Abstract

Elevated plasma concentrations of lipoprotein(a) [Lp(a)] have been identified as an independent risk factor for vascular diseases including coronary heart disease and stroke. In the current study, we have examined the binding and degradation of recombinant forms of apolipoprotein(a) [r-apo(a)], the unique kringle-containing moiety of Lp(a), using a cultured cell model. We found that the incubation of human hepatoma (HepG2) cells with an iodinated 17 kringle-containing (17K) recombinant form of apo(a) resulted in a two-component binding system characterized by a high affinity ($K_d = 12$ nM), low capacity binding site, and a low affinity ($K_d = 249$ nM), high capacity binding site. We subsequently determined that the high affinity binding site on HepG2 cells corresponds to the LDL receptor. In the HepG2 cell model, association of apo(a) with the LDL receptor was shown to be dependent on the formation of Lp(a) particles from endogenous LDL. Using an apo(a) mutant incapable of binding to the high affinity site through its inability to form Lp(a) particles (17KΔLBS7,8), we further demonstrated that the LDL receptor does not participate in Lp(a) catabolism. The low affinity binding component observed on HepG2 cells, familial hypercholesterolemia (FH) fibroblasts and human embryonic kidney (HEK) 293 cells may correspond to a member(s) of the plasminogen receptor family, as binding to this site(s) was decreased by the addition of the lysine analogue epsilon-aminocaproic acid. The lysine-dependent nature of the low affinity binding site was further confirmed in HepG2 binding studies utilizing r-apo(a) species with impaired lysine binding ability. We observed a reduction maximum binding capacity for 17K r-apo(a) variants lacking the strong lysine binding site (LBS) in kringle...
IV type 10 (17KΔAsp) and the very weak LBS in kringle V (17KΔV). Degradation of Lp(a)/apo(a) was found to be mediated exclusively by the low affinity component on both HepG2 cells and FH fibroblasts. Fluorescence confocal microscopy, using the 17K r-apo(a) variant fused to green fluorescent protein, further confirmed that degradation by the low affinity component on HepG2 cells does not proceed by the activity of cellular lysosomes. Taken together, these data suggest a potentially significant route for Lp(a)/apo(a) clearance in vivo.
Acknowledgements

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<tr>
<td>17K</td>
<td>wild type, 17 kringle-containing recombinant form of apo(a)</td>
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<tr>
<td>17KΔAsp</td>
<td>17K containing Asp56 to Ala mutation in KIV&lt;sub&gt;10&lt;/sub&gt; domain</td>
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<tr>
<td>17KΔLBS&lt;sub&gt;7,8&lt;/sub&gt;</td>
<td>17K lacking weak lysine binding sites in KIV&lt;sub&gt;7&lt;/sub&gt; and KIV&lt;sub&gt;8&lt;/sub&gt;</td>
</tr>
<tr>
<td>17KΔCys</td>
<td>17K lacking free cysteine residue in KIV&lt;sub&gt;9&lt;/sub&gt;</td>
</tr>
<tr>
<td>17KΔV</td>
<td>17K lacking KV domain</td>
</tr>
<tr>
<td>α&lt;sub&gt;2&lt;/sub&gt;MR</td>
<td>alpha-2-macroglobulin receptor</td>
</tr>
<tr>
<td>ε-ACA</td>
<td>epsilon-aminocaproic acid</td>
</tr>
<tr>
<td>apo(a)</td>
<td>apolipoprotein(a)</td>
</tr>
<tr>
<td>apoB-100</td>
<td>apolipoprotein B-100</td>
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<tr>
<td>apoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>apolipoprotein E-deficient</td>
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<tr>
<td>ASGPR</td>
<td>asialoglycoprotein receptor</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum binding capacity</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CpB</td>
<td>carboxypeptidase B</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Opti-MEM</td>
<td>serum free, conditioned medium</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Pg</td>
<td>plasminogen</td>
</tr>
<tr>
<td>r-apo(a)</td>
<td>recombinant apo(a)</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TC</td>
<td>tyramine cellobiose</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular-cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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<tr>
<td>VLDLR</td>
<td>very low-density lipoprotein receptor</td>
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Chapter 1

Introduction

1.1 Structure of Lipoprotein(a) and Apolipoprotein(a)

Originally discovered by Kåre Berg in 1963 (1), lipoprotein(a) [Lp(a)] (Figure 1-1) is nearly identical to low-density lipoprotein (LDL) in both lipid composition and the presence of apolipoproteinB-100 (apoB-100). Lp(a) is distinguishable from LDL by the presence of a unique glycoprotein moiety called apolipoprotein(a) [apo(a)], which is covalently attached to the apoB-100 component of LDL by a single disulfide bond (2). Apo(a) has a unique structure compared to other apolipoproteins in that it is hydrophilic, highly carbohydrate-rich and lacks amphipathic alpha helices (3,4). The cloning of apo(a) from a liver cDNA library revealed a striking homology between the amino acid sequence of apo(a) and that of the fibrinolytic serine protease zymogen plasminogen (5). The similarity of apo(a) to plasminogen results in many shared properties of these two molecules, including affinity for lysine residues in biological substrates (6,7). Unlike plasminogen, apo(a) cannot form an active protease (8), which underlies the ability of apo(a) to antagonize plasminogen function (9-11).

Human apo(a) possesses 10 subclasses of plasminogen kringle IV-like domains (designated KIV types 1-10), which are differentiated from one another on the basis of their amino acid sequence (Figure 1-2) (5). The various apo(a) KIV types have been found to exhibit between 75-84% amino acid identity with plasminogen kringle IV (5). Each of apo(a)’s 10 kringle IV-like domains exhibits a characteristic tri-looped structure, with its 80 amino acid frame held by three conserved disulphide bonds (12). These
Figure 1-1. Structural Representation of Lipoprotein(a). Lp(a) constitutes a unique class of plasma lipoproteins found in human plasma. It consists of an LDL-like moiety covalently linked to apo(a) by a single disulfide bond. The LDL component of Lp(a) consists of a phospholipid (PL) and free cholesterol (FC) monolayer, surrounding an inner core of triglycerides (TG), cholesteryl esters (CE) and a single molecule of apoB-100. The apo(a) component of Lp(a) surrounds the LDL-like moiety and is not associated with the LDL homologue. Analysis of a liver cDNA sequence in 1987 by McLean and colleagues (5) revealed a striking homology between apo(a) and the fibrinolytic plasma proenzyme plasminogen. [Reproduced, with permission, from Koschinsky ML (3).]
Apo(a) possesses 10 subclasses of plasminogen kringle IV-like domains (designated KIV types 1-10), which can be differentiated from one another on the basis of amino acid sequence. KIV1 and KIV3-10 are all represented in a single copy in apo(a), while KIV2 is present in a variable number of copies (3 to >40), contributing to Lp(a) isoform size heterogeneity. KIV5-8 each contain a weak lysine binding site (LBS), of which those in KIV7-8 associate with the LDL-like component of Lp(a) through non-covalent interactions prior to covalent Lp(a) assembly. KIV9 contains a free cysteine residue that is involved in covalent linkage between apo(a) and apoB-100. KIV10 possesses a strong LBS and has been found to interact with biological substrates such as fibrin. In the carboxyl-terminal region of apo(a), there is a plasminogen KV-like domain, followed by a serine protease-like domain. The protease domain in apo(a) is incapable of being cleaved by plasminogen due to the presence of critical amino acid substitutions. As such, the protease domain of apo(a) is inactive, which accounts for the ability of Lp(a) to antagonize plasminogen function. [Figure adapted from Koschinsky and Marcovina (13).]
Krin ge domains are further separated by a region of approximately 30 amino acid residues referred to as the interkringle region. This region functions in adjoining adjacent kringle s and may provide flexibility to the quaternary structure of apo(a) (14). Kringle IV types 1 and 3-10 are all present in single copy in apo(a) (15), while the kringle IV type 2 (KIV2) sequence is present in identically repeated copies varying in number from 3 to >40 (Figure 1-3) (16,17). The variable number of KIV2 repeats results in isoform heterogeneity, which is the hallmark of apo(a) structure. The KIV2 repeat region thus confers size heterogeneity to Lp(a) particles, with molecular masses of apo(a) in these particles ranging in the human population from <200 to >800 kDa (18,19). Several of the apo(a) KIV domains are of particular interest in terms of functionality. The KIV5-8 domains of apo(a) each possess weak lysine binding sites (LBS) \(K_d \sim 230 \mu M\), and those contained in KIV7-8 mediate the initial non-covalent interaction between apo(a) and N-terminal lysine residues (Lys680 and Lys690) in apoB-100 prior to covalent Lp(a) assembly (20-22). Apo(a) KIV9 contains a free cysteine residue. Activity by an as yet unidentified extracellular oxidase enzyme catalyzes the formation of the sole covalent bond between apo(a) KIV9 and apoB-100 (2,23). Apo(a) KIV10 possesses a stronger LBS \(K_d \sim 30 \mu M\), relative to those previously mentioned in KIV5-8; the LBS in KIV10 may mediate interactions with key biological substrates including fibrin (20,24). The KV domain contains a weak LBS and may account for the immunological and proinflammatory properties of apo(a) that contributes to the progression of atherosclerosis (25). Finally, the apo(a) glycoprotein contains a protease-like domain which is catalytically inactive due to an arginine to serine substitution in apo(a) that
Figure 1-3. Relationship Between Apo(a) and Plasminogen. The domain organization of apo(a) is shown relative to that of the plasma proenzyme plasminogen. Plasminogen consists of an amino-terminal tail domain (T), five different kringle domains (sequentially numbered I through V) and a serine protease domain (P). Apo(a) contains multiple copies of sequences resembling plasminogen KIV, followed by a single copy of sequences resembling the plasminogen kringle V and protease domains. Apo(a) contains 10 subclasses of plasminogen KIV-like domains, numbered 1 through 10. Each KIV domain is present in a single copy with the exception of KIV2, which can be repeated between 3 and >40 times, accounting for the size heterogeneity of this lipoprotein. The percent amino acid identity shared between the domains of plasminogen and apo(a) is indicated above. [Figure adapted from Koschinsky ML (3).]
removes the cleavage site recognized by plasminogen activators such as tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (8). Interestingly, the preceding study demonstrated that even if the normal cleavage site was restored, the apo(a) protease domain remains catalytically inactive due to the deletion of nine amino acids within a highly conserved region of many different serine proteases (26).

1.2 Determination of Plasma Lipoprotein(a) Levels

Plasma Lp(a) concentrations vary over 1000-fold in the human population, ranging from <0.1 to >100 mg/dL. Evidence has suggested that the rate of Lp(a) formation, as opposed to its removal from the circulation, appears to be the primary determinant of plasma Lp(a) levels (27). In Caucasians, the apo(a) gene itself accounts for ~90% of the observed variation in plasma Lp(a) levels (28), while only ~78% of the variation in African Americans can be explained by the gene (29). Unlike LDL, Lp(a) levels are relatively resistant to conventional lipid-lowering by diet or exercise, as well as by most pharmacological interventions, including statin therapy (30). Niacin, or vitamin B₃, has been identified as the only pharmaceutical capable of consistently reducing Lp(a) levels (31). A clinical study testing the effectiveness of niacin as a treatment for hypercholesterolemia found that subjects receiving daily doses of niacin displayed a 35% reduction in Lp(a) plasma levels after the 26th week of treatment (31).

A general inverse correlation exists in the human population between apo(a) isoform size and plasma Lp(a) concentrations (19,32,33). This correlation may arise
from the less efficient secretion of larger apo(a) isoforms from hepatocytes, reflecting longer retention times in the endoplasmic reticulum and subsequent increased intracellular degradation (34). However, the general inverse relationship cannot account for ethnic variations in plasma Lp(a) levels and the existence of a threshold above 20 KIV repeats for which the correlation does not apply (35,36). Accordingly, the size of the apo(a) gene alone cannot account for population-wide differences in Lp(a) levels. It has been postulated that in addition to the size of the apo(a) gene, \textit{cis}-acting elements present in the apo(a) gene may influence transcription of this lipoprotein (37). Alternately, the relationship between apo(a) isoform size and mRNA transcript stability, translational efficiency and efficiency of assembly into Lp(a) particles may also contribute to the inverse correlation; the contribution of these processes to Lp(a) levels remains to be determined (3).

1.3 Atherosclerosis and Lipoprotein(a) Pathogenicity

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries (38). In Westernized societies, atherosclerosis is the primary cause of heart disease and stroke, resulting in approximately 50% of all deaths (38). The progression of atherosclerosis involves the focal thickening of the arterial intima, resulting in the formation of atherosclerotic lesions (39). Atherogenesis is stimulated by the invasion and retention of lipid-laden molecules, such as LDL, in the intimal layer of the artery. These lesions consist of inflammatory and immune cells, connective-tissue elements, lipids and cellular debris (40). As the lesions
become more advanced, a dense collection of smooth muscle cells (SMCs) proliferate and migrate from the arterial media to the intima, where they produce extracellular matrix (ECM) components that enclose a core of lipids and necrotic debris (38). Eventually, advanced lesions can grow sufficiently large to block blood flow, where rupture of the lesion often results in the formation of a thrombus and subsequently, myocardial infarction or stroke. Interestingly, Lp(a) has also been observed in the arterial intima at the site of atherosclerotic lesions using immunohistochemical methods (41), where it preferentially accumulates compared to LDL alone (42). The extent to which Lp(a) accumulates in lesions is directly correlated with plasma levels of this lipoprotein (42). Since Lp(a) is similar to both plasminogen and LDL, researchers have suggested that Lp(a) may contribute to the progression of atherosclerosis through either proatherogenic or prothrombotic mechanisms (43). Mechanisms that are potentially proatherogenic include increases in macrophage foam cell formation, SMC proliferation and migration, monocyte chemoattractant activity and endothelial cell (EC) adhesion molecule expression (44,45). In terms of prothrombotic effects, apo(a) has been shown to increase platelet responsiveness (46), while both in vitro and in vivo studies have shown that Lp(a)/apo(a) can inhibit fibrin clot lysis (47-49), as well as tPA-mediated plasminogen activation on the surface of fibrin (9,50).

Endothelial cells are the most proximal structure to direct blood flow in the vasculature, functioning as a selectively permeable barrier between blood and tissues (38). The movement of mildly oxidized LDL across ECs and into the arterial intima triggers cellular activation, characterized by the production of a variety of adhesion
molecules, including vascular-cell adhesion molecule-1 (VCAM-1) (39). Up-regulation of VCAM-1 has been observed in response to hypercholesterolemia and as a result of EC interaction with an array of cytokines, including tumor necrosis factor-α (TNF-α) and interleukins-1, 2, 3 and 6 (39). VCAM-1 molecules serve as adhesive sites, promoting the recruitment of lymphocytes and monocytes to the inflammatory region. Lp(a) itself has been found to induce monocyte chemotactic activity, mediated by the apo(a)-dependent release of chemokine CCL1 (I-309) (51). Further studies have also shown that Lp(a) stimulates the expression of intercellular adhesion molecule-1 (ICAM-1) (52), VCAM-1 and E-selectin (53), promoting the binding of inflammatory cells to the dysfunctional endothelium. As such, it has become well-accepted that Lp(a) promotes endothelial dysfunction, an important early step in atherogenesis (54).

Once leukocytes are recruited to the ECs overlying the lesion, further cytokines and growth factors are produced in the inflamed intima, inducing monocytes to infiltrate arterial plaques and to differentiate into macrophages (39). The accumulated LDL is then subjected to oxidative and enzymatic modifications within the intima, leading to particle uptake by macrophages and the creation of foam cells. The inner core of the lesion is thus comprised of foam cells and extracellular lipid droplets, while a cap of smooth muscle cells and a collagen matrix forms the exterior component (39). Necrosis of macrophage and smooth muscle cell-derived foam cells then leads to the formation of a necrotic core and the accumulation of extracellular cholesterol (55). Most cases of infarction involve the formation of an occlusive thrombus upon the rupture of a vulnerable plaque. During plaque rupture, abundant immune cells within the lesion
produce cytokines and enzymes that weaken the fibrous cap, leading to rupture of the plaque and the release of platelet-adhesive factors, phospholipids and tissue factors into the blood (39). The recruitment of tissue factors and platelets quickly trigger the coagulation cascade, resulting in thrombus formation in the vessel lumen, with consequent disruption in blood flow.

Although Lp(a) has been identified as a risk factor for coronary heart disease (CHD), its mechanism of action remains poorly understood. Interactions between Lp(a) and plaque elements may be mediated through binding of apo(a) to a number of matrix components such as fibrin, fibronectin and laminin (56). Interestingly, the strong LBS in KIV\textsubscript{10} of apo(a) has recently been discovered to mediate increased endothelial cell contraction and permeability via a Rho/Rho kinase-dependent pathway (57). In addition, oxidative effects on Lp(a) might be increased by the inflammatory environment of arterial lesions (54). The similarity of Lp(a) to LDL may further allow it to contribute to cholesterol deposition, or it might mediate an antifibrinolytic effect due to its similarity to plasminogen. Although apo(a) has been shown to inhibit tPA-mediated plasminogen activation on the surface of fibrin, the mechanism has been controversial, with competitive (58), uncompetitive (50,59) and an equilibrium template model (9) utilized to describe the observed inhibition. It is also proposed that apo(a) upregulates plasminogen activator inhibitor-1 (PAI-1) expression in ECs, thus preventing cleavage of the plasminogen proenzyme (60). Also, Lp(a) has been found to inhibit smooth muscle cell migration and proliferation by inhibiting plasmin formation, and thus the plasmin-mediated activation of transforming growth factor-β (TGF-β) (61,62). Since
plasminogen activation and fibrinolysis are critical in many cellular processes, including those involved in atherothrombosis, knowledge of the exact mechanisms by which apo(a)/Lp(a) exert their downstream effects are crucial in cardiovascular disease prevention.

1.4 Lipoprotein(a) Assembly

Lp(a) assembly, in its simplest terms, can be described as the 1:1 stoichiometric association between apo(a) and the apoB-100 moiety of LDL to form covalent Lp(a) particles (63). The spatial location of Lp(a) assembly was first classified as an extracellular process based on *in vitro* studies utilizing human hepatoma (HepG2) cells, stably transfected with a 17-kringle containing form of recombinant apo(a) [r-apo(a)]. In these studies, only free apo(a) was observed in 17K-transfected cellular lysates, while *bona fide* Lp(a) particles were detected in the corresponding conditioned medium (2). Further analysis from a number of different groups subsequently confirmed these findings by reconstituting Lp(a) in tissue culture from r-apo(a) and either purified LDL or LDL present in the plasma (64,65). Overall, these findings suggested that the covalent assembly of Lp(a) does not require the activity of enzymes within the intracellular secretory pathway, including protein disulfide isomerase that catalyzes disulfide bond formation in the ER during protein folding.

Lp(a) assembly proceeds as a two-step process in which initial non-covalent interactions between apo(a) and apoB-100 precede specific disulfide bond formation (Figure 1-4) (65). Early studies using a variety of r-apo(a) variants identified a key role
for KIV$_{5,8}$; the ability of lysine and lysine analogues to inhibit Lp(a) formation further suggested that the weak LBS in these kringle mediated Lp(a) assembly (66,67). Additional studies using point mutations in r-apo(a) to abolish the weak LBS in KIV types 6, 7 and 8 demonstrated that the LBS in KIV$_8$, and to a lesser extent, KIV$_7$, were required for non-covalent Lp(a) assembly (68). The interaction between the weak LBS in KIV$_{7,8}$ and apoB-100 was found to be facilitated through specific N-terminal lysine residues (Lys680 and Lys690) in apoB-100, where mutation of these residues to alanine in a synthetic peptide abolished the lysine-dependent interaction with apo(a) (22).

The covalent linkage between apo(a) and apoB-100 is mediated by a seventh unpaired cysteine at position 4057 in the published sequence of apo(a), located in apo(a) KIV$_9$ (2). Several studies employing site-directed mutagenesis suggest that Cys4326 in apoB-100 is involved in disulfide bond formation with apo(a) (69,70). However, these findings are in conflict with earlier data from biophysical and biochemical studies, which propose the involvement of Cys3734 in apoB-100 in covalent binding to apo(a) (6,71,72). Despite the discrepancy with respect to the participating cysteine residue in apoB-100, the initial non-covalent interaction is thought to properly align the cysteines in both apo(a) and apoB-100 prior to disulfide bond formation. Once aligned, disulfide bond formation was initially thought to occur spontaneously. However recent findings have suggested the presence of an extracellular oxidase enzyme in the conditioned medium of HepG2 cells that enhances the rate of disulfide bond formation (23). This activity is likely conferred by a protein because it is heat-sensitive and it is retained in the concentrate following ultracentrifugation through a 5 kDa cutoff filter (23). However,
Figure 1-4. Two-Step Model for Lp(a) Assembly. The first step in Lp(a) assembly involves a high-affinity ($K_d \approx 20$ nM) non-covalent interaction between apo(a) and apoB-100, where lysine binding sites in apo(a) KIV types 7 and 8 interact with N-terminal lysine residues in apoB-100. This initial step can be inhibited by lysine and lysine analogues, such as epsilon-aminocaproic acid ($\varepsilon$-ACA). The second step in the assembly process involves disulfide bond formation between apo(a) and apoB-100 and has been found in vitro to be regulated by the presence of an extracellular oxidase enzyme. [Reproduced, with permission, from Koschinsky ML (3).]
subsequent dialysis of the concentrate revealed a loss in specific activity, suggesting the requirement for a small molecule cofactor (23). To date, the identity of the enzyme remains unknown, however its kinetic properties have been well documented (23).

1.5 Lipoprotein(a) Catabolism

1.5.1 Overview

Currently, the pathway for Lp(a) catabolism remains largely unknown. However, it seems that the process of Lp(a) catabolism does not contribute significantly to plasma Lp(a) levels in humans (28,29). In fact, the source of most of the variability in plasma Lp(a) concentrations has been attributed to the rate of Lp(a) production (27). The steps of Lp(a) production that may be subject to regulation include synthesis, secretion and Lp(a) formation.

Recent work by Becker and co-workers (23) has revealed that the rate of covalent Lp(a) assembly is regulated by an extracellular oxidase enzyme, which may present a possible target for the pharmacological lowering of plasma Lp(a) levels. However, until the catabolic pathway for Lp(a) is determined, it is not possible to consider manipulating Lp(a) uptake as a means to decrease plasma levels in vivo. Numerous efforts have thus been made to characterize Lp(a) tissue uptake for the purpose of identifying the major organ(s) responsible for clearance. The following sections will describe the findings from in vivo studies which highlight the notion that the liver plays a primary role in Lp(a) catabolism. Additionally, evidence from in vitro as well as clinical studies will be
presented, which imply a minor contribution by the kidneys in the catabolism of Lp(a). Finally, a thorough examination of candidate Lp(a) receptors will be summarized, where the uptake mechanism and the nature of the receptor-ligand interactions will be highlighted.

1.5.2 Hepatic Uptake of Lipoprotein(a)

Identification of the organ(s) regulating Lp(a) catabolism has resulted in contradictory findings. To date, the most convincing evidence supporting liver-mediated catabolism has emerged from two in vivo studies elegantly designed to directly measure Lp(a) tissue uptake. Prior to examining these reports however, it should be noted that Lp(a) is only present in humans, Old World Monkeys and the European hedgehog (73). Since in vivo systems for Lp(a) study are limited, the following studies employ the use of the rat and the mouse, both of which have become attractive models for analyzing Lp(a) clearance despite the fact that neither species produces endogenous Lp(a). In the case of the rat, reports previously demonstrated that human LDL was taken up by rat hepatic receptors (74). For mice, the clearance of LDL has been shown to be faster than that of Lp(a), and the relative fractional catabolic rate (FCR) of LDL compared to Lp(a) is similar to that observed in humans (27,75,76). Additionally, low-density lipoprotein receptor (LDLR) deficiency in both humans and mice reduces the LDL FCR to ~50% of normal, while only reducing the Lp(a) FCR by ~10% (76).

The first study supporting hepatic Lp(a) uptake was performed in 1988 by Ye and co-workers (74), who utilized a rat model to determine the sites and mathematical
distribution of Lp(a) tissue uptake. After labeling human Lp(a) with a non-hydrolyzable tritiated ether and injecting the substrate into the rat, it was noted that the greatest proportion of uptake (28.5%) was in the liver, whereas the kidney accounted for less than 0.5% of the injected dose. The remaining label was recovered in the small intestines (9.6%), lungs (0.94%), spleen (2.7%) and adrenals (0.19%) (74). The group never tested the urine, feces or plasma to mathematically account for the entire quantity of injected Lp(a).

Similarly, Cain and co-workers (76) performed experiments using $^{125}$I-tyramine cellobiose- (TC) labeled human Lp(a) in a heterologous mouse model. Since the tyramine cellobiose ligand is incapable of being degraded intracellularly, the radiolabel served to identify tissues mediating Lp(a) catabolism. Following tail vein injection and a subsequent 24-hour incubation period, the animals were euthanized and tissue uptake of radiolabeled Lp(a) was quantified. Interestingly, highly comparable results were obtained in this study when measured against the work of Ye et al. (74), as hepatic uptake accounted for 21.3% of the injected dose, while the kidney only accounted for a small fraction of tissue uptake (1.3%) (76). Combined values for the stomach, spleen, adrenals, testes, heart, lungs and brain were <2% of the injected dose, while the remainder of the radiolabeled material was recovered in the urine and plasma (76).

In a parallel study, this group also injected $^{125}$I-TC-apo(a) into the mouse and found that approximately 35% of the injected dose was recovered in the liver, while over 20% was present in the urine (76). Since several studies have reported the presence of apo(a) fragments in human urine (77,78), numerous groups have suggested that these
fragments are formed extrarenally and are subsequently excreted by the kidney (78,79). Interestingly, Mooser et al. (78) injected human Lp(a) into mice and detected only trace amounts of apo(a) in the urine by ELISA; apo(a) fragments were not identified in either the urine or the plasma by immunoblot analysis. Taken together, the above evidence presents a convincing case that the liver is the main site of Lp(a) catabolism in vivo.

1.5.3 Lipoprotein(a) Clearance by the Kidney

Evidence suggesting a role for the kidney in Lp(a) catabolism began with a variety of clinical observations in patients diagnosed with impaired kidney function. Initially, Kostner et al. (77) identified a correlation between patients with renal disease and elevated concentrations of plasma Lp(a). On average, plasma Lp(a) concentrations were found to be significantly higher in patients (33.6 mg/dL), compared with control subjects (22.3 mg/dL), where the degree of Lp(a) elevation was found to be inversely correlated with the glomerular filtration rate (80). Additionally, since the quantity of urinary apo(a) excretion is dependent on plasma Lp(a) levels, kidney patients were also found to excrete less apo(a) when compared to the normal population (77). Interestingly, the Kostner group also reported that the fraction of Lp(a) cleared by the kidney accounts for a maximum of 1% of total plasma apo(a) catabolism (81), thus implying only a minor role for the kidney in Lp(a) clearance.

Observations by Reblin and co-workers (82) have also supported a role for the kidney in Lp(a) clearance. Utilizing a heterologous rat model, the group demonstrated that when human Lp(a) is injected in rats, accumulation of apo(a) occurs in the proximal
tubular cells of the kidney and apo(a) fragments are further excreted in the urine. Along the same lines, Kronenberg and co-workers (83) demonstrated that in patients undergoing coronary angioplasty, Lp(a) levels were lower in the renal vein (18.7 ± 20.3 mg/dL) than those in the ascending aorta (20.1 ± 21.6 mg/dL), suggesting that Lp(a) is removed in the renal circulation. Interestingly, the same group was unable to detect Lp(a) or intact apo(a) in the urine from healthy probands, but detected smaller fragments using antibodies against apo(a) (83). These observations coincide with those of Mooser et al. (78), who intravenously injected purified, urinary human apo(a) fragments into mice and observed the rapid appearance of these same fragments in the urine. Once again, this suggests that urinary apo(a) fragments are formed extrarenally and are then excreted by the kidney (79).

Further evidence supporting a role for the kidney in mediating Lp(a) clearance is again evident in the work of Kronenberg and colleagues (84), who reported a general reduction in total plasma Lp(a) in renal patients following kidney transplantation surgery. On average, plasma concentrations were reduced from 25.9 ± 28.7 mg/dL to 17.9 ± 25.5 mg/dL 3 weeks after surgery. Interestingly, only patients with high-molecular weight isoforms exhibited a significant decrease in Lp(a) plasma concentrations (84). One mechanism potentially responsible for the initial Lp(a) elevation could have been due to a lack of protein retention by the kidney, resulting in a compensatory increase in liver gene expression (84). Since apo(a) is synthesized exclusively by hepatocytes, Kostner and co-workers (77) suggested that an overall increase in protein synthesis by the liver may be required to compensate for the reduced ability of the kidney to retain protein during
glomerular filtration. However, the Lp(a) fractional catabolic rate has been reported to be significantly lower in kidney function-impaired patients (0.164 ± 0.114 d\(^{-1}\)) than in healthy controls (0.246 ± 0.067 d\(^{-1}\)) (\(p = 0.042\)) (85). Accordingly, since elevated plasma Lp(a) concentrations are correlated with a loss of renal function, as opposed to a reduction in formation, the hypothesis linking renal deficiency to enhanced protein synthesis has been proven to be misleading.

Additional evidence suggesting that the kidney plays a role in Lp(a) clearance is apparent in clinical studies involving hemodialysis patients, where impaired renal function has been found to reduce Lp(a) clearance, while the rate of Lp(a) formation remains unchanged (85). As mentioned above, these findings also indicate a longer plasma Lp(a) retention time in hemodialysis patients than in controls with normal kidney function (85).

Overall, the above studies suggest a minor contribution by the kidneys in Lp(a) catabolism in individuals with normal renal function. Unfortunately, direct evidence for renal uptake of Lp(a) in humans is lacking and thus a regulatory role for the kidney in mediating plasma Lp(a) levels remains uncertain (86).
1.6 Lipoprotein(a) Catabolic Receptors

1.6.1 Candidate Receptors: An Overview

Several receptors that mediate the cellular binding and internalization of lipoproteins possessing apoB-100 have been postulated as candidate receptors for Lp(a) clearance. These receptors include the LDL receptor (LDLR) (87-90), the LDL receptor-related protein/\(\alpha_2\)-macroglobulin receptor (LRP/\(\alpha_2\)MR) (91), the very low-density lipoprotein receptor (VLDL) (92) and the megalin/gp330 receptor (93). Additionally, since the apo(a) component of Lp(a) is heavily glycosylated (94) and shares a high amino acid identity with plasminogen (5), the asialoglycoprotein receptor (ASGPR) (76,86) and members of the plasminogen receptor family (95,96) have further been examined as potential catabolic receptors specific for the apo(a) component of the Lp(a) particle. The following section will review the candidate receptors (Figure 1-5) in terms of their physiological role and as potential players in Lp(a)/apo(a) clearance.

1.6.2 LDL Receptor

The similarity of Lp(a) to LDL, both in terms of lipid composition and the presence of apoB-100, has triggered the widespread analysis of the LDLR as a clearance pathway for Lp(a). Under normal physiological conditions, the LDLR is expressed at a high level on the surface of the liver and accounts for over 70% of the total-body uptake of LDL (97). The LDLR represents a high affinity pathway for the removal of LDL from the circulating plasma by recognizing the apoB-100 component of LDL, which is embedded in the outer phospholipid of the lipoprotein particle (98). Alternately, the
Figure 1-5. Schematic Representation of Proposed Pathways for Lp(a) Catabolism. Apo(a) is synthesized and secreted by the liver, after which it associates with LDL in a 1:1 molar ratio to form circulating Lp(a) (black arrows). Although the detailed mechanism for Lp(a) catabolism remains largely unknown, the liver seems to be the major organ involved in Lp(a) clearance (solid red arrow) \( (74,76) \). Additionally, the kidney (dashed arrow) and a variety of peripheral tissues (dashed arrow) have been suggested as potential contributors to the removal of circulating Lp(a). For all routes of catabolism, candidate clearance receptors are listed. [Reproduced, with permission, from Albers et al. \( (86) \).]
LDLR can recognize the apolipoprotein E (apoE) component found in chylomicron remnants and VLDL remnants (99).

Following synthesis in the rough endoplasmic reticulum and modification in the Golgi apparatus, LDL receptor complexes cluster in clathrin-coated pits on membrane surfaces (100). Upon binding to LDL-cholesterol, the coated pits invaginate to form endocytic vesicles, which then fuse to form larger organelles known as endosomes (100). In the acidic environment of the endosome, a conformational change occurs in the receptor that triggers its dissociation from LDL (100). The receptor is then either degraded in the endosome, or recycled via an endocytic vesicle to the membrane surface where the neutral pH causes it to revert back to its native conformation (100). The dissociated LDL is then delivered to a lysosome, where the protein component is hydrolyzed to amino acids and the cholesteryl esters are hydrolyzed by an acidic lipase, thereby liberating free cholesterol (100).

The contribution of the LDLR to Lp(a) catabolism is controversial based on data from both in vitro and in vivo systems. Some studies examining the interaction between Lp(a) and the LDLR in cell culture have provided in vitro evidence supporting a role for the LDLR in Lp(a) catabolism (88,90). Perhaps the most striking evidence for a physiological role for the LDLR in Lp(a) clearance comes from a study by Hofmann and co-workers (101), who utilized transgenic mice overexpressing the LDLR. The group injected human Lp(a) into these mice and observed a marked increase in Lp(a) clearance in the transgenic mice compared to the wild type mice (101). These findings were further supported by Utermann and co-workers (102), who reported that plasma Lp(a) levels
were elevated 3-fold in patients with the heterozygous form of familial hypercholesterolemia (FH), a condition where individuals possess one mutant copy of the \textit{LDLR} gene.

Convincing evidence arguing against a significant contribution by the LDLR in Lp(a) clearance comes from the analysis of the FCR of Lp(a) in subjects who were either homozygous or heterozygous for FH (103). In this study, the catabolism of Lp(a) was not significantly different in homozygous FH patients (0.251 d\(^{-1}\)), heterozygous FH patients (0.254 d\(^{-1}\)) and control subjects (0.287 d\(^{-1}\)), thus suggesting that the LDLR is not a physiologically relevant route for Lp(a) catabolism in humans. Further evidence refuting a role for the LDLR in Lp(a) clearance is apparent in the work of Cain and co-workers (76), who utilized \textit{LDLR}\(^{-/-}\) knockout mice to measure the FCR of \(^{125}\)I-TC-human Lp(a). In an elegant experiment, the group noted that the catabolic rate of LDL was significantly slowed in \textit{LDLR}\(^{-/-}\) mice, but that plasma Lp(a) clearance in the knockout mice was similar to that observed in wild type mice. Interestingly, the group also discovered that the introduction of a molar excess of apo(a) significantly inhibited Lp(a) clearance in wild type mice, thus providing strong evidence to suggest that the apo(a) moiety of Lp(a) is the major ligand mediating hepatic clearance of Lp(a) (76).

\subsection*{1.6.3 LDL Receptor-Related Protein / \(\alpha_2\)-Macroglobulin Receptor}

Chylomicrons are synthesized by the intestine to transport dietary triglyceride and cholesterol (104). While in circulation, the triglycerides in these particles are hydrolyzed by lipoprotein lipase (LPL), producing cholesterol-enriched chylomicron remnants (104).
These remnants are cleared rapidly and efficiently from the plasma by the liver, and their cholesterol converted to bile acids and excreted, or used in membrane or hepatic lipoprotein biosynthesis (104). Very low-density lipoproteins (VLDLs) are synthesized in the liver and like chylomicrons, fulfill a lipid transport function (105). The triglycerides in these particles are also acted on by LPL in the plasma and generate small, cholesterol-enriched lipoproteins known as intermediate-density lipoproteins (IDLs) (105). LDL particles are the end product of VLDL catabolism and are the major cholesterol-transporting lipoprotein in the plasma (104). Approximately half of the VLDL remnants are cleared directly by the liver through an apoE-mediated process (105), while the remainder are converted to LDL containing only apoB-100 and are cleared as described in the preceding section.

The LDL receptor-related protein (LRP), also referred to as the $\alpha_2$-macroglobulin receptor ($\alpha_2$MR), represents a second class of hepatic receptor that participates in lipid metabolism by recognizing chylomicron remnants associated with apoE (105). The LRP interacts with apoE-containing lipoproteins, but not with apoB-containing LDL (104). Since a definitive role for the LDLR in Lp(a) clearance has been elusive, the LRP has been suggested as a potential catabolic receptor for Lp(a). Work by Marz and co-workers (91) has observed through ligand blotting that high molecular weight isoforms of apo(a) preferentially bind to the LRP over the LDLR, thus suggesting a role for the LRP in Lp(a) clearance. Additionally, van Barlingen and co-workers (106) demonstrated that Lp(a) could be enriched with apoE and that this resulted in increased lipoprotein lipase-enhanced binding to proteoglycans. Because heparin sulfate proteoglycans sequester
remnant lipoproteins in the perisinusoidal space of the liver (107), coupled with the fact that the LRP binds and mediates the catabolism of LPL (108), it has been suggested that the LRP is a potential clearance receptor for Lp(a) (106). Interestingly, these observations have been strengthened by the work of Devlin and co-workers (109), who demonstrated that the KIV\textsubscript{5-8} fragment of apo(a) inhibits the hepatic clearance of cholesterol-rich remnant lipoproteins in a transgenic mouse line. This \textit{in vivo} observation suggests that apo(a) potentially competes with the LRP or the LDLR for remnant lipoprotein clearance (109).

The most convincing evidence against the LRP as candidate receptor for Lp(a) clearance comes from the work of Cain and co-workers (76), who utilized apoE deficient mice (\textit{ApoE} \textsuperscript{-/-}) and examined the fractional catabolic rate of Lp(a) in this knockout mouse model. In \textit{ApoE} \textsuperscript{-/-} mice, Cain et al. (76) found that Lp(a) plasma clearance was slightly accelerated compared to that in wild type mice, and there was a small increase in hepatic uptake. Interestingly, these \textit{in vivo} observations coincided with earlier findings from \textit{in vitro} experiments, in which the addition of \(\alpha\)-2-macroglobulin failed to decrease total specific binding of apo(a) to cultured HepG2 cells (110). Taken together, these studies suggest that Lp(a) catabolism is not primarily facilitated through the acquisition of apoE and the subsequent degradation by the LRP/\(\alpha\textsubscript{2}MR\).

\subsection*{1.6.4 VLDL Receptor}

The VLDL receptor (VLDLR) is a member of the LDL receptor family and functions as a peripheral remnant lipoprotein receptor, binding apoE-containing
triglyceride rich lipoproteins (111). Unlike the LDLR, the VLDLR is barely detectable in the liver and is instead expressed abundantly in fatty acid-active tissues (heart, skeletal muscle and fat), the brain and macrophages (112). Evidence suggesting a role for the VLDLR in Lp(a) clearance is presented in the work of Argraves et al. (92), who demonstrated that fibroblasts expressing the VLDLR were able to mediate the endocytosis and lysosomal degradation of Lp(a) more effectively than fibroblasts lacking this receptor. Additionally, the group determined through LDL competition studies that the apo(a) component of Lp(a) mediates binding to the VLDLR (92). Nonetheless, previous studies (74,76) have downplayed a primary role for this receptor in vivo due to its non-hepatic distribution, although it remains a possibility that a small fraction of Lp(a) is catabolized by this remnant lipoprotein receptor.

1.6.5 Megalin/gp330 Receptor

Megalin/glycoprotein 330 (gp330) is a member of the LDLR family and is expressed in a number of resorptive, polarity‐differentiated epithelia that are heavily engaged in receptor‐mediated endocytosis (93). The receptor is most abundant in the kidney, lung, thyroid, brain, yolk sac, inner ear and mammary gland (113). Ligands include elements of lipoprotein metabolism, such as LDL, apoE and apoB-100 (114,115), and of the blood clotting and fibrinolytic systems, including plasminogen and plasminogen activator‐inhibitor complexes (116). Since megalin/gp330 is highly abundant in the kidney and has previously been shown to bind both plasminogen and apoB-100 (114,116), it has been postulated as a catabolic receptor for Lp(a) in this organ.
Interestingly, work by Niemeier and co-workers (93) has revealed that the uptake and degradation of radiolabeled Lp(a) by a megalin/gp330 expressing cell line was, on average, 2-fold higher than that of control cells. The group also demonstrated that purified megalin/gp330, immobilized on a sensor chip, directly bound Lp(a) in a Ca\(^{2+}\)-dependent manner (93). Together, these results suggest a potential clearance mechanism for Lp(a) and may account for the kidney-mediated degradation of Lp(a).

### 1.6.6 Asialoglycoprotein Receptor

The asialoglycoprotein receptor (ASGPR) is a hetero-oligomeric endocytic receptor predominantly expressed on the surface of hepatocytes (117). The primary physiological function of the receptor is to remove and degrade desialylated circulating proteins, thus making it a candidate receptor for Lp(a) due to the extensive sialylation of the apo(a) component (94). Since sialic acid residues usually cap the non-reducing terminal of glycoproteins, the enzymatic removal of these residues by neuraminidases exposes galactose residues, which are readily recognized by the ASGPR (117). The distribution of mammalian neuraminidases is quite vast, as this group of enzymes has been characterized in hepatocytes, erythrocytes, monocytes and platelets, as well as in the blood plasma (118). In the event that apo(a) becomes desialylated, it may become a potential ligand for the ASGPR. In order to assess the role of the ASGPR in Lp(a) clearance, Cain and co-workers (76) injected mice with asialofetuin, a known ligand of the ASGPR, simultaneously with radiolabeled Lp(a). They reported that plasma Lp(a) clearance was essentially unaffected by the presence of the competing ligand. In
contrast, the addition of an unlabeled molar excess of asialofetuin significantly reduced its own clearance, thus acting as a positive control for the experiment and confirming the notion that the ASGPR does not play a significant role in Lp(a)/apo(a) catabolism (76).

1.6.7 The Plasminogen Receptor Family

Plasminogen (Pg) receptors represent a ubiquitous class of proteins, whose expression has been characterized in a number of cell types, including smooth muscle cells, endothelial cells, fibroblasts and monocytes (119). Binding analysis of plasminogen to a number of cell types has revealed a very high capacity for binding, where up to $10^7$ receptors per cell have been identified depending on the cell type (120-122). To date, three potential functions of these receptors have been identified. Firstly, the receptors serve as a means of localizing the plasminogen zymogen to the cell surface prior to its activation (119). Secondly, the rate of plasminogen activation has been found to be enhanced when the zymogen is bound to its cell surface receptor (123). Finally, plasmin bound to the plasminogen receptor appears to be protected from inactivation by α2-antiplasmin (120).

The interactions of plasminogen with specific plasmin substrates, inhibitors and regulatory molecules are mediated by lysine binding sites within the plasminogen molecule (119). Plasminogen binding to its cellular receptors thus requires these LBS to be unoccupied (119). Correspondingly, studies have shown that the binding of radiolabeled plasminogen to human umbilical vein endothelial cells (HUVECs) is inhibited in a dose-dependent manner by the lysine analogue ε-aminocaproic acid (ε-
ACA) (123). Interestingly, no single protein molecule can account for the entire capacity of any given cell to bind plasminogen (124). The implied heterogeneity of the plasminogen receptor family has been demonstrated in studies employing the use of carboxypeptidase B (CpB), an enzyme which catalytically removes carboxyl terminal arginines and lysines. Felez and colleagues (125) observed that CpB treatment reduced plasminogen binding to monocyteoid cells by 60%, while cell surface-dependent enhancement of plasminogen activation was reduced by >95%. This suggests that although several distinct molecules may contribute to plasminogen binding, the subset of plasminogen receptors responsible for the enhancement of plasminogen activation have an exposed carboxyl-terminal lysine that is sensitive to CpB. Additionally, Hawley and co-workers (124) demonstrated that CpB treatment of monocytes, followed by ligand blotting with $^{125}$I-plasminogen, revealed α-enolase, actin and annexin II as potential plasminogen-binding proteins. Curiously, each binding partner was inhibited by CpB treatment to a varying extent. Accordingly, it is entirely possible that although plasminogen receptors are heterogeneous with respect to their sequences, those that function as profibrinolytic plasminogen receptors on cell surfaces maintain a specific arrangement of lysine residues in the carboxyl-terminal (124).

Since the apo(a) component of Lp(a) possesses a number of LBS and shares a high degree of amino acid identity with plasminogen (5), the plasminogen receptor family has been proposed to play a role in Lp(a) clearance. Much of this early speculation was based on in vitro evidence identifying Lp(a) as a potential plasminogen receptor family binding partner. Correspondingly, an early study by Miles and co-
workers (126) demonstrated that Lp(a) competes with plasminogen for receptors on the endothelial cell surface. Similarly, Hajjar et al. (95) reported that Lp(a) interferes with endothelial cell fibrinolysis by inhibiting plasminogen binding and thus plasmin formation. Currently, the most direct link between the plasminogen receptor and Lp(a) clearance has come from the work of Tam and co-workers, (110) who demonstrated that plasminogen receptors present in both HepG2 cells and human fibroblasts are capable of mediating the uptake and degradation of both Lp(a) and apo(a). Collectively, the above studies suggest a potential role for the plasminogen receptor in Lp(a) clearance. However, whether or not the plasminogen receptor family represents a significant catabolic route in vivo remains unclear.

1.7 Hypothesis

The major hypotheses which form the basis of this thesis are as follows:

1) Specific sequences in apo(a) mediate the non-covalent association of Lp(a) with hepatocytes.

2) Interactions between apo(a) and its catabolic receptor(s) are lysine-dependent.

1.8 Rationale and Research Objectives

The classification of elevated plasma concentrations of Lp(a) as an independent risk factor for coronary heart disease and stroke has provided the rationale for studies that focus on the plasma reduction of this lipoprotein. Since the catabolic pathway for Lp(a) clearance remains largely unknown, it is not possible to consider manipulating uptake as
a means to reduce plasma Lp(a) levels. Prior to beginning this thesis, it was evident in
the literature that the liver is the major organ involved in Lp(a) clearance, and that the
apo(a) moiety of Lp(a) is the major ligand mediating catabolism (76). Accordingly, the
specific objectives of this research project were developed as follows:

1) To perform *in vitro* binding analysis of a variety of apo(a) variants to HepG2
cells and FH fibroblasts.

2) To analyze the strong and weak lysine binding sites in apo(a) and to determine
their role in the plasma clearance of Lp(a).

3) To visualize and quantify the degradation of apo(a) in HepG2 cells and FH
fibroblasts.

4) To isolate and characterize the receptor(s) directly responsible for
Lp(a)/apo(a) catabolism.
Chapter 2
Experimental Procedures

2.1 Cell Culture

Human hepatoma (HepG2) cells were obtained from ATCC (#HB-8065, Manassas, VA, USA) and were grown in minimal essential media (MEM; Gibco, Burlington, ON, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS; ATCC) and 1% (v/v) antibiotic solution (Gibco). Human embryonic kidney (HEK) 293 cells (127) were maintained in MEM containing 5% (v/v) FBS (HyClone, Logan, UT, USA) and 1% (v/v) antibiotic solution (Gibco). Primary, familial hypercholesterolemic (FH) fibroblasts, obtained from a 9 year old LDL receptor defective Caucasian male, were purchased from Coriell Institute (#GM03040, Camden, NJ, USA) and were grown in MEM (Gibco) supplemented with 15% (v/v) FBS (HyClone), 2 mM L-glutamine and 1% (v/v) antibiotic solution (Gibco). FH fibroblasts were obtained at passage 17 and were used up to passage 26. All cells were grown at 37°C in 5% CO₂ and the medium was exchanged every 2nd or 3rd day.

2.2 Construction and Expression of Recombinant Apo(a) Variants

Construction of plasmids in the pRK5 expression vector that encode the following r-apo(a) variants have been previously described: 17K (wild type) (94), 17KΔAsp(D56A) (10), 17KΔLBS7,8 (68), 17KΔCys and KIV₅₋₈ (128) and 17KΔV (9). All expression
plasmids were used to generate stably expressing HEK 293 cell lines as previously described (94).

2.3 Purification of Recombinant Apo(a) Variants

All recombinant apo(a) variants were purified from the conditioned medium of stably expressing HEK 293 cell lines by lysine-Sepharose affinity chromatography as previously described (9). Briefly, conditioned medium (Opti-MEM) harvested every 3 days from the stably transfected cells grown in roller bottles was collected and loaded over a 50-mL column containing lysine-Sepharose CL-4B resin (Amersham Biosciences, Baie d’Urfe, QC, Canada), pre-equilibrated with phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 10.14 mM Na$_2$HPO$_4$, 1.38 mM KH$_2$PO$_4$, pH 7.4). The column was washed thoroughly with PBS containing 500 mM NaCl and was eluted with 200 mM ε-aminocaproic acid (ε-ACA) in the same buffer. Fractions were analyzed spectrophotometrically at 280 nm and protein-containing fractions were pooled and dialyzed three times against HEPES buffered saline (HBS; 20 mM HEPES pH 7.4 containing 150 mM NaCl) for 2 h at 4°C. The resulting solution was pre-concentrated using polyethylene glycol 20’000 (Fluka Analytical, Oakville, ON, Canada) and 1 mL aliquots were centrifuged at maximum speed for 5 min using a Beckman Micromax Thermo IEC centrifuge. The precipitated protein pellet was discarded and the supernatants were collected and concentrated using an Amicon Ultra centrifugal filter device (Millipore, Etobicoke, ON, Canada) with a molecular weight cutoff of 30 kDa. The protein concentration was determined spectrophotometrically (corrected for Rayleigh
scattering) for all apo(a) variants, with the exception of the 17K-GFP fusion construct, using the following molecular weights and molar extinction coefficients: 17K wild type \((M_r \sim 278,219; \varepsilon_{280 \text{ nm}} = 2.07 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\); 17K\(\Delta\)Asp (D56A) \((M_r \sim 278,219; \varepsilon_{280 \text{ nm}} = 2.07 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\); 17K\(\Delta\)LBS7,8 \((M_r \sim 278,219; \varepsilon_{280 \text{ nm}} = 2.07 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\); 17K\(\Delta\)Cys \((M_r \sim 278,219; \varepsilon_{280 \text{ nm}} = 2.07 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\); 17K\(\Delta\)V \((M_r \sim 269,978; \varepsilon_{280 \text{ nm}} = 2.11 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\); KIV\_5-8 \((M_r \sim 56,000; \varepsilon_{280 \text{ nm}} = 2.10 \text{ mg}^{-1} \text{ mL} \text{ cm}^{-1})\) (9). The concentration of the 17K-GFP protein was determined using the Bio-Rad protein assay. All proteins were assessed for purity by SDS-PAGE, with the use of 2.5-15% gradient gels, under reduced (containing 10 mM dithiothreitol) and non-reduced conditions, followed by silver staining. All purified recombinant apo(a) variants exhibited single bands, with an upward shift in mobility in the reduced samples as has been previously reported (94). Aliquots of the purified proteins were stored at -70ºC prior to use.

### 2.4 Lipoprotein-Depleted Serum

Lipoprotein-depleted serum (LPDS) was prepared by adjustment of the fetal bovine serum (ATCC) density from 1.006 g/mL to 1.21 g/mL with NaBr according to the following equation:

\[
\text{NaBr (g)} = \frac{\text{mL FBS} \times (\rho_{\text{final}} - \rho_{\text{initial}})}{(1 - 0.245 \times \rho_{\text{final}})}
\]

where \(\rho_{\text{final}} = 1.21 \text{ g/mL}\), \(\rho_{\text{initial}} = 1.006 \text{ g/mL}\) and 0.245 mL/g represents the partial specific volume of NaBr.
Sealable centrifuge tubes (Beckman Coulter, Mississauga, ON, Canada) were filled with the heavy FBS solution, soldered shut and spun at 60,000 rpm for 20 h (4°C) in a Beckman L8-70 Ultracentrifuge equipped with the Type 70.1 Ti Rotor. The serum was resolved into two distinct layers, where the less dense lipoprotein-containing fraction was carefully removed with a syringe. The lipoprotein-free layer was dialyzed extensively against HBS at 4°C and aliquots were stored at -20°C prior to use.

2.5 Protein Iodination

Recombinant lipoproteins were radiolabeled with Na$^{125}$I (Perkin Elmer, Woodbridge, ON, Canada) by the chemical oxidation method (129). Prior to iodination, 300 to 500 μg of recombinant protein was added to 150 μL of iodination buffer (200 mM Tris-HCl pH 7.4 containing 100 mM NaCl). Two IODO-BEADS (Pierce, Ottawa, ON Canada) were then washed once with 1 mL of iodination buffer for 1 min and were then suspended in 500 μL of iodination buffer and 1 mCi of Na$^{125}$I. Following a 5 min incubation at room temperature, the radioactive iodine solution was added to the previously prepared recombinant protein solution. The resulting mixture was incubated at room temperature for 10 min and was subsequently quenched by the addition of 10 μL of 1 M sodium metabisulfite. The radiolabeled sample was separated from the free iodine label using a 10 mL Econo-Pac 10DG desalting column (Bio-Rad, Mississauga, ON, Canada) equilibrated with HBS. Fractions (500 μL) were collected and 5 μL aliquots were measured for associated radioactivity using a 1275 MiniGamma counter (LKB Wallac, Turku, Finland). Protein-containing fractions were pooled and the
concentration was determined spectrophotometrically (corrected for contributions from Rayleigh scattering). Radiolabeled samples were stored at 4°C for a maximum period of 3 weeks prior to use.

2.6 Bicinchoninic Acid Protein Quantification Assay

Protein standards ranging in concentration from 0 to 2000 μg/mL were prepared by diluting 10 mg/mL of BSA (New England BioLabs, Mississauga, ON, Canada) in HBS. Each unknown protein sample (10 μL), in addition to 10 μL of each standard, were then added in duplicate to the wells of a 96-well plate (Costar, Lowell, MA, USA). The bicinchoninic acid (BCA) detection reagent was then immediately prepared by mixing BCA Reagent A (Pierce) with BCA Reagent B (Pierce) in a 50:1 volumetric ratio. The resulting detection reagent (200 μL) was added to each well and the biuret reaction was allowed to proceed for 30 min at 37°C. Following incubation, the light blue to violet samples were measured in the linear absorbance range at 562 nm using a SpectraMax M2e Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) running SoftMax Pro Software (Molecular Devices). A linear standard curve was constructed using the BSA standards and the unknown protein concentrations were determined by mathematical interpolation.

2.7 Bio-Rad Protein Assay

Protein quantification was performed in an analogous manner to the BCA assay with the following exceptions. Protein standards were prepared from a 1 mg/mL BSA
stock solution and ranged in concentration from 0 to 0.5 mg/mL. The dye reagent concentrate (Bio-Rad) was diluted 1:4 in HBS and acted as the assay detection reagent. Each protein sample (10 μL) and 200 μL of diluted concentrate were added in duplicate to the wells of a 96-well plate. Following incubation for at least 5 min at room temperature, absorbance measurements were performed at 595 nm.

2.8 Ligand Binding Assay

Binding experiments were performed using confluent HepG2 cells, HEK 293 cells or FH fibroblasts in 12- or 24-well tissue culture plates (Costar). Prior to each experiment, the growth media was removed and replaced overnight with MEM containing 10% lipoprotein-depleted serum (LPDS) prepared as described above. Cells were pre-chilled on crushed ice for 30 min, at which point the medium was removed and varying concentrations of $^{125}$I-recombinant apo(a) variants (17K, 17KΔAsp, 17KΔV, 17KΔCys, 17KΔLBS7,8 and KIV$_{5-8}$) were added. In some experiments, varying concentrations of $\varepsilon$-ACA were included in the reaction to test the lysine-dependency of binding to the cell surface. After incubation for 3 h at 4°C, the binding reaction was terminated by removing the medium, and the cells were washed twice with ice-cold PBS containing 0.2% BSA, followed by 2 final washes with PBS alone. The cells were then dissolved in 1 M NaOH and the associated radioactivity was determined using a Model 1275 MiniGamma counter (LKB Wallac). Total cellular protein was measured using the Pierce BCA protein assay described above. Total binding was analyzed by plotting the total counts/mg cell protein versus the total counts corresponding to free ligand. Non-
specific binding was calculated by incubating the cells in the same manner as described above with various concentrations of $^{125}$I-BSA. Total $^{125}$I-BSA counts/mg cell protein was plotted versus the total counts corresponding to free BSA. The slope of the plot was determined by linear regression, and the corrected specific counts bound/mg cell protein was determined as follows:

(1) Total Binding Slope = \left( \frac{\text{Total Counts Bound / mg Cell Protein}}{\text{Total Counts Free}} \right)

(2) Non-Specific Binding Slope = \left( \frac{\text{BSA Counts Bound / mg Cell Protein}}{\text{BSA Counts Free}} \right)

(3) Specific Binding = \left( \left( \text{Total Binding Slope} \right) - \left( \text{Non-Specific Binding Slope} \right) \right) \times \text{Total Counts Free}

Substitution of equations (1) and (2) into (3) yields the final calculation for specific binding:

\left( \frac{\text{Total Counts Bound / mg Cell Protein}}{\text{Total Counts Free}} - \frac{\text{BSA Counts Bound / mg Cell Protein}}{\text{BSA Counts Free}} \right) \times \text{Total Counts Free}

The specific binding curve was subjected to non-linear regression analysis utilizing the program GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA), enabling the calculation of the $K_d$ and $B_{\max}$ values.

2.9 **Apo(a) Degradation Assay**

Cellular degradation of recombinant apo(a) was performed in a manner analogous to the ligand binding assay described above, with the following modifications. Confluent
HepG2 cells or FH fibroblasts were incubated with varying concentrations of $^{125}$I-labeled r-apo(a) (17K, 17KΔLBS7,8) at 37°C for the indicated time periods. Following incubation, the medium was removed from each cell monolayer and the cells were washed with PBS as described earlier. Apo(a) degradation was determined by measurement of the trichloroacetic acid- (TCA) soluble radioactivity released into the medium. TCA-soluble radioactivity was analyzed for water-soluble (small peptides and amino acids) and chloroform-soluble (iodine) radioactivity as previously described by Goldstein and co-workers (130). Briefly, 2 volumes of ice cold 10% TCA were added to 1 volume of cellular medium in order to precipitate intact proteins. After incubation at 4°C for 30 min, the precipitated protein was removed by centrifugation and 1 mL of the resulting supernatant was combined with 10 μL of 40% (w/w) KI and 40 μL of 30% (w/w) H$_2$O$_2$. The samples were mixed thoroughly and incubated at room temperature for 5 min, during which time the hydrogen peroxide converted $[^{125}\text{I}]$ iodide ions to $[^{125}\text{I}]$ iodine. Next, 2 volumes of chloroform were added to each sample to extract the free $[^{125}\text{I}]$ iodine into the organic phase by vortex agitation. After 15 min at room temperature, aliquots from the upper aqueous layer were counted to determine total radioactivity released into the medium. A correction was applied for non-specific ligand degradation by subtracting TCA-soluble counts in a cell-free environment from total radioactivity for each data point. Total protein normalization was performed using the BCA assay as described above, where cell monolayers were dissolved in 1 M NaOH following extensive PBS washing.
2.10 LysoTracker Lysosomal Degradation

HepG2 cells (250,000) were seeded on a Delta T dish (Bioptechs, Butler, PA, USA) and were grown to sub-confluency. Prior to the experiment, the growth medium was replaced overnight with phenol red-free MEM (Gibco) containing 10% LPDS. The cells were then incubated for 2 h at 37°C in phenol red-free MEM supplemented with 50 nM of the LysoTracker Red DND-99 probe (Molecular Probes #L7528, Burlington, ON, Canada). Following three PBS washes, 500 nM of 17K-GFP in phenol red-free MEM was added to the dish. The protein was allowed to bind to the cell surface for 1 h at 4°C. Fluorescence imaging was then performed at 37°C for varying times up to 4 h using a Leica TCS SP2 Multiphoton Confocal Microscope (Leica Microsystems, Bannockburn, IL, USA). Images were captured using the Leica Confocal Software and time-lapse movies were created using Image-Pro Plus 6.0 (MediaCybernetics, Bethesda, MD, USA) and ImageJ (NIH, Bethesda, MD, USA).

2.11 Statistical Analysis

All statistical analyses were performed using the unpaired two-tailed Student’s \( t \)-test.
Chapter 3
Results

3.1 Binding of Recombinant Apo(a) to Cultured Human Hepatoma Cells

Previous studies analyzing the *in vivo* tissue uptake of Lp(a) have demonstrated that the liver is the primary metabolic organ mediating the catabolism of this lipoprotein (74,76). Additionally, reports have indicated that the apo(a) component of Lp(a) is the primary ligand that mediates Lp(a) hepatic uptake and plasma clearance (76). Taken together, these findings suggest an interplay between hepatic cell surface receptors and apo(a) in the process of Lp(a) catabolism. As such, we began our investigation by developing an *in vitro* biophysical assay to quantify the cell surface binding properties of r-apo(a) variants to cultured human hepatoma (HepG2) cells. However, since we utilized a non-equilibrium method for binding analysis, it was first necessary to ensure that binding was saturable in our cell system. As anticipated, the binding of an iodinated 17-kringle containing recombinant form of apo(a) (17K) to HepG2 cells was found to be saturable as a function of time (Figure 3-1). This experiment was conducted by applying a 120 nM concentration of $^{125}$I-17K to the cells and allowing the system to equilibrate at 4ºC for set time periods. Following radioactive measurements, a direct plot of bound apo(a) ligand versus time illustrated that a plateau was reached in the system at approximately the 3 h mark. Since binding was found to be saturable as a function of time, the 3 h time point was utilized as the minimum incubation period for all subsequent binding assays.
Figure 3-1. Time-Dependent Binding of $^{125}$I-17K to HepG2 Cells. Confluent cell monolayers were incubated overnight in MEM containing 10% lipoprotein-depleted serum (LPDS). The cells were pre-chilled on crushed ice for 30 min, the medium was removed and a constant concentration of $^{125}$I-17K (120 nM) was added to the cells in MEM. After incubation at 4°C for the indicated time periods, 2 PBS washes containing 0.2% BSA were performed, followed by 2 final washes with sterile PBS. The cells were lysed with 1 M NaOH and the radioactivity associated with the cells was subsequently determined. The results represent the mean ± SEM of two independent experiments performed in duplicate.
In order to characterize the binding of apo(a) to HepG2 cells, increasing concentrations of $^{125}$I-17K were added to the cells for 3 h at 4°C. The resulting plot of specifically bound r-apo(a) versus the concentration of free ligand produced a curve which resembled a rectangular hyperbola (Figure 3-2). Upon Scatchard transformation of the saturation curve, a non-linear plot was obtained which suggests a deviation from single-site binding (Inset; Figure 3-2). The resulting Scatchard plot appeared concave, where as the ligand concentration increased, the plot curved down towards the origin. The specific binding curve was further subjected to non-linear regression analysis; the plot was resolved into two-components using Equation (1):

$$v = \frac{B_{max} [L]}{K_{d1} + [L]} + \frac{B_{max} [L]}{K_{d2} + [L]}$$

One of the binding components had a $K_d$ of 12 nM, and the other had a $K_d$ of 249 nM. The maximum binding capacities ($B_{max}$) of the high and low affinity sites were further calculated to be 0.0463 and 0.436 pmol r-apo(a)/mg cell protein, respectively. Taken together, these data indicated the presence of both a high affinity, low capacity binding site as well as a low affinity, high capacity binding site.

Previous observations by Tam and co-workers (3) have suggested that free apo(a) is capable of binding to hepatocyte cell surfaces and that this interaction is largely lysine-dependent. Since our cell binding model contained at least two classes of binding sites, we proposed that binding to one of these sites might be entirely lysine-dependent. Accordingly, competition binding assays were performed where increasing concentrations (0-50 mM) of the lysine analogue, $\varepsilon$-ACA, were added to the $^{125}$I-17K
Figure 3-2. Binding of $^{125}$I-17K to HepG2 Cells. Experiments were performed using confluent cell monolayers in 24-well tissue culture plates. Binding at 4°C was measured for 3 h as described in the Experimental Procedures section. The curve represents specific binding (total minus non-specific binding); non-specific binding was quantified using $^{125}$I-labeled BSA. The results represent the mean ± SEM of three independent experiments performed in duplicate. The inset contains a representative Scatchard plot of the binding curve. For simplicity, only mean values are presented in the Scatchard plot. The Scatchard plot is displayed as a visual aid only, as binding parameters were calculated by subjecting the specific binding curve to non-linear regression analysis.
binding reaction. Following radioactive measurements, we found that ε-ACA concentrations as low as 2.5 mM were capable of abolishing approximately 40% of specific binding of r-apo(a) to cultured HepG2 cells (Figure 3-3). Interestingly, the extent of ε-ACA inhibition of specific binding was essentially the same for all concentrations tested.

Despite the observation that ε-ACA reduced the cell surface binding of apo(a), the extent of inhibition caused by ε-ACA corresponding to each of the two individual classes of binding sites identified in Figure 3-2 remained unclear. Therefore, specific apo(a) domain analysis was required in order to identify the specific lysine binding sites (LBS) in apo(a) involved in cell surface binding.

3.2 Critical Domains in Apo(a) that Mediate its Binding to Cultured HepG2 Cells

To elucidate the specific sequences in apo(a) required for binding to HepG2 cells, a series of r-apo(a) variants (both deletion and point mutations) were utilized in the ligand binding assay (Figure 3-4). For use in quantitative r-apo(a) binding analysis, the following apo(a) variants were expressed in human embryonic kidney (HEK) 293 cells and purified from the conditioned medium as described in the Experimental Procedures: 17K, 17KΔAsp, 17KΔV, 17KΔLBS7,8, 17KΔCys and KIV5-8. Each of the r-apo(a) variants were further subjected to SDS-PAGE analysis followed by silver staining to assess sample purity.

We examined the effects of r-apo(a) species with reduced lysine binding capacity to bind to HepG2 cells. Purified r-apo(a) variants, namely the 17KΔAsp and 17KΔV
Figure 3-3. The Effects of ε-ACA on the Binding of $^{125}$I-17K to HepG2 Cells. Competition binding assays were carried out at 4°C for 3 h as described in the Experimental Procedures section. HepG2 cell monolayers were incubated with 400 nM $^{125}$I-labeled-17K in the presence of the indicated concentrations of ε-ACA. Specific binding in the absence of ε-ACA was arbitrarily set at 100%. The results represent the mean ± SEM of two independent experiments performed in duplicate.
**Figure 3-4. Schematic Representation of Recombinant Apo(a) Variants Utilized in the Proposed Studies.** The 17K recombinant apo(a) construct shown on the top line represents a physiologically relevant apo(a) isoform and contains 17 kringle IV-like repeats, where 1-10 indicates the 10 subclasses of plasminogen kringle IV-like domains, KV denotes the V-like sequences and P represents the inactive protease domain. The bar over KIV9 indicates the position of the free cysteine residue in apo(a) that is involved in covalent linkage with apoB-100 in the Lp(a) particle. The black circle (●) within a kringle indicates the disruption of a lysine binding site via site directed mutagenesis, while the triangle (▼) represents the mutation of a cysteine to a tyrosine residue. The GFP domain indicates the presence of a sequence encoding a green fluorescent protein fluorophore isolated from the jellyfish *Aequorea victoria.*
(lacking the strong LBS in KIV10 and the very weak LBS in KV respectively), were iodinated and increasing concentrations of either ligand were incubated with confluent HepG2 cells for 3 h at 4°C. Following radioactive measurements, the quantity of bound r-apo(a) was determined and plotted against the concentration of free ligand (Figure 3-5A). Compared to the 17K binding curve, we observed a reduction in specific binding capacity for both the 17KΔAsp and 17KΔV apo(a) variants. To test whether or not we had successfully eliminated the binding to one class of sites, we performed Scatchard transformational analysis on the saturation curves (Figure 3-5B). Like the 17K Scatchard plot, the 17KΔAsp and 17KΔV plots appeared concave in nature, once again indicating the presence of multiple classes of ideal binding sites. We further characterized the affinities and capacities of these sites by subjecting each binding curve to non-linear regression analysis using the two-site model (Table 3-1). The average reduction in \( B_{\text{max}} \) between the two binding components for the 17KΔAsp and 17KΔV apo(a) variants was approximately 38% and 63% respectively, compared to the 17K control. Curiously, \( K_d \) values for both the high and low affinity sites remained relatively unchanged, as the 17KΔAsp and 17KΔV apo(a) variants showed comparable binding affinities to that of the 17K r-apo(a) control.
Figure 3-5. Binding Analysis of $^{125}$I-17KΔAsp and $^{125}$I-17KΔV to HepG2 Cells. (A) Binding of iodinated 17K (○), 17KΔAsp (■) and 17KΔV (▲) to HepG2 cells was performed for 3 h at 4°C as described in the Experimental Procedures. The results represent the mean ± SEM of three independent experiments performed in duplicate. (B) Scatchard transformation of the binding curves in Panel A are shown. For ease of presentation, only representative plots are shown.
Table 3-1. Binding Affinities and Capacities of Recombinant Apo(a) Variants to HepG2 Cells. Calculated $K_d$ and $B_{max}$ values were determined from non-linear regression analysis of the data in Figures 3-2, 3-5A, 3-6A and 3-7A. Values are based on three independent experiments ± standard error.

<table>
<thead>
<tr>
<th>Apo(a) Variant</th>
<th>$K_d$ Low (nM)</th>
<th>$B_{max}$ Low (pmol r-apo(a)/mg cell protein)</th>
<th>$K_d$ Hi (nM)</th>
<th>$B_{max}$ Hi (pmol r-apo(a)/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17K wt</td>
<td>249 ± 57</td>
<td>0.436 ± 0.017</td>
<td>12 ± 10</td>
<td>0.046 ± 0.027</td>
</tr>
<tr>
<td>17KΔAsp</td>
<td>231 ± 76</td>
<td>0.289 ± 0.024</td>
<td>2 ± 4</td>
<td>0.027 ± 0.014</td>
</tr>
<tr>
<td>17KΔV</td>
<td>208 ± 48</td>
<td>0.175 ± 0.011</td>
<td>1 ± 1</td>
<td>0.015 ± 0.005</td>
</tr>
<tr>
<td>KIV_{5,8}</td>
<td>217 ± 86</td>
<td>0.632 ± 0.060</td>
<td>1 ± 2</td>
<td>0.073 ± 0.038</td>
</tr>
<tr>
<td>17KΔLBS7,8</td>
<td>84 ± 10</td>
<td>1.042 ± 0.040</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>17KΔCys</td>
<td>506 ± 275</td>
<td>1.385 ± 0.230</td>
<td>16 ± 16</td>
<td>0.164 ± 0.107</td>
</tr>
</tbody>
</table>
Although a reduction in maximum binding capacity was observed with the 17KΔAsp and 17KΔV apo(a) variants, these experiments tested the functionalities of only two of the six characterized LBS in apo(a). In order to thoroughly examine the remaining four LBS, we attempted to quantify the binding of the KIV\textsubscript{5-8} variant to cultured HepG2 cells, where each of the KIV\textsubscript{5-8} sequences contains a weak LBS. Cells were incubated for 3 h at 4°C in the presence and absence of 50 mM ε-ACA and specific binding as a function of increasing concentrations of \textsuperscript{125}I-labeled KIV\textsubscript{5-8} was determined. The binding curves shown in Figure 3-6 demonstrate a dramatic reduction in specific binding for reactions supplemented with 50 mM ε-ACA. Interestingly, Scatchard transformation of the binding curves resulted in two very distinct plots. In the case of the KIV\textsubscript{5-8} control, we observed a concave curve, signifying binding of the KIV\textsubscript{5-8} variant to multiple classes of sites. However, the Scatchard plot for KIV\textsubscript{5-8} supplemented with 50 mM ε-ACA appeared linear, indicating single-site binding. Non-linear regression analysis of the KIV\textsubscript{5-8} binding curve further revealed high and low affinity $K_d$ values of 1 ± 2 nM and 217 ± 86 nM, respectively. Interestingly, the calculated $K_d$ value from the binding curve produced in the presence of ε-ACA was found to be 260 ± 37 nM, which closely resembles the low affinity binding component ($K_d = 249$ nM) on HepG2 cells.

The preceding result suggested that the LBS in the KIV\textsubscript{5-8} variant are critical for binding to the high affinity site on HepG2 cells. Since the LBS in KIV\textsubscript{7} and KIV\textsubscript{8} are responsible for the initial non-covalent interaction between apoB-100 and apo(a) prior to covalent Lp(a) assembly (20-22), we assessed the binding to HepG2 cells of a full length apo(a) variant lacking these functional LBS domains (17KΔLBS\textsubscript{7,8}). We also examined
Figure 3-6. Effects of \( \varepsilon \)-ACA on the Binding of \(^{125}\text{I}\)-KIV\(_{5-8}\) to HepG2 Cells. (A) Binding of \(^{125}\text{I}\)-labeled KIV\(_{5-8}\) to the cells, in the presence (○) and absence (●) of 50 mM \( \varepsilon \)-ACA, was performed for 3 h at 4°C as described in the Experimental Procedures. Binding curves shown in Panel A are representative plots from three independent experiments. The values shown represent the mean of a single experiment performed in duplicate. (B & C) The bottom panels represent the Scatchard transformations for the binding curves shown in Panel A.
the binding of an apo(a) variant which lacks the free cysteine residue in KIV\textsubscript{9} (17K\textsubscript{ΔCys}), thus disrupting covalent bond formation with apoB-100 during the second step of Lp(a) assembly. The binding of these iodinated species to HepG2 cell monolayers is shown in Figure 3-7. Scatchard transformational analysis of the saturation binding curves revealed that the 17K\textsubscript{ΔCys} species was capable of binding to both the high and low affinity sites ($K_{d \text{ HI}} = 16 \pm 16$ nM, $K_{d \text{ LOW}} = 506 \pm 275$ nM), while the 17K\textsubscript{ΔLBS7,8} apo(a) variant was only capable of binding to the low affinity site ($K_{d \text{ LOW}} = 84 \pm 10$ nM). Overall, these results suggest that a non-covalent Lp(a) particle (mediated by the binding of KIV\textsubscript{7} and KIV\textsubscript{8} to specific lysine residues in apoB-100) is required for association with the high affinity component and that the identity of this site may in fact be the LDLR. Interestingly, the data further imply that the non-covalent LDL-apo(a) complex is stable enough to allow for binding to the high affinity component in the absence of disulfide bond formation between apo(a) and apoB-100.

3.3 Binding of $^{125}$I-17K to Cultured FH Fibroblasts and HEK 293 Cells

To investigate whether or not LDL was required in the binding of r-apo(a) to the high affinity component, we examined the association of r-apo(a) with fibroblasts derived from a Caucasian male diagnosed with familial hypercholesterolemia (FH). Since these cells possess genetically defective LDL receptors (unlike HepG2 cells), we were able to directly assess a role for the LDLR in our two component binding system. Binding of $^{125}$I-labeled 17K was performed for 3 h at 4°C and a specific binding curve was generated (Figure 3-8A). The Scatchard transformation (Figure 3-8B) produced a linear plot, and
Figure 3-7. Binding of $^{125}$I-17KΔLBS7,8 and $^{125}$I-17KΔCys Assembly Mutants to HepG2 Cells. (A) Binding of $^{125}$I-labeled 17KΔLBS7,8 (●) and 17KΔCys (●) was performed for 3 h at 4°C as described in the Experimental Procedures. Binding curves presented in the top panel represent the mean values of three independent experiments performed in duplicate ± SEM. (B & C) The bottom panels represent the corresponding Scatchard transformations for the aforementioned binding curves. For ease of presentation, only mean values are used in the Scatchard plots.
non-linear regression analysis of the saturation curve estimated a $K_d$ of 190 ± 29 nM and a $B_{\text{max}}$ of 2.4 ± 0.15 pmol r-apo(a)/mg cell protein. Once again, the calculated $K_d$ value was found to resemble the low affinity binding component ($K_d = 249$ nM) previously observed on HepG2 cells. We also performed ε-ACA competition assays using FH fibroblasts to assess the lysine dependency of low affinity binding in these cells. We found that ε-ACA concentrations between 2.5 and 50 mM abolished approximately 30% of r-apo(a) specific binding (Figure 3-9), indicating a greater non-lysine dependent binding component corresponding to this site. Interestingly, the extent of inhibition by ε-ACA in FH fibroblasts (30%) was found to be similar to that observed in HepG2 cells (40%), although experiments in the HepG2 cell system included contributions from both the high and low affinity sites.

In the next series of experiments, we analyzed the binding of r-apo(a) to wild type HEK 293 cells, a cell line incapable of endogenous LDL production and expression of the LDLR. Accordingly, HEK 293 cells were pre-incubated with LPDS overnight and specific binding as a function of increasing concentrations of $^{125}$I-17K was determined. The resulting saturation curve is shown in Figure 3-10, where a plateau was observed at approximately 0.4 pmol r-apo(a)/mg cell protein. Scatchard transformation of the binding curve resulted in a linear plot (Inset; Figure 3-10) and non-linear regression analysis further revealed that $^{125}$I-17K bound exclusively to a site with a $K_d$ of 240 ± 58 nM, resembling the low affinity component ($K_d = 249$ nM) in the HepG2 cell model.
Figure 3-8. Binding of $^{125}$I-17K to Cultured Human FH Fibroblasts. (A) Experiments were performed using confluent cell monolayers in 24-well tissue culture plates. Binding at 4ºC for 3 h was measured as described in the Experimental Procedures. The curve represents specific binding (total minus non-specific binding); non-specific binding was quantified using $^{125}$I-labeled BSA. The results represent the mean ± SEM of three independent experiments performed in duplicate. (B) A representative Scatchard transformation of the data in Panel A is shown. For ease of presentation, only mean values are included.
Figure 3-9. The Effects of ε-ACA on the Binding of $^{125}$I-17K to FH Fibroblasts. Competition binding assays were carried out at 4°C for 3 h as described in the Experimental Procedures chapter. Human FH fibroblast cell monolayers were incubated with 100 nM $^{125}$I-labeled-17K in the presence of the indicated concentrations of ε-ACA. Specific binding in the absence of ε-ACA was arbitrarily set at 100%. The results represent the mean ± SEM of four independent experiments carried out in duplicate.
Figure 3-10. Binding of $^{125}$I-17K to Cultured HEK 293 Cells. Experiments were performed using confluent cell monolayers in 24-well tissue culture plates. Binding at 4°C for 3 h was measured as described in the Experimental Procedures. The curve represents specific binding (total minus non-specific binding); non-specific binding was quantified using $^{125}$I-labeled BSA. The data shown are the mean of one representative experiment performed in duplicate. The inset contains the Scatchard transformation of the binding curve.
3.4 Cellular Degradation of $^{125}$I-Labeled Recombinant Apo(a)

To assess the catabolic potential of both the high and low affinity binding sites on HepG2 cells, we quantified the degradation of iodinated 17K and 17KΔLBS7,8 at 37°C over a 4 h period. Degradation was determined by measurement of the total trichloroacetic acid- (TCA) soluble radioactivity released to the medium at each time point. TCA-soluble radioactivity was further analyzed for water-soluble radioactivity, where iodotyrosine counts served as a direct measurement of degraded r-apo(a). Over time, we observed an increasing amount of degradation of both the 17K and 17KΔLBS7,8 species (Figure 3-11A), however no significant differences ($p = 0.48$) were found to exist between the two curves. Additionally, we attempted to evaluate the ability of the low affinity site in the FH fibroblast cell line to degrade $^{125}$I-17K (Figure 3-11B). Once again, we observed increasing degradation of apo(a) as a function of time, thus suggesting that the low affinity site is capable of both binding and degrading apo(a).

3.5 Non-Lysosomal Degradation of 17K-GFP by HepG2 Cells

In order to determine if apo(a) undergoes lysosomal degradation, we utilized fluorescence confocal microscopy to examine the interaction between r-apo(a) and cellular lysosomes. Cells were first incubated with 50 nM of the LysoTracker Red probe to fluorescently label acidic cellular components, including the lysosomes and transport vesicles. A 17K isoform of apo(a), fused to a green fluorescent protein fluorophore (17K-GFP), was then allowed to bind to the cell surface for 1 h at 4°C. Imaging was subsequently performed for 4 h at 37°C, where time-lapse photographs were captured.
once per minute. At time zero, we observed an increase in apo(a) binding to the HepG2 cell surface, where most apo(a) was located extracellularly (Figure 3-12). After 120 min of incubation, apo(a) continued to accumulate on cell surface receptors and some 17K-GFP was observed to translocate across the cellular membrane. Interestingly, we observed apo(a) association and dissociation throughout the entire cell culture dish, indicating that binding was reversible. At the 150 and 180 min time points, peak catabolic activity was observed, as the amount of apo(a) associated with the membrane became reduced, however minimal co-localization was observed. Following 240 min of incubation, catabolic activity was reduced in the region of interest, as most of the initially bound apo(a) had been actively degraded or had dissociated from the cell surface. Once again, minimal co-localization was observed between apo(a) and cellular lysosomes. Overall, these observations suggest that cellular lysosomes play a minimal, if any, role in the degradation of apo(a) in HepG2 cells following a series of binding and internalization events.
Figure 3-11. Degradation of $^{125}$I-17K and $^{125}$I-17KΔLBS7,8 by HepG2 Cells and FH Fibroblasts. (A) Degradation of iodinated 17K (---) and 17KΔLBS7,8 (○○○) by HepG2 cells was performed as described in the Experimental Procedures. Degradation was calculated as TCA-soluble radioactivity released to the medium after incubation at 37°C for the indicated time intervals. A cell free blank control experiment was also performed to account for any non-specific ligand degradation. (B) Degradation of $^{125}$I-17K by FH fibroblasts was performed as described above and in the Experimental Procedures chapter. Results are the mean ± SEM of four independent experiments performed in duplicate.
Figure 3-12. Non-Lysosomal Degradation of 17K-GFP by HepG2 Cells. HepG2 cellular lysosomes were stained with LysoTracker red and incubated with 500 nM 17K-GFP as described in the Experimental Procedures. Lysosomal degradation, defined by yellow co-localization in the overlay, was examined in the region of interest (ROI, yellow circle) at each of the indicated time points. The ROI was arbitrarily selected prior to the experiment as the focal point for the confocal microscope. Images are representative of three independent experiments.
Chapter 4
Discussion

4.1 Analysis of Apo(a) Binding to HepG2 Cells

In the present study, we utilized the human hepatoma cell line, HepG2, in order to analyze the binding and degradation of a recombinant 17-kringle containing form of apo(a) (17K). The HepG2 cell model was deemed appropriate for our \textit{in vitro} work based on its ability to express the LDLR, the LRP and the plasminogen receptor family, along with their respective ligands (131-133). Additionally, the HepG2 cell line has been used extensively in lipoprotein binding studies, where other groups have attempted to measure the association of apo(a), LDL and Lp(a) with hepatocyte cell surface receptors (110,134-136).

In our study, we found that the binding analysis of 17K r-apo(a) to HepG2 cells yielded a two-component binding system through which apo(a) could be internalized and degraded. Scatchard analysis of the binding curve revealed a concave-shaped plot which represents a deviation from ideal binding. The reason for the concave shape can be explained by the existence of more than one class of ideal binding sites with distinctive $K_d$ and $B_{\text{max}}$ values. For low quantities of bound ligand, the higher affinity sites become preferentially occupied because the higher affinity $K_d$ value predominates. As the amount of bound ligand increases, binding to the lower affinity sites predominates because the high affinity sites are mostly occupied. Since the slope of the Scatchard plot is equal to the negative reciprocal of the $K_d$, binding to the higher affinity sites is
represented by a steeper negative slope on the curve, while binding to the lower affinity sites is reflected by a flatter, less negative slope. Besides the existence of more than one class of apo(a) binding sites on HepG2 cells, it should be noted that the concave behavior of the Scatchard plot could also represent a negatively cooperative binding system or lattice-ligand interactions. However, previous studies analyzing the binding of apo(a) to HepG2 cells have confirmed that apo(a) acts as a ligand for two classes of binding sites, one with a $K_d$ of 10 nM, and the other with a $K_d$ of 305 nM (110). Using non-linear regression analysis, we observed a similar binding profile in our system, as the first class of receptors was found to conform to a high affinity ($K_d = 12$ nM), low capacity binding site (0.0463 pmol r-apo(a)/mg cell protein), while the second class of receptors consisted of a number of low affinity ($K_d = 249$ nM), high capacity (0.436 pmol r-apo(a)/mg cell protein) binding sites. Although the calculated $K_d$ values agree with the findings of Tam and co-workers (110), we report a 9.4-fold difference in $B_{max}$ between the high and low capacity binding components, in comparison to the 3-fold difference previously observed. One explanation for this inconsistency could be based on the differences in computational methods utilized to calculate the $K_d$ and $B_{max}$ values. Interestingly, analysis of the binding data from Tam and colleagues (110) using our non-linear regression algorithm revealed the same 9.4-fold $B_{max}$ ratio between the high and low capacity binding components, suggesting that differences in mathematical measurement in fact caused the discrepancy. In our study, we utilized non-linear regression analysis of the binding curve to elucidate the reported binding values. However, Tam and colleagues (110) calculated their binding parameters by resolving the two-component Scatchard plot using the
methods of Rosenthal (137). Since the y-axis of the Scatchard plot represents the concentration of bound ligand divided by the concentration of free ligand, the inversion of small free ligand concentrations can greatly distort errors, resulting in larger bound ligand values. Accordingly, it is likely that Tam and colleagues (110) overestimated the $B_{\text{max}}$ of the high affinity binding site, thus contributing to their smaller calculated distribution between the high and low affinity sites.

4.2 Apo(a) Clearance is not Mediated by the High Affinity LDL Receptor

The high affinity binding site was identified as the LDL receptor based on apo(a) domain analysis and ligand binding assays performed in HEK 293 cells and human FH fibroblasts. In the HepG2 cell system, we determined that one amino acid substitution in each of the KIV$_7$ (E56G) and KIV$_8$ (E56G) domains, which abolish the weak LBS present in each of these kringle (68), was indirectly required for high affinity binding. By inhibiting the non-covalent interactions between apo(a) and apoB-100 with the 17KΔLBS7,8 mutant, apo(a) was unable to interact with LDL which, in turn, prevented the subsequent association of Lp(a) with the LDLR. Although these experiments were performed in serum-free medium, we propose that a vanishingly small amount of LDL was produced endogenously by the HepG2 cells during the 3 h incubation, while a trace amount of LDL could have remained bound to the LDLR prior to the incubation with iodinated r-apo(a). To confirm the role of LDL and the LDLR in our system, we analyzed the binding of r-apo(a) to HEK 293 cells, a line which lacks endogenous LDL production and expression of the LDLR. Interestingly, we observed binding to one class
of sites resembling the low affinity component on HepG2 cells. Similarly, binding analysis in FH fibroblasts, a line with genetically defective LDLRs, also revealed the presence of a single binding component similar to the one identified on HepG2 cells. Interestingly, binding analysis in normal fibroblasts revealed a two-component binding system only when iodinated apo(a) was supplemented with LDL, suggesting participation by the LDLR (110). In addition, the 12 nM $K_d$ value that we had calculated for the high affinity binding site was identical to the $K_d$ value found by Izem and co-workers (135), for the binding of $^{125}$I-LDL to HepG2 cells. Taken together, these results suggest that our HepG2 binding data captured the interaction between the LDL component of Lp(a) formed in our assay, and the LDLR.

Our proposed model for apo(a) high affinity binding is consistent with the findings of Kostner (136), who observed that the interaction between Lp(a) and HepG2 cells is mediated through the LDLR in what was previously described as a “hitch-hiking” process. Although we propose that in our system, apo(a) covalently interacts with apoB-100 prior to uptake by the LDLR, we believe that this mechanism is irrelevant in vivo and that this observation reflects an artifact in our in vitro system. Since apo(a) binding assays were performed in serum-free medium, our cell system did not accurately reflect the fact that LDL is in vast excess of apo(a)/Lp(a) in vivo. In addition, the 3 h incubation time at 4°C only enabled the HepG2 cells to produce vanishingly small amounts of LDL within the time-frame of our study. Accordingly, we were likely observing the interaction between apo(a) and a small number of LDL particles secreted by HepG2 cells prior to the experiment. These particles probably persisted through the pre-experimental
wash included in our binding protocol by remaining bound to the LDLR following the aspiration of MEM supplemented with 10% LPDS. Interestingly, previous work by Tam and co-workers (110) has shown that the pretreatment of HepG2 cells with heparin removes residually bound LDL, thus abolishing apo(a) binding to a high affinity site (\(K_d = 10\) nM) on HepG2 cells.

*In vivo*, the optimal concentration of LDL in healthy individuals is approximately 100 mg/dL (138), a value which is between 4- and 5-fold greater than average plasma Lp(a) levels (139). Based on mathematical distributions, we would expect a majority of LDLR binding sites *in vivo* to be occupied by LDL, as opposed to Lp(a). More importantly, the work of Cain and co-workers (76) has demonstrated that the FCR of Lp(a) remains unchanged in LDLR \(^{-/-}\) knockout mice, thus strongly refuting a catabolic role for this receptor. Correspondingly, our *in vitro* data is consistent with these findings, as the 17K and 17K\(\Delta\)LBS7,8 r-apo(a) variants displayed no significant difference from each other \((p = 0.48)\) in degradation, thus implying that the LDLR plays a very minimal, if any, role in Lp(a) catabolism.

### 4.3 Low Affinity Apo(a) Binding Appears Ubiquitous and Heterogeneous

The low affinity binding component on HepG2 cells \((K_d = 249\) nM) was found to resemble, at least in terms of affinity, the single binding component observed on HEK 293 cells \((K_d = 240\) nM) and on human FH fibroblasts \((K_d = 226\) nM). In the present study, we demonstrated that low affinity binding was inhibited by the lysine analogue \(\varepsilon\)-ACA. However, we were unable to completely abolish specific binding to this site using
KIV\textsubscript{5-8} supplemented with ε-ACA, or with the 17KΔAsp and 17KΔV mutants. It should be pointed out that experiments using KIV\textsubscript{5-8} do not specifically test the LBS within these kringles in the context of the full length 17K apo(a) variant, which might make a difference in low affinity binding. Additionally, the discrepancy between the reported KIV\textsubscript{5-8} \(B_{\text{max Hi}}\) value (Table 3-1), and the ε-ACA-dependent reduction in KIV\textsubscript{5-8} specific binding (Figure 3-6), was likely due to the wide variation observed in \(B_{\text{max}}\) among KIV\textsubscript{5-8} experimental repeats. Since KIV\textsubscript{5-8} represents a truncated r-apo(a) variant, the number of tyrosine residues available for iodination are less abundant. Accordingly, the signal to noise ratio is much smaller for KIV\textsubscript{5-8}, in comparison to full length r-apo(a) variants, thus offering an explanation for the variation in KIV\textsubscript{5-8} \(B_{\text{max}}\) between experiments. In order to curb this problem in the future, binding samples could be counted for a longer period of time in the gamma counter, thus reducing the magnitude of error associated with each measurement, particularly at low concentrations of KIV\textsubscript{5-8} ligand.

We have generated data to support the possibility that the low affinity site may represent a ubiquitous receptor class, as we identified this binding component in three separate human cell lines. Interestingly, our findings suggest that some apo(a) mutants with impaired lysine binding ability can bind to the low affinity site better than others, as shown with the 17KΔAsp (\(B_{\text{max}} = 0.289 \pm 0.024\) pmol r-apo(a)/mg cell protein) and 17KΔV (\(B_{\text{max}} = 0.175 \pm 0.011\) pmol r-apo(a)/mg cell protein) apo(a) variants. A possible explanation for this discrepancy could be based on the notion that the low affinity component is itself, comprised of multiple distinct low affinity sites with similar \(K_d\) values. Mathematically, each class would individually conform to the Scatchard model,
however their sum would not. Unfortunately, the Scatchard transformation would be unable to discriminate between these individual classes of sites. Since each site would hypothetically possess a similar $K_d$ value, the resulting Scatchard plot would superficially appear as a single binding component, yet consist of multiple finite sites. Accordingly, analysis of the $B_{max}$ values for each LBS mutant might be the only method for comparing the ability of each r-apo(a) variant to interact with the low affinity component. To simplify these studies, ligand binding assays could be performed with HEK 293 cells or FH fibroblasts to eliminate the non-linear regression estimation of the high affinity component.

Another explanation for the variation in binding between the 17KΔAsp and 17KΔV mutants could be based upon the conformational differences between these apo(a) species. Previous work by Becker and co-workers (140) has demonstrated that in the presence of ε-ACA, apo(a) undergoes a conformational change from a closed to an open structure that is characterized by an increase in the radius of gyration, an alteration of domain stability and an enhancement of covalent Lp(a) formation. Although ε-ACA is a well characterized inhibitor of Lp(a) assembly, low concentrations of this ligand (100 μM) were found to significantly enhance Lp(a) formation by altering the conformation of apo(a) (140). Interestingly, this group further determined that the closed conformation of apo(a) is maintained by intermolecular interactions between sequences within the amino- and carboxyl-terminal halves of the molecule, where a major role for the LBS in KIV$_{10}$ was identified (141). Since the 17KΔAsp apo(a) variant lacks the strong LBS in KIV$_{10}$, it is entirely possible that this construct was unable to remain in a closed conformation, thus
altering its binding ability to cell surfaces. Additionally, loss of the entire KV domain in the 17KΔV mutant could have even greater structural implications, thus explaining its reduced binding capacity to HepG2 cells, compared to the 17KΔAsp mutant. Conversely, Becker and colleagues (141) previously observed that kringle domains within the KIV5-8 region were unable to adopt a closed conformation that could be converted to an open form upon binding to ε-ACA. These findings are consistent with our theory in that the reduction in KIV5-8 binding was likely due to a disruption in apo(a) non-covalent interactions with apoB-100, rather than due to a change in structural conformation. In order to assess the conformational theory, analytical ultracentrifugation experiments could be performed, where comparison of the sedimentation coefficients between the KIV5-8, 17KΔAsp and 17KΔV constructs could be utilized to validate or refute the observed differences in binding.

4.4 Evidence for the Plasminogen Receptor Family as the Low Affinity Binding Site

The ability of plasminogen to compete with apo(a)/Lp(a) for binding to the plasminogen receptor family (95,110,126) has created much speculation about the role of these receptors in Lp(a) clearance. It is currently unknown, however, if these interactions serve a catabolic function for Lp(a) in vivo. The interactions between plasminogen and its specific substrates, inhibitors and receptors have been found to be mediated by lysine binding sites within the plasminogen molecule (119). Accordingly, plasminogen binding to a number of cell types, including monocytes, endothelial cells and fibroblasts, has been shown to be abolished by high concentrations of ε-ACA (121,123,142).
In the present study, we demonstrated that apo(a) can bind to a low affinity component in three distinct cell lines, where specific binding was found to be reduced by ε-ACA. Additionally, we have compiled preliminary data to suggest that the low affinity component on FH fibroblasts is capable of apo(a) degradation, suggesting that this receptor may play a significant catabolic role \textit{in vivo}. These observations agree with the work of Tam and colleagues (110), who demonstrated that free apo(a) can be cleared by a plasminogen receptor present on human fibroblasts. In order to validate this initial observation, additional apo(a) degradation assays need to be performed using a greater quantity of FH fibroblasts, perhaps in 6-well plates, as the variability in our data set (Figure 3-11B) is likely due to the relatively low amount of cells available to participate in degradation. Regardless, the ubiquitous nature of the receptor in question, along with its sensitivity to ε-ACA and plasminogen, has led us to believe that the identity of the low affinity component may represent a member of the plasminogen receptor family. Interestingly, work by Miles and Plow (143) has shown that gangliosides (sialic acid containing glycosphingolipids) inhibit plasminogen binding to certain cells in a structurally specific manner. In addition, $^{125}$I-plasminogen binds specifically and saturably to insolubilized gangliosides, thus potentially mediating the interaction between plasminogen and cell surface receptors (143). Since a panel of gangliosides have been shown to reduce plasminogen binding to varying degrees, it would be interesting to pre-incubate cells with a range of gangliosides and then perform the apo(a) degradation assay to test if catabolism could be inhibited under these conditions.
The heterogeneity of plasminogen receptors could further act as an explanation for the discrepancy in the capacity of the low affinity binding component observed between the 17KΔAsp and 17KΔV apo(a) variants. Since membrane proteins expressing C-terminal basic residues promote plasminogen binding (124), a number of different proteins could fall into this broad receptor class. In the event that the low affinity component comprises multiple binding sites, each with a relatively similar $K_d$ value, it is possible that individual apo(a) species bind to separate plasminogen receptors on the same cell type. While current attempts to purify and isolate these protein receptors have been met with a number of complications, recent work by Miles and colleagues (144) have identified a previously uncharacterized plasminogen receptor utilizing multidimensional protein identification technology. The group first biotinylated membrane proteins with exposed Asp or Glu residues, and then subjected membrane extracts to affinity chromatography on plasminogen-Sepharose. Biotinylated plasminogen-binding proteins were further captured on immobilized avidin, eluted, digested and identified by mass spectrometry. In theory, a similar approach could be utilized with apo(a), where potential binding partners could be identified by apo(a)-Sepharose affinity chromatography, followed by mass spectrometry.

4.5 Physiological Relevance of Apo(a) Binding to Endothelial Cells

The presence of plasminogen receptors on endothelial cell (EC) surfaces is critical for the regulation of fibrinolysis. Much of this regulation is achieved through the controlled assembly of proteins including plasminogen, tissue-type plasminogen activator
(tPA) and urokinase-type plasminogen activator (uPA) (145). Because of the close structural homology between Lp(a) and plasminogen, Lp(a) has been found to interfere with the binding of plasminogen to ECs, thus disrupting plasminogen activation (95). Furthermore, Lp(a) has been found to bind non-covalently to fibrin and ECs, ($K_d = 6-600$ nM) where these interactions were inhibited up to 35% by lysine analogues such as ε-ACA (146). Based on our findings, it is quite reasonable to suggest that the low affinity binding component observed on HepG2 cells and FH fibroblasts is also present on ECs. If this in fact were the case, then the ubiquitous receptor on ECs might contribute to apo(a) uptake and degradation, although no direct evidence currently exists in the literature detailing such a mechanism. Furthermore, the Lp(a) binding site on ECs could also play a role in the deposition of Lp(a) on EC surfaces and as a result, enhance the atherogenic effects of Lp(a). Additionally, Lp(a) could possibly attenuate fibrinolysis by occupying plasminogen binding sites on ECs, thus inhibiting the tPA-mediated conversion of plasminogen to plasmin.

4.6 Apo(a) Clearance is not Mediated by the LRP/\(\alpha_2\)MR

It has been proposed that the LRP/\(\alpha_2\)MR plays a prominent role in the plasma clearance of Lp(a) (91). We have provided evidence to exclude the LRP/\(\alpha_2\)MR as the low affinity site for apo(a) based on the binding and degradation analysis of r-apo(a) in FH fibroblasts. Since fibroblasts are incapable of synthesizing apoB-100, only free apo(a) can act as a ligand for these cell surface receptors. Accordingly, because apoE cannot facilitate the interaction between apoB-100 and the LRP/\(\alpha_2\)MR in this cell line, it
is improbable for apo(a) to be cleared in this manner. Our findings are consistent with the work of Cain and co-workers (76) who observed that Lp(a) plasma clearance was only slightly accelerated in ApoE−/− mice, thus refuting a catabolic role for the LRP. Along the same lines, Tam and co-workers (110) have demonstrated that antibodies against the binding domain of the LRP/α2MR had no significant effect on r-apo(a) binding to human fibroblasts. In contrast, a study by Marz et al. (91) suggested that the LRP may play a role in the clearance of high molecular weight Lp(a) isoforms. An explanation for this discrepancy could be based on the possibility that their preparation of Lp(a) might have contained apoE, thus facilitating its own subsequent uptake and clearance by the LRP.

4.7 Maintenance of Lp(a) Levels In Vivo

Using in vitro cell culture models, we have demonstrated that the plasma clearance of Lp(a) in vivo may be mediated by a low affinity receptor present on HepG2 cells and FH fibroblasts. Fluorescence confocal microscopy has further enabled us to confirm that apo(a) binding is reversible and that degradation is carried out by a lysosomal-independent process in HepG2 cells. To date, the physiological mechanism for the determination of plasma Lp(a) levels, along with the pathophysiology of Lp(a), remain unclear. Although apo(a) isoform size is inversely correlated with plasma Lp(a) levels, even individuals with the same apo(a) isoform size have been found to exhibit widely varying Lp(a) concentrations (75). In addition, the substantial variation in Lp(a) levels among these individuals was found to be caused by differences in Lp(a) production
rates, not catabolism (75). These observations have led us to believe that the maintenance of Lp(a) levels likely occurs at the level of the apo(a) gene itself, through the action of cis- or trans-acting factors (147). Alternately, the apo(a) size polymorphism could modulate apo(a) gene transcription, mRNA stability, protein translation, post-translational stability or secretion from hepatocytes (27). In contrast to Lp(a), LDL plasma regulation has been well characterized in the human population because of genetic diseases such as familial hypercholesterolemia (FH), in which subjects have deficiencies in LDL receptor function. Since FH leads to a loss-of-function phenotype in vivo, this has been useful in the understanding of LDL metabolism. Perhaps once a clearer picture of the physiological role of Lp(a) is established, researchers will be in a better position to speculate on how plasma Lp(a) levels are determined.

4.8 Conclusions

The present study has demonstrated the binding of apo(a)/Lp(a) to distinct high and low affinity receptor classes on HepG2 cell surfaces. Binding to the high affinity component was found to require the formation of an Lp(a) particle. However only the formation of non-covalent interactions between LDL and apo(a) were identified as a prerequisite for high affinity receptor binding. We have further characterized the high affinity site as the LDL receptor, which was shown indirectly to play a minimal role, if any, in Lp(a) degradation. The second receptor class was characterized as a low affinity, lysine-dependent binding component, capable of internalizing and degrading Lp(a)/apo(a). The receptor class was found to be ubiquitous, as we observed binding to
this site in HepG2 cells, FH fibroblasts and HEK 293 cells. We have proposed that this low affinity binding component may belong to the plasminogen receptor family. Overall, our data suggest that Lp(a) plasma clearance *in vivo* is regulated by a low affinity, high capacity receptor(s) belonging to the plasminogen receptor family, however the precise identity of this novel receptor(s) remains unknown.
References


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