuria as control. A paired t-test between two measurements of oxLDL antibody concentrations in serum against concentrations of the same antibodies isolated from LDL-IC was conducted. Analysis of covariance (ANOCOVA, Dunnett-Hsu correction) with normal albuminuria as control while adjusting for gender, age, HbA1c among three UAE groups was performed as indicated. Additional details about statistical analysis can be found in the footnotes for table 4 and on the legends for figures 10-13.

Chapter 3

Results and Discussion

Part 1: Characterization of Oxidized LDL Antibodies

Part 1: Introduction

In recent years, the role of atherogenic lipoproteins in human disease has been the object of intense research. This research has led to increased attention focusing on the free radical-mediated oxidative modification of lipoproteins and their participation in the development of macrovascular disease (atherosclerosis) and microvascular disease (glomerulosclerosis), two diseases found commonly in diabetic individuals (Cooper et al., 1997; Nathan, 1993). *In vitro* studies have revealed the apparent susceptibility of LDL to oxidation by endothelial cells, smooth muscle cells, macrophages, and renal mesangial cells (Cathcart et al., 1985; Morel et al., 1984; Parthasarathy et al., 1986; Steinbrecher et al., 1984b; Wheeler et al., 1994); and that oxidized LDL is present in atheromatous and glomerulosclerotic lesions (Itabe et al., 1994; Lee and Kim, 1998; Magil, 1999; Schlondorff, 1993; Yla-Herttuala et al., 1994). These observations have led to the belief that oxLDL is somehow involved in both processes.

Although atherosclerosis is generally recognized as a macrovascular disease and nephropathy is primarily a microvascular disease, they share several pathogenic similarities supported by a growing body of evidence suggesting that atherosclerosis and glomerulosclerosis are indeed analogous pathological processes (Diamond, 1991; Moorhead et al., 1997). OxLDL is a suspected active participant in both processes, and several theories have been proposed to explain how it appears to contribute to both processes. OxLDL is atherogenic (Steinberg and Witztum, 1999). It is known to induce chemotaxis and the recruitment of monocytes into the vascular intima, where monocyte-derived macrophages avidly ingest it via several types of scavenger receptors, greatly enhancing intracellular cholesterol accumulation and foam cell formation (Steinberg, 1993; Yla-Herttuala, 1998), with foam cells being well recognized hallmarks of both lesions (Guijarro and Keane, 1994; Kamanna et al., 1997; Lopes-Virella and Virella, 1998; Magil, 1999; Schwartz et al., 1991). Additionally, oxLDL causes endothelial cell dysfunction by upregulating expression of cell adhesion molecules, effectively enhancing the adhesion/interaction of monocytes and endothelial cells; and is known to be cytotoxic to cultures of vascular endothelial cells, glomerular endothelial cells, mesangial cells, and smooth muscle cells (Berliner et al., 1990; Jeng et al., 1993; Kamanna et al., 1999; Morel et al., 1983; Shiozawa, 2000; Steinberg and Witztum, 1990).

It is common knowledge that post-translational modification of LDL renders the

lipoprotein immunogenic, enabling it to interact with the immune system and generate the formation of antibodies (Steinbrecher et al., 1984a). In fact, autoantibodies binding to various epitopes of oxLDL have been described in humans, rabbits, and mice (Lopes-Virella et al., 1999; Orchard et al., 1999; Orekhov et al., 1991b; Palinski et al., 1994; Palinski et al., 1989; Parums et al., 1990). Interestingly, anti-oxLDL antibodies and LDL-IC have been detected in human sera with relatively high frequency, and the atherogenic potential of LDL-IC is attested to by a large body of experimental data suggesting that incubation of LDL-IC and human monocyte-derived macrophages severely disrupts the cell's cholesterol metabolism, leading to massive lipid accumulation in these macrophages (Gisinger and Lopes-Virella, 1992; Gisinger et al., 1991; Griffith et al., 1988; Klimov et al., 1985; Klimov et al., 1988; Lopes-Virella et al., 1991; Orekhov et al., 1991a; Orekhov et al., 1991b; Palinski et al., 1989; Salonen et al., 1992; Tertov et al., 1990a; Virella et al., 1993). In our laboratory, we have conclusively shown that co-incubation of LDL-IC and monocyte-derived macrophages induce foam cell formation more efficiently than any other known stimulus. Additionally, work from our research group has demonstrated the ability of LDL-IC to activate macrophages and to cause cytokine release (activate macrophages), while simultaneously promoting foam cell formation through a paradoxical increase in LDL receptors and scavenger receptors (Ghosh et al., 1996; Gisinger and Lopes-Virella, 1992; Lopes-Virella et al., 1991; Virella et al., 1995).

In light of these observations, it seems logical that there would be keen interest in determining if oxLDL antibody titers are clinically useful in either reflecting or predicting the severity index of atherosclerosis and/or glomerulosclerosis. Surprisingly, studies examining an association of oxLDL antibody titers and nephropathy are sparse and the results conflicting. While some groups have reported a positive correlation between oxLDL antibody titers and nephropathy, others have reported no significant differences in oxLDL antibody levels between groups with and without nephropathy (Bellazzi et al., 1993; Korpinen et al., 1997; Leinonen et al., 1998). Similar studies in atherosclerosis are not only more numerous, but have fueled spirited discussions. Many of these studies have reported elevated autoantibody titers in subjects with increased cardiovascular risk factors or clinically manifest atherosclerosis, including coronary artery disease, myocardial infarction, peripheral vascular disease, and hypertension (Bergmark et al., 1995; Branch et al., 1994; Heitzer et al., 1996; Holvoet et al., 1999; Maggi, 1994; Puurunen et al., 1994). In contrast, an equally impressive number of studies, including observations from our laboratory, have found no correlation between the titers of circulating oxLDL antibodies and clinical indicators of atherosclerosis; and, interestingly, one group even reported a negative correlation (Festa et al., 1998; Lopes-Virella et al., 1999; Maggi, 1994; Mironova et al., 1997; Orchard et al., 1999; Virella et al., 1993).

These conflicting data may be due, at least in part, to small sample size,

sampling demographics, or to differences in screening methodologies. It is hoped that large-scale clinical studies currently underway and standardization of oxLDL antibody testing protocols may help resolve these issues. However, the relationship between oxLDL antibody titers and the severity of atherosclerosis, or glomerulosclerosis, is undoubtedly complex and may involve the interaction of one or more of the following scenarios: 1) Accurate determination of the overall burden of atherosclerosis/glomerulosclerosis is problematic, leaving the real possibility that non-invasive measurements do not accurately reflect the extent of disease; 2) Measuring free oxLDL antibody titers may be inaccurate because an unknown quantity of oxLDL antibodies may be bound to circulating antigens, forming LDL-IC, as has been reported by our research group in subjects with diabetes (Lopes-Virella et al., 1999; Mironova et al., 2000; Mironova et al., 1997); 3) OxLDL antibodies may combine with oxLDL in circulation or in tissues other than arteries and the kidneys, allowing the arteries and kidneys to remain relatively free of pathological changes in spite of high levels of oxLDL antibody. In light of these potential pitfalls, and the fact that more than one of the above processes may be involved, it is surprising that an appreciable number of studies found a correlation between atherosclerosis, or glomerulosclerosis, and oxLDL antibody titers at all.

Therefore, we decided to isolate and characterize free and IC-bound oxLDL antibodies from human sera to confirm their presence and to attempt to find

answers to these conflicting reports. This stimulus has led to considerable effort from our research group directed toward isolating and characterizing human oxLDL antibodies from serum and oxLDL-IC. The task at hand is ongoing, and began with the isolation and characterization of oxLDL antibodies from the serum of eight subjects (Mironova et al., 1995), and subsequently was expanded to include the characterization of oxLDL antibodies isolated from serum and IC from 46 subjects. The following results report the findings of this portion of our study.

Part 1: Results

Characterization of free serum oxLDL antibodies: oxLDL antibodies were isolated from the serum of 46 subjects with type 1 diabetes, using a column containing oxLDL cross-linked to Sepharose, as described in Materials and Methods. Serum concentrations of oxLDL immunoglobulin isotypes (Figure 7) and IgG subclasses (Figure 8) were determined by radial immunodiffusion (RID). The predominant immunoglobulin isotype was IgG (83%), exceeding IgM (14%) by a factor of 6. The amount of IgA (3%) oxLDL antibodies isolated from serum was minimal. Quantification of IgG subclasses demonstrated a predominance of IgG1 and IgG3, with IgG1 accounting for 72% of the total IgG levels and IgG3 making up 21%. The average affinity of free oxLDL antibodies was determined via the dissociation constant (Kd) and found to be $1.13 \pm 0.13 \times 10^{-8}$ mol/L.

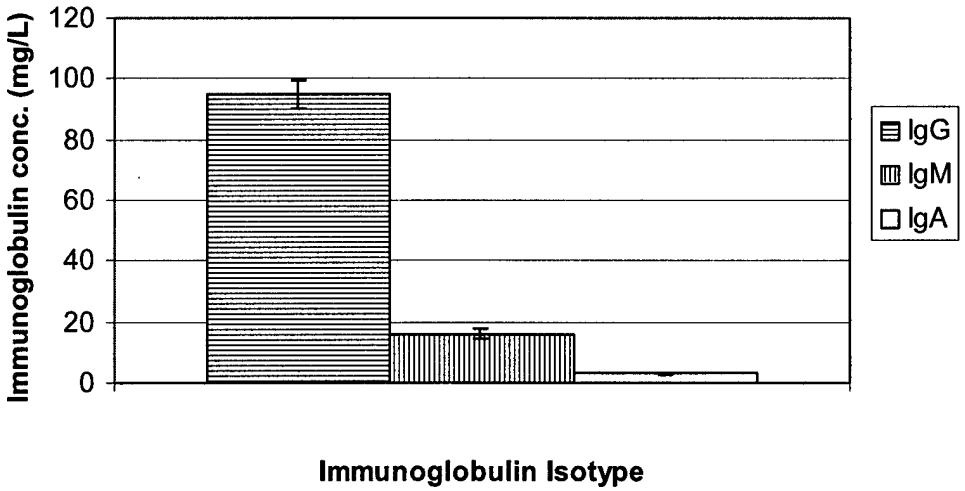

Figure 7. Immunoglobulin isotypes of oxLDL antibodies isolated from serum of human type 1 diabetic subjects (N=46). Antibodies were isolated using an oxLDL cross-linked to Sepharose column. Immunoglobulin isotypes were determined by radial immunodiffusion. The data in the graph is expressed as mean (mg/L) ± SEM.

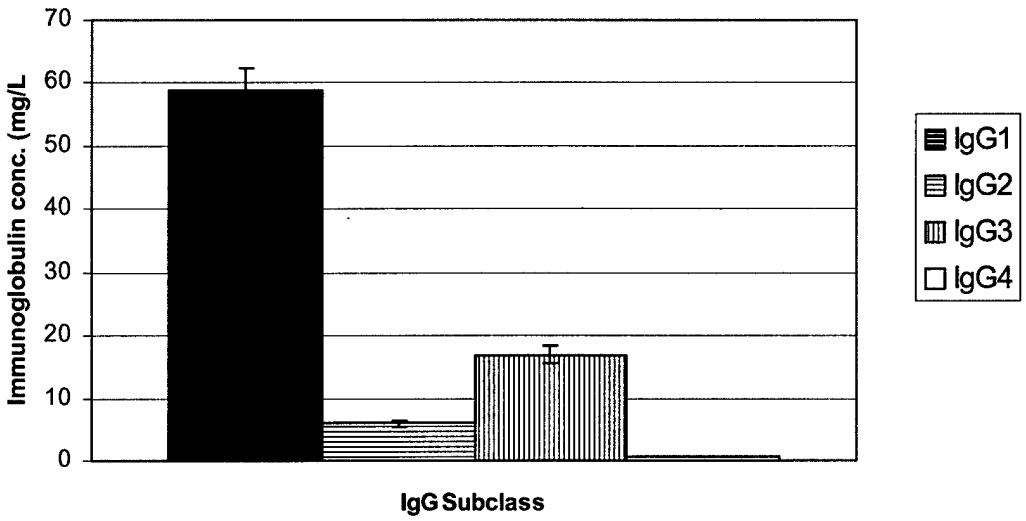

Figure 8. IgG subclass distribution of oxLDL antibodies isolated from serum of human type 1 diabetic subjects (N=46). Antibodies were isolated using an oxLDL cross-linked to Sepharose column. IgG subclasses were determined by radial immunodiffusion. The data in the graph is expressed as mean (mg/L) ± SEM.

Characterization of oxLDL antibodies isolated from immune complexes:

The isolation of oxLDL antibodies from immune complexes was a complex multiple-step process that began with the precipitation of soluble immune complexes present in serum, using a final concentration of 4% PEG. After precipitating this heterogeneous mixture of immune complexes, we purified polyclonal IgG from the immune complexes, and then measured anti-oxLDL activity in the purified IgG. This was not an easy task, and involved a modification of a technique developed previously in our laboratory (Mironova et al., 1997). This technique utilized a protein G column (Pharmacia) to separate IgG from immune complexes. However, the apparent affinities of oxLDL antibodies purified in this manner were lower than the affinities of serum (free) antibodies [unpublished data], and the purified IgG fraction was found to contained LDL. To overcome this limitation, we capitalized on the observation that a high salt buffer could disrupt the antigen:antibody interaction of oxLDL-IC, while having a relatively minimal effect on the binding of IgG to protein G. This process allowed us to capture immune complex-associated IgG on a protein G column, dissociate oxLDL, and recover oxLDL-free IgG antibodies for further testing. Using this protocol, we purified IgG oxLDL antibodies from IC, and determined their Kd. We found that the avidities of oxLDL IgG antibodies isolated from immune complexes appeared to be higher than the affinities of free oxLDL antibodies isolated from serum ($0.86 \pm 0.1 \times 10^{-8}$ vs. $1.13 \pm 0.1 \times 10^{-8}$ mol/L; $p < 0.05$). Additionally, we were able to determine the IgG subclass

distribution of oxLDL antibodies (Figure 9) from the IC-derived IgG isolates of 9 subject's sera by purifying the oxLDL antibody fraction of IgG, using oxLDL cross-linked to Sepharose column, and found it not significantly different than that of free oxLDL antibodies isolated from serum.

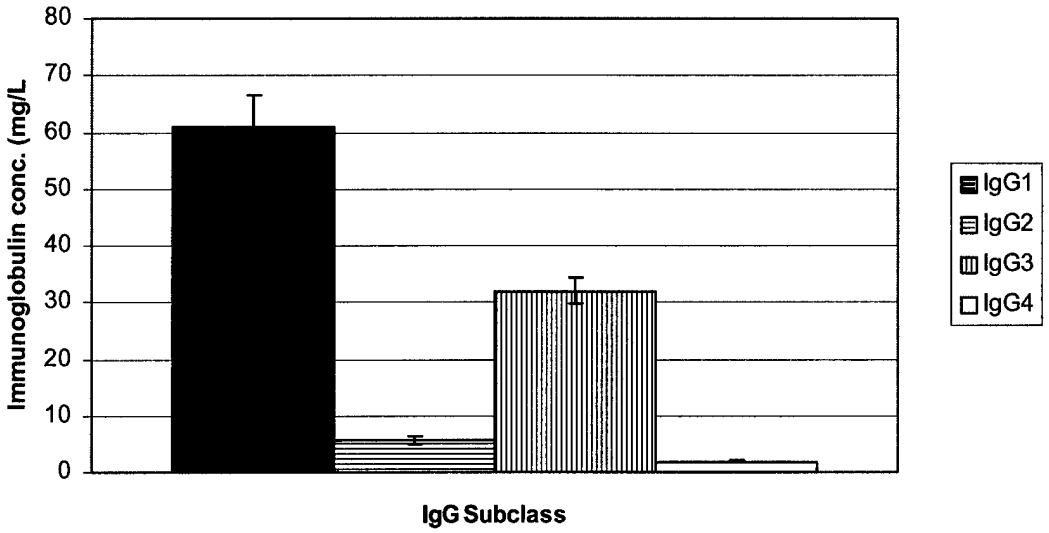

Figure 9. Immunoglobulin IgG subclasses oxLDL antibodies isolated from PEG-precipitated immune complexes of human type 1 diabetic subjects (N=9). IgG subclasses were determined by radial immunodiffusion, using IgG antibodies purified from PEG-precipitated immune complexes by a combination of protein G affinity chromatography in oxLDL-specific antibodies using an oxLDL cross-linked to Sepharose. The data in the graph is expressed as mean (mg/L) ± SEM.

Part 1: Summary

Isotypic characterization of free oxLDL antibodies isolated from serum demonstrated that IgG was the primary isotype, accounting for more than 80% of the isolated oxLDL antibodies, followed by small amounts of IgM and minute amounts of IgA. The IgG subclass distribution observed in the purified oxLDL antibodies showed predominance of IgG1 and IgG3, with similar profiles being observed in oxLDL antibodies isolated from IC. We view this finding as significant because these IgG subclasses are efficient activators of complement, and interact with the three types of FcγR; therefore, appear capable of forming immune complexes with proinflammatory properties (Virella, 2001), and warrant further investigation. An EIA-based determination of oxLDL avidities showed that oxLDL antibodies isolated from serum had higher dissociation constants (Kd) than those observed in oxLDL antibodies isolated from immune complexes ($Kd = 1.13 \pm 0.13 \times 10^{-8}$ mol/L vs. $0.86 \pm 0.06 \times 10^{-8}$ mol/L; $p<0.05$), indicating that immune complexes are formed with oxLDL antibodies of higher avidity than that of antibodies that remain in circulation.

Collectively, these findings indicate a possible pathogenic role for oxLDL antibodies and the oxLDL-IC they help form. Our data strongly suggest that oxLDL antibodies are predominantly of isotypes and subclasses able to interact with Fc receptors on phagocytic cells and to activate complement, therefore, are considered proinflammatory. The observation that the avidity of antibodies

isolated from soluble IC exceeds the avidity of free antibodies isolated from serum suggests that antibodies of oxLDL are of sufficiently high avidity to form stable IC. These observations, furthermore, indicate that LDL-IC formation may help explain the discrepant association of oxLDL antibody levels and atherosclerosis/glomerulosclerosis. It seems reasonable that these IC concentrations may have a stronger correlation with vascular disease than that of oxLDL antibody levels alone. Considering the potentially proinflammatory nature of the antibodies involved in oxLDL-IC formation, further studies examining the association of oxLDL-IC and vascular disease are needed, as well as *in vitro* investigations examining the potential role of IC in atherosclerotic and glomerulosclerotic disease models.

Part 2: Antioxidized LDL-Containing Immune Complexes in Diabetic Nephropathy

Part 2: Introduction

It is well known that people with type 1 diabetes are at increased risk of developing microvascular and macrovascular diseases (Lloyd et al., 1996; Maser et al., 1991; Orchard et al., 1990; Raskin, 1994), with macrovascular disease being the leading cause of morbidity and mortality in this group. In toto, these complications translate into an increased likelihood of ischemic heart disease, stroke, amputation, kidney failure, and blindness. Interestingly, nephropathy, a microvascular complication of diabetes, has been shown to accelerate atherosclerosis in patients with diabetes, and has been identified as a significant independent risk factor for macrovascular disease (De Cosmo et al., 1997; Ishimura et al., 2001; Jensen et al., 1987), suggesting that the two processes are interrelated. Several hypotheses have been put forward to explain how nephropathy accelerates the development of macrovascular disease. The most commonly accepted hypothesis postulates that abnormalities in lipoprotein metabolism secondary to nephropathy lead to the formation of atherogenic lipoproteins and, as a consequence, to the accelerated development of atherosclerosis (Groop et al., 1996; Hirano, 1999). Some of the better characterized lipoprotein changes in nephropathy include LDL glycation and oxidation – the former enhancing the formation of oxidized LDL (oxLDL) as well

as of advanced glycated end-products (AGE)-LDL (Salahudeen et al., 1997). OxLDL and AGE-LDL play a role not only in the development of atherosclerosis but also in the development of nephropathy (Diamond, 1991; Raptis and Viberti, 2001; Witztum, 1994a). Our present study is mainly focused on the role of oxidized LDL, independently, and as a component of oxLDL-containing immune complexes (oxLDL-IC), in the pathogenesis of diabetic nephropathy.

OxLDL has been demonstrated in both atherosclerotic plaques and in glomerulosclerotic lesions, where it appears to be actively involved in lesion formation. It stimulates monocyte endothelial cell interactions (Berliner et al., 1990; Shi et al., 2000), and acts as a chemoattractant, recruiting macrophages into atheromatous as well as into glomerulosclerotic lesions (Diamond, 1991; Kamanna et al., 1999; Kamanna et al., 1996; Kaplan and Aviram, 1999; Takaku et al., 1999; Witztum, 1994b). After recruitment of macrophages into the lesions, these cells take up oxLDL and are transformed into foam cells, hallmarks of both lesions (Itabe et al., 1994; Kamanna et al., 1997; Lopes-Virella and Virella, 1998; Magil, 1999). Besides inducing accumulation of foam cells in both atherosclerotic and glomerulosclerotic lesions, oxLDL induces cell proliferation (smooth muscle cells in atherosclerotic lesions and mesangial cells in glomerulosclerosis) and causes expansion of extracellular matrix in both processes via mechanisms that are both complex and poorly defined (Diamond, 1991; Han and Pak, 1999). Although the exact mechanisms are not known,

macrophages undoubtedly occupy a pivotal role in these processes based on the observation that they secrete a variety of cytokines, including transforming growth factor-β which contributes to the expansion of the extracellular matrix via fibronectin, collagen, and proteoglycan synthesis; and IL-1β which is known to induce proliferation of vascular smooth muscle cells and mesangial cells (Ku et al., 1992; Nathan, 1987; Tesch et al., 1997).

One property of oxLDL that has attracted considerable attention is its immunogenicity, as reflected by the formation of oxLDL autoantibodies (Lopes-Virella and Virella, 1998; Lopes-Virella et al., 1999; Steinbrecher et al., 1984a). Evidence for the involvement of humoral immune factors including autoantibodies against modified forms of LDL in atherogenesis has been supported by data from experimental models and clinical studies (Bergmark et al., 1995; Griffith et al., 1988; Kiener et al., 1995; Klimov et al., 1988; Lopes-Virella et al., 1991; Lopes-Virella and Virella, 1994a; Mironova et al., 1997; Orchard et al., 1999). In humans, LDL-containing immune complexes (LDL-IC) are present in the blood of subjects with and without demonstrable vascular disease (Klimov et al., 1988; Mironova et al., 1997; Tertov et al., 1990a; Tertov et al., 1990b), and appear to contribute to atherosclerosis via enhanced intracellular cholesterol and cholesteryl ester accumulation in macrophages, as well as their ability to activate macrophages (Klimov et al., 1988; Lopes-Virella and Virella, 1996; Tertov et al., 1990a; Tertov et al., 1990b; Virella et al., 1995). Studies

performed in our laboratory (Griffith et al., 1988; Lopes-Virella et al., 1991) have shown that co-incubation of human monocyte-derived macrophages with heterologous LDL-IC prepared with human LDL and rabbit anti-LDL antibodies is the most efficient mechanism for inducing foam cell formation. Similar observations have also been reported using immune complexes isolated from human serum (Mironova et al., 2000; Mironova et al., 1997). In contrast, studies examining the possible correlation between the levels of circulating oxLDL antibodies and clinical evidence of atherosclerosis have yielded contradictory and inconclusive results (Bergmark et al., 1995; Festa et al., 1998; Klimov et al., 1988; Lopes-Virella et al., 1999; Maggi, 1994; Orchard et al., 1999). Furthermore, the pathogenic role of oxLDL antibodies has recently been questioned in two animal studies, wherein the immunization of LDL-receptor deficient rabbits (Palinski et al., 1995) and apo-E deficient mice (George et al., 1998) with MDA-LDL appeared to have a protective atherogenic effect. The interpretation of these results to suggest an atherogenic protective role for humoral immunity to oxLDL in humans (Wick and Xu, 1999) is problematic because the studies were performed using genetically altered animals, the titers of antibodies in immunized animals were higher than those measured in nonimmunized animals, the isotype and avidity of elicited antibodies was not characterized, and a later study demonstrated that the protective effect of immunization was not due to oxLDL antibody production (Freigang et al., 1998). The lack of correlation between concentrations of circulating antibodies and

parameters indicative of atherosclerosis in humans may be explained, at least in part, by the interference of circulating immune complexes in oxLDL antibody assays. We have previously reported an inverse correlation between the serum concentrations of oxLDL antibodies and of LDL-containing immune complexes (Lopes-Virella et al., 1999; Mironova et al., 1997). Thus, the paradoxical inverse correlation between circulating oxLDL antibodies and vascular disease may result from the underestimation of oxLDL antibody levels due to the presence of large concentrations of circulating oxLDL-IC (Lopes-Virella et al., 1999; Mironova et al., 1997). Also, the antibodies responsible for IC formation could be of higher avidity than those that remain free in serum. As a consequence, the resulting immune complexes would be relatively stable and more able to cause inflammatory reactions in sites of formation or deposition. In contrast, the free circulating antibodies, of lower avidity, may not even play a pathogenic role. The direct correlation observed between LDL-IC levels and the development of coronary heart disease reported by us in a previous study certainly supports this interpretation of the experimental facts (Lopes-Virella et al., 1999).

An attractive hypothesis to explain the better correlation between LDL-IC and atherosclerosis is that the oxLDL antibodies responsible for IC formation are of higher avidity than those that remain free in serum. As a consequence, the resulting immune complexes would be relatively stable and more able to cause inflammatory reactions in sites of formation or deposition. In contrast, the free

circulating antibodies, of lower avidity, may not even play a pathogenic role.

In light of the hypothesis that atherosclerosis and glomerulosclerosis share pathogenic factors (Diamond, 1991; Kamanna et al., 1997), and of our previous observation of a positive correlation between atherosclerosis and LDL-IC, along with a negative correlation with free oxLDL antibodies, we decided to investigate if the levels and characteristics of LDL-IC correlated with diabetic nephropathy. In the current study, we have tested the hypothesis that nephropathy is associated with the concentration of circulating immune complexes containing oxLDL, not with free antibody against oxLDL, that the avidity of antibodies leading to immune complex formation is higher than the avidity of those antibodies remaining free in circulation, and that diabetic subjects with increased albuminuria have oxLDL antibodies in their LDL-IC of higher avidity than patients without albuminuria. To answer these objectives, we have determined in 105 subjects with type I diabetes, subdivided into 3 groups according to their degree of albuminuria (an index of nephropathy), the concentration and avidity of free circulating oxLDL antibodies, as well as the concentration and avidity of these antibodies in circulating immune complexes.

Part 2: Results

The clinical and laboratorial characteristics of the 3 subgroups of subjects studied are summarized in table 4. The three subgroups were closely matched, by design, for gender, age, and degree of glycemic control at the beginning of the study. Significant differences in their lipid profile were observed, as expected. As shown in table 4, when compared to the subjects with normoalbuminuria, the subjects with macroalbuminuria had increased levels of total cholesterol and triglycerides that reached statistical significance, while the patients with microalbuminuria has significant lower levels of HDL-cholesterol than those with macroalbuminuria or normoalbuminuria. The levels of circulating oxLDL antibodies in the 3 subgroups studied were increased in the groups with microalbuminuria and macroalbuminuria relative to the normoalbuminuric group. However, the increase did not reach statistical significance ($p=0.0885$). A trend analysis comparing UAE with concentrations of circulating oxLDL antibodies also failed to achieve statistical significance ($p=0.0520$).

TABLE 4 – Characteristics of selected study subjects from DCCT/EDIC cohort

	Normoalbuminuric	Microalbuminuric	Macroalbuminuric	*p*
N	43	29	33	
Sex (male/female)	33/10	23/6	25/8	ns
Age (years)	40±8	40±8	40±8	ns
HbA_1c(%)	8.7±1.1	9.2±1.3	9.0 ±1.3	ns
Chol (mg/dL)	189±33	188±33	215±40	<0.003
HDL (mg/dL)	56±12	49±10	57±15	<0.05
LDL (mg/dL)	117±30	117±25	130±26	ns
TGL (mg/dL)	81±53	114±69	139±85	<0.002
Anti-oxLDL (mg/dL)	44±5	54±9	72±13	ns

Data are means ± SD. Statistical significance was determined by an ANOVA among three matched groups with contrast to normal albuminuria as a control group, testing within-group differences.

Measurement of oxLDL antibodies, cholesterol and apolipoprotein B was performed in immune complexes isolated by precipitation with 3.5% of PEG. There was no significant difference in the concentrations of oxLDL antibodies in the PEG precipitates (data not shown). However, as shown in figure 10A, the mean total cholesterol concentration in PEG precipitates was higher in the macroalbuminuric (272±20 mg/L) and microalbuminuric (244±20mg/L) groups, compared to the normoalbuminuric group (204±18 mg/L). The difference between the cholesterol content of IC in normoalbuminuric and macroalbuminuric groups reached statistical significance ($p<0.03$). Additionally, levels of apolipoprotein B in PEG precipitates showed similar increases in the macroalbuminuric (134±10 mg/L) and microalbuminuric (110±11 mg/L) groups compared to the normoalbuminuric group (99±9 mg/L), with the difference reaching statistical significance when the macroalbuminuric and normoalbuminuric groups were compared ($p<0.03$, figure 10B). These data indicate a direct correlation between the levels of LDL-IC and UAE.

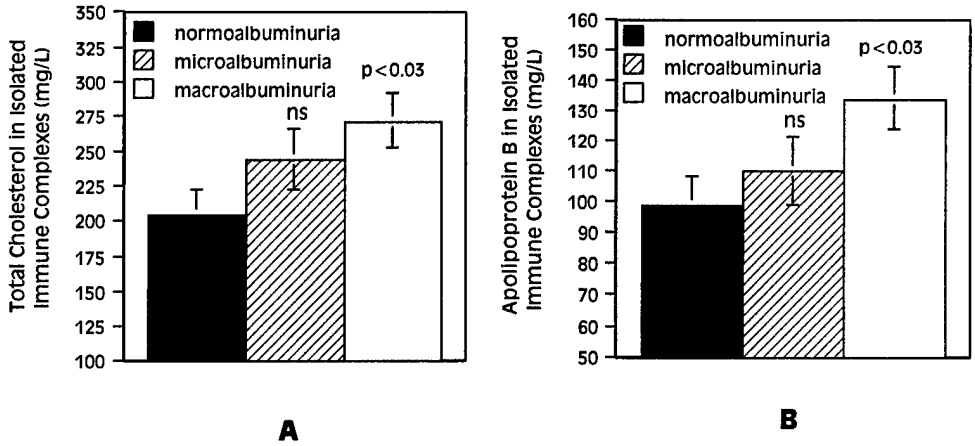

Figure 10: Total cholesterol (A) and Apolipoprotein B (B) levels in polyethylene glycol-precipitated immune complexes of 105 type 1 diabetic subjects with varying degrees of nephropathy: 43 subjects had normoalbuminuria, 29 had microalbuminuria, and 33 subjects had macroalbuminuria. 0.5 mL serum was precipitated with polyethylene glycol and cholesterol was extracted using a chloroform:methanol mixture (2:1, v/v). Cholesterol levels were determined using gas chromatography. Apolipoprotein B levels were determined using quantitative immunoelectrophoresis. Results are given in mg/L and data is expressed as mean ± SEM. Statistical significance was determined by an analysis of covariance (ANOCOVA) with normal albuminuria as control group when adjusted for gender, and age, and HbA1c. A significant difference was observed between the contents of cholesterol and apolipoprotein B in PEG-precipitated IC between the group of patients with macroalbuminuria and the group of patients with normal levels of urinary albumin.

The presence of cholesterol and apolipoprotein B in PEG precipitates does not prove that the precipitated LDL is oxLDL. However, we have obtained strong indirect evidence for the presence of oxLDL in PEG precipitates by isolating IgG antibodies specific for oxLDL from PEG precipitates. We started by fractionating PEG precipitates by affinity chromatography using immobilized streptococcal protein G. Using conventional chromatography conditions that involved washing unbound proteins from the protein G column with 20 mM sodium phosphate buffer, pH 7.0, small concentrations of cholesterol are uniformly present in the IgG fraction eluted with 0.2M glycine-HCl buffer, presumably indicating the retention of lipoprotein-IgG complexes in the column. The presence of LDL-IC in the IgG eluates is undesirable because it potentially interferes with the assay of oxLDL antibody avidity. To avoid this problem, we modified the elution protocol so that we could dissociate LDL-IC while retaining the absorbed IgG, wash out the dissociated LDL, and elute antigen-free IgG (table 5). To this end, we washed the protein G column with 0.1 M NaHC03 pH 8.3 buffer containing 0.5M NaCl. Under these conditions, the IgG eluted with 0.2M glycine-HCl, pH 2.7 was virtually devoid of cholesterol (cholesterol was undetectable in three out of four eluates). The IgG fractions obtained with both protocols contained oxLDL antibodies, measured by our competitive immunoassay. The recoveries of IgG and of oxLDL antibody did not differ significantly with the two different elution protocols. It must be noted that the recovery of oxLDL antibody was in apparent excess of the amount loaded in the column, this being at least in part, a

consequence of the interference of IC in the assay of oxLDL antibody. Also, when the concentrations of oxLDL antibodies in serum and PEG precipitated immune complexes were compared, the levels measured in the IgG fraction isolated from the PEG precipitates were significantly higher than the levels measured in serum (Figure 11). The presence of significant amount of oxLDL antibodies in the purified IgG fraction confirmed our assumption that PEG-precipitated circulating immune complexes contain oxLDL complexed with its corresponding antibody in subjects with type 1 diabetes. Interestingly, we detected small amounts of oxLDL antibodies in protein G washout fractions, most probably corresponding to immune complexes containing immunoglobulin isotypes not bound by protein G (primarily IgA and IgM).

Table 5. Comparison of elution protocols for isolation of IgG from PEG precipitates of four different individuals. A PEG precipitate was fractionated in a protein G column in triplicate under two sets of elution conditions.

	Original elution protocol[a]		**Modified elution protocol[b]**	
Initial load	Cholesterol*	480[¶]	Cholesterol*	480[¶]
	IgG*	1000[¶]	IgG*	1000[¶]
	ox-LDL Ab*	60[¶]	ox-LDL Ab*	60[¶]
Wash-out	Cholesterol*	366 ± 40	Cholesterol*	443 ± 97
	IgG*	<18	IgG*	<18
	ox-LDL Ab*	12.3 ± 1.3	ox-LDL Ab*	24.9 ± 0.5
Final eluate	Cholesterol*	2.9 ± 2.3	Cholesterol*	0.2 ± 0.3
	IgG*	768 ± 51	IgG*	797 ± 110
	ox-LDL Ab*	127 ± 61	ox-LDL Ab*	131 ± 41

[a] Wash-out with 20 mM sodium phosphate buffer, pH 7.0 followed by elution with 0.2M glycine-HCl, pH 2.5

[b] Wash with 0.1 M NaHC03 pH 8.3 + 0.5M NaCl, followed by elution with 0.2M glycine-HCl, pH 2.5.

* All values are means, expressed in micrograms ± SD (n=4)

[¶] Concentrations measured in the original precipitate

Ab = antibody

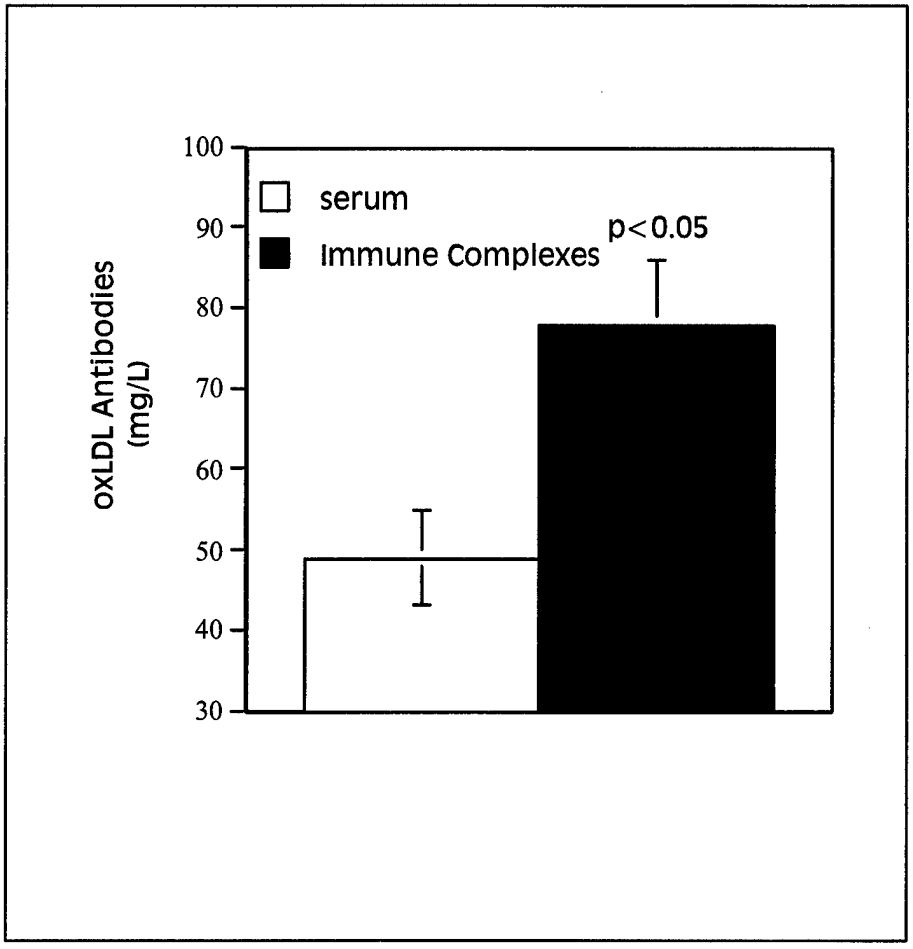

Figure 11. OxLDL antibody levels in serum and polyethylene glycol-precipitated immune complexes of type 1 diabetic subjects (N=47 paired serum and PEG-IC samples). OxLDL antibody levels were determined by competitive enzyme immunoassay in serum and in IgG purified from polyethylene glycol-precipitated immune complexes using protein G chromatography. The assays were calibrated with serial dilutions of serum with known concentration of oxLDL antibody and calculated in mg/L. The data in the graph is expressed as mean ± SEM. Statistical analysis was performed using a paired t-test of oxidized LDL antibody concentrations in serum against concentrations of the same antibodies isolated from immune complexes.

We then investigated whether or not the avidity of oxLDL antibodies isolated from sera was comparable in the three subgroups of subjects studied and whether or not it would differ from the avidity of oxLDL antibodies contained in immune complexes. As shown in table 6 the Kd values of oxLDL antibodies isolated from patient's sera were virtually undistinguishable. A comparison of Kd values from serum antibodies and antibodies present in immune complexes showed the later to be of higher avidity (serum antibody Kd = 1.13±0.13 07 x 10^{-8} mol/L; immune complex antibody Kd = 0.86±0.06 x 10^{-8} mol/L mol/L; $p<0.05$). The average Kd of IgG antibodies isolated from PEG-precipitated IC from patients with UAE equal or greater than 30 mg/24 hr (microalbuminuric and macroalbuminuric) was lower than the average Kd of IgG antibodies isolated from PEG-precipitated IC from patients with UAE below 30 mg/24 hr (normoalbuminuric). The differences between the Kd values measured in the three different groups did not reach statistical significance, but a statistically significant difference ($p<0.05$) was observed when the Kd values of the patients with normoalbuminria were compared with those from a combined group, including all patients with abnormal albuminuria (Figure 12).

Table 6. Kd values measured in oxLDL antibodies isolated from serum and IgG isolated from PEG-precipitated immune complexes

Variable	Patient Groups	N	Mean	SE	*p*	*p**
Serum oxLDL Antibodies Kd (10^{-8} mol/L)	Macroalbuminuric	14	1.58	0.24	ns	ns
	Microalbuminuric	11	1.48	0.27	ns	ns
	Normoalbuminuric	22	1.51	0.19	Reference	Reference
Immune Complex oxLDL Antibodies Kd (10^{-8} mol/L)	Macroalbuminuric	14	0.87	0.11	ns	ns
	Microalbuminuric	11	0.84	0.12	ns	ns
	Normoalbuminuric	22	1.13	0.08	Reference	Reference

*p is adjusted for gender, age, and HbA1c.

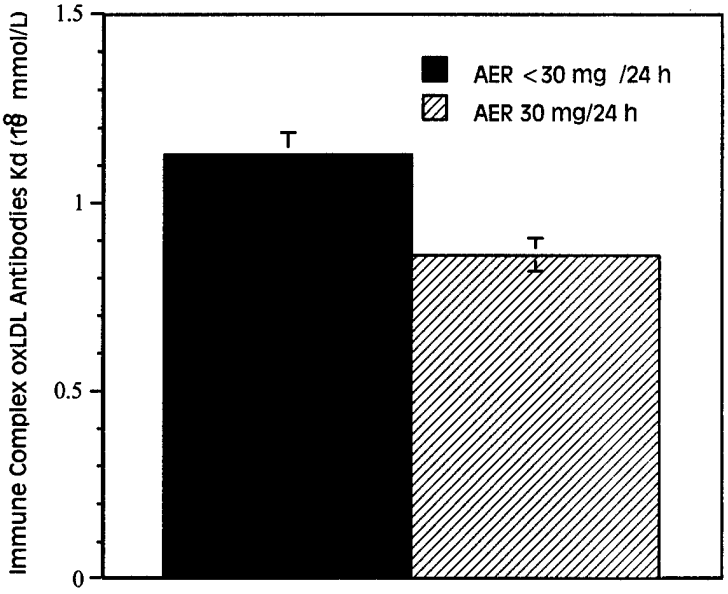

Figure 12. OxLDL antibody Kd values measured in IgG fractions purified from PEG-precipitated IC obtained from the sera of type 1 diabetics with UAE under 30 mg/24 hr (n=22) and equal or greater than 30 mg/24 hr (n=25). IgG was purified from polyethylene glycol-precipitated immune complexes using protein G chromatography, and Kd levels were determined by competitive ELISA (see methods). Results are expressed in 10^{-8} moles per liter. Statistical analysis was performed using analysis of covariance while adjusted for gender, age, and HbA1c.

Part 2: Discussion

In spite of the prevalence of nephropathy in type 1 diabetes and its associated morbidity and mortality, the pathogenic mechanisms responsible for its development are still poorly understood. However, there is substantial data suggesting that the development of nephropathy in type 1 diabetes mimics, from a pathogenic point of view, that of systemic atherosclerosis (Diamond, 1991; Gin et al., 2000; Kamanna et al., 1997; Moorhead et al., 1997), and that lipids such as oxLDL are major contributors to the deterioration of renal function (Al-Shebeb et al., 1988; Bosmans et al., 2001; Gin et al., 2000; Lee and Kim, 1998). The pathogenic mechanism proposed to explain how hyperlipidemia leads to nephropathy involves the infiltration of LDL, due to increased capillary permeability, into the glomerular mesangium followed by LDL oxidation by mesangial cells (Wheeler et al., 1994). As a consequence, there is recruitment and accumulation of macrophages (Jeng et al., 1993; Kamanna et al., 1999; Kamanna et al., 1996; Suzuki et al., 2001), phagocytosis of the oxLDL, and transformation of these cells into lipid-laden foam cells—a hallmark of diabetic nephropathy (Magil, 1999). Ultimately, these macrophages become activated, exacerbating the progression of nephropathy through the release of cytokines, followed by proliferation of mesangial cells and expansion of the glomerular extracellular matrix (Moorhead et al., 1997; Schreiner, 1991; Shiozawa, 2000).

In contrast to nephropathy, the pathogenic role of modified lipoproteins in atherosclerosis is well established. It has been investigated from two different angles: the direct pro-atherogenic effect of oxLDL (Diamond, 1991; Lopes-Virella and Virella, 1998; Witztum, 1994b) and the effect of the immune complexes formed as a consequence of the immune response directed against neoepitopes resulting from lipoprotein modification (Lopes-Virella and Virella, 1998; Lopes-Virella et al., 1999). The development of accurate assays for free oxLDL antibodies (Koskinen et al., 1998; Lopes-Virella et al., 2000; Mironova et al., 1997) and design and implementation of techniques for the isolation of oxLDL antibodies (Lopes-Virella and Virella, 1994a; Virella et al., 2000) allowed the investigation of the possible connection between oxLDL antibodies and various manifestations of atherosclerosis. The majority of isolated oxLDL antibodies have been characterized as belonging to the IgG isotype, predominantly of the IgG1 and IgG3 subclasses, and are of moderate to low avidity, two to three logs lower than the average avidity of polyclonal rabbit anti-apolipoprotein B antibodies (Virella et al., 2000).

Support for the atherogenic role of oxLDL antibodies was initially found in some studies showing a positive correlation between increased circulating oxLDL antibody levels and atherosclerotic vascular disease (Bergmark et al., 1995; Klimov et al., 1988; Maggi, 1994), as well as in the isolation of oxLDL and its corresponding antibodies from atheromatous lesions (Itabe et al., 1994; Palinski

and Witztum, 2000; Steinberg et al., 1989). However, our group, as well as others, has obtained negative data in studies examining the correlation between circulating oxLDL antibody concentrations and atherosclerosis (Hulthe et al., 1998; Virella et al., 1993). Even more perplexing are the reports showing that the serum concentrations of antibodies to oxLDL are lower in people with diabetes-related complications than in those without (Festa et al., 1998; Orchard et al., 1990). Palinski et al. raised further questions concerning the pathogenic role of oxLDL antibodies with a study suggesting that immunization of LDL receptor deficient rabbits with homologous MDA-LDL had a protective effect relative to atherogenesis (Palinski et al., 1995). George et al. (George et al., 1998) reported similar observations after immunizing apo-E deficient mice with MDA-LDL. These observations have been taken out of context to imply that humoral immunity to oxLDL has general protective effects in humans (Wick and Xu, 1999); ignoring species differences; that the laboratory animals used in these experiments were genetically altered; that the titers of antibodies resulting from deliberate immunization were considerably higher that those measured in non-immunized animals; and that no attempt was made to compare IgG subclass distribution or avidity values between spontaneous and elicited antibodies. Also ignored was the fact that later studies comparing the effects of immunization of LDL receptor deficient mice with MDA-LDL and native LDL demonstrated that the protective effect of immunization was unrelated to the production of oxLDL antibodies (Freigang et al., 1998).

While the pathogenic significance of circulating oxLDL antibodies is questionable, as demonstrated by the contradictory results mentioned above, we have concentrated our efforts in studying the possible pathogenic role of LDL-containing IC. The putative role of oxLDL-IC in atherosclerosis is supported by both *in vivo* and *in vitro* data. *In vivo*, oxLDL and antibodies reacting with modified lipoprotein particles are known to be components of atherosclerotic plaques, each having been identified and purified from such lesions, as mentioned earlier (Palinski and Witztum, 2000; Steinberg et al., 1989). *In vitro* experiments demonstrated that LDL-IC interact with the high avidity Fc receptor (FcγRI) of monocytes and macrophages (Lopes-Virella et al., 1997a) and are extremely potent activators of human macrophages, leading to cholesterol ester accumulation, unregulated expression of LDL receptors, and release of pro-inflammatory cytokines, metalloproteinases and reactive oxygen species (Griffith et al., 1988; Huang et al., 1999; Kiener et al., 1995; Klimov et al., 1988; Tertov et al., 1990b; Virella et al., 1995). This was the impetus for re-examining the question of the pathogenic role of IC in atherosclerosis, focusing specifically on LDL-containing IC.

Tertov et al. proposed in 1990 that LDL-containing IC played a pathogenic role in atherosclerosis based on *in vitro* data demonstrating that cholesterol-rich PEG precipitates had atherogenic properties when incubated with human macrophages (Tertov et al., 1990a). This led the same group to propose that

LDL-IC could be used as markers of atherosclerosis (Orekhov et al., 1991a). Later publications on the same topic had conflicting conclusions. Boullier et al., using an enzyme immunoassay for LDL-IC detection, reported that LDL-IC were not increased in subjects suffering from coronary atherosclerosis (Boullier et al., 1995). In contrast, Ahmed et al., using a similar assay, reported a higher prevalence of circulating IC in diabetic patients with vascular complications (Ahmed et al., 1999). Our initial studies failed to clarify the issue, when we proved that atherogenic IC could be isolated not only from the sera of subjects with diabetes, but also from the sera of normal controls (Mironova et al., 1997). However, data from a prospective study of a group of type I diabetic subjects demonstrated that LDL-IC were risk factors for coronary heart disease in these patients (Lopes-Virella et al., 1999; Orchard et al., 1999).

A suspected correlation between ox LDL antibodies and diabetic nephropathy was first investigated by Korpinen et al., but they found no association between serum levels of oxLDL antibodies and diabetic nephropathy or nephropathy-related macroangiopathy (Korpinen et al., 1997). In our current study we were also unable to show a significant correlation between free, circulating oxLDL antibody levels and proteinuria. However, when we focused our attention in LDL-IC levels we obtained evidence supporting the involvement of LDL-IC as pathogenic factors in diabetic nephropathy, as reflected by abnormal albuminuria. Significantly higher levels of cholesterol and apolipoprotein B were

detected in the PEG precipitates of subjects with abnormal UAE, particularly those with macroalbuminuria. But more significantly, we were able to purify antigen-free IgG from PEG precipitates and demonstrate that it contained oxLDL antibodies, and that these antibodies were of higher avidity than free, circulating oxLDL antibodies. Furthermore, we have shown that the avidity of antibodies contained in PEG precipitates was significantly higher in subjects with abnormal UAE values. The nature of the LDL molecules found in the PEG precipitates could not be established directly by the performed assays of cholesterol and Apo-B. No fully satisfactory method for specific assay of oxLDL in small samples, such as PEG precipitates, is available. Attempts to develop immunoassays have not been successful because of the lack of adequate commercially available polyclonal or monoclonal antibodies. Chemical assays, on the other hand, require considerable quantities of lipoprotein that cannot be obtained from clinical samples. However, the demonstration of oxLDL antibodies in the PEG precipitates strongly supports the hypothesis that the PEG-precipitable IC contain oxLDL.

This, to our knowledge, is the first report showing evidence that supports a pathogenic role for oxLDL-containing IC in diabetic nephropathy. Our data further suggest that the pathogenic potential of oxLDL IC is directly related to oxLDL antibody avidity. The correlation between antibody pathogenicity and avidity agrees with classical observations published by Winfield et al. who

demonstrated in the late 1970s that the avidity of antibodies eluted from deposited IC was significantly higher than the avidity of circulating antibodies of the same specificity (Winfield et al., 1977). The fact that IC containing IgG oxLDL antibodies of higher avidity predominated in patients with micro and macroalbuminuria is likely to be a reflection of the fact that as the antibody avidity increases, the ability to form stable complexes is enhanced. The formation of stable IC involving biologically active antibodies (IgG1 and IgG3 particularly) is, in turn, a requirement for the activation of pro-inflammatory mechanisms (Theofilopoulos and Dixon, 1979). From previous work conducted in our laboratory we know that oxLDL antibodies are predominantly of the IgG1 and IgG3 isotypes (Mironova et al., 1996), able to activate the classical pathway of the complement system and to engage all known types of Fcγ receptors (Virella and Wang, 1998).

The process by which oxLDL-IC mediate glomerular damage is not clear. OxLDL has been shown to induce proliferation of mesangial cells, a prominent feature of diabetic nephropathy (Moorhead et al., 1997), and cause the expansion of the extracellular matrix (Diamond, 1991; French et al., 1967). In turn, mesangial cells have been shown to oxidize LDL (Wheeler et al., 1994). In the case of human macrophages, the higher atherogenic potential of ox-LDL IC relative to free oxLDL seems to be related to the engagement of FcγRI by the IC (Lopes-Virella et al., 1997a). Human mesangial cells have been reported to

express this same receptor after activation by interferon-γ (Uciechowski et al., 1998). It remains to be determined if uptake of oxLDL IC through this receptor can activate mesangial cells to the same extent as macrophages. If it does, the question of the *in vivo* cellular source of interferon-γ will arise. As in the pathogenesis of large vessel atherosclerosis, it is likely that a variety of immune cells are involved in the process. Activated T cells reacting with oxLDL and hsp-60-derived epitopes have been detected in atheromatous lesions (Stemme et al., 1995). A similar involvement at the glomerular level could account for the release of interferon-γ and mesangial cell activation. These and many other questions will have to be explored to reach a better understanding of the pathogenic role of oxLDL-IC in diabetic nephropathy.

Part 3: Effects of Homologous Human OxLDL-Containing Immune Complexes IC on Human Macrophage-like Cell Line, THP-1 Cells

Part 3: Introduction

During the last 20 years, research in cardiovascular disease has accumulated a large body of evidence suggesting an important role for low density lipoprotein-containing immune complexes (LDL-IC) in the pathogenesis of atherosclerosis, as reviewed by Lopes-Virella and Virella in 1998 (Lopes-Virella and Virella, 1998). Supporting the proatherogenic role of LDL-IC are reported observations that atherosclerotic lesions contain both oxLDL and LDL antibodies (Itabe et al., 1994; Palinski and Witztum, 2000; Steinberg et al., 1989; Steinbrecher et al., 1990). Epidemiological data has been obtained suggesting that soluble LDL-IC are detected in the serum of subjects with coronary artery disease (CAD) at levels significantly higher than subjects without CAD (Lopes-Virella et al., 1999; Orchard et al., 1999; Orekhov et al., 1991a; Tertov et al., 1990a), with data obtained in a prospective study involving diabetic subjects suggesting that increased levels of LDL-IC were predictive of CAD (Lopes-Virella et al., 1999; Orchard et al., 1999). Additionally, soluble LDL-IC isolated from the serum of subjects with coronary heart disease, by chromatography using immobilized staphylococcal protein A or precipitation with polyethylene glycol, have been shown to be atherogenic, as indicated by their ability to induce intracellular accumulation of cholesterol and cholesteryl ester (CE) in pericardial

macrophages and smooth muscle cells (Klimov et al., 1988; Tertov et al., 1990a).

Transformation of human monocyte-derived macrophages into lipid-laden foam cells is a key manifestation of the atherosclerosis process (Gerrity, 1981; Schaffner et al., 1980). Therefore, it should come as no surprise that the role of LDL-IC in foam cell formation has received significant attention. Beginning in the 1980s, researchers in our group began investigating the effects of LDL-IC on human monocyte-derived macrophages, and later on, we extended these observations to the human monocytic cell line THP-1, a widely used human macrophage model (Auwerx, 1991; Lopes-Virella et al., 1997a). Numerous studies in our laboratory have shown that co-incubation of human monocyte-derived macrophages with LDL-IC prepared with human native LDL and rabbit anti-human LDL antibodies cause intracellular CE accumulation, and subsequent foam cell formation more efficiently than any other known mechanism of foam cell formation (Griffith et al., 1988; Lopes-Virella et al., 1991). The massive intracellular increase in cholesterol and CE accumulation and impaired cholesterol homeostasis observed in THP-1 cells incubated with LDL-IC mirror the effects of LDL-IC on human monocyte-derived macrophages, substantiating the use of this cell line as a model of human monocyte-derived macrophages (Huang et al., 1997).

Substantial data has been accumulated, suggesting that the intracellular accumulation of cholesterol and CE in macrophages incubated with LDL-IC, and resultant foam cell formation, is the result of a complex series of actions triggered by LDL-IC. For example, the results of studies conducted in our laboratory indicate that the massive CE accumulation is due to a combination of increased uptake and impaired intracellular metabolism of the ingested LDL component of the LDL-IC (Lopes-Virella et al., 1991). We have demonstrated that the amount of LDL taken up by human macrophages at the end of 5 hours is an impressive 7-fold higher when the LDL is complexed with antibody than when cells are incubated with native LDL alone. In addition to enhanced ingestion of LDL-IC, we have also observed that there is a delay in degradation of LDL when it is complexed with IgG. Pulse-chase experiments have shown that degradation of IC-complexed LDL takes up to 48 hours, and that as the LDL is hydrolyzed; cholesterol is released and re-esterified by ACAT leading to intracellular cholesterol and CE accumulation (Lopes-Virella et al., 1991). Taken together, these observations demonstrate the ability of LDL-IC to cause intracellular lipid accumulation by at least two mechanisms: increased lipid ingestion, and decreased lipid clearance.

Thus, there is solid evidence suggesting a role for LDL-IC in the pathogenesis of atherosclerosis. Unfortunately, the vast majority of these studies have been performed using LDL-IC prepared with human LDL and rabbit hyper-immune

anti-sera against human LDL, or an ill-defined heterogeneous mix of human LDL-containing IC and other IC isolated from human sera by PEG precipitation. The experimental designs have left unresolved the question of whether LDL-IC prepared with heterologous antibodies of much higher avidity than the naturally occurring human antibodies truly represent the effect of homologous LDL-IC on human macrophages. There is also some uncertainty regarding what types of LDL-IC isolated from human serum are responsible for foam cell formation in macrophages. In this paper, we report the results of a series of experiments supporting our contention that homologous LDL-IC prepared with human oxLDL and human antibodies to oxLDL (oxLDL-IC) are metabolized by human macrophages similarly to heterologous LDL-IC prepared with human LDL and hyperimmune rabbit anti-human LDL serum. We believe that these data prove that human oxLDL autoantibodies can form atherogenic LDL-IC and validates the continued use of LDL-IC prepared with human LDL and heterologous anti-LDL antibodies to study the interactions of human monocyte-derived macrophages and LDL-IC.

Part 3: Results

The protocol developed for preparing and isolating homologous human oxLDL-IC involved multiple steps. We began by isolating oxLDL antibodies from human sera, using a column containing oxLDL cross-linked to Sepharose. The eluted antibodies were then dialyzed and incubated overnight at 4°C with varying concentrations of copper-oxidized LDL to allow formation of oxLDL-IC. Unlike the insoluble immune complexes formed with heterogeneous mixtures of human LDL and LDL-hyperimmune rabbit sera, which are relatively simple to make and purify, oxLDL-IC formed with human oxLDL and purified human oxLDL antibodies are soluble complexes, and cannot be isolated by simple centrifugation. To overcome this obstacle, we first prepared soluble oxLDL-IC by incubating oxLDL with purified human oxLDL antibodies overnight at 4°C. We then added polyethylene glycol (PEG), at a final concentration of 4%, to the antigen-antibody mixture to precipitate the soluble immune complexes. After overnight incubation at 4°C, we obtained oxLDL-IC precipitates that were isolated by centrifugation, reconstituted in PBS, and subsequently analyzed. Figure 13 shows the results of a precipitation curve prepared with different antigen-antibody ratios and identical PEG concentrations (4%). The curve indicates that optimal yields of oxLDL-IC complexes are obtained when 150 μg oxLDL is mixed with 400 μg/ of purified oxLDL antibodies.

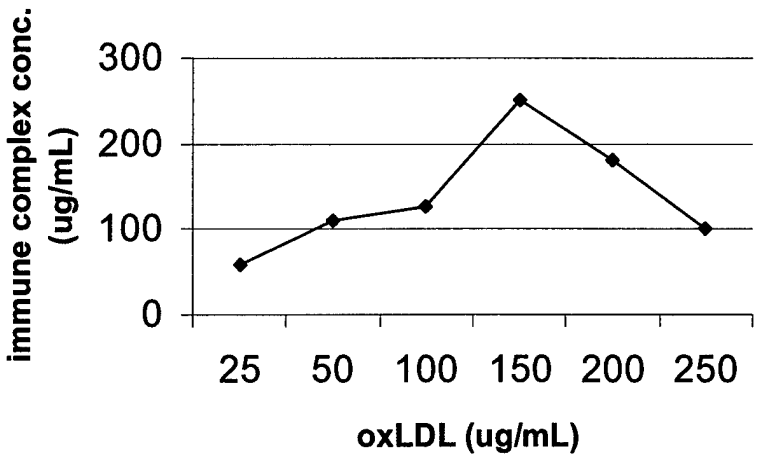

Figure 13. Homologous human oxLDL-IC precipitation curve. Variable concentrations of oxLDL isolated from human serum were added to 1 mL of purified human oxLDL antibody aliquots containing 400μg/mL of protein. After overnight incubation, addition of 4% PEG, and a second overnight incubation, precipitates were separated by centrifugation, and protein concentration was determined in resuspended precipitates by the Lowry method (Lowry et al., 1951).

In order to rule out nonspecific precipitation of proteins by PEG, we next designed an experiment to compare the protein concentrations precipitated from oxLDL alone, oxLDL + an irrelevant antibody (rabbit anti-Rho), oxLDL + human oxLDL antibody, and oxLDL + LDL-hyperimmune rabbit antibody. In this experiment, oxLDL alone and oxLDL + the irrelevant antibody served as negative controls, while oxLDL + LDL-hyperimmune rabbit antibody served as a positive control. In all conditions, both the oxLDL concentration and antibody concentration were kept constant, at 150 μg/mL and 400 μg/mL, respectively, as determined by the Lowry method. The results are graphically represented in figure 14, and demonstrate that oxLDL-IC are formed when oxLDL is mixed with purified oxLDL antibodies, and that PEG precipitates minimal amounts of uncomplexed proteins/lipoproteins.

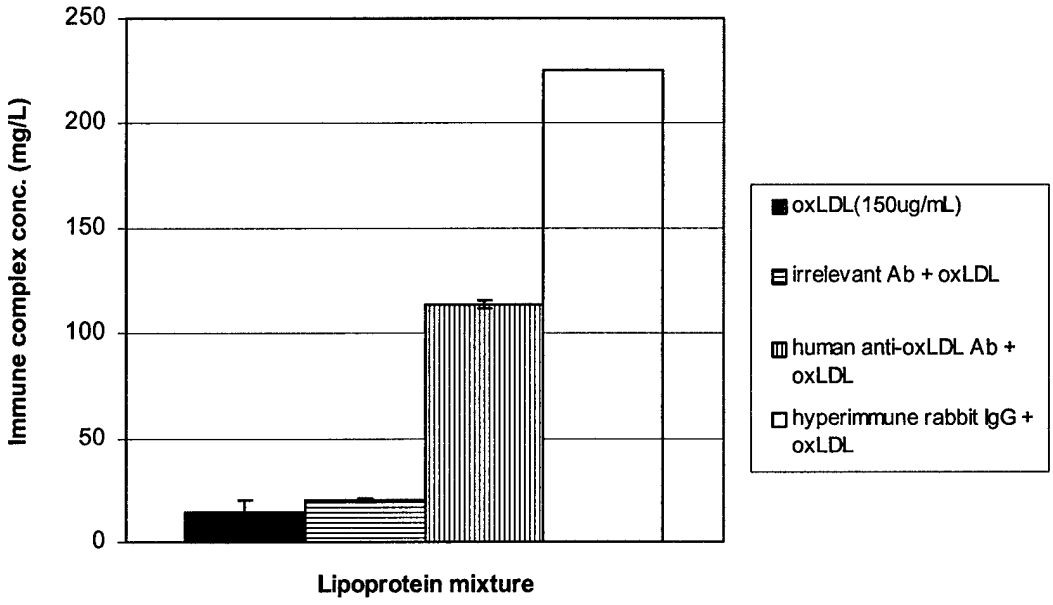

Figure 14. Specificity of oxLDL-IC precipitation by 4% PEG. Copper-oxidized LDL alone, oxLDL plus anti-Rho (irrelevant) antibodies, oxLDL plus purified human oxLDL antibodies, and oxLDL plus purified hyperimmune rabbit IgG was precipitated with 4% PEG, and precipitate concentrations were compared by protein Lowry. All conditions used a final volume of 1 mL in PBS, 150μg oxLDL, and 400 μg of the antibody indicated.

Having successfully established a method to reproducibly prepare and purify homologous human oxLDL-IC, we then carried out experiments to determine if these complexes would alter cholesterol metabolism, resulting in cholesterol and cholesteryl ester accumulation in macrophages, as has been observed in macrophages incubated with LDL-IC prepared with human LDL and hyperimmune rabbit anti-human LDL antibodies. To this end, we transformed monocytic THP-1 cells to macrophages by treatment with 160 nmol/L phorbol 12-myristate 13-acetate (PMA) for 3 days, and incubated the THP-1 macrophage-like cells with varying concentrations of human homologous oxLDL-IC or oxLDL alone. During incubation of these macrophages with free oxLDL or with oxLDL-IC, it was found that the majority of concentrations studied (50-200 mg oxLDL/oxLDL-IC per mL) caused an increase in total cholesterol (Figure 15A). And we observed that the increase in both components of total cholesterol (free cholesterol and CE) was much more pronounced in cells incubated with oxLDL-IC, compared to those incubated with free oxLDL (Figure 15A). Beginning at 50 μg/mL, intracellular CE accumulation in cells incubated with oxLDL-IC was increased more than 2-fold higher than cells incubated with free oxLDL alone. Figure 15B, shows that the accumulation of intracellular CE occurred in a dose-dependent manner, indicating the ability of oxLDL-IC to dramatically alter cholesterol metabolism and lead to foam cell formation more efficiently than oxLDL alone.

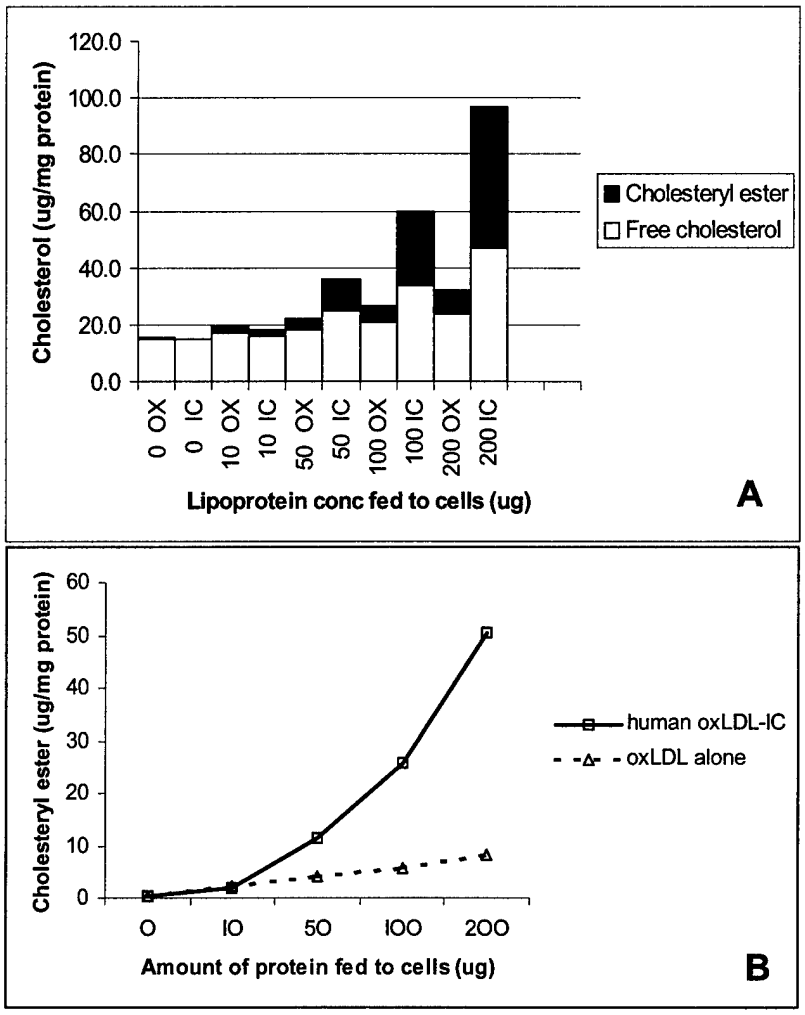

Figure 15. Cholesterol accumulation in THP-1 cells incubated with variable concentrations of oxLDL (OX) and oxLDL-IC (IC). Each point represents the average of three experiments performed in duplicate or triplicate. Cells were incubated with 0-200 μg/mL oxLDL or oxLDL-IC for 48 hours, and cholesterol concentrations were determined by GC.

During incubation of macrophages with 100 μg/mL of oxLDL or oxLDL-IC prepared with either human oxLDL antibodies or rabbit anti-human LDL-hyperimmune serum (Figure 16), we observed an increase in intracellular cholesterol accumulation in cells incubated with free or complexed oxLDL, supporting the atherogenic potential of oxLDL. Although oxLDL alone is capable of causing foam cell formation, as attested by the 2-fold increase in intracellular total cholesterol and 8-fold increase in CE, these data indicate a more profound enhancement in intracellular cholesterol accumulation in macrophages incubated with oxLDL-IC. Cells incubated with homologous oxLDL-IC prepared with purified human oxLDL antibodies and oxLDL nearly doubled the intracellular cholesterol levels observed in those incubated with oxLDL alone, and showed a 3-fold increase in CE, closely approaching the lipid accumulation observed in cells incubated with heterologous oxLDL-IC prepared with anti-human LDL-hyperimmune rabbit serum and oxLDL. Taken together, these data strongly suggest that oxLDL-IC prepared with human oxLDL antibodies produce similar results to those observed when using heterologous LDL-IC prepared with LDL-hyperimmune rabbit serum. More importantly, we have shown for the first time that the oxLDL-IC likely to be encountered in humans are have more atherogenic potential than oxLDL alone.

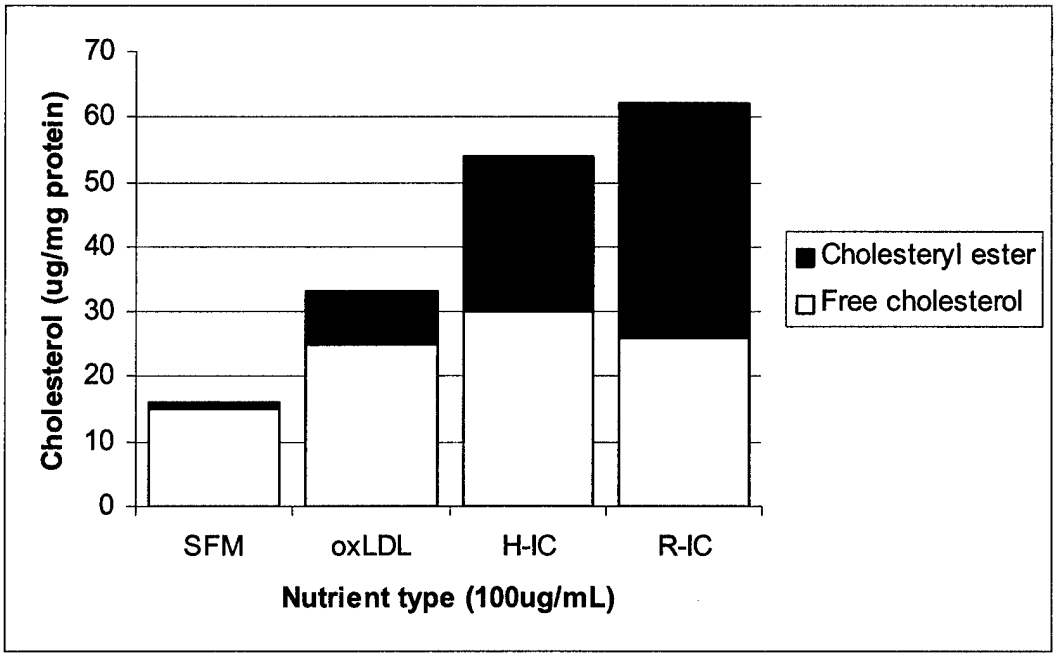

Figure 16. Cholesterol mass (µg/mg cell protein) in THP-1 cells incubated with for 48 hours with SFM (n=16), 100 µg oxLDL (n=10), 100 µg human oxLDL-IC (H-IC) (n=28), or 100 µg rabbit oxLDL-IC (R-IC) (n=12), that had been precipitated by incubation with 4% PEG (18 hr, 4°C). The results are expressed as mean of 4-7 experiments with samples run in duplicate or triplicate in each experiment.

Part 3: Conclusions

It is well known that the accumulation of CE in macrophages, and subsequent transformation into foam cells, is enhanced by incubation of these cells with modified forms of LDL (Brown et al., 1980) and that incubation of macrophages with heterologous LDL-IC prepared with human LDL and anti-human LDL-hyperimmune rabbit sera drives intracellular CE accumulation more efficiently than incubation with modified LDL alone (Griffith et al., 1988; Lopes-Virella et al., 1997a; Lopes-Virella et al., 1997b; Virella et al., 1995). Furthermore, Klimov et al. (Klimov et al., 1988) demonstrated that lipoprotein-antibody autoimmune complexes isolated from human serum using sucrose density gradient ultracentrifugation induced an approximate 3-fold CE increase in murine macrophages when compared to the same cells incubated with lipoprotein alone. Concurrently with the work of Klimov, data from our group strongly implicated the participation of Fc-γ receptors in LDL-IC uptake by macrophages by demonstrating decreased CE accumulation in cells whose Fc-γ receptors were blocked (Griffith et al., 1988), and we later proved that the receptors primarily involved in this process were FcγRI (Lopes-Virella et al., 1997a). Unfortunately, these studies, though they convincingly showed that LDL-IC lead to CE accumulation in macrophages, could be criticized by the fact that some authors used macrophages isolated from non-human sources (Klimov et al., 1988), while others used LDL-IC prepared with LDL antibodies from a different species (Griffith et al., 1988; Lopes-Virella et al., 1997a), or used human LDL-IC whose

constituent components were not well defined (Klimov et al., 1988). Some of the problems presented by these design flaws are obvious, while others are less obvious. First, with regard to the type of macrophages used, non-human macrophages may, or may not accurately reflect their human counterpart. Though these cell lines are cultivated more easily than native human monocyte-derived macrophages, there may well be species to species differences, therefore, human macrophages are obviously preferred. Second, using heterologous LDL-IC prepared with LDL-hyperimmune rabbit sera and human LDL may be problematic. Since rabbit antibodies are of higher avidity than human LDL antibodies (Mironova et al., 1996) and recognize a wider range of epitopes on LDL, they form insoluble LDL-IC. Since the corresponding homologous LDL-IC are soluble, one cannot be sure that macrophages incubated with these heterologous LDL-IC complexes react similarly to those incubated with homologous LDL-IC. Third, data produced showing CE accumulation in macrophages incubated with LDL-IC isolated from human sera (Klimov et al., 1988) are difficult to interpret because the type of antigen involved, as well as the antibodies involved in the formation of these complexes is unknown. These studies inadequately answer the question of what type of LDL modification (glycation, oxidation, advanced glycated end products, etc.) drives antibody formation and, therefore, fail to identify and/or characterize the type of LDL-IC involved. Finally, the use of native LDL as a negative control may not be the best choice as a baseline measurement for several reasons. It is common

knowledge that LDL becomes atherogenic only after it is modified (Steinberg and Witztum, 1999), that this modification confers immunogenicity (Steinbrecher et al., 1984a), and that LDL-IC are much more efficient than native LDL in their ability to cause foam cell formation in macrophages (Griffith et al., 1988; Klimov et al., 1988; Lopes-Virella and Virella, 1998). Since native LDL is not immunogenic, and does not participate in formation of immune complexes, it seems reasonable to suggest that a modified form of LDL known to participate in formation of LDL-IC is a better control in these experiments.

In the present study, we are the first to report the successful *in vitro* production and purification of human homologous oxLDL-IC, using oxLDL and oxLDL antibodies isolated from human serum (Figure 14). OxLDL was chosen as the antigen for this group of experiments because it occurs *in vivo* and has been observed in foam cells and atherosclerotic plaques (Itabe et al., 1994; Lee and Kim, 1998; Magil, 1999; Palinski et al., 1995; Palinski and Witztum, 2000; Steinberg et al., 1989). We then embarked on a series of experiments aimed at overcoming the afore mentioned potential problems encountered by using heterologous LDL-IC by using human monocyte-derived THP-1 macrophages incubated with homologous oxLDL-IC, and used oxLDL as our baseline control, rather than native LDL alone. We observed an accumulation of cholesterol esters when human monocyte-derived THP-1 macrophages were incubated with homologous oxLDL-IC that approached levels observed in macrophages

incubated with oxLDL-IC prepared with anti-human LDL-hyperimmune rabbit sera (Figure 16). Furthermore, this accumulation of cholesterol esters was consistently higher than that observed in macrophages incubated with oxLDL alone (Figure 15), with cholesterol ester concentrations ranging from 3 to 6-fold higher than those observed in cells incubated with oxLDL alone.

In summary, these data provide conclusive evidence that homologous oxLDL-IC prepared with human oxLDL and purified human oxLDL antibodies are actively ingested by macrophages. Moreover, when homologous oxLDL-IC are incubated with human monocyte-derived THP-1 macrophages, they appear to result in transformation of these cells into foam cells, approaching the efficiency observed in earlier studies where macrophages were incubated with heterologous LDL-IC (Gisinger and Lopes-Virella, 1992; Griffith et al., 1988; Klimov et al., 1985; Lopes-Virella et al., 1997b). Together, these data demonstrate that homologous oxLDL-IC not only cause cholesterol and cholesteryl ester accumulation in macrophages, but also are significantly more efficient in their atherogenic potential than oxLDL alone. These observations support the hypothesis presented by Lopes-Virella et al. that oxidized LDL-containing immune complexes (oxLDL-IC) are active participants in foam cell formation and atherosclerosis (Lopes-Virella and Virella, 1998). Moreover, these data support the continued use of heterologous LDL-IC, prepared with human LDL and LDL-hyperimmune rabbit serum, in studies of the effects of LDL-IC on human monocyte-derived macrophages, foam cell formation, and atherogenesis.

Chapter V

Summary and Conclusions

Patients with diabetes mellitus tend to develop a multitude of microvascular and macrovascular complications, with heart disease being the number one cause of death in diabetes (Breyer, 1992; CDC, 1999; Cooper et al., 1997; Kamanna et al., 1997; Lloyd et al., 1996; Maser et al., 1991; Nathan, 1993; NCHS/CDC, 2000; Orchard et al., 1990). Accruing evidence suggests that two major vascular complications of diabetes, nephropathy and atherosclerosis, may share pathogenic mechanisms (Figure 17), with nephropathy involving mainly small vessels (capillaries, arterioles, and small arteries), while atherosclerosis is primarily a disease of large arteries (Diamond, 1991; Hirano, 1999; Kamanna et al., 1997; Moorhead et al., 1997). Although diabetes is a well-defined risk factor for atherosclerosis and nephropathy, the mechanisms responsible for lesion pathogenesis in these subjects are multifaceted and have not been totally defined. However, given the fact that glycated proteins are commonly formed in subjects with diabetes and that glycated proteins are more susceptible to oxidation (Mullarkey et al., 1990; Nathan, 1996; Wolff, 1987), it is not surprising that investigations of the role of modified proteins and lipoproteins in vascular disease pathogenesis have been intense. Two key participants in

atherosclerosis and nephropathy that have received considerable attention are oxLDL and foam cells (Itabe et al., 1994; Kamanna et al., 1997; Kreisberg, 1998; Lopes-Virella and Virella, 1998; Magil, 1999; Palinski and Witztum, 2000; Shiozawa, 2000), with the altered metabolism of diabetes believed to be responsible for increased oxidant stress and subsequent oxidation of LDL (Brownlee et al., 1984; Cooper et al., 1997; Hunt et al., 1993; Lopes-Virella and Virella, 1998; Mullarkey et al., 1990; Wolff, 1987). Mechanisms by which oxLDL may contribute to atherosclerosis and glomerulosclerosis are numerous. OxLDL is chemotactic for monocytes, and is ingested by monocyte-derived macrophages via scavenger receptors, bypassing the normal negative feedback regulatory loop observed when native LDL is ingested via the LDL receptors. As a result, oxLDL leads to foam cell formation, a prominent feature of both atherosclerosis and glomerulosclerosis (Diamond, 1991; Lopes-Virella and Virella, 1998; Magil, 1999; Parthasarathy et al., 1986; Steinberg and Witztum, 1999).

In addition to the well-demonstrated ability of oxLDL to induce the transformation of macrophages into foam cells, it has also been shown to be cytotoxic to vascular endothelial cells, thus able to damage the endothelium and contribute to the progression of atherosclerosis (Cathcart et al., 1985; Morel et al., 1984). Macrophages may, in turn, contribute to the oxidation of LDL by generating free radicals or through the myeloperoxidase pathway (Heinecke, 1998; Hunt and

Wolff, 1991l). In addition, macrophages are likely to promote atherosclerosis and glomerulosclerosis by secreting various potent cytokines that profoundly influence other vascular cells, including endothelial cells and T cells (Hansson, 1997; Schreiner, 1991; Terkeltaub et al., 1998). Macrophages further contribute to atherosclerotic lesion expansion by secreting transforming growth factor-β, which causes expansion of the extracellular matrix by upregulating production of fibronectin, collagen, and proteoglycan synthesis (Berliner et al., 1990). In both atherosclerosis and glomerulosclerosis, fibrosis probably continues independently of the initial stimulus.

Many groups researching this area have devoted their efforts to dissecting the effects of oxLDL in atherogenesis and glomerulosclerosis (Berliner et al., 1990; Diamond, 1991; Guijarro and Keane, 1994; Haberland et al., 1982; Haberland et al., 1988; Heinecke, 1998; Hirano, 1999; Jeng et al., 1993; Kamanna et al., 1999; Maggi, 1994; Moorhead et al., 1997), but there is also solid evidence that oxLDL is immunogenic, supported by numerous reports of detectable antibodies to oxLDL and MDA-LDL (an epitope of oxLDL) in human sera by a variety of research groups, including our own (Bellazzi et al., 1993; Bergmark et al., 1995; Korpinen et al., 1997; Lopes-Virella et al., 1999; Maggi, 1994; Salmon et al., 1987; Salonen et al., 1992; Virella et al., 2000; Virella et al., 1993). Furthermore, support for a pathogenic role for LDL autoantibodies comes from the observations of researchers showing that LDL-containing IC prepared *in vitro* or

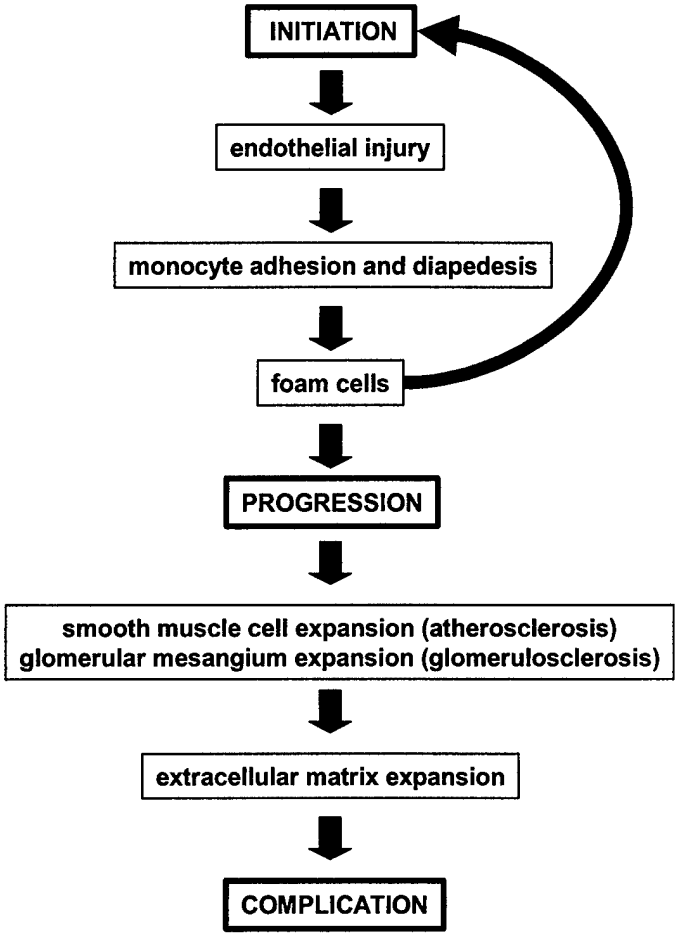

Figure 17. Analogies of atherosclerosis and glomerulosclerosis. Both processes are triggered by endothelial insult, followed by an influx of monocytes. The monocytes then transform into lipid-laden macrophages (foam cells), and appear to secrete a variety of pro-inflammatory products, thus causing further endothelial insult. As the disease progresses, there is smooth muscle cell or mesangial cell expansion in atherosclerosis and glomerulosclerosis, respectively, along with expansion of the extracellular matrix. Left unimpeded, complications arise. (Diamond, 1991; Nathan, 1987).

isolated from human serum can induce intracellular accumulation of cholesterol esters (Gisinger and Lopes-Virella, 1992; Gisinger et al., 1991; Griffith et al., 1988; Klimov et al., 1985; Klimov et al., 1988; Lopes-Virella et al., 1991; Tertov et al., 1990a; Virella et al., 1995). Our group has pioneered research in this area, focusing on the active role of antibodies to modified forms of LDL and the LDL-immune complexes (LDL-IC) they form in atherogenesis and diabetes (Lopes-Virella et al., 1996; Lopes-Virella et al., 1997b; Lopes-Virella and Virella, 1998; Virella et al., 1995). Subsequently, we have convincingly demonstrated the atherogenic potential of heterologous oxLDL-IC prepared with human oxLDL and anti-human LDL-hyperimmune rabbit serum (Gisinger and Lopes-Virella, 1992; Gisinger et al., 1991; Griffith et al., 1988; Lopes-Virella et al., 1991). Our *in vitro* studies have shown that stimulation of human monocyte-derived macrophages with insoluble heterologous LDL-IC led to an unparalleled transformation of these cells into foam cells (Griffith et al., 1988), as well as their activation (Virella et al., 1995). These observations documented that exposure of human macrophages to LDL-IC led to increased uptake and impaired degradation of LDL (Lopes-Virella et al., 1991), along with enhancement of a variety of cellular functions, including the release of pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor (TNF) (Virella et al., 1995). These activated macrophages can have pathological significance in that the secreted cytokines contribute to vascular injury by upregulating the expression of adhesion molecules, thus increasing the adherence of leukocytes to the

endothelial cells (Lopes-Virella et al., 1982) and promoting their migration into the subendothelial space. Further substantiating the activation of macrophages by LDL-IC, we demonstrated the ability of LDL-IC to cause respiratory burst in human macrophages (Virella et al., 1995).

The observations described above strongly suggest that modulation of the immune system plays an important role in the pathogenesis of atherosclerosis and glomerulosclerosis, and prompted us to further characterize the involvement of LDL-IC and macrophages in these processes. Our studies were roughly divided into three aims. We sought to 1) characterize of free and immune complex-bound oxLDL antibodies; 2) determine if LDL-IC were associated with nephropathy, as is the case with atherosclerosis; and 3) determine the atherogenicity of homologous oxLDL-IC prepared with human oxLDL and purified human anti-oxLDL antibodies.

The in-depth characterization of human oxLDL antibodies reported in this dissertation was a complex process that required the isolation and purification of both soluble (free) and immunocomplexed (bound) oxLDL antibodies from serum. OxLDL antibodies isolated from both serum and LDL-IC were predominantly IgG (primarily subclasses IgG1 and IgG3), followed by IgM and minute levels of IgA. These IgG subclasses are known to activate phagocytic cells and the complement system, therefore, their presence in LDL-IC supports a

proinflammatory role in atherosclerosis and glomerulosclerosis. We also observed that the avidity of oxLDL antibodies isolated from immune complexes was significantly higher than the avidity of unbound antibodies of the same specificity isolated from serum, suggesting that there is a threshold of antibody avidity required for the formation of stable LDL-IC, and that antibodies of oxLDL are of sufficiently high avidity to form stable IC. Antibodies of lower avidity remain in the circulation and are probably non-pathogenic. Collectively, these findings indicate a possible pathogenic role for oxLDL antibodies and the oxLDL-IC they help form. These observations, furthermore, indicate that LDL-IC formation may help explain the discrepant association of oxLDL antibody levels and atherosclerosis/glomerulosclerosis. Indeed, it is reasonable to expect that these IC concentrations may have a stronger correlation with vascular disease than that of oxLDL antibody levels alone.

With regard to the second undertaking of this research project, three observations sparked our interest, causing us to investigate if LDL-containing immune complexes were associated with diabetic nephropathy. First, our literature review revealed that diabetes dramatically increases an individual's risk for all types of vascular disease (Cooper et al., 1997; Nathan, 1993). Although there is little doubt that a positive relationship between hyperglycemia and microvascular disease exists, hyperglycemia alone is not sufficient to cause the observed pathology (Raptis and Viberti, 2001; Wolff, 1987). Second, it has been

successfully argued that glomerulosclerosis, the root cause of diabetic nephropathy, is an analogous process to atherosclerosis (Diamond, 1991; Diamond, 1994; Moorhead et al., 1997); with oxLDL and foam cells actively participating in the development of diabetic nephropathy (Jeng et al., 1993; Kamanna et al., 1999; Kamanna et al., 1996; Magil, 1999; Suzuki et al., 2001), as well as in atherosclerosis (Lopes-Virella and Virella, 1998). Third, an active involvement of LDL-IC in atherosclerosis is supported by *in vitro* observations of the conversion of monocyte-derived macrophages into foam cells by LDL-IC, the activation and release of proinflammatory cytokines by macrophages incubated with LDL-IC, and demonstration of a positive correlation between clinical manifestations of atherosclerosis and LDL-IC levels in subjects with diabetes (Lopes-Virella et al., 1997b; Orchard et al., 1999).

If atherosclerosis and glomerulosclerosis are indeed analogous processes, and LDL-IC are active participants in atherosclerosis, we hypothesized that a similar positive correlation would exist between nephropathy and LDL-IC. This was indeed the case. When we compared various components of LDL-IC, including LDL surrogate-markers such as cholesterol and apolipoprotein B (apoB), along with free and IC-bound oxLDL antibodies in diabetic subjects with varying degrees of nephropathy, we made several interesting discoveries. Our analysis of PEG-precipitated soluble immune complexes for LDL surrogate markers, cholesterol and apoB, showed a positive correlation between urine albumin

excretion (an indicator of the severity of nephropathy) and LDL-IC. Additionally, we isolated oxLDL antibodies from the PEG-precipitated immune complexes. Although PEG precipitates immune complexes irrespective of the antigen/antibody composition, the isolation of oxLDL antibodies from IC that also contained apoB and cholesterol firmly established the presence of oxLDL-IC as components of these complexes. Lastly, we demonstrated that the avidity of oxLDL antibodies isolated from PEG-precipitated immune complexes was higher in immune complexes isolated from subjects with advanced nephropathy (macroalbuminuria), compared to those with no detectable nephropathy (normoalbuminuria), suggesting that the stability of oxLDL-IC may play a role in nephropathy.

Although these findings may be interpreted to suggest that oxLDL-IC actively participate in the pathogenesis of both atherosclerosis and glomerulosclerosis, we fully realize that mere association does not equal causation. However, data obtained in a small cohort of type 1 diabetic subjects (n=98), the Pittsburgh Epidemiology Study of Complications, strongly favors a causal effect of LDL-IC in atherosclerosis since the elevation of LDL-IC preceded the appearance of CHD (Lopes-Virella et al., 1997b; Orchard et al., 1999). In fact, the baseline levels of LDL-IC obtained at the beginning of the study, when the patients were free of macrovascular disease, were significantly higher in the group that eventually developed macrovascular complications than in a matched case-

control group who did not develop macrovascular disease. These data require confirmation via large-scale prospective studies of this type, wherein markers of LDL-IC are quantitated in the sera of subjects destined to progress to nephropathy, or other measurable indicators of vascular disease. If these markers are found to be present at increased levels compared to non-progressors, the causative role of LDL-IC in the vascular disease of interest will be strengthened.

Turning to our third goal, we wished to address the issue of whether or not homologous oxLDL-IC, made with human oxLDL and purified human oxLDL antibodies, would cause foam cell formation in human monocyte-derived macrophages, as had been observed using LDL-IC made with anti-human LDL-hyperimmune rabbit serum (Gisinger et al., 1991; Griffith et al., 1988; Lopes-Virella et al., 1991; Lopes-Virella and Virella, 1998). To accomplish this task, we were able to draw upon the expertise within our group in the area of human oxLDL antibody isolation (Mironova et al., 1996; Virella et al., 2000), and purified relatively large quantities of oxLDL antibodies from human serum. Using these antibodies, we developed a novel process for synthesizing and purifying human homologous oxLDL-IC using a modification of Zubler's classical method of soluble immune complex isolation, with 4% polyethylene glycol (Zubler et al., 1977). Upon isolation of immune complexes, we then co-incubated these immune complexes with cells from the human monocyte-derived macrophage-

like cell line, THP-1, and determined intracellular cholesterol and cholesteryl ester accumulation. Our findings showed a massive intracellular accumulation of cholesterol and cholesterol esters in THP-1 cells incubated with homologous human oxLDL-IC that was several fold higher than that of cells incubated with oxLDL alone, and approached that of cells incubated with heterologous LDL-IC made with hyperimmune rabbit sera. Added to this, parallel experiments carried out by Koskinen and others in our group showed increased TNF expression in THP-1 cells incubated with human red blood cells that had been coated with oxLDL and purified oxLDL antibodies (unpublished data), strongly suggesting that these IC are capable of activating macrophages. These observations strengthen the hypothesis that LDL-IC modulate the human immune system in a proinflammatory manner, thus demonstrating the potential to exacerbate the development and progression of atherosclerosis (Lopes-Virella and Virella, 1998; Lopes-Virella et al., 1999). These observations are significant on several levels. First, and foremost, these findings strongly implicate oxLDL-IC in the pathogenesis of atherosclerosis and glomerulosclerosis. Second, these findings validate the continued use of heterologous LDL-IC prepared with LDL-hyperimmune rabbit sera in future studies of this nature. Third, this is the first published report describing preparation of human homologous oxLDL-IC, along with its effect on human macrophages.

As is always the case, much work remains to be done. The mechanisms behind

the accelerated development of vascular diseases such as atherosclerosis and nephropathy in type 1 diabetes are poorly understood. Therefore, the definition of predictors that could lead to the identification of patients at risk for accelerated development of atherosclerosis and/or nephropathy could represent a major advancement in the treatment of these patients. If the levels or characteristics of LDL-IC are proven to have such predictive power, simpler methods for their identification can be developed. Patients identified by these predictors should benefit from treatment with immunomodulators aimed at decreasing the production of pathogenic LDL-IC.

Accordingly, the precise nature of the LDL modifications associated with the synthesis of antibodies of sufficient avidity as to form stable LDL-IC that may be proinflammatory, and thus contribute to both nephropathy and macrovascular complications deserves investigation. Quantification of the various types of naturally occurring LDL modifications in circulation will require raising antibodies to these antigens, and the development of enzyme immunoassays (EIAs). These EIAs can then be used to screen retrospectively for these modifications in the baseline sera/IC samples of patients from the DCCT cohort who either developed atherosclerosis and/or nephropathy and a matched group of patients who remained free of complications. This data will be used to determine if high concentrations of LDL-containing some of these modifications in the baseline sera and/or IC are predictive of the development of nephropathy and/or

atherosclerosis. Further studies designed are needed to characterize immunogenic modifications of LDL (glycation, MDA-lysine, AGE/ALE) and determine if the presence of these modifications predict the development of vascular complications are often observed in people with diabetes. Similarly, the antibodies associated with these modified forms of LDL need to be characterized by isotype and avidity to determine if a specific subset is predictive of future glomerulosclerotic or atherosclerotic pathology. The definition of such predictors could lead to the identification of patients in which treatment with immunomodulatory statins (Chen et al., 2002) or stronger immunomodulators, such as hydroxychloroquine (Wallace, 1996; Wallace et al., 1993), would prevent the development of micro- and macrovascular complications. Lastly, the proinflammatory and atherogenic potential of LDL-IC formed between various modifications of LDL and their corresponding antibodies needs to be tested *in vitro* by incubating these LDL-IC with macrophages and mesangial cells and then determining intracellular cholesterol and cholesterol ester accumulation, as well as release of cytokines, specifically TNF, IL1β, IL6 and GM-CSF.

In conclusion, diabetes mellitus is a disease associated with serious vascular complications. And we have presented data suggesting that oxLDL-IC may play a critical role in the pathogenesis of both microvascular and macrovascular disease. Specifically, we are the first to report the positive association of oxLDL-IC and diabetic glomerulosclerosis, and the design of a novel protocol for

preparing purified homologous human oxLDL-IC. Additionally, we have reported the results of a series of experiments that strongly implicate the ability of these complexes to drive macrophage foam-cell transformation, a pathological component of both atherosclerosis and glomerulosclerosis. These data form a very strong body of evidence implicating oxLDL-IC as a culprit in vascular disease, and present, for the first time, proof that IC formed from human oxLDL and purified human oxLDL antibodies modulate this process in a negative direction more so than oxLDL alone. At this point, these observations represent the conceptual framework for on-going investigations that, if fruitful, promise to illuminate new perspectives for prevention and intervention of both micro- and macrovascular complications of diabetes mellitus.

Appendix 1

Diabetes mellitus

Diabetes mellitus (DM), is a disorder of glucose metabolism that progressively compromises the function of virtually every organ system in the human body as its secondary complications inexorably develop (CDC, 1999; Nathan, 1993; NCHS/CDC, 2000) (Table 1), and has the notoriety of being the fourth leading cause of death by disease in the United States (Table 7). Diabetes is characterized by hyperglycemia and affects approximately 15.7 million people (or 5.9% of the population) in the United States, with an estimated economic cost to our nation recently reaching $98 billion dollars per year (ADA, 1998). Two major forms of diabetes, type I diabetes and type II diabetes, are distinguished by the need for exogenous replacement of insulin. People with type I diabetes, previously termed insulin-dependent diabetes mellitus (IDDM), account for approximately 10% of DM in the United States. This form of diabetes usually appears abruptly before the age of 40, with typical symptoms of thirst, excessive urination, increased appetite, rapid weight loss, and high levels of sugar in the urine. In type I diabetes, there is destruction of the pancreatic beta-cells in the islets of Langerhans, resulting in an absolute deficiency of the hormone, which necessitates exogenous insulin replacement therapy. Type II diabetes, previously known as non-insulin-dependent diabetes mellitus (NIDDM), accounts for approximately 90% of DM in the United States, and usually occurs gradually in people over the age of 40 who are overweight. It is caused by a quantitative decrease in insulin production or

Table 7. Estimated number of deaths for the 10 leading causes of death: United States, 2000 (NCHS/CDC, 2000)

Rank	Cause of death	Total number	Percentage of deaths
1	Heart disease*	709,894	38.0
2	Cancer*	551,833	29.6
3	Stroke	166,028	8.9
5	Chronic lower respiratory disease*	123,550	6.6
4	Accidents	93,592	5.0
6	**Diabetes***	68,662	3.7
7	Pneumonia/Influenza	67,024	3.6
8	Alzheimer's Disease	49,044	2.6
9	Kidney disease*	37,672	2.0
10	Septicemia	31,613	1.7
All causes		1,898,912	100.0

*Causes of death due to disease

or impaired insulin sensitivity, or a combination of the two. The majority of subjects afflicted with type II diabetes can be managed with diet and exercise alone; however, when this approach fails, treatment with oral hypoglycemic agents or insulin is indicated (ADA, 2000).

DM complications.

Clearly, the quality of life for people with diabetes is diminished as a consequence of increased lifetime risk for development of long-term vascular complications including both microvascular disease (nephropathy, retinopathy, and neuropathy) and macrovascular disease (atherosclerosis, ischaemic heart disease, cerebrovascular disease, and peripheral vascular disease) (Breyer, 1992; Cooper et al., 1997; Kamanna et al., 1997; Lloyd et al., 1996; Lopes-Virella and Virella, 1998; Maser et al., 1991; Nathan, 1993; Orchard et al., 1990; Raskin, 1994). These complications mean that patients with diabetes are more likely to have heart attacks, strokes, amputations, kidney failure, and blindness than the general population; with accelerated and aggressive atherosclerosis being the leading cause of morbidity and mortality in diabetes (Rubin et al., 1992)(Table 1 & 8).

Table 8. Complications of diabetes in the United States (CDC, 1999; NCHS/CDC, 2000)

Deaths

- Diabetes is the cause of an estimated 65,000 deaths among Americans and is a contributing cause in another 95,000 deaths annually.

Heart Disease and Stroke

- People with diabetes have a two to four-fold increase in heart disease and are two to six times more likely to have a stroke than people who do not have diabetes.

Kidney Disease

- Diabetes is the single most common cause of end-stage renal disease (ESRD - where a person requires dialysis or a kidney transplant to live).

- Thirty to forty percent of those with diabetes develop ESRD.

- Currently over one-third of all patients with ESRD have diabetes and this number is expected to reach 50% if the current rate of increase continues.

Blindness

- Diabetes is the leading cause of blindness among adults 20 to 74 years of age.

Amputations

- Diabetes is the underlying cause of the majority of leg and foot amputations.

Appendix 2.

Diabetic nephropathy: natural history without pharmacological intervention.

For reasons that remain elusive, a significant percentage, but not all people with diabetes develop diabetic nephropathy. Additionally, although the cumulative incidence of nephropathy appears to be similar in both type I and type II diabetes (Hasslacher et al., 1989), nephropathy in type I diabetes tends to present in a more homologous fashion than that in type II diabetes; therefore, this discussion will focus primarily on un-arrested nephropathy in type I diabetes.

Nephropathy is recognized as one of the most severe metabolic components of long-term diabetes (Nathan, 1996), with the lifetime risk of developing nephropathy being approximately 30-40% for type I diabetes and 25-60% for type II diabetes (Hasslacher et al., 1989; Viberti et al., 1992). If pharmacological intervention with angiotensin-converting enzyme (ACE) inhibitors and antihypertensives is not introduced early in the following timeline, all people destined to develop diabetic nephropathy are expected to progress to end stage renal disease (ESRD) through the various stages illustrated in figure 18 (Breyer, 1992; Mogensen et al., 1983).

Early in the course of diabetes, the vast majority of affected people experience functional changes in the kidney, such as hyperfiltration and trace amounts of

albumin (referred to as microalbuminuria). If pharmacological intervention is not instituted, renal morphological changes occur within the next few years. Seven to 15 years after onset of diabetes, patients destined for nephropathy reach the next stage, termed "incipient nephropathy." During this period, predictors of diabetes-related nephropathy such as hyperfiltration, hypertension (HTN), poor glycemic control, and microalbuminuria (urinary albumin excretion of 30-300 mg/day) occur. Approximately 17 years after the onset of type I diabetes, macroalbuminuria (urinary albumin excretion of >300 mg/day) is manifested in those 30-40% of type I diabetes patients destined to develop overt diabetic nephropathy. Once these levels of albumin excretion are reached, renal function inexorably declines, with 50% of patients reaching ESRD within 7 years of onset of proteinuria. Ultimately, ESRD requires replacement of renal function using dialysis or renal transplant.

What is common amongst all people with nephropathy is a high rate of cardiovascular morbidity and mortality. In fact, myocardial infarction is recognized as the leading cause of death in these patients (Degoulet et al., 1982; Wheeler, 1996). The mechanisms responsible for the increased cardiovascular morbidity and mortality in this group of people remain somewhat elusive, but are believed to involve the atherogenic lipid profile that commonly accompanies nephropathy.

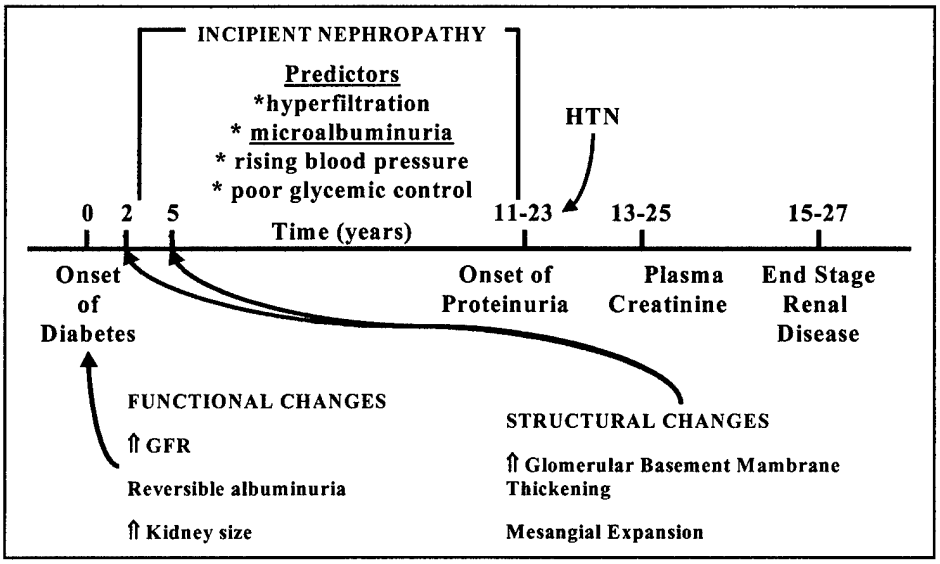

Figure 18. The natural history of nephropathy in type I diabetes without pharmacological intervention (Breyer, 1992).

Nephropathy and atherogenic lipids

Nephrotic hyperlipidemia is well documented. In 1860, Virchow first recorded its presence in medical literature (Virchow, 1860), with his description of degeneration in the kidneys of patients with Bright's disease; later Munk introduced the term "lipid nephrosis" (Munk, 1916). In 1936, interstitial and arterial "lipidosis" was described in people with diabetes (Kimmelsteil and Wilson, 1936) and later, glomerular "lipidosis" was noted in intercapillary lesions in diabetes (Wilens et al., 1951). We now know that the albuminuria associated with diabetic nephropathy is positively correlated with an atherogenic lipid profile and macrovascular disease (Guijarro and Keane, 1994; Moorhead et al., 1996; Wheeler, 1996). Namely, albuminuria is associated with increasing blood levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, apolipoprotein B, triglycerides, and reduced levels of high-density lipoprotein (HDL) cholesterol. Whether these lipid abnormalities are a cause, a consequence, or an epiphenomenona of diabetic nephropathy is unknown. However, it is known that as plasma albumin levels decrease due to albuminuria, hepatocytes synthesize excess amounts of very low density lipoprotein (VLDL) which is carried in the circulation by apolipoprotein B (apo-B) (Moorhead, 1991). Once in the peripheral circulation, lipases further reduce the VLDL to LDL. Paradoxically, as nephropathy-related LDL synthesis increases there is an accompanying slower than normal metabolism of LDL in the peripheral circulation (Warwick et al., 1990). The turnover rates of VLDL and LDL are slowed for reasons which

include decreased activity of two enzymes, lipoprotein lipase (LPL) and lecithin cholesterol acyltransferase (LCAT). The reasons for the decreased enzyme activity are unknown, but may be partly due to urinary loss of cofactors (Warwick et al., 1990). Nevertheless, plasma levels of LDL cholesterol are increased, thus increasing the risk of cardiovascular disease.

Appendix 3.

Atherosclerosis.

Atherosclerosis is a multifactorial disease that has the notoriety of being responsible for more than 50% of all mortality in developed nations, and is well recognized as the primary contributor to the pathogenesis of heart attack, stroke, and gangrene of the extremities (Ross, 1993). Various risk factors associated with the disease are listed in table 9.

Atherosclerotic lesion formation is characterized by a gradual focal progressive accumulation of cells, along with extracellular matrix and lipids in the intima of mid-size to large arteries. Danger comes when the resulting lesion either occludes the vessel or ruptures, thus triggering vascular occlusion via thrombosis. The earliest morphological evidence of disease has been appropriately termed the "fatty streak" due to the accumulation of lipid-laden macrophages and T lymphocytes within the innermost layer of the artery wall (Faggiotto and Ross, 1984; Gerrity, 1981; Joris et al., 1983). In 1973, Ross et al. formally proposed the response-to-injury hypothesis, suggesting that atherosclerosis might be precipitated by one or more insults to the endothelial lining of mid-size and large arteries (Ross and Glomset, 1973). This hypothesis has been modified as more data have become available (Lopes-Virella et al., 1997b; Lopes-Virella and Virella, 1998; Ross, 1986; Ross, 1993; Steinberg, 1993).

Table 9. Risk factors associated with atherosclerosis (American-Heart-Association, 2001)

Non-Modifiable	*Modifiable*
- Family History	- Diabetes
- Age	- Sedentary lifestyle
- Gender	- Cigarette smoking
	- Hypertension
	- Hyperlipidemia
	- Poor nutrition
	- Obesity

Events contributing to the formation of atherosclerotic lesions, as proposed by this hypothesis are as follows (Figure 19): The endothelium is injured. The injury may be mechanical, most notably at branch points in the arterial tree due to hypertension and/or shear stress; or it may be chemical, caused by oxidized low density lipoproteins, for example. Alternatively, the injury may be immunological, or due to viral or bacterial infection, or due to the effect of toxins. Indeed, the etiology of atherogenic injury is not mutually exclusive and may well be a combination of one or more of these insults. Nevertheless, after the initial injury there is trapping of lipoproteins and increased adherence of circulating monocytes and T lymphocytes to the damaged endothelium, followed by migration of the monocytes into the subendothelial space. Once in the arterial intima, the monocytes differentiate into macrophages, secrete pro-inflammatory cytokines, release oxygen active radicals that contribute to oxidation of sequestered lipoproteins, ingest and metabolize modified lipoproteins, and are transformed into lipid-laden foam cells. The foam cells, along with smooth muscle cells and T lymphocytes are primary components of the lesions appropriately described as "fatty streaks". Left uninterrupted, the fatty streak expands with alternating layers of smooth muscle cells and lipid-engorged macrophages, forming more advanced fibro-fatty lesions, ultimately resulting in formation of a fibrous plaque. At some point, perhaps as a consequence of excessive release of matrix metalloproteinases by activated macrophages (Huang et al., 1999), the plaque may destabilize and rupture, triggering a

thrombogenic episode that can either occlude the vessel or cause progression of the atherosclerotic lesion. Foam cell formation is recognized as a key component of atherosclerosis and, interestingly, is suspected to play an equally important role in nephropathy (Diamond, 1991; Magil, 1999; Moorhead et al., 1997).

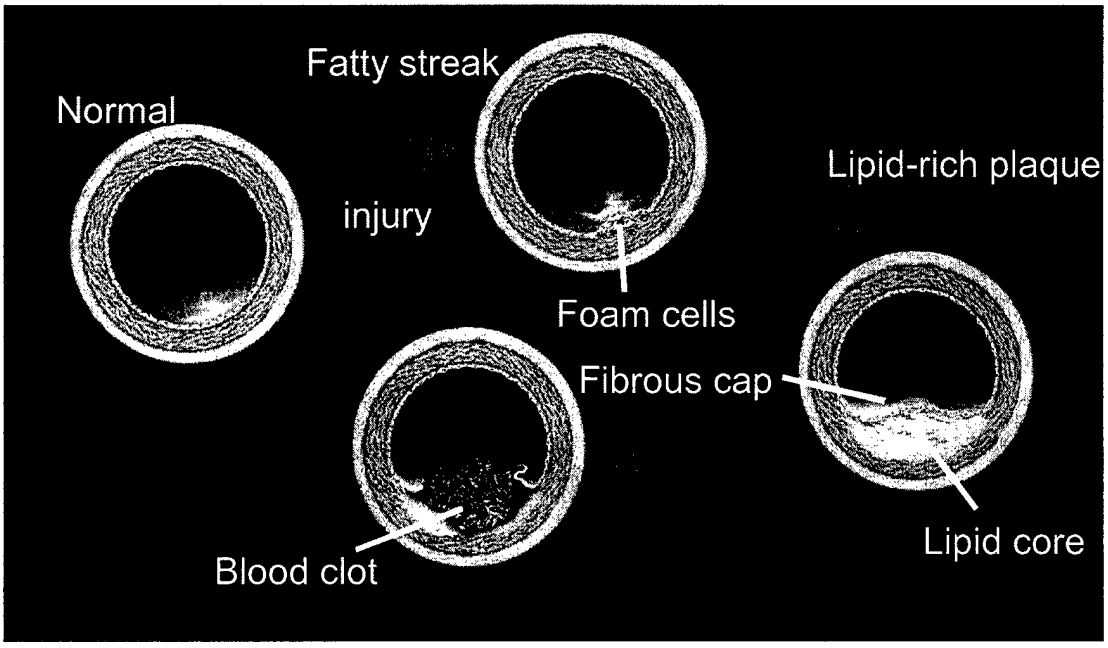

Figure 19. The response to injury hypothesis, adapted from Ross, 1999 (Ross, 1999). Atherosclerotic lesions arise in response to endothelial dysfunction that is initiated by an injury to the endothelium. The trapping of lipoproteins follows Endothelial injury and migration of monocytes into the subendothelial space, where the monocytes transform into lipid engorged macrophages (foam cells), establishing the lesion known as a fatty streak. Progression of this lesion results in a lipid-rich plaque, which may later become unstable and rupture, triggering blood clot formation.

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