STUDIES ON VARIATIONS IN LIPOPROTEIN(a) [Lp(a)] STRUCTURE AND PROPERTIES

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TO

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DECLARATION

I, **Kalaivani V**, hereby declare that I had personally carried out the work depicted in the thesis entitled "**Studies on Variations in Lipoprotein(a) [Lp(a)] Structure and Properties**". No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that Mrs. Kalaivani V, in the Department of Biochemistry of this institute, has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled "**Studies on Variations in Lipoprotein(a) [Lp(a)] Structure and Properties**" was carried out under my direct supervision. No part of thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from the Institutional Ethics Committee/Institutional Animal Ethics for carrying out the study.

P. S. Appukuttan

04 January 2014

The thesis entitled

STUDIES ON VARIATIONS IN LIPOPROTEIN(a) [Lp(a)] STRUCTURE AND PROPERTIES

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Kalaivani V

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LIST OF ABBREVIATIONS

ApoB	Apolipoprotein B 100
Apo(a)	Apolipoprotein(a)
ApoE	Apolipoprotein E
CHD	Coronary heart diseases
CVD	Cardiovascular vascular diseases
CLGG	Cross-linked guar gum
4-CN	4-Chloro-1-naphthol
Con A	Concanavalin A
CRD	Carbohydrate recognition domain
Da	Dalton
DCFH-DA	Dichloro-fluoresceine diacetate
DEAE-Sephadex	Diethylaminoethyl Sephadex
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme linked lectin assay
FITC	Flourescene isothiocyanate
GAG	Glycosaminoglycan
HDL	High density lipoprotein
HRP	Horse radish peroxidase

HSA	Human serum albumin
HS	Heparan sulphate
HSPG	Heparan sulphate-containing proteoglycans
IC	Immune complex
IgA1	Immunoglobulin A1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JL1	Lipid fraction of jacalin precipitate of plasma
JSL1	Lipid layer of supernatant after jacalin precipitation of plasma
kDa	Kilo Daltons
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
Lp(a)	Lipoprotein(a)
Lp(-a)	Lipoprotein(a) minus apolipoprotein(a)
2-ME	2-mercaptoethanol
+NH	Heat inactivated neuraminidase
OD	Optical density
OL1	Total lipid layer of untreated plasma
OPD	Ortho-phenylenediamine
oxLDL	Oxidized low density lipoprotein
oxLp(a)	Oxidized lipoprotein(a)
oxPLs	Oxidized phospholipids

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (20 mM with150 mM NaCl), pH 7.4
PBS-T	PBS containing 0.05% Tween 20
PE	Pre-eclampsia
PEG	Polyethylene glycol, PEG-6000
PLL	Plasma lipoprotein(a)-containing lipids
PVDF	Polyvinylidene diflouride
RBC	Red blood cell
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cells
RPMI	Roswell Park Memorial Institute
T / TF antigen	Thomsen-Friedenreich antigen (Gal β 1→3 GalNAc-)
TBE	Tris- Borate- EDTA buffer (0.05 M Tris, 0.025 M boric acid, 0.003 M disodium salt of EDTA), pH 8.7
TEMED	N,N,N',N'- tetramethyl ethylene diamine
U-10%	upper 10% lipid layer of plasma after ultracentrifugation
U-20%	upper 20% lipid layer of plasma after ultracentrifugation
U-70%	upper 20% lipid layer of plasma after ultracentrifugation
VLDL	Very low density lipoprotein

SYNOPSIS

Plasma lipoprotein(a) [Lp(a)] is an independent risk factor for cardiovascular disease, stroke, aneurism, peripheral vascular diseases and Alzheimer's disease. The characteristic feature of Lp(a) is the presence of an apolipoprotein(a) [apo(a)] subunit which is joined by a lone disulfide bond to an apolipoprotein B 100 subunit (apoB), that is part of an LDL molecule. Apo(a) is very rich in O-linked oligosaccharides containing sialylated form of T antigen [Gal β 1→3 GalNAc] and occurs only in higher mammals such as man and old world monkeys. The size of the LDL component is constant while the apo(a) particle is highly polymorphic among individuals due to variation in kringle IV type 2 repeats of its apo(a) subunit. To date 34 Lp(a) isoforms have been identified in human plasma. In 1987, Utermann et al., identified the presence of at least two different apo(a) isoforms in a single individual and a strong inverse correlation between the plasma Lp(a) level and apo(a) size.

While most investigations focussed on the thrombogenic action of Lp(a) due to the structural homology of apo(a) to plasminogen leading to the inhibition of fibrinolysis pro-atherogenic role of Lp(a) could arise mainly due to the retention and accumulation of Lp(a) on vascular endothelium. Though Lp(a) deposits have been identified in coronary vessels, plaque tissue in atherosclerotic cerebral vessels, placenta and glomeruli in various forms of renal diseases, the molecular mechanisms of Lp(a) attachment to these tissue sites is largely unknown. The recognition by the most prominent carbohydrate-binding cell surface protein, galectin-1 of O-glycans in

Lp(a) may be one of the possible route of Lp(a) entry into these cells. Curiously more Lp(a) is sequestered into atherosclerotic plaques than LDL though in plasma LDL molecules far outnumber Lp(a). Moreover in plasma Lp(a) exist in association with free circulating LDL to form Lp(a):LDL adduct. The present work aims to study how Lp(a) size heterogeneity affects its intra-molecular structure, its interaction with other LDL molecules to form Lp(a):LDL adduct and its recognition by the host protein, galectin-1.

The present work was based on the following objectives;

1. Development of an efficient method for Lp(a) isolation to characterize Lp(a) isoforms.
2. Elucidation of differential response of antibodies and lectins towards Lp(a) isoforms.
3. Study of association of Lp(a) with LDL to form Lp(a):LDL adduct.
4. Delineation of molecular features of Lp(a) that affect Lp(a):LDL adduct formation.
5. To check whether extra LDL in Lp(a):LDL adduct helps the latter bind to LDL receptor.
6. To check whether Lp(a) in adduct mediates its uptake through tissue lectin, galectin-1.
7. Development of inhibitors of Lp(a)-galectin-1 recognition.

The following experimental approaches were employed to achieve the above objectives.

1. Lp(a) was isolated from outdated/discarded plasma by newly standardized protocol which includes affinity precipitation with jacalin, release of jacalin by sugar treatment, ultracentrifugation to sequester free lipoproteins, resolution of lipoproteins by Tris-borate EDTA (TBE) electrophoresis and electroelution and finally identification by ELISA using specific antibodies.
2. De novo formation of Lp(a):LDL adduct was demonstrated by two protocols. In one microplate coated TBE-purified Lp(a) was treated with purified biotin-labeled LDL and bound LDL was detected by avidin-HRP. In the other, adduct formed between TBE-purified Lp(a) and LDL in solution was treated with biotin-labeled jacalin, the jacalin-adduct complex was captured on streptavidin-coated microplates and attached LDL was quantitated by ELISA using specific antibodies.
3. Human monocyte-macrophage culture (9 days old) and cultured rat cardiac fibroblasts (75% confluent P3 passage cells from *Sprague - Dawley* strain) were used to check the binding of Lp(a):LDL adduct to LDL receptor.
4. Binding of Lp(a):LDL adduct to immobilized human galectin-1 was demonstrated by ELISA. Here fluorescently labeled LDL was used to form de novo adduct with TBE purified Lp(a). Biotin labeled galectin-1 was captured on streptavidin-coated microplates to obtain an immobilized form of the lectin akin to that on cell surfaces.
5. Glycopeptides with O-linked sugars were prepared from human plasma proteins by pronase-mediated proteolysis followed by ion-exchange chromatography on DEAE-Sephadex A-50 and elution with 250 mM NaCl.

6. The ability of glycopeptides to block Lp(a) - galectin-1 interaction was checked by inhibitive ELISA using microplate-coated Lp(a) and HRP-labeled galectin-1.

The protocol developed in this study is superior to the conventional method of Lp(a) isolation since Lp(a) with high molecular weight and often low in plasma concentration were also isolated, unlike conventional method which could often isolate only the major isoform from the same plasma. Out of 58 plasma samples tried 83% showed two Lp(a) isoforms each representing a different apo(a) phenotype. Five different clusters of apo(a) size have been reported starting from F (smaller than apoB), through B (equal to apoB in size), S1, S2, S3, S4 to S5 (all larger than apoB) in the increasing order of size. Apo(a) and apoB bands isolated from five different individuals were detected by immunoblotting. The ratio of mobility of apoB subunit [mB] to that of apo(a) [m(a)] was used as an index of molecular size of apo(a), since apoB size remains constant among Lp(a) isoforms. Apart from the detection of four different apo(a) isoforms larger than apoB (S band phenotype) a notable feature of the result was the detection of B band apo(a) which is equal in size to apoB. Fold purification of the isolated Lp(a) was 788 times more than in plasma indicating the elimination of other plasma protein impurities since in most plasma samples total protein concentrations are about 800 times more than that of Lp(a).

The purified Lp(a) was then characterized for its size and differential affinity to antibodies and lectins. Lp(a) is one of the best human glycoconjugate ligand detected so far for galectin-1, an endogenous lectin expressed on endothelial cells. O-

Glycans of apo(a) being rich in T antigen, the large isoforms of Lp(a) showed greater affinity to galectin-1. Present results indicate that in free form large Lp(a) molecules are more likely to get deposited on endothelial cell surface as well as on plaques, kidney mesangial cells and placenta through its interaction with cell surface galectin-1.

Even though Lp(a) contains two N-glycosylated concanavalin A (Con A)-binding proteins (apo(a) and apoB), it showed a lower affinity to Con A compared to LDL for a given amount of protein. Moreover the steady decrease in availability of apoB for recognition by anti-apoB with increase in apo(a) size among Lp(a) isoforms indicated masking of apoB subunit by the extended apo(a) subunit. Availability of antigenic epitopes as well as N-linked oligosaccharides of apoB in Lp(a) for binding of anti-apoB and Con A respectively increased considerably following separation of apo(a) and apoB chains by breaking the disulfide bridge between them using DTT, the increase being proportional to the size of ap(a). It followed that masking of apoB by apo(a) was more in large Lp(a) molecules and that apoB mediated processes such as LDL receptor binding is likely to be suppressed more, the larger the Lp(a).

Further results showed that Lp(a) dominated over LDL in the fraction of plasma lipoproteins that was complexed with other proteins while it was a minor component among total plasma lipoproteins. In circulation Lp(a) had been shown earlier to exist in non-covalent association with LDL by engagement with the apoB subunit of the latter giving rise to Lp(a):LDL adduct. Lp(a) can also form immune complex (Lp(a)-IC) with various naturally occurring anti-carbohydrate antibodies that have been reported. Titration with PEG showed that all LDL-free Lp(a)

molecules precipitated at 0.8% PEG and that the fraction of total Lp(a) precipitable with 0.8% PEG decreased with apo(a) size. Thus small Lp(a) molecules were relatively more free. Results suggested a greater tendency of large Lp(a) to associate with LDL or antibodies to form Lp(a):LDL adduct or Lp(a)-IC. Further results indicated that adduct formation was independent of the quantitative or qualitative nature of plasma LDL whereas the sialic acid moieties in O-glycans of apo(a) were crucial in stabilizing LDL attachment since adduct formation decreased after desialylation of Lp(a) by neuraminidase treatment. Large Lp(a) isoforms were found to have greater tendency to form adduct with LDL compared to the small one. Adduct formation with the more hydrophobic LDL made large Lp(a) molecules in plasma more hydrophobic and less precipitable with PEG. At the same time it was also found that Lp(a) molecules with the same size could make Lp(a):LDL adduct of differing stoichiometry resulting in differential precipitability with PEG. Moreover, increase in rate of adduct formation with apo(a) size was faster than the rate of increase in O-glycan content accompanying apo(a) elongation.

Cell culture studies showed that presence of extra LDL in Lp(a):LDL adduct did not make the latter a ligand for LDL receptor probably due to LDL receptor binding domain of apoB of additional LDL molecules in the adduct being involved in adduct formation with Lp(a). But Lp(a):LDL adduct was found to bind immobilized human galectin-1 in trials. This result offered a possible route through which these lipoproteins may get deposited on tissue sites. Results also suggested that by their increased capacity to get associated with LDL molecules and consequent increased

delivery of LDL to cells like macrophages, large Lp(a) molecules may be individually more pathogenic than small Lp(a).

Since carbohydrate-dependent uptake of Lp(a) to tissues mediated by galectin-1 is a distinct possibility, search for an efficient inhibitor to block this interaction is relevant. For this purpose, glycopeptides with O-linked sugars were prepared from plasma proteins. The resulting glycopeptides were free of detectable N-glycans, but rich in O-glycans and used as an inhibitor of Lp(a)-galectin-1 interaction. Result indicated that plasma O-glycan-containing glycopeptides were several fold more inhibitory towards carbohydrate-dependent recognition of Lp(a) by galectin-1, than was lactose, the best known inhibitor of galectin-1.

In conclusion the present work offers an ideal protocol for the isolation of Lp(a) isoforms from plasma. Even though deposition of lipoproteins in endothelial cell surfaces leading to atherosclerosis was found to be a gradual process starting from the early childhood the reason behind this is not completely understood. The greater masking of apoB subunit in large Lp(a) indicates the dominant role of apo(a) of Lp(a) in physiological as well as pathological function. Attachment of extra LDL particles at a rate faster than the increase in apo(a) size and capture of the adduct by host galectin-1 offer a possible route of deposition of lipoproteins on vessel walls since galectin-1 is ubiquitously expressed in all tissue sites even from the early embryonic stage. Thus the present work mainly focuses on the atherogenic potential of Lp(a) distinct from the proposed thrombogenic potential. The study also offers the possibility of human plasma-derived O-glycan-rich glycopeptides to effectively reverse Lp(a) entry into perivascular tissue.

INTRODUCTION

INTRODUCTION

Lipoprotein(a) [Lp(a)] represents a heterogenous class of plasma lipoprotein particles having a core of neutral lipids and a protein component consisting of an apoB subunit covalently linked to a unique protein called apolipoprotein(a) [apo(a)] (Chiesa et al., 1992). Apo(a) consists of repetitive protein segments called kringles homologous to that in plasminogen. Lp(a) is highly polymorphic and to date 34 Lp(a) isoforms with apparent molecular mass ranging from 300 to 800 kDa have been identified in human plasma (McLean et al., 1987, Lackner et al., 1991, Marcovina et al., 1993). Another peculiar feature of apo(a) is that it is highly enriched in O-linked oligosaccharides containing T antigen [Gal β 1→3 GalNAc] (Kratzin et al., 1987, Garner et al., 2001). Lp(a) is a milestone in mammalian evolution as it occurs only in higher mammals such as man and old world monkeys (Lawn et al., 1995). There is a strong inverse correlation between plasma Lp(a) level and apo(a) size and the low molecular weight/high plasma titre phenotype is suggested to be more pathogenic (Erqou et al., 2010). Even though both pro-thrombotic and pro-atherosclerotic effects of Lp(a) have been suggested (Kamstrup et al., 2010) it is, however, still unclear whether Lp(a) promotes cardiovascular disease primarily via increased thrombosis or atherosclerosis. Though most of the researchers focussed on the thrombogenic action of Lp(a) due to its structural homology to plasminogen leading to the inhibition of fibrinolysis recent studies exploring the association of genetically elevated Lp(a) levels with risk of venous thrombosis have produced conflicting results (Sofi et al., 2007, Kamstrup et al., 2012). Lp(a) deposits have been identified in coronary vessels (Rath et al., 1989),

plaque tissue in atherosclerotic cerebral vessels (Jamieson, D.G., 1995), placenta (Salafia et al., 1998) and in glomeruli in various forms of renal disease (Sato et al., 1993). But the molecular mechanism of sequestration of Lp(a) as well as the role of apo(a) size polymorphism in its structure, pathogenesis and biological fate remains unclear. The recognition by cell surface protein, galectin-1 of O-glycans in Lp(a) is one possible route of Lp(a) entry into these cells (Chellan et al., 2007). In this context the present work aims to study the role of Lp(a) size heterogeneity in its intra-molecular structure and its interaction with other plasma proteins as well as galectin-1.

For the study of the role of size polymorphism in pathogenesis as well as the fate of Lp(a) in plasma it is imperative to obtain Lp(a) polymorphs in purified form. But particle heterogeneity makes it difficult to isolate Lp(a) from other plasma lipoproteins by density criteria only (Pfaffinger et al., 1991). So we initiated our study with the standardization of a new protocol for the isolation of Lp(a) isoforms in order to structurally and functionally characterize them. In the 'Results part – I', affinity precipitation of plasma with the lectin, jacalin was employed to isolate Lp(a) isoforms differing in size and abundance by exploiting the strong affinity of jacalin for T antigen present in core-1 type O-glycan structure in Lp(a) (Tachibana et al., 2006). The new protocol is superior to the conventional method of Lp(a) isolation since it is efficient in the isolation of all Lp(a) isoforms (Lp(a) isoform of F form, B form and S form category) from plasma.

As Lp(a) isoforms of various sizes in pure form was available by the new protocol, we studied the availability of antigenic as well as glycan epitopes of apo(a)

and apoB subunits of Lp(a) isoforms as a function of apo(a) size for recognition by respective antibodies or N-and O-glycan specific lectins (Results in part – II). The steady decrease in availability of apoB with respect to increase in apo(a) size showed the masking of apoB subunit in Lp(a) by the extended apo(a) subunit suggesting the suppression of apoB-mediated processes such as LDL receptor binding. Moreover the superior binding of large Lp(a) isoforms to galectin-1 indicated its chance of preferential deposition on endothelial cell surface as well as on plaques, kidney mesangial cells and placenta where galectin-1 is ubiquitously expressed.

Though Lp(a) can exists in non-covalent association with LDL to form Lp(a):LDL adduct (Trieu et al., 1990) or as immune complex (Lp(a)-IC) with various naturally occurring anti-carbohydrate antibodies (Geetha et al., 2013), effect of differences in molecular size of Lp(a) on its own fate or on its physiological role remains unclear. Results part – III is a detailed study of the composition of Lp(a):LDL adduct as well the role of both Lp(a) and LDL in adduct formation whereas 'Results part – IV' deals with the physiological consequence of adduct in circulation. Results showed that Lp(a) dominated in lipoprotein complexes in plasma while it was a minor component among total plasma lipoproteins. This observation was similar to the preferential accumulation of Lp(a) to LDL in plaques (Pepin et al., 1991). Inverse correlation of free Lp(a) fraction in plasma with Lp(a) size indicated greater tendency of large Lp(a) to associate with LDL or antibodies to form Lp(a):LDL adduct or Lp(a)-IC. Results also showed that adduct formation was independent of the quantitative or qualitative nature of plasma LDL whereas the sialic acid moieties in O-glycans of apo(a) were crucial in stabilizing LDL

attachment. Cell culture studies (in Results part – IV) showed that presence of extra LDLs in adduct did not make the latter a ligand for LDL receptor probably due to LDL receptor binding domain of apoB of LDL being involved in adduct formation with Lp(a). But the binding of Lp(a):LDL adduct to immobilized galectin-1 through Lp(a) offered a possible route through which these lipoproteins may get deposited on tissue sites. So the last part of this study (Results part – IV) deals with the search for an efficient inhibitor to block this interaction. Glycopeptides with O-linked sugars were prepared from plasma proteins by proteolysis followed by ion-exchange chromatography and were found to be several fold more inhibitory to Lp(a)–galectin-1 recognition than lactose which was the best known naturally occurring inhibitor for galectin-1.

In summary this work analyses intra-molecular interactions in the Lp(a) macromolecule, its association with LDL as well as recognition *in vitro* of Lp(a) complexes by cell surface molecules cognate to structural components of Lp(a) or its complexes. Most circulating Lp(a) molecules being in adduct form with LDL with several LDL molecules attached to each Lp(a) molecule, physiologically the recognition of these adducts by cell surface galectin-1 may have a prominent role in the deposition of these lipoproteins in endothelial cell surfaces and other tissue sites since galectin-1 is ubiquitously expressed in all tissue sites. The new protocols standardized in this study help the isolation of Lp(a) isoforms to extend the understanding of structural as well as functional features of Lp(a) and offer effective non-antigenic inhibitors for lipoprotein deposition mediated by galectin-1.

REVIEW OF LITERATURE

Lipoprotein(a) - An Atherogenic Lipoprotein Phenotype

1. Introduction

The importance of cholesterol in the development of atherosclerosis and coronary heart disease (CHD) has been shown by numerous epidemiological investigations with specific emphasis on low density lipoprotein (LDL) (Packard et al., 2000). Another risk factor is lipoprotein(a) [Lp(a)] which has gained great clinical interest since its discovery as a major independent risk factor for atherosclerosis and cardiovascular disease (Berg et al., 1974). However, this could not be confirmed in several prospective studies carried out in the 1990s. But the application of new methods, more recent studies demonstrated an association of *LPA* genotypes with risk of myocardial infarction and strongly supports Lp(a) as a direct cause of cardiovascular disease (Nordestgaard et al., 2010, Erqou et al., 2010, Kamstrup et al., 2012, Kamstrup et al., 2013).

1.1.Historical background

For many years Lp(a) was considered as a variant of low density lipoproteins (LDL). It was discovered by the Norwegian physician investigator Kare Berg in 1963 at the University of Oslo, in an attempt to demonstrate individual antigenic differences in human beta-lipoproteins by immunizing rabbits with a human LDL fraction (Berg, 1963). He obtained heteroimmune rabbit sera that, after adsorbing with LDL, detected an antigen in some but not all individuals because in addition to producing antibodies against LDL, the rabbits also produced an antibody against a variant form of LDL. This new antigen system was given the name Lp, referring to

lipoprotein; positive “reactors” were designated Lp(a+), and negative reactors Lp(a-). At this time the Lp(a) antigen was discovered in 30-40% of the subjects studied in different populations and was considered to be a qualitative genetic trait and a genetic variant of LDL (Berg and Wendt, 1964).

Later, it was found that lipoprotein containing the Lp(a) antigen had significantly more carbohydrate than did LDL and, unlike LDL, had pre-beta electrophoretic mobility. In contrast to the pre-beta very low density lipoprotein (VLDL) Lp(a) sinks upon ultracentrifugation. So it became referred to as the “sinking pre-beta lipoprotein” (Albers et al., 1975). Later, the Lp(a) antigenic determinant was shown to reside in a distinct high molecular weight glycoprotein, designated apolipoprotein(a) [apo(a)], that has little affinity for lipid (Gaubatz et al., 1983). Numerous studies since then have found that Lp(a) is immunochemically and physicochemically distinct from LDL.

1.2.General structure

Lp(a) is a spherical particle with a molecular weight of 4.6-5.6 million Da which is approximately twice as that of LDL and represents a heterogeneous class of lipoprotein particles (Fig.1) having a core of neutral lipids and a protein moiety consisting of one subunit of apoB100 covalently linked by a disulfide bond to one subunit of apo(a) (Koschinsky et al., 1993). Chemically, Lp(a) consists of approximately 30% protein, 10% carbohydrates, 37% cholesterol + cholesteryl esters, 18% phospholipids and 5% triglycerides (Kostner and Kostner, 2002). Even though LDL-like moiety of Lp(a) is virtually indistinguishable from free LDL (Utermann, 1989) most of the plasma Lp(a) belong to the density range of 1.055 to

1.100 g/ml instead of 1.006-1.063 g/ml (density range for LDL), apparently due to presence of additional protein subunit in the former.

Apolipoprotein(a) [apo(a)] – One of the most polymorphic glycoprotein in humans

Apo(a), the characteristic glycoprotein component of Lp(a) has a rather unique structure. Nascent apo(a) contains a 19-residue signal peptide that is released during processing to yield a mature protein. The elucidation of the primary structure of apo(a) is an important milestone in Lp(a) research. The cDNA-derived sequence of apo(a) shows exceptional homology with plasminogen, the zymogen of the fibrinolytic enzyme plasmin (McLean et al., 1987). Apo(a) consists of repetitive protein segments homologous to structures in plasminogen, and called "kringles", i.e. stretches of approximately 110 amino acids forming a secondary structure which resembles "Danish kringles" (McLean et al., 1987). Kringle structures have been classified as types IV and V (Scanu and Edelstein, 1995). In contrast to plasminogen which has a single copy of kringle IV, apo(a) consists of ten different kringle IV types which are numbered from type 1-10 (Fig.1) based on the amino acid sequence (McLean et al., 1987, Morrisett et al., 1990). All of them are present as a single copy except for kringle IV type 2 which occur in identical repeats of 3 to 40 copies due to allelic variations in the number of sequences encoding kringle IV in the apo(a) gene (Marcovina et al., 1993) which forms the basis of the Lp(a) size heterogeneity evident in the human population and determines the size of apo(a) and influence the plasma Lp(a) level (Utermann, 1989, Lackner et al., 1991). The smallest apo(a) isoform contains the protease domain, kringle V and 11 kringle IV types of which kringle IV type 1 and type 3-10 are unique in their primary structure, whereas kringle

IV type 2 is present in 2 identical copies. Large apo(a) isoforms are formed by increase in the number of kringle IV type 2's; the largest apo(a) described so far having 52 kringle IV type 2's.

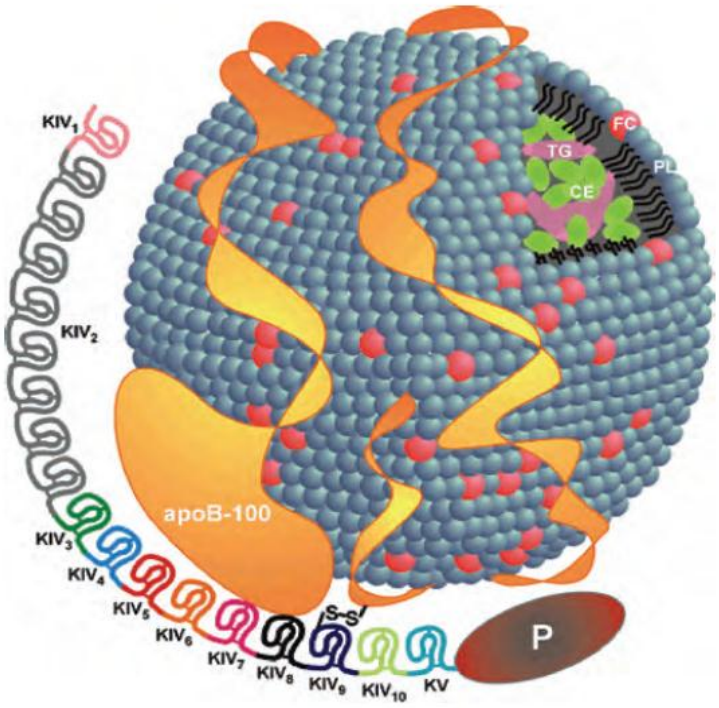


Fig.1. Schematic representation of structure Lp(a) (Koschinsky and Marcovina, 2004): Lp(a) consists of an LDL-like moiety covalently linked to apo(a). The LDL-like moiety is composed of a central core of triglycerides (TG) and cholesteryl esters (CE) surrounded by phospholipids (PL), free cholesterol (FC), and a single molecule of apoB100. Apo(a) contains 10 different types of plasminogen kringle IV- like repeats as well as regions homologous to the kringle V (KV) and protease (P) regions of plasminogen. All apo(a) isoforms contain a single copy of kringle IV type 1 (KIV_1), and kringle IV type 3-10 (KIV_3 - KIV_{10}) while the number of kringle IV type 2 (KIV_2) is variable in its repeat number which form the molecular basis of apo(a) isoform size heterogeneity. The kringle V and inactive protease domains are located at the C-terminus of the molecule. Apo(a) is joined to apoB by a single disulfide bond involving an unpaired cysteine residue in kringle IV type 9. Also depicted is the non-covalent interaction between apoB and apo(a) kringle IV types 7 and 8, which plays a role in Lp(a) assembly.

The size heterogeneity of apo(a) was first described by R. Duvic et al. (1985) with the recognition of 3 different polymorphs. Utermann et al. (1987) categorised apo(a) isoforms into six different groups differing in molecular mass, designated F, B, S1, S2, S3 and S4 (which are increasing in size with respect to that of apoB) and an inverse relationship between apo(a) size and Lp(a) serum concentrations. In 1990 Gaubatz et al. (1990) resolved 11 different apo(a) polymorphs, while 23 apo(a) isoforms were distinguished by SDS-agarose gel electrophoresis and immunoblotting (Kamboh et al., 1991) and 19 apo(a) alleles have been demonstrated by pulse-field gel electrophoresis of DNA and genomic blotting (Lackner et al., 1991). In these two studies the frequency of the double-band phenotypes was reported to be 77% and 94%, respectively. Marcovina et al. (1993) reported 34 different apo(a) isoforms in a cohort of which 74% contained two apo(a) isoforms. Later another isoform category, S5, was identified (Emanuele et al., 2003).

Kringles of apo(a)

The kringle motifs occur not only in apo(a) and plasminogen but also in other proteins of the fibrinolytic and coagulation systems. In apo(a), all the kringles except kringle IV type 1, kringle IV type 2, and kringle V are 77 amino acid residues in length. Kringle IV type 1 and kringle IV type 2 contain 78 amino acids and kringle V has 79. Each kringle is a triple loop structure containing six highly conserved cysteine residues stabilized by three intra-kringle disulfide bonds that are Cys1-6, Cys2-4 and Cys3-5 pairs (Fig.2).

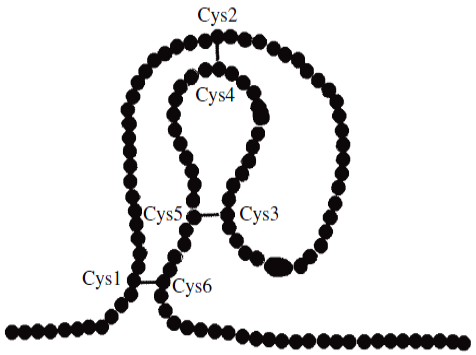


Fig.2. General representation of a tri-looped kringle structure (Mc Lean et al., 1987): Amino acids of a kringle structure, organized into three loops by disulfide bonds between the cysteine residues (C). The shape is maintained by three pairs of cysteine disulfide bonds

Kringle IV type 9 contains, in addition, an unpaired cysteine in position 4057, which is the only Cys with free –SH group in apo(a), the site where apo(a) forms a disulfide bridge with Cys 4326 of apoB in Lp(a) (McCormick et al., 1995). Each kringle IV domain contains a "lysine-binding pocket". The kringle IV type 10 and the region encompassing kringle IV type 5 to kringle IV type 8 have a high binding affinity for lysine, lysine analogs, and fibrin(ogen). These domains are important for mediating the initial interaction of apo(a) with apoB in the first step of Lp(a) assembly and for the binding of Lp(a) to other proteins, such as plasminogen receptors and fibrin, which may contribute to the atherogenicity of Lp(a).

Linkers

The adjacent kringles of apo(a) are joined together by interkringle regions called linkers whose function is still largely undetermined. It is likely, however, that they influence the structural flexibility of the kringle domains and the overall activity of apo(a). Contrary to the linkers joining the identical kringle IV type 2 repeats, those

between non-identical kringles differ in the length of the peptide chain and composition. The linkers between type 2 repeats each has 36 amino acids. Linkers connecting kringle IV type 6 to kringle IV type 7 and kringle IV type 10 to kringle V contain 28 and 26 amino acids, respectively. Each linker is identified according to the lowest number of the two kringles it connects. Linker regions are predicted to be heavily glycosylated with a predominance of O-glycosylation. Linker 4 is not predicted to have glycosylation sites. In contrast, linker 7 is highly O-glycosylated and also contains one N-glycosylation site (Hansen et al., 1997).

Glycosylation in apo(a)

A major post-translational modification to the nascent apo(a) peptide is glycosylation, as up to 25-30% of the weight of the protein is carbohydrate in a molar ratio of 3:7:5:4:7 for mannose, galactose, galactosamine, glucosamine and sialic acid respectively (Fless et al., 1986). The results of both carbohydrate analysis and peptide sequence information suggest the existence of N-linked as well as O-linked glycosylation sites (Fless et al., 1986, White et al., 1993), together with a high sialic acid content which may be a component of either glycan (Fless et al., 1986). Each kringle IV contains one possible N-linked glycosylation site at the sequence Asn-Leu-Thr. This site is not present in kringle V or in the protease region. The most comprehensive characterization of the sugars in apo(a) was published by Garner et al. (2001) who demonstrated that 17% of the oligosaccharide structures consist of two major arginine-linked N-oligosaccharides which are complex biantennary structures in either a mono or disialylated state (Garner et al., 2001). From the carbohydrate molar ratios, the existence of 14 N-linked oligosaccharides were

predicted by Fless et al. (1986) in their apo(a) isoform under study. Studies of apo(a) glycopeptides as well as the high content of Ser and Thr residues in the interkringle revealed that the O-glycans are clustered in the kringle IV linker domains (Scanu and Edelstein, 1995). Moreover, based on the knowledge of the molecular weight of apo(a) preparation, its high content in sialic acid, galactose, galactosamine and absence of fucose, a content of 36 O-linked oligosaccharides was predicted (Fless et al., 1986). Approximately 80% of the Ser/Thr O-linked oligosaccharides present in all apo(a) isoforms are represented by the monosialylated core-1 type structure, NeuNAcα2-3Galβ1-3GalNAc and the remaining 20% consist of disialylated and nonsialylated O-glycans (Fless et al., 1986).

Currently, there is limited information concerning the possible functional importance of glycosylation of apo(a) in the context of Lp(a) assembly and metabolism. Apo(a) O- (and N-) glycans play additional functional roles, for example in intracellular processing, maintaining the tertiary structure of apo(a), and preventing aggregation. The distribution of O-linked glycosylation sites over a large portion of apo(a) imparts a high degree of hydrophilicity which also ensures that the bulk of apo(a) is extended out into the aqueous phase (Phillips et al., 1993). Apo(a) glycosylation modulates the rate at which apo(a) is processed through the secretory pathway without affecting the ability of the secreted material to covalently associate with apoB of LDL (Bonen et al., 1998). The presence of O-glycans protects apo(a) from protease digestion (Van den Steen et al., 1998). In the event of desialylation newly exposed galactose residues on apo(a) may render it a ligand for the macrophage asialoglycoprotein receptor (Keesler et al., 1994).

Apolipoprotein B 100 (apoB) – an apolipoprotein common to both Lp(a) and LDL

ApoB, which constitutes approximately 20% of the LDL particle by weight, is the only protein component of LDL consisting of 4,536 amino acid residues with a molecular mass of about 550 kDa (Segrest et al., 2001). There are two isoforms of apoB in mammals, which are coded for by the same gene. A full-length transcript produces a very large protein, of 4536 amino acids, known as apoB100 (since it is 100% of the possible length). A specific enzyme system present in some cells edits the mRNA coding for apoB to introduce a stop codon about half-way along. This transcript codes for a shorter protein, apoB48 (2152 amino acids) so-called because it is 48% of the length of apoB100 (Wang et al., 2003a). In humans the intestine secretes apoB48 and the liver apoB100, and these proteins are therefore specific markers of chylomicrons and VLDL, respectively. The NH_2- terminal and the COOH-terminal end of apoB in LDL are in close proximity to each other and that the NH_2-terminal region forms a protrusion with a compact globular structure (Chatterton et al., 1991). It is now generally accepted that apoB is wrapped around the surface of the LDL particle and thus stabilizes the structure of the protein–lipid complex. In a more recent computational model based on a secondary structure analysis of apoB, a so called “pentapartite” structure was elaborated, proposing five consecutive domains that are enriched with amphipathic secondary structural elements, termed NH_2-$\beta\alpha_1$-β_1-α_2-β_2-α_3-COOH (Fig.3) and, to date, this concept serves as the basis for the structure of apoB (Segrest et al., 2001). This structure consists of one globular N-terminal structure, two domains of amphipathic β-sheets

and two domains of amphipathic α-helices. The amphipathic β-sheet domains form very strong lipid-binding structures.

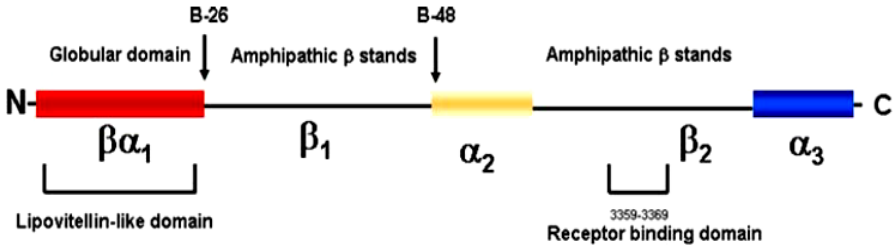


Fig.3. Schematic diagram of the pentapartite structural model of apoB (Segrest et al., 2001): The N-terminal globular lipovitellin-like domain corresponds to about 26% of the amino acid sequence, followed by amphipathic β-strands containing the receptor-binding domain. The C-terminal region is mainly α-helical.

The NH_2-terminal domain of apoB is not tightly associated with lipids and contains most of the Cys residues of apoB in the form of disulfide bonds resulting in a very compact folding of this region and resembles the lipid-binding pocket in lipovitellin from lamprey (Mann et al., 1999). Essential functional roles of the N-terminus include the modulation of the interactions of apoB with lipases and with scavenger receptors on macrophages (Kreuzer et al., 1997). The N-terminal 22–29% of apoB is essential for the co-translational assembly of lipoprotein particles in the liver (Burch and Herscovitz, 2000). The second, β_1-domain, encompassing residues 1,000–2,000, being irreversibly lipid-associated, is predominantly composed of amphipathic β-strands. Together with the next domain (α_2-domain), both are most likely responsible for the accommodation of apoB on the particle surface and for the intimate association with the lipid environment (Shelness et al., 2003). Apart from this, little information is available on the functional role or on structural features of

these sequences. A cluster of basic amino acids (residues 3,359–3,369) within the β_2-domain preceding the COOH-terminal end interacts with the anionic sites of the LDL-receptor and forms the receptor-binding motif. Receptor-binding activity is more likely associated with conformational changes of apoB (Boren et al., 1998). The highly conserved receptor-binding site is stabilized by the interaction of Arg3500 with Trp4369, indeed a single point amino acid mutation (Arg3500 to Glu3500) completely abolishes receptor recognition (Boren et al., 1998).

Human apoB has 19 potential N-glycosylation (Asn-X-Ser/Thr) sites, of which 17 are found to be glycosylated, five with high-mannose type oligosaccharides, and the rest with mono- or bi- antennary sialylated LacNAc-terminating oligosaccharides (Harazono et al., 2005, Garner et al., 2001). The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL (Vukmirica et al., 2002). Seven of the N-glycans are predicted to occur close to the LDL–receptor binding region of apoB and seem to have an important role in receptor binding (Vukmirica et al., 2002). However, one study reported that they do not appear to play a significant role in the binding of apoB to the LDL receptor (Shireman and Fisher, 1979).

1.3.Genetic architecture

Apo(a) and plasminogen genes (*PLG* gene) are linked together and found on human chromosome 6q26-27 which are organized in a head to head configuration separated by about 40 kb and apo(a) gene shares 80% sequence identity to *PLG* (McLean et al., 1987, Magnaghi et al., 1994). The *PLG* gene has one each of the kringle (K) sequences KI, KII, KIII, KIV and KV together with a protease domain

while the human apo(a) gene contains a hydrophobic signal sequence followed by a highly repetitive kringle IV sequence domain and skips KI, KII and KIII, (Lackner et al., 1991) followed by a single copy of kringle V, and an inactive protease domain (McLean et al., 1987). Apo(a) was first sequenced on a gene obtained from a patient with 37 tandemly arrayed copies of kringle IV repeats whose apo(a) mRNA was 14 kilobase (kb) and contained 4529 amino acids with a 19 amino acid signal sequence (McLean et al., 1987, Lackner et al., 1991). Of the 37 kringle IV sequence in the apo(a) cDNA clone, 29 were identical in sequence and were designated 'type A' repeats (kringle IV-A). Four of the repeats differed in sequence from kringle IV-A by 3 nucleotides (but no amino acids) and were referred to as 'type-B' repeats (kringle IV-B). The first (kringle IV type 1), as well as the last eight kringle IV encoding sequences (kringle IV type 30 to kringle IV type 37), were unique and present in all individual suggesting that these kringles are invariant in the population. It differed from the kringle IV-A repeats by 10 to 71 b (McLean et al., 1987). This also suggested a functional role for these invariable kringle units in the apo(a) protein. In addition, a new type designated 2C (kringle IV-2C) was also reported (Enkhmaa et al., 2011). Each kringle IV repeat is 342 base pair long constituted by 5.5 kilobase DNA sequence unit consisting of two exons (160 and 182 base-pairs) and two introns (4 and 1.2 kilobases), respectively (Lackner et al., 1993) and encodes 114 amino acids. The eleven types of kringles are each coded by two separate exons with introns inserted at positions as in the plasminogen gene, i.e., in the middle and at both ends of each kringle (Gavish et al., 1989).

Each individual contains two alleles of the apo(a) gene which may be different in size. It has been found that individual alleles contain from 12 to 40

similar or identical kringles, encoding proteins that range in apparent molecular mass from; 250,000 to 800,000 kDa (Lackner et al., 1993). So far 38 different apo(a) alleles belonging to seven different genetic apo(a) isoforms, designated $Lp(a)^{F}$, $Lp(a)^{B}$, $Lp(a)^{S1}$, $Lp(a)^{S2}$, $Lp(a)^{S3}$, $Lp(a)^{S4}$ and $Lp(a)^{S5}$ have been revealed with pulsed field gel electrophoresis (PFGE) reflecting varying numbers of kringle IV type 2 repeats (Lackner et al., 1993, Trommsdorff et al., 1995). Null alleles have been identified, in which one allele has an exceedingly large number of kringle IV type 2 repeats and does not produce a secreted protein (Gaw et al., 1994). Baboons and humans are similar in terms of properties of Lp(a) and apo(a) and nine isoforms of apo(a) have been identified in baboons and are designated A (the largest) through L (the smallest) (Rainwater and Lanford, 1989). The genetically determined kringle IV type 2 repeat size affects the final size of the apo(a) protein. The average heterozygosity at the apo(a) structural locus due to an expressed hypervariable region within the coding sequence of gene was 94%, which is the highest value for any protein polymorphism reported to date (Kamboh et al., 1991). The only other example of protein polymorphism which has demonstrated hypervariable expressed coding sequences is the human tumor-associated epithelial mucin-type glycoproteins encoded by the PUM locus (Swallow et al., 1987) which has been shown to have at least 10 discrete allele products. The biochemical effect and biological significance of polymorphisms of apo(a) on the function of circulating Lp(a) is still unclear but a highly significant inverse correlation was found between the molecular weight of apo(a) and the plasma Lp(a) concentration (Utermann, 1989, Lackner et al., 1993). As there are individuals with one or two apo(a) phenotypes or isoforms, the

combined expression of both alleles determines an individual's plasma Lp(a) level (Edjeme-Ake et al., 2008).

1.4. Role of genetic polymorphism of *LPA* gene in the skewed distribution of Lp(a) level

Lp(a) plasma level vary considerably, from almost undetectable to greater than 100 mg per dL (< 1 mg per dL to > 100 mg per dL) within the human population and there is generally a skewed distribution, with a high prevalence of the low concentrations with more than two thirds of the population having levels lower than 20 mg per dL (Marcovina et al., 1996, Murase et al., 2007). Lp(a) levels differ by up to a 1000-fold between different ethnic groups, and are higher in Hispanics and Blacks than in non-Hispanic Caucasians and Asians (Sandholzer et al., 1991). The greatest ethnic variation is observed in high molecular weight apo(a) polymorphs (Lackner et al., 1991). The smaller isoforms are relatively rare in Caucasians and are associated with higher Lp(a) concentrations, whereas the larger isoforms are rather common and are associated with lower Lp(a) concentrations (Utermann, 1989). Serum Lp(a) levels are not significantly affected by external factors such as nutrition, smoking, drinking status, or the use of drugs and are known to remain unchanged during long periods of life (12 years, or more than 20 years) (Erqou et al., 2010). As Lp(a) is an acute-phase reactant the high Lp(a) level observed in centenarians (Thillet et al., 1998) might be ascribed to age-associated chronic inflammation. Moreover a recent study showed HIV disease activity as a modulator of Lp(a) and allele-specific apo(a) levels (Enkhmaa et al., 2013). Other studies have shown the influence of gender, race and even diet in the plasma Lp(a) level (Rubenfire et al.,

2012). Several types of polymorphisms of *LPA* gene, size as well as sequence changes both in the coding and regulatory sequences, have been reported to influence the variability of Lp(a) concentration, including different kind of repeat polymorphisms and single nucleotide polymorphism (SNP).

A. Repeat polymorphism

a) Variable number of transcribed kringle IV type 2 repeats (KIV-VNTRs)

The most prominent size polymorphism of the protein is the variable number of kringle IV type 2 repeats. The *LPA* locus accounts for 70–90% of the variability in Lp(a) levels in worldwide populations, with the kringle IV size polymorphism accounting for approximately half of this effect (Ober et al., 2009). Despite the strong association between Lp(a) lipoprotein level and number of kringle IV repeats, a 200-fold difference in the Lp(a) lipoprotein concentration has been observed between individuals with the same kringle IV repeat number (Perombelon et al., 1994) suggesting the role of other polymorphisms in deciding circulating plasma Lp(a) level. Moreover, the apo(a) gene of same size can give rise to Lp(a) in very different plasma levels suggesting differences in the promoter region of the gene, or in other sequences that alter the transcription or translation of the mRNA, or the transport of the protein that impact importantly on allele expression.

b) Variable number of pentanucleotide repeats (PNRs)

A pentanucleotide repeat (PNR) polymorphism consisting of varying numbers of a TTTTA repeat has been shown at position ± 1373 bp from the apo(a) translational start site (ATG site) of *LPA* (Wade et al., 1993). This PNR polymorphism in the promoter region may account for 3-14% of the inter-individual

variations in plasma concentration in Caucasians with a number of TTTTA repeats ranging from 4 to 12 (Ferreira et al., 2003). A negative correlation between the number of pentanucleotide TTTTA(n) repeat (PNR) sequences in the regulatory region of the apo(a) gene and Lp(a) level has also been observed in Caucasians and Indians but not in African Americans (Pati and Pati, 2000). However, significant linkage disequilibrium was noted between the PNR number and kringle IV-VNTR.

B. Single nucleotide polymorphism (SNPs)

Recent studies have identified common SNPs in *LPA* as strongly associated with Lp(a) levels independent of the kringle repeat, explaining up to 36% of the trait variance in populations (Clarke et al., 2009, Lanktree et al., 2010, Ober et al., 2009) . A significant association was found between *LPA* common SNPs and Lp(a) levels across multiple, diverse populations. Of the 19 *LPA* SNPs genotyped, 17 are located in introns. SNPs affecting Lp(a) plasma concentrations also exist in the apo(a) kringle IV type 2 domain (Parson et al., 2004). Strong linkage disequilibrium between SNP markers and the PNR polymorphism has been revealed (Brazier et al., 1999). In addition to this SNP markers were also demonstrated in the 5-prime region of apo(a) gene as well as in exon regions (Ichinose, 1992). In one study an association between Lp(a) levels as well as CAD and an exonic SNP, rs3798220, located in the protease domain of *LPA* was reported (Luke et al., 2007). Moreover two non-expressed SNPs named “null” alleles have been reported (Ogorelkova et al., 1999). In addition, the contribution of other genes in regulating Lp(a) levels has also been described (Enkhmaa et al., 2011). For example, the positive association of the common 2174G/C polymorphism of the human interleukin-6 (IL-6) gene with

elevated Lp(a) concentrations (≥ 60 mg/dl) suggests that Lp(a) serum levels are regulated by IL-6 in humans (Schultz et al., 2010).

1.5. Evolutionary perspective

Lp(a) has an unusual species distribution and its existence is restricted to old world monkeys, great apes, and humans, but not in other animals, with one intriguing exception: the insectivore, hedgehog (La laud, P. M., 1998). Based on the rate of sequence substitution and comparison of the 3' untranslated regions of the apo(a) and plasminogen genes (87% identity in both the human and rhesus sequences), the apo(a) gene may have arisen as recently as 40,000,000 years ago. This is approximately the time of the separation of old and new world monkey lineages. The apo(a) gene is a duplicated version of the plasminogen gene followed by exon shuffling like deletions, multiplications, and single base substitutions (McLean et al., 1987, Tomlinson et al., 1989). As a result exons encoding the final 3 residues of the signal prepeptide, the amino-terminal preactivation peptide (or "tail") and kringles I to III of plasminogen were deleted in human apo(a). Subsequently, there followed extensive multiplication of the kringle IV-like sequences. Apo(a) and plasminogen are thus paralogous genes as they are derived from a common ancestor (Lackner et al., 1993). In contrast an independent creation of an apo(a) like gene by remodelling a duplicated plasminogen gene distinct from the evolution of primate apo(a) has been suggested for hedgehog lineage (Lawn et al., 1995). Expansion and contraction of the intragenic kringle repeat sequences by homologous recombination is probably still occurring and is a likely cause of the size variation of apo(a) among individuals (McLean et al., 1987).

1.6. Metabolism

a) Assembly, synthesis and secretion from endoplasmic reticulum

Metabolic studies in vivo and in vitro have suggested that in spite of the remarkable similarities between Lp(a) and LDL, the former is secreted in the lipid-free apo(a) form into circulation (Sandholzer et al., 1992). mRNAs for apo(a) and apoB100 are independently processed and the proteins are secreted by the liver and not by the intestine or other sources (Linton et al., 1991). After secretion assembly of Lp(a) particles occurs extracellularly (McCormick et al., 1995) or in part at the cell surface. Studies to date suggest that generally the assembly proceeds in two steps. First step involves multiple noncovalent interactions between weak lysine-binding sites within the apo(a) kringle IV type 7-8 and lysine residues (K680 and K690) within the amino terminal of apoB resulting in the formation of a tight non-covalent complex (Becker et al., 2004b). This ε-amino caproic acid sensitive step serves to position Cys4057 of apo(a) close to carboxy terminal cysteine Cys4326 of apoB (McCormick et al., 1995) to form an interchain disulfide bridge in the second step independent of the endoplasmic reticulum resident enzyme protein disulfide isomerase (PDI) (Chiesa et al., 1992).

The rate of covalent Lp(a) assembly is regulated by the conformational status of apo(a) (i.e., “closed” or “open”) by altering the rate constant for disulfide bond formation but not the affinity of apo(a) for apoB. The closed conformation of apo(a) negatively regulates covalent Lp(a) assembly by restricting the access of apoB to the free cysteine residue in apo(a) kringle IV type 9. The rate of covalent Lp(a) assembly is inversely related to apo(a) isoform size (Becker et al., 2004a). Human apo(a) associates selectively with LDL and not with human-HDL or VLDL or mouse LDL

(Chiesa et al., 1992) due to structural differences between human and mouse LDL and changes in apoB conformation indicating that the peptide sequence of apoB known to interact with apo(a) is highly conserved between species.

Apo(a) undergoes extensive post-translational modifications in the endoplasmic reticulum including the formation of 3 intra-disulfide bonds in each kringle, addition of O-linked glycans and conversion of *N*-linked sugars to the complex form. Indeed, newly synthesized apo(a) requires a prolonged period (30–60 min) to fold (White, 1997) and interacts with multiple endoplasmic reticulum chaperone proteins (White et al., 1999). Apo(a) allelic variants vary considerably in the extent of their intracellular retention and degradation. In particular, large apo(a) isoforms tend to be secreted less efficiently than small isoforms, accounting at least partially for the inverse correlation between apo(a) size and plasma Lp(a) levels (White et al., 1994). In addition, the absence of detectable plasma Lp(a) associated with some ("null") apo(a) alleles is explained by the production of apo(a) proteins which are unable to exit the endoplasmic reticulum and are completely retained inside the cells and degraded (White et al., 1999).

b) Catabolism

In contrast to the fairly good information on Lp(a) biosynthesis the site and mode of Lp(a) catabolism is unknown. Although a close correlation between the fractional catabolic rate of Lp(a) and LDL was obtained (Krempler et al., 1980, Krempler, 1980), no relationship was found between the serum levels of these two lipoproteins (Albers et al., 1975). LDL receptor (LDLR) is a cell surface glycoprotein with an apparent molecular weight of 155 to 160 kDa and consists of 839 amino acids in length and which mediates the binding, internalization, and

subsequent degradation of plasma LDL and is the primary pathway for removal of cholesterol from the circulation (Slater et al., 1984). This transmembrane protein contains two complex N-linked glycans and approximately 18 mucin-like O-linked oligosaccharide chains and has 5 domains (Fig.4).

(i) LDLR repeat domain

The LDLR type A repeat domain which is responsible for ligand binding by the LDL receptor accommodates apoB of LDL (Shireman et al., 1977) and lipoproteins containing apoE (Mahley et al., 1977). The LDLR repeat domain consists of seven homologous repeats each contains approximately 40 amino acids.

(ii) Epidermal growth factor (EGF) receptor domain

The epidermal growth factor (EGF) receptor homology domain consists of three repeated sequences that are homologous (35% homology) to the EGF receptor. This domain may contribute to ligand release from the receptor following internalization once it encounters the low pH of the lysosome (Rudenko et al., 2002).

(iii) O-linked glycosylation domain

This is a 58 amino acid section that is rich in serine/threonine and thought to serve as a spacer region between the EGF receptor domain and the plasma membrane. Approximately 70-85% of these oligosaccharides are clustered in an extracellular domain near the transmembrane domain of the receptor (and are designated the "clustered O-linked chains"), while the rest are apparently dispersed at undefined locations possibly first and second domains N-terminal to the clustered domain (Cummings et al., 1983) which may be indispensable for the LDL receptor. The O-linked sugars on the receptor itself are essential for maintaining normal

receptor stability and function by preventing proteolytic cleavage of the extracellular domains of these proteins (Kozarsky et al., 1988).

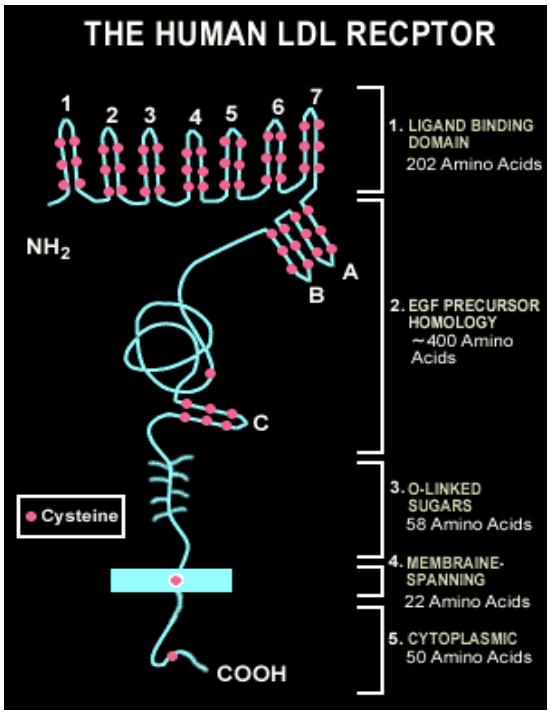


Fig.4. A schematic diagram illustrating the 5 domains of the low density lipoprotein receptor (LDLR) (Goldstein and Brown, 2009): 1) LDLR type A repeat domain consists of seven homologous repeats 2) epidermal growth factor (EGF) receptor homology domain containing the β-propeller sub-domain 3) O-linked glycosylation domain 4) transmembrane domain 5) cytoplasmic domain required for the proper sorting of the receptor following synthesis.

(iv) Transmembrane domain

This consists of an approximately 25 amino acid section enriched in hydrophobic amino acids that serves to anchor the LDLR within the plasma membrane.

(v) Cytoplasmic domain

This consists of a 56 amino acid and glycine residue at position 823 that is required for the proper sorting of the receptor to the plasma membrane following synthesis.

The interaction between receptor and ligand is electrostatic with acidic residues in the LDLR binding domain interacting with basic residues on the apoB (Mahley et al., 1980). Despite the fact that an integral part of Lp(a) consists of apoB, the LDL receptor does not appear to be significantly involved in Lp(a) catabolism (Reblin et al., 1997) due to the possible masking of apoB recognition sites by specific Lp(a) motifs leading to altered steric effects (Armstrong et al., 1990). However one study suggested that LDL receptor preferentially internalizes low molecular weight Lp(a) (Marz et al., 1993).

Several additional members of the LDL receptor family have been identified which may function in Lp(a) catabolism. These members include the LDL receptor–related protein (LRP), gp330/megalin, and the VLDL receptor. The LRP is highly expressed by the liver, and one in vitro study suggested that it may act as a receptor for high molecular weight isoforms of Lp(a) (Marz et al., 1993), but other in vitro studies do not support this notion. Of all known LDLR gene family members, the VLDL receptor (VLDLR) is closest in structure to that of LDLR. The widespread distribution and expression of this receptor in the vascular wall of (endothelial cells of) normal vessels and macrophages in atherosclerotic lesions (Argraves et al., 1997) as well as the detection of Lp(a) in these sites (Rath et al., 1989) also suggest its role in endocytosis of Lp(a).

Megalin/gp330 is expressed in a number of sites such as proximal tubule epithelia in the kidney, type II pneumocytes of the lung, epithelial cells of the thyroid, parathyroid and ependymal cells and choroid plexus in the brain, mammary gland, inner ear, retina, and yolk sac that are heavily engaged in receptor-mediated endocytosis (Lundgren et al., 1997). Megalin/gp330 was first identified as the major

autoantigen in Heymann's nephritis, a rat model for membranous glomerulonephritis (Kerjaschki et al., 1992) and represents the only member of the LDLR gene family that has been shown to bind plasminogen as well as apoB (Stefansson et al., 1995). These binding properties, in combination with high-abundance in the kidney prompted to speculate its role in Lp(a) catabolism and it was found to be capable of binding and mediating the cellular uptake and degradation of Lp(a) through a direct molecular interaction in a Ca^{2+}-dependent and isoform independent manner (Niemeier et al., 1999). Moreover increased plasma Lp(a) levels in patients with renal failure and presence of apo(a) in human urine (Doucet et al., 2000) suggest the role of kidney in Lp(a) catabolism. But animal experiments showed that liver is the major organ responsible for the plasma clearance of Lp(a) mediated by apo(a) and that the kidney plays a very minor role under normal conditions. The existence of one or more receptors in the liver that bind to Lp(a) through the kringle IV type 5–8 region of apo(a) and mediate the plasma clearance of remnant lipoproteins was also suggested (Devlin et al., 2005). In animals, neuraminidase treatment of Lp(a) resulted in a very fast removal from plasma suggesting that asialo-Lp(a) is bound and catabolized by liver through galactose-specific asialoglycoprotein receptor (ASPGR). The less efficient interaction of Lp(a) with the LDL receptor compared to LDL leads to prolonged circulation times and to partial desialylation of Lp(a), which in turn is rapidly recognized and catabolized by ASPGR (Hrzenjak et al., 2003).

1.7. Biological functions

As Lp(a) is present ubiquitously in all populations, there is a need to search the evolutionary benefit(s) of Lp(a) to humans and other mammals that have it. When considering the physiological role of Lp(a) it should be noted that apo(a), rich

in kringle domains, and LDL, rich in cholesterol, are structural elements from two different functional systems, which has led to the suggestion that Lp(a) may bridge the two systems also in a functional sense (Utermann et al., 1987).

Presence of apo(a) in Lp(a) may be responsible for many of the unique structural and functional characteristics that distinguish Lp(a) from LDL, such as the carbohydrate-induced water solubility of apo(a) in contrast to apoB which is not water soluble (Scanu and Fless, 1990). Through the His–X–His sequence within apo(a) kringle IV types 5–8, apo(a) can mediate the binding of metal ions from solution and protect LDL from copper-initiated oxidation (Hill et al., 2003) and makes Lp(a) less susceptible to oxidation. The small pool of cholesterol associated with Lp(a) may act as a 'quick response' reserve for areas where rapid cell turnover, active membrane biosynthesis, acute inflammatory processes or such as wound healing occurs (von Zychlinski et al., 2011), a function that may become pathological when plasma levels of Lp(a) are high.

Lp(a) is regarded as a surrogate for ascorbate (Vitamin C) as the large extracellular deposition of Lp(a) represents a biological defensive mechanism in human occlusive CVD, a degenerative condition induced by chronic vitamin C deficiency (Rath and Pauling, 1990). Even though a decrease in elevated Lp(a) level as well as prevention of the accumulation of Lp(a) in the arterial wall and the consecutive development of atherosclerosis by vitamin C have been found in animal models (Linus Pauling et al, 1990), there were no other reliable clinical or biological evidence regarding this aspect. Additionally, high-dose of ascorbate supplementation (40 mg per kg body weight per day) in patients with premature coronary heart

disease have no clinically important lowering effect on plasma Lp(a) (Bostom et al., 1995). Taken together, this evidence suggests that, although vitamin C might represent a powerful agent against heart disease, its biological relationship with Lp(a) needs further investigation.

Lp(a) displays unequivocal growth-factor-like properties, promoting the growth of human umbilical vein endothelial cells (hUVECs) (Takahashi et al., 1996), and enhancing the proliferation of human vascular smooth cells (hVSMCs) in culture by inhibiting the activation of transforming growth factor-β (Grainger et al., 1994). Moreover, apo(a) belongs to a family of growth factors evolved from a common ancestral kringle-containing serine protease (Donate et al., 1994). Another physiological role of Lp(a) is to preferentially bind and transport pro-inflammatory oxidised phospholipids (oxPLs) derived from oxidized lipoproteins, or from apoptosis during inflammation and oxidative stress, or during the mobilization of oxPLs from tissues during plaque rupture or during lesion regression (Bergmark et al., 2008). As Lp(a) is associated with the enzyme platelet-activating factor hydrolase (PAF-AH) (Tsimikas et al., 2007) it may possibly even detoxify oxPL (Tsimikas et al., 2007). Lp(a) is also able to neutralize the biological effects of lipopolysaccharide (LPS) during endotoxemia and gram-negative infections, by reducing the endotoxin-stimulated tumor necrosis factor (TNF) production by human peripheral blood mononuclear cells (PBMC) as does LDL (Netea et al., 1998). Lp(a) has been implicated in acute inflammatory response (Topciu Shufta et al., 2010) playing a role in reducing inflammation or promoting tissue repair by inhibiting neutrophil recruitment, suggesting that apo(a) is a cell-specific suppressor of the inflammatory

response (Hoover-Plow et al., 2009). A clinical study of myocardial infarction reported that hyperlipoproteinemia(a) was associated with a decrease in coronary collateral circulation (Aras et al., 2006). Thus, Lp(a) may also suppress angiogenesis in cases with inflammation. As kringle domains of apo(a) are highly homologous to plasminogen residues with a simultaneous lack of protease activity, Lp(a) may have an anti-angiogenic effect. Furthermore, some evidence is emerging that Lp(a) has anti-tumor properties (Yu et al., 2005, Lippi et al., 2007a).

2. Pathological significance and clinical manifestations of Lp(a)

Apart from its elusive role in cardiovascular and cerebrovascular diseases, a lot of studies have shown Lp(a) as a pathogenic molecule in other diseases also. Some of them are described below:

2.1.In tumor angiogenesis and cancer

Even though the exact mechanism of action of Lp(a) in cancer is not known, it was suggested that the kringle structure of apo(a) may have a prominent role as it is highly homologous to angiostatin, a degraded product of plasminogen, and exerts an anti-neoplastic effect by inhibiting angiogenesis (Yi et al., 2009). As angiogenesis is necessary for re-endothelialization following vascular injury, suppression of angiogenesis by apo(a) may also contribute to the atherogenicity of apo(a) (Trieu and Uckun, 1999). But a truncated apo(a) protein with only six kringle IV repeats did not exhibit such an effect suggesting the higher atherogenicity of higher molecular weight isoforms. Without any prospective study regarding the association between Lp(a) and cancer, whether Lp(a) is advantageous to cancer patients or not remains uncertain.

2.2.In diabetes

There has been an alarming increase in the rate of mortality and morbidity in patients with insulin-dependent diabetes mellitus (IDDM) due to coexisting dyslipidemia, atherosclerosis and coronary artery disease (CAD) (Jensen et al., 1987). Even though LDL and HDL have been studied extensively in patients with IDDM, there are conflicting reports on the relationship between Lp(a) levels and type 2 diabetes. Though subjects with IDDM have been found to have higher (Singla et al., 2009), or lower levels of Lp(a) (Haffner et al., 1992) than normoglycemic control subjects, other studies have not found any association (Chico et al., 1996). Some reports suggest Lp(a) as a strong independent predictor of CHD risk in type-2 diabetic (Qasim et al., 2011), while one recent study failed to demonstrate such an association (Qi et al., 2012). There are also contradictory results in the involvement of apo(a) phenotypes in diabetes (Hirata et al., 1995). A positive correlation between insulin sensitivity and Lp(a) levels was observed in normoglycemic men (Haffner et al., 1995). Specifically, the age-related risk of diabetes and resistance to insulin were lower for subjects with Lp(a) levels above 46 mg per dL (Boronat et al., 2012), while some previous population-based studies failed to demonstrate such a relationship (Inoue et al., 1997). However, some recent studies have shown an inverse relationship between Lp(a) levels and type 2 diabetes (Mora et al., 2010). There is no obvious explanation for this inverse correlation between Lp(a) and both diabetes and insulin resistance. Because Lp(a) levels are determined mainly by genetic mechanisms, one possibility is that genetic polymorphisms associated with increased levels of Lp(a) are in linkage disequilibrium with gene(s) that protect against insulin

resistance. Similarly there are conflicting reports on the association between Lp(a) levels and diabetic micro vascular complications like nephropathy, retinopathy and neuropathy and diabetic foot problems like gangrenous foot lesions (Chopra et al., 2007, Tseng 2009, Chandni and Ramamoorthy, 2012). Because vascular risk is directly related to the duration of diabetes, the possible contribution of elevated Lp(a) levels to higher vascular risk among type 2 diabetes demands investigation in future clinical trials.

2.3.In cerebrovascular disease, migraine and stroke

A significantly higher Lp(a) level in cerebrovascular disease group suggest that Lp(a) is not only a risk factor for cardiovascular disease but also for cerebrovascular disease (Smolders et al., 2007). Studies provide contradictory findings regarding Lp(a) as a predictor of ischemic stroke as well as the association of Lp(a) isoforms. Even though some studies failed to show any associations of Lp(a) and stroke (Albucher et al., 2000, Price et al., 2001), a lot of recent studies favour such an association (Teber et al., 2010, Georgiadis et al., 2007). Two studies addressing this topic demonstrated that small-sized apo(a) isoforms were over-represented in stroke patients (Milionis et al., 2006, Zambrelli et al., 2005), whereas another report found no difference in the apo(a) isoform distribution between young stroke patients and control subjects (Peynet et al., 1999). Apo(a) has been shown to be deposited in the atherosclerotic plaques of cerebral vessels mainly within the endothelial cell and sub-endothelial cell layers and correlated well with the degree of cerebral atherosclerosis (Jamieson et al., 1995). The biological mechanisms that underlie the association between Lp(a) and stroke may be related to increased

coagulation, impaired fibrinoloysis, and inflammation (Klein et al., 2008), which contributes to both proatherogenic and prothrombotic/antifibrinolytic processes.

Migraine is a common chronic and multifactorial neurovascular disorder that is characterized by recurrent attacks of disabling headache. It has been suggested to be an independent risk factor for stroke (Cinzia et al., 2009) and associated with cerebrovascular and cardiovascular events. The risk of cerebrovascular events is increased with a migraine attack frequency of greater than 12 episodes per year. High Lp(a) levels represent a risk factor for migraine and provide evidence of a link between this biologic marker and migraine in predisposing to stroke, but high Lp(a) levels do not affect the clinical features of the disease hypothesizing a novel link between migraine and stroke (Cinzia et al., 2009, Teber et al., 2010, Teber et al., 2011). A significant gender-specific effect was also noted with high Lp(a) in women linked to a 3.8 times greater risk for migraine compared with controls, whereas there was a non-significant relationship in men (Cinzia et al., 2009).

2.4. In neurodegenerative diseases

As cardiac and cerebrovascular diseases are recognized risk factors for dementia (Nyenhuis and Gorelick, 1998), it is significant to study the role of Lp(a) in neurodegenerative diseases. Role of increased Lp(a) serum concentrations in stroke (Zenker et al., 1986) suggests its role in determining clinical Alzheimer's disease (AD) and vascular dementia (Urakami et al., 2000). Correlation of serum Lp(a) with incidence of Alzheimer's disease varied among reports (Solfrizzi et al., 2002). Higher Lp(a) levels were observed in patients with vascular dementia (VD) and large artery stroke compared with patients with Alzheimer's dementia, cerebral

hemorrhage, and lacunar infarcts. But in some studies elevated Lp(a) levels do not appear to be a major determinant of cognitive impairment in the elderly (Sarti et al., 2001). Abnormally high serum levels of Lp(a) due to specific increases in low molecular weight apo(a) isoforms in patients with CVD and VD suggest that Lp(a) should be one of the indicators that distinguish VD from dementia of the Alzheimer type (DAT). Both Lp(a) and apoE are considered multifunctional proteins with expanding roles in the pathogenesis of vascular and late-onset Alzheimer's disease (Solfrizzi et al., 2002). The presence of apo(a) in human brain as well as in the cerebrospinal fluid (CSF) of patients with blood-brain barrier (BBB) dysfunction has been noted (Koch et al., 2001). But as both LDL and Lp(a) are not produced in this tissue, BBB dysfunction is expected to permit the transmigration of Lp(a) from serum into the CSF (Pepe et al., 2006). A similar isoform distribution was seen in both serum and cerebrospinal fluid (Pepe et al., 2006).

Some studies indicate a possibility of dual inverse effects of Lp(a) on the occurrence of Japanese late-onset Alzheimer's disease, in which high Lp(a) levels suppress the Alzheimer's pathological process and promote ischemic changes in the cerebral white matter by an unknown mechanism. Several lines of evidence linking clinical expression of AD with cerebral infarct suggest that Lp(a) is an additional risk factor for late onset AD in ε4 carriers, while this lipoprotein may protect against the disease in non-carriers older than 80 years (Mooser et al., 2000). Furthermore, clinical and epidemiological data have shown that chronic inflammation appears as a precursor of symptomatic AD (McGeer and McGeer, 1995), suggesting another possible link between increased serum Lp(a) and AD as increased Lp(a)

concentration has been found in a number of clinical and subclinical chronic inflammatory disorders (Baggio et al., 1998).

2.5.In perivascular diseases

Concerning the relationship between Lp(a) levels and peripheral vascular disease (PVD), many studies have provided interesting findings. There are strong evidence that Lp(a) is a significant independent risk factor for PVD and that elevated Lp(a) levels may be associated with more severe forms of PVD (Jones et al., 2007).

3. Mechanisms of Lp(a) pathogenecity – pro-thrombotic, pro-atherogenic or both?

Despite ample data from conventional mechanistic studies demonstrating both pro-thrombotic and pro-atherosclerotic effects of Lp(a) (Kamstrup, 2010) it is, however, still unclear whether Lp(a) promotes cardiovascular disease primarily via increased thrombosis or atherosclerosis. Most of the researchers focussed on the thrombogenic action of Lp(a) due to its structural homology to plasminogen leading to the inhibition of fibrinolysis (Nordestgaard et al., 2010). Apo(a) interferes with an important positive feedback reaction in fibrinolytic cascade and forms a quarternary complex with plasminogen, tPA, and fibrin that is catalytically inefficient in conversion of Glu-plasminogen to active Lys-plasminogen relative to the ternary complex formed in the absence of apo(a) (Fredenburgh and Nesheim, 1992). The kringle IV type 10 LBS, kringle V domain and the amino terminus (kringle IV type 1 to 4) of apo(a) were identified as critical for this inhibition. The anti-fibrinolytic effect of Lp(a) in subjects with two apo(a) isoforms may depend not only on the total

plasma level of Lp(a) but also on the relative concentration of the small apo(a) isoform. Some of the researchers found that such an inhibition is independent of apo(a) isoform size and hence that the increased risk associated with low molecular weight isoforms of apo(a) cannot be ascribed to the ability of apo(a) to inhibit fibrinolytic cascade (Angles-Cano et al., 2001). Another mechanism by which Lp(a) may exert its prothrombotic effect is attenuation of the activation of TGF-ß by the inhibition of plasmin formation at the surface of endothelial cells. TGF-ß has been shown to play an important role in the inhibition of smooth muscle cell migration and proliferation and is activated by local plasmin generation (Grainger et al., 1993). Even though endogenous thrombolysis is attenuated by apo(a) (Biemond et al., 1997), some studies have explored the association of genetically elevated Lp(a) levels with risk of venous thrombosis in the general population with conflicting results (Kamstrup et al., 2012).

Unique properties that apo(a) confers to Lp(a) independent of its similarity to plasminogen include the ability of apo(a)/Lp(a) to affect platelet function (Barre, 2007), to contribute to endothelial dysfunction (Rodie et al., 2004), to inhibit the clearance of chylomicron remnant particles (Devlin et al., 2005), and in the preferential binding of oxidized phospholipid adducts through one of the kringle motifs in apo(a) (Tsimikas et al., 2005). These unique structural features of Lp(a) suggest this lipoprotein has atherogenic potential. Moreover a recent study has suggested that apo(a) can induce proinflammatory and proatherosclerotic effects through modulation of vascular endothelial cell function (Cho et al., 2013). Thus Lp(a) may provide a link between thrombosis and atherosclerosis (Foody et al.,

2000). But it is not yet known, however, whether Lp(a) has a role in the early phases (initiation, development) or late phases (thrombosis) of occlusive arterial disease, or whether the associated cardiovascular risk is mediated by some other mechanism. The pro-atherogenic role of Lp(a) is mainly due to the retention and accumulation of Lp(a) on vascular endothelium (Kronenberg and Utermann, 1994). The molecular properties of apo(a) are important in determining the atherogenicity of Lp(a). Moreover, Lp(a) deposits, like LDL, have been identified in coronary vessels (Rath et al., 1989), plaque tissue in atherosclerotic cerebral vessels (Jamieson, D.G., 1995), placenta (Salafia CM, 1998) and in glomeruli in various forms of renal disease (Sato et al., 1993). Accumulation of Lp(a) in the vessel wall promotes cholesterol accumulation in macrophages forming foam cells and subsequent fatty streaks (Maher and Brown, 1995) which eventually leads to complications like atherosclerosis, glomerulosclerosis (Sato et al., 1993) and acute atherosis (Salafia CM, 1998) in pre-eclampsia.

3.1.Cardiovascular disorders : atherosclerosis

Genetic studies demonstrated an association of *LPA* single-nucleotide polymorphisms, associated with elevated Lp(a) plasma levels, with an increased risk of atherosclerosis (Nordestgaard et al., 2010, Erqou et al., 2010), carotid artery stenosis (Ronald et al., 2011), coronary atherosclerosis (Momiyamaa et al., 2012) femoral atherosclerotic stenosis (Kamstrup PR., 2010), but not with risk of venous thrombosis (Kamstrup et al., 2012, Momiyamaa et al., 2012). The involvement of Lp(a) in the pathogenesis of atherosclerosis was initially suggested by the presence of apo(a) in atherosclerotic lesions with apo(a)/apoB ratio far higher than in plasma

(Hoff et al., 1994). This suggested a preferential accumulation of Lp(a) in plaques (Pepin J. M., 1991). A positive association between the carotid intima-media thickness (CIMT) (one of the best accepted surrogate indices reflecting local and generalized atherosclerosis, associated with cardio- and cerebro-vascular morbidity and mortality) and plasma Lp(a) level have been reported (Calmarza et al., 2012), while others reported no significant association (Mika Kivima¨ki et al., 2011). Interestingly one of the recent studies which focus on the implications of a low Lp(a) level has shown that Lp(a) levels were inversely correlated with the carotid intima media thickening (CIMT) in asymptomatic female subjects within plasma Lp(a) concentration <30 mg per dL (Kazuhiko Kotania, 2012), which suggested that subjects with a low Lp(a) level may have a predisposition to carotid atherosclerosis. Molecular mechanisms of Lp(a) attachment to endothelial, smooth muscle or macrophages remain un-elucidated. The recognition by cell surface galectin-1 of O-glycans in Lp(a) may be one of the possible mechanisms of Lp(a) entry into these cells (Chellan et al., 2007).

3.2.Renal pathology : glomerulosclerosis

Atherosclerotic cardiovascular disease (CVD) is one of the major cause of morbidity and mortality in patients with chronic kidney disease (CKD) (Chawla and Krishnan, 2009). In addition to its role in cardiovascular disease, high plasma levels of Lp(a) have been described to be associated with renal pathology (Rao et al., 2010). As kidney may play a role in Lp(a) catabolism, this elevated Lp(a) level is may be due to the decrease in Lp(a) clearance in renal patients (Frischmann et al., 2007). The general increase in protein synthesis caused by the liver due to proteinuria or an

activated acute-phase response due to malnutrition and/or chronic inflammation is likely another explanation for the elevated Lp(a) level in patients with renal disease (Topciu Shufta et al., 2010). Another hypothesis is that the disease itself is responsible for a metabolic "milieu" that increases hepatic production of Lp(a) or reduces its catabolism in unknown sites; indeed, a rapid decrease in Lp(a) levels was observed after renal transplantation (Rosas et al., 2008). Interestingly the change in Lp(a) level in CKD is apo(a) phenotype dependent and a more pronounced increase in the plasma Lp(a) level was found in those patients expressing predominantly high molecular weight isoforms of apo(a) (Kronenberg et al., 1994).

Lp(a) deposits are found in diseased glomeruli (Sato H et al., 1993) leading to a condition similar to atherosclerosis and known as glomerulosclerosis (Diamond and Karnovsky, 1992). In vitro experiments showed the binding and uptake of native but not oxidised Lp(a) by human mesangial cells leading to its proliferation and thereby contributing to the progression of glomerulosclerosis (Greiber et al., 1996, Kramer-Guth et al., 1996). Even though Lp(a) is not a ligand for the LDL receptor in mesangial cells its tight binding to extracellular matrix suggest that it is not uptake of Lp(a) by mesangial cells but trapping by matrix proteins that contributes to the generation of glomerular apo(a) deposits (Greiber and Wanner, 1997). Further prospective studies will be necessary to confirm these observations.

3.3.Pre-eclampsia (PE): acute atherosis

Pre-eclampsia (pregnancy-induced hypertension) is a medical condition in which hypertension arises in pregnancy from 20 weeks gestation in association with significant amounts of protein in the urine and thought to be associated with

generalized endothelial dysfunction. All over the world PE is the 3rd leading cause for maternal mortality and the 7th leading cause for the perinatal mortality (Redman and Sargent, 2005). There is a seven fold risk for atherosclerotic disease in women with past history of pre-eclampsia.

In pre-eclampsia a distinctive feature of the vascular damage in the placental bed is arterial lesions of the spiral arteries consisting of fibrinoid necrosis, accumulation of foam cells or lipid laden macrophages in the decidua, fibroblast proliferation and a perivascular infiltrate causing reduced placental perfusion (Meekins et al., 1994a). Because of the resemblance to early stages of atherosclerotic lesions, pre-eclamptic lesions were termed "acute atherosis" (Robertson et al., 1967). The term "acute" referred to the fact that this lesion develops over a relatively short time period (during the pregnancy), in contrast to the classic atherosclerosis lesion. Increased Lp(a) levels induced plaque formation on placental vessels leading to reduced uteroplacental perfusion (Berg et al., 1994, Wang et al., 1998). An increased Lp(a) deposition was demonstrated in the walls of placental bed spiral arteries of normal pregnancies that have undergone atherosis but less than in pre-eclamptic pregnancies (Meekins et al., 1994b). Even though Lp(a) phenotype does not contribute significantly to the pathogenesis of pre-eclampsia, uterine vascular Lp(a) deposition is considered as a marker of chronic vascular damage which appears to accompany a wide range of normal and pathological obstetric conditions. However controversy still exists in obstetric literature regarding to the role of Lp(a) in pre-eclampsia one recent study implicate higher Lp(a) levels in pre-eclampsia (Parvin et al., 2010).

Three hypotheses about the mechanisms by which Lp(a) may be associated with pre-eclampsia have been described (Manten et al., 2005): (a) Lp(a), as an acute-phase reactant, transporting cholesterol to sites of endothelial damage for reparation, temporarily increases during pregnancy in response to a greater extend of endothelial injury. After delivery, pre-eclampsia subsides and Lp(a) concentrations return to baseline levels. (b) In cases of severe pre-eclampsia, there is even more extensive endothelial damage and consequently a higher consumption of Lp(a). These women will have lower concentrations of Lp(a). (c) High baseline concentrations of Lp(a), which are genetically determined, may induce or contribute to the development of pre-eclampsia by promoting endothelial dysfunction. Pregnancy is a hypofibrinolytic state which might be necessary for normal placentation (Greer et al., 1994). Pregnancies complicated by pre-eclampsia show an exaggerated inhibition of fibrinolysis (Sattar et al., 2000) which, in-part, results from an increase in plasma plasminogen inhibition in which Lp(a) has a role. So Lp(a) might cause pre-eclampsia mediated by its effects on trophoblast invasion, the fibrinolytic process, blood coagulation, plaque formation, endothelial and mononucleated cell functions (Meekins et al., 1994a).

4. Possible mechanisms through which Lp(a) become pro-atherogenic

Several mechanism(s) have been suggested by which Lp(a) contributes to the above mentioned atherogenic processes which leads to its accumulation on various tissues. Some of them are describing below;

4.1. Oxidative modification

The existence of oxidized phospholipids on Lp(a) in the circulation has been reported to be strongly associated with coronary artery disease (Tsimikas et al., 2005). Lp(a) binds oxPLs and may participate in the transfer of oxPLs (Tsimikas et al., 2005). Lp(a) could undergo oxidative modifications similar to LDL that makes it more atherogenic than native Lp(a). However the susceptibility of Lp(a) to oxidation was reported to be either decreased (Sattler et al., 1991) or increased (Naruszewicz et al., 1992) in an isoform dependent manner in comparison with LDL due to the higher content of sialic acid in apo(a). In spite of this greater resistance to lipid peroxidation, immobilized Lp(a) may finally become oxidized and thus participate in the development of atherosclerotic lesions.

4.2. Immune complex formation

Lp(a) might trigger an immune response leading to the production of auto antibodies and subsequently to the formation of immune complexes (Wang et al., 2003b). A significant positive relationships were found between Lp(a) immune complex (Lp(a)-IC) and Lp(a) levels (Wang et al., 2003b). The presence of oxLp(a) and Lp(a)-IC in newborns and children as well as its increased concentration in patients with coronary heart disease and rheumatoid arthritis suggest an important role of Lp(a)-IC in atherosclerosis (Wang et al., 2009). The plaque-deposited apo(a) also showed the characteristic of immunoglobulins suggesting the possibility of association of apo(a) fragments with human IgG both in plasma and tissue extracts (Hoff et al., 1994). A chronic *Chlamydia pneumoniae* infection and a high plasma Lp(a) level might influence and aggravate aortic heart valve sclerosis via the

formation of circulating immune complexes with *C. pneumoniae* IgG antibody (Gladera et al., 2003). More recent work in this laboratory has shown that anti-α-galactoside antibody (anti-gal) is chiefly involved in immune complex formation with Lp(a) in all plasma samples examined (Geetha et al., 2013).

4.3. Interaction of Lp(a) with LDL to form Lp(a):LDL adduct

Just like LDL Lp(a) can also form insoluble aggregates (Yashiro et al., 1993) which appeared as leaf-like sheets (Xu, 1998) and possess multiple lysine-binding sites which make them more efficient than individual Lp(a) monomers in binding to the extracellular matrix of human arterial intima. Three kinds of interactions are potentially responsible for Lp(a) aggregation, namely, interactions between LDLs, between apo(a) and LDL, and/or between apo(a)s (Gaubatz et al., 2001). Apo(a) is longer than the diameter of the LDL sphere making it to sterically interact with apo(a)s from other Lp(a) molecules and to bring the LDL spheres together, forming aggregates (Xu, 1998). Thus each apo(a) molecule potentially can bind to two neighbouring LDL spheres and there will be one more unoccupied docking site on each LDL sphere available for other apo(a)s to bind to. As a result, the LDL spheres of Lp(a)s chained together by apo(a)s and form a giant Lp(a) aggregate (Xu, 1998).

Very little is known about the composition of Lp(a):LDL complexes and the governing factor of this interaction. Since the initial interaction of apo(a) with LDL involves several non-covalent lysine binding sites of apo(a) and lysine residues of apoB (Becker et al., 2004b) the same interaction may also have a role in the Lp(a):LDL interaction (Ye et al., 1988). Both hydrophobic and ionic interaction have been suggested for the apo(a)/Lp(a):LDL formation with the predominance of

hydrophobic forces. Therefore, the binding site for Lp(a) was predicted to be an exposed hydrophobic domain on apoB (Trieu and McConathy, 1990). The apo(a) moiety of Lp(a) is responsible for the binding of Lp(a) to other apoB-containing lipoproteins (apoBLp) including LDL (Trieu et al., 1991, McConathy and Trieu, 1991). As a result of this initial non-covalent interaction, the apo(a) protein winds around the LDL particle. But after strong shearing in the blood (Weisel et al., 2001) the apo(a) protein undergoes a conformational change and the bulk of apo(a) extends out into solution away from the lipoprotein surface, remaining linked to the LDL particle by the disulfide bond, where it may interact with other ligands (Phillips et al., 1993). This leads to the possibility of the existence of a series of complexes between Lp(a) and differentially sized apoB-containing particles in plasma. Since the association of apoB with lipoprotein particles is non-exchangeable and responsible for the stabilization of the particles in the hydrophilic environment of the blood, the huge Lp(a):LDL complexes may also perform the same function.

Lp(a) can also form insoluble complexes induced by Ca^{2+} cross-bridges between different Lp(a) particles mediated by sialic acids on apo(a) (Yashiro et al., 1993). Even though other lipoproteins also possess sialic acids, they did not form insoluble complexes with Ca^{2+} (Gaubatz et al., 1983) as efficiently as Lp(a). A comparatively higher sialic acid content and a unique flexible extended open conformation of apo(a) (Fless et al., 1986) may be critical for this interaction. Akira Yashiro et al., (1993) were first to report the binding of apoB containing lipoproteins such as LDL and VLDL to Lp(a) in Ca^{2+}-induced insoluble complexes, as evidenced by their co-precipitation with Lp(a). Due to the strong surface negative charge acetyl LDL is smarter than native LDL in complex formation with Lp(a) at physiologic

concentrations of Ca^{2+} (5 mM) through ionic interaction mediated by the positive charge on LDL (Yashiro et al., 1993). However, this interaction differs from the Lp(a):LDL interaction reported by Trieu et al., (1991), which requires the interaction of apo(a) with lysine residues on LDL, rather than Ca^{2+}. Since the putative receptor binding domain of apoB is lysine-rich (Mahley et al., 1980), the interaction of Lp(a) with this domain of apoB could significantly diminish (Armstrong et al., 1985) resulting in the persistence of these complexes in plasma which ultimately leads to its accumulation at various tissues. However, some studies show that apo(a)/Lp(a) stimulates the binding of LDL to fibroblasts several fold by an LDL receptor-independent mechanism (Kostner and Grillhofer, 1991).

Several in vitro studies have provided evidence for the presence of Lp(a)-TRL (lipoprotein(a)-triglyceride-rich lipoprotein complex) in which Lp(a) is noncovalently bound (Marcoux et al., 1997) and Lp(a) with higher molecular weight apo(a) polymorphs (i.e. > 500,000 kDa) appear to be preferentially bound to TRL both in native and reconstituted Lp(a)-TRL complexes (Gaubatz et al., 2001). A sub-species of Lp(a) containing apoE rich in triacylglycerol, distributed mainly in the VLDL and IDL size range was also noted (Bard et al., 1992).

5. Possible routes of Lp(a) sequestration in vascular endothelium

Sub-endothelial matrix (SEM) of the vessel wall is a dense mesh composed of proteins and proteoglycans (PG). Lp(a) may interact ionically with one or several of the intima extracellular matrix proteins and PG resulting in its retention on sub-endothelial matrix.

5.1. Interaction with proteoglycans on arterial wall

Proteoglycans (PGs) are structurally diverse with respect to core proteins and the number, size, and monosaccharide sequence of the attached glycosaminoglycan (GAG) chains. PG contains three major classes of negatively charged glycosaminoglycans: heparan sulfate (HS), chondroitin sulfate, and dermatan sulfate which may be involved in the specific retention of Lp(a). Several studies indicate that the composition and content of glycosaminoglycans change during lesion development. Progressing lesions have an intima rich in chondroitin sulfate proteoglycans of the versican type with a special affinity for apoB-containing particles while there is a decrease in the amount of heparan sulfate proteoglycans (HSPG) (Volker et al., 1990). Many agents are implicated in extracellular matrix remodeling during atherogenesis by affecting endothelial HSPG including lesion macrophages which synthesise matrix-degrading metalloproteases (Gallis et al., 1995), OxLDL or its component lysolecithin which degrade HSPG through the production of a heparanase-like activity (Sivaram et al., 1995), heparin etc. Because HSPG are in a network with fibronectin, collagen type IV and other matrix adhesion proteins loss of HSPG is an atherosclerosis-promoting process which results in increased Lp(a) binding due to unmasking of lipoprotein binding sites (Vlodavsky et al., 1996). Alternatively, since apo(a) is a negatively charged protein, removal of negatively charged HS in the matrix may decrease charge repulsion, leading to increased binding. Thus, the presence of HSPG may be beneficial for the artery because it prevents retention of several atherogenic lipoproteins in the matrix. Lp(a) binds to decorin, a well characterized PG with a protein core and a single GAG

chain, through an electrostatic interaction between GAG and apoB (Hurt-Camejo et al., 1997). There is a second binding mode which is hydrophobic in nature and involves apo(a) and the decorin protein core mediated via its C-terminal domain that comprises kringles IV type 5 to IV type 10, kringle V, and the protease region independent of the lysine binding site of apo(a) (Klezovitch et al., 1998).

5.2.Lp(a) and extracellular matrix proteins

The preferential retention of Lp(a) among lipoprotein particles in intima of lesions in contrast to its low abundance in plasma (Nielsen et al., 1996) can be explained by the binding of Lp(a) to purified matrix proteins via both apo(a) and apoB suggesting an efficient trapping mechanism in the arterial intima for Lp(a) (Van der Hoek et al.,1994). Major SEM proteins include collagens, fibrinogen, fibronectin, laminin, and vitronectin. Fibronectin binds and co-localizes with plasminogen, Lp(a), and recombinant apo(a) via its heparin binding domain to the lysine binding sites of plasminogen or apo(a) (Van der Hoek et al., 1994) in early atherosclerotic lesions and plaques. Laminin, a major component of basal membrane, present in SEM is a heparin-binding protein, has been shown to bind Lp(a) (Pillarisetti et al., 1997) through apo(a) independent of apo(a) isoform size (D'Angelo et al., 2005). A high degree of co-localization of Lp(a) and laminin was found in human atherosclerotic carotid artery specimens, but not in non-atherosclerotic sections suggesting a role of laminin in selective retention of Lp(a) in the atherosclerotic intima (D'Angeloa et al., 2005).

Lp(a) and LDL can also associate with macromolecules residing temporally in the extracellular and pericellular matrix, such as lipoprotein lipase (LPL) or

sphingomyelinase which is mediated by positively charged clusters of arginine and lysine domains of apoB (Auerbach et al., 1999). Lp(a), and to a lesser extent, LDL, after getting bound to the matrix by interacting with proteoglycans, create new binding sites, probably by self-aggregation and increased the further binding of apoB-containing lipoproteins to the matrix. Moreover LDL bound tightly to ECM after pre-saturation with Lp(a) as the preliminary binding of Lp(a) to the ECM leads to complex formation with additional components of the apolipoproteins and to fusion of the particles (Trieu V,1995). These hypothetical complexes may have restricted capacity to return to the circulation across the endothelial barrier and may lead to their focal accumulation and eventual associations with the arterial intima (Trieu V, 1995).

5.3. Interaction of Lp(a) with cells involved in atherosclerotic event: endothelial cells and monocytes/macrophages

Inflammatory changes within the vessel wall and the retention of Lp(a) leading to atherosclerosis involves the active participation of various cell types such as monocytes/macrophages, endothelial cells etc.

5.3.1. Interaction with monocytes/macrophages

The migration of peripheral circulating monocytes into tissues is another important process in the formation of atherosclerotic plaques. Lp(a) modulates the gene expression of blood monocytes thereby inducing a pro-inflammatory response of these cells (Buechler et al., 2001). The interaction of Lp(a) with macrophages is crucial since lipid-laden macrophages (foam cells) are a prominent feature of atherosclerotic lesion. Very few studies have shown the binding of Lp(a) to

macrophages with limited efficiency of internalization. For the first time Bottalico LA et al. (Bottalico et al., 1993) revealed an apo(a)/Lp(a) receptor activity, different from known lipoprotein receptors solely dependent on the apo(a) moiety which is upregulated in macrophages by cholesterol loading in an isoform independent way. Moreover macrophage metalloelastase (MMP-12) secreted by inflammatory macrophages cleaves, in vitro, apo(a) between kringles IV type 4 and IV type 5 (Edelstein et al., 1999).

5.3.3. Interaction with endothelial cells: Role of endothelial expressed lectin, galectin-1

Expression studies of the entire galectin family in human endothelial cells showed that multiple galectins are expressed by endothelial cells and also provided evidence that the level and localization of galectin expression is affected by the activation status of the endothelial cell, both in vitro and in vivo (Thijssen et al., 2008). Galectins, formerly known as S-type lectins or galaptins are a phylogenetically conserved family of lectins defined in 1994 as a shared consensus of amino-acid sequences of about 130 amino acids and the carbohydrate recognition domain (CRD) responsible for β-galactoside binding (Hirabayashi et al., 1996). A total of 15 galectins have now been found in mammals, but only 12 galectin genes are found in humans and were named from galectin-1 to 12. Apart from source all galectins were dimers with subunit molecular weights of about 15,000 Da (Barondes, 1984). Galectins have been classified into three groups according to their structure: prototypical, chimeric, and tandem repeat (Fig.5).

(i) prototypical galectins contain a single CRD and are presumed to form non-covalent dimers under physiological conditions.

(ii) chimeric galectins consists of 29-35 kDa subunits, of which galectin-3 is the only known species found in vertebrates. Galectin-3 is characterized by having a single CRD and a large amino-terminal domain, which is rich in proline, glycine, and tyrosine residues, where it may contribute to self-aggregation.

(iii) tandem-repeat galectins consists of at least two CRDs within a single polypeptide. They are bridged or linked by a small peptide domain which can range from 5 to more than 50 amino acids in length.

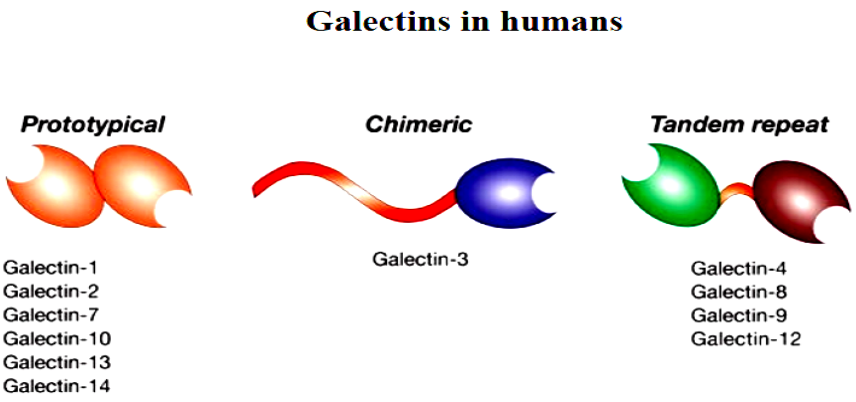


Fig.5. Different types of galectins in humans (Hirabayashi et al., 2001): Human galectins have been classified into three groups according to their structure: prototypical, chimeric, and tandem repeat. The carbohydrate-recognition domain (CRD) of most galectins is approximately 130 amino acids, and this is indicated by the oval domain.

In humans genes encoding galectin-1 *LGALS1* (Lectin, galactoside-binding, soluble) have been mapped to the q12-q13 region of the chromosome 22 (Mehrabian et al., 1993). Given the role of galectin-1 in cell-matrix adhesion, it is likely that the

extracellular galectin-1 is required for the attachment and migration of activated endothelial cells over the extracellular matrix (Thijssen et al., 2006). Thomsen-Friedenreich antigen with or without sialic acid cover (T antigen) has been found to be a potent ligand for galectin-1 (Sangeetha and Appukuttan, 2005). Thus among serum IgA1 (Sangeetha and Appukuttan, 2005) and Lp(a) (Chellan et al., 2007a), T antigen containing O-linked oligosaccharides were recognized by galectin-1. Galectins achieve a stable interaction with their ligands through their multivalency, as binding to multiple ligand leads to increase in their avidity (Ahmad et al., 2004). Importantly, the affinity for galectin ligands is similar and independent of the number of CRDs in the molecule when the galectins are immobilized to a solid surface (Hirabayashi et al., 2002) and forms a crystal latticework. Due to their carbohydrate binding as well as protein binding property they perform various functions both in extracellular and intracellular region. Extracellular functions of galectins depend on their multivalent glycan binding to form cross linked complexes with various glycan groups of glycoproteins and/or glycolipids on the surface of various cell types (Brewer et al., 2002). Galectins may interact with glycoproteins in the extracellular matrix to form a glycoprotein lattice or act as soluble ligands to cross-link the carbohydrates of surface proteins evoking signal transduction.

5.3.3.1.Pathological significance of galectin-1-glycoprotein interaction in atherosclerosis and myocardial infarctions

Galectin-1 is least heard-of in atherosclerosis research so far. Nevertheless, recent studies extend the list of biological activities of galectin-1 from immune responses and tumor growth to hemostasis and thrombosis. At least four members of the galectin family are present in advanced human atherosclerotic lesions

(Papaspyridonos et al., 2006), particularly galectin-1 and -3, have been implicated in the development of atherogenesis (Chellan et al., 2007b, Nachtigal et al., 2008). Galectin-1 is expressed by numerous cell types of the cardiovascular system including endothelial cells (Thijssen et al., 2008), smooth muscle (Moiseeva et al. 2003; Chellan et al. 2007), and cells of the monocyte/macrophage lineage (Fulcher et al., 2009, Malik et al., 2009). Galectin-1 affects SMC adhesion in a dose- and β-galactoside-dependent manner by interacting with β1 integrin on the cell surface of SMCs inducing outside-in signalling (Moiseeva et al., 2003). Reports have shown that galectin-1 may act as chemoattractant at sites of inflammation in vivo and binds Lp(a) (Chellan et al., 2007) through its extended T antigen structure contributing to disease processes such as atherosclerosis. Galectin-1 binds dendritic cell (DC) surface glycoconjugates such as CD2, CD43, and CD45 in a lactose-dependent manner and activates human monocyte-derived dentritic cells (MDDCs) to become phenotypically and functionally mature DCs and also induces cell surface marker expression. Furthermore, some studies have detected galectin-1 expression in DCs (Fulcher et al., 2009) which increased upon DC maturation. On the other hand galectin-1 behaves like a stereotypic chemoattractive protein which is only chemoattractive for monocytes in a dose dependent as well as CRD dependent manner but not for macrophages (Malik et al., 2009) thereby playing a crucial role in the initiation of atherosclerosis by recruiting monocytes to the vessel wall.

In contrast to galectin-1, the effect of endothelial cell activation on galectin-3 expression is not as clear and may depend on the specific microenvironment. However galectin-3 is up-regulated in the aorta of hypercholesterolemic rabbits and in human atherosclerotic lesions (Papaspyridonos et al., 2006). The noticeable

activation of *LGALS3* gene in cells involved in the development of atherosclerosis establishes a strong relationship between the expression of this factor and the development of plaques (Arar et al., 1998). While its precise function in atherosclerosis remains to be investigated, several lines of evidence suggest that this lectin could play a role in control of cell adhesion. An important difference between galectin-1 and galectin-3 is that in contrast to the former the latter has been shown to be a chemoattractant for both monocyte and macrophage in a lactose dependent manner indicating the importance of carbohydrate binding domain in this response (Sano et al., 2000). The synthesis and surface expression of galectin-3 was found to be upregulated during the differentiation and activation of monocytes and macrophages suggesting a role for this lectin in the inflammatory response. By activating macrophages and possibly other cell types within the atherosclerotic plaque, galectin-3 may propagate the release of pro-inflammatory mediators that ensure a continuous influx of monocytes within the vessel wall suggesting galectin-3 as a "macrophage activation" marker (Liu et al., 1995) and a novel upstream key target in anti-inflammatory strategies for the treatment of atherosclerosis (Papaspyridonos et al., 2008). Production of galectin-3 in SMC could act as an anti-adhesive factor in reducing the interactions between integrins and matrix glycoproteins thus facilitating their migration and proliferation in the neointima. Galectin-3 might act as a pattern recognition receptors (PRR) on macrophages, by mediating internalization of modified lipoproteins (Zhu et al., 2001) and thus participate in the formation of foam cells and also get up-regulated in atherosclerotic foam cells (Kim et al., 2003) as well as lipid laden aortic smooth muscle cells. A protective role for galectin-3 in the uptake and effective removal of modified

lipoproteins with concurrent down regulation of pro-inflammatory pathways responsible for atherosclerosis initiation and progression had been also suggested (Iacobini et al., 2009).

In addition to galectin-1 and galectins-3, galectin-2 and galectins-8 are also reported to be important players in atherosclerosis. Macrophages of human atherosclerotic lesion express galectin-2, which binds to lymphotoxin –α and amplifies the inflammatory cascade (Ozaki et al., 2004). Interestingly a single nucleotide polymorphism in the *LGALS2* gene, which affects its transcription, is associated with increased susceptibility to myocardial infarction in the Japanese population (Ozaki et al., 2004). Endothelial cells also express galectin-8 which has a role in triggering platelet activation (Romaniuk et al., 2012) suggesting a roles in hemostatic/thrombotic processes.

5.3.3.2. Functional antagonists of galectin-1 - glycoprotein interaction

Due to the essential and multifunctional role of galectins and/or their binding glycoconjugates (glycoproteins or glycolipids) in various pathological processes, various candidate inhibitors of galectin-glycoconjugate recognition have been proposed as potential anti-galectin drugs with anti-inflammatory, as anti-cancer and may be anti-atherogenic activity. Most of the antagonists tried are based on derivatives of lactose. The multivalent design approach was exploited to synthesis lactulose amine compounds (essentially polymethylene-spaced dilactosamine derivatives) that demonstrated apparently selective effects in different events linked to tumor cell apoptosis, cell aggregation, angiogenesis and endothelial cell

morphogenesis. But weak galectin-binding affinities, cross-reactivity and the probability of eliciting various unwanted side effects of these compounds make them less advantageous (Rabinovich et al., 2006).

Other potential galectin antagonists include dietary polysaccharides, peptide mimetics and synthetic low molecular weight glycoamine analogs (Fru-D-Leu and Lac-L-Leu). Peptides bind somewhere on the surface of the galectin and directly on the carbohydrate-binding site, because they carry a net positive charge like the natural carbohydrate-ligands. Glycoamines, isolated from human and mouse sera consist of penta- or hexa- saccharides generally bound to primary or secondary amine group by covalent binding and inhibit adhesion and aggregation in vitro and *in vivo* (Sorme et al., 2003).

Modified citrus pectin (MCP), water soluble modified natural polysaccharide fiber derived from citrus fruit, specifically inhibits galectin-3 in tumor growth and metastasis, is of interest as an anti-cancer agent (Nangia-Makker et al., 2002). The smaller complex oligosaccharide units of MCP can combine with the carbohydrate binding domain of galectin-3 and interfere with its binding to specific cell surface receptors. In addition, recent findings described the synthesis of wedge-like glycodendrimers with two, four and eight lactose moieties using 3,5 di-(2-aminoethoxy) benzoic acid as the branching unit (Liu FT and Rabinovich GA., 2005). These compounds successfully inhibited the binding of galectin-1 to a highly glycosylated matrix. Recently a variety of high affinity and stable galectin inhibitors were designed including a galectin-3 inhibitor based on 3'-derivatization of N-

acetyllactosamine with an inhibitory potenty of ~50 times greater than N-acetyllactosamine (Salameh et al., 2005).

One study compared a series of oligosaccharides that are homologous to natural carbohydrate chains of galectin ligands and demonstrated that oligosaccharide derivatives especially allyl lactoside 3 can differentially inhibit binding of galectin-1 or -3 (Iurisci et al., 2009) and could serve as a basis for further structure optimization and site-specific modifications to obtain effective and safe pharmacological inhibitors. But the ability of these glycomimetics to affect other galectin-independent mechanisms warrants further studies. However, before galectin-1-based therapeutic agents can be extrapolated to clinical settings, a more thorough understanding of the mechanisms involved in galectin functions is required.

6. Lp(a) lowering therapy

In 2010 new international guidelines recommended screening for elevated Lp(a) in people at moderate to high risk of atherosclerotic cardiovascular disease (CVD) (Nordestgaard et al., 2010) as recent findings have significantly strengthened the notion of Lp(a) as a causal risk factor for CVD (Enkhmaa et al., 2011). Indeed, irrespective of the underlying biological mechanisms for the athero-thrombotic potential of this lipoprotein, over recent years, the major focus has been on identification of suitable therapies for hyperlipoproteinemia(a). There is no generally accepted drug treatment for Lp(a) lowering, although some drugs have been described to be effective.

Unfortunately, Lp(a) has proven resistant to statins and traditional lipid-lowering therapies, except high doses of niacin (Tziomalos et al., 2009). Niacin is

one of a few modalities capable of meaningfully decreasing Lp(a), on average by 30%–40% along with other risk factors such as triglycerides and LDL cholesterol, and to raise levels of HDL cholesterol (Chapman et al., 2010). Despite the lack of evidence from clinical trials, the European Atherosclerosis Society (EAS) has recently recommended treatment with niacin (1-3 g orally per day) for persons with a high-risk Lp(a) defined as ≥50 mg per dL who have established coronary heart disease (CHD) (Nordestgaard et al., 2010, Brown et al., 2010).

Although concentrations of Lp(a) in plasma are highly resistant to changes from environmental factors (McCormick, 2004), diet, age, sex, and treatment with standard lipid lowering drugs they can be influenced by certain metabolic abnormalities and pharmacological agents. Alcohol intake significantly reduces Lp(a) (Valimaki et al., 1991). Weight loss has also been found to lower Lp(a) level although no relationship of adiposity to Lp(a) concentration was observed (Haffner et al., 1992). Red wine has potential anti-atherogenic effects as it specifically reduces Lp(a) and LDL (Sharpe et al., 1995). Ethanol-extracted soy protein is another possible therapy to lower Lp(a) (Meinertz et al., 2002). Xuezhikang, a Chinese red-yeast rice dietary supplement is an extract of cholestin and contains a family of monacolin-related substances, one of which is a naturally occurring lovastatin, in addition to unsaturated fatty acids and other substances (Heber et al., 1999). Xuezhikang has been reported to markedly lower levels of fasting Lp(a) total cholesterol and triglyceride and also reduce postprandial Lp(a) and triglyceride, although the lipid modulating mechanism may be complex (Liu et al., 2003).

Faster removal of free apo(a) not complexed to LDL (Hobbs et al., 1994) led to the use of *N*-acetyl cysteine as an Lp(a) reducing agent (Gavish and Breslow, 1991) because it prevents the oxidation of cysteine to cystine and thus blocks the formation of the disulfide bond between apo(a) and apoB in vitro thereby interfering with the assembly of Lp(a) formation with reduction of Lp(a) levels by only 7%. A recent study revealed that the humanized monoclonal antibody against the interleukin-6 (IL-6) receptor, tocilizumab, decreased Lp(a) concentrations in humans (Schultz et al., 2010).

Other Lp(a)-lowering therapies have been pursued, including lipid apheresis and the use of antisense oligonucleotide (Merki et al., 2011, Parhofer, 2011). The most aggressive approach, to date, for lowering Lp(a) is that based on apheresis techniques (Berthold et al., 2013) (Safarova et al., 2013). Other suitable approaches for lowering Lp(a) include double filtration and traditional hemodialysis (Lippi et al, 2007b) . Although all these strategies are successful under some circumstances they are expensive and cumbersome compared with more conventional drug therapies (Lupattelli et al., 2010).

AIMS AND OBJECTIVES OF THE STUDY

AIMS AND OBJECTIVES OF THE STUDY

1. **Development of an efficient method for Lp(a) isolation to characterize Lp(a) isoforms.**

2. **Elucidation of differential response of antibodies and lectins towards Lp(a) isoforms.**

3. **Study of association of Lp(a) with LDL to form Lp(a):LDL adduct.**

4. **Delineation of molecular features of Lp(a) that affect Lp(a):LDL adduct formation.**

5. **To check whether extra LDL in Lp(a):LDL adduct help the latter bind to LDL receptor.**

6. **To check whether Lp(a) in adduct mediate its uptake through tissue lectin, galectin-1.**

7. **Development of inhibitors of Lp(a)-galectin-1 recognition.**

MATERIALS AND METHODS

1. Materials

Coommassie brilliant blue G-250 and R-250, 1-O-methyl α-D-galactopyranoside, galactose, lactose, fetuin, bovine serum albumin, Tween-20, horse radish peroxidase (HRP) type II, orthophenylene diamine (OPD), acrylamide, N N`-methylene bisacrylamide, tetramethylene diamine (TEMED), 2-mercaptoethanol (2-ME), sodium dodecyl sulphate (SDS), Tris, glycine, boric acid, disodium salt of EDTA, ammonium per sulphate, riboflavin, bromophenol blue, divinyl sulphone, cyanogen bromide (CNBr), potassium borohydride, iodoacetamide, epicholorohydrin, potassium bromide (KBr), 1-flouro-dinitrobenzene (FDNB), sodium periodate, polyethylene glycol-6000 (PEG-6000), agarose, soluble guar gum, Biogel P4, Sepharose 4B, Sephadex A-50, Sephadex G-50, DEAE-Sephadex A-50, dithiothreitol (DTT), copper sulphate, L-proline, L-lysine, ε-aminocaproic acid (EACA), phenylmethanesulfonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), benzamidine hydrochloride, polyvinylidene difluoride (PVDF), 4-chloro-1-naphthol (4-CN), flouroisothiocyanate (FITC), avidin (from egg white), neuraminidase from *Clostridium perfringens*, pronase from *Streptomyces griesues*, wheat germ agglutinin (WGA), Histopaque 1077, ficoll, sodium diazotroate, RPMI 1640, penicillin, streptomycin, gentamicin, 2',7'-dichloro-fluoresceine diacetate (DCFH-DA), sterile Whatman syringe filter (25 mm diameter, 0.2 µ pore size), concentration unit (CENTRIPLUS; 10000 Da molecular weight cut off), trypan blue and oil red O stain were purchased from Sigma-Aldrich (India), Bangalore. Streptavidin and sulpho NHS-LC biotin were purchased from Pierce. U-bottom 96-well polystyrene microtitre plates were purchased from Laxbro, New Delhi. Polystyrene 96-well ELISA plates (MAXISORP) and sterile 4-well tissue culture

plates (17 mm) were purchased from Nunc, Denmark. Antibodies to human apo(a), apoB, IgG, IgA and IgM raised in goat were purchased from Dako, Denmark. All other chemicals used in solvents and buffers were of analytical grade and obtained from local sources.

The seeds of jack fruit (*Artocarpus integrifolia*) and jack beans (*Canavalia ensiformis*) were obtained locally. Out-dated (discarded) plasma samples were collected from the Department of Blood Transfusion Services of this institute with institutional ethical board approval (SCT/IEC-511). Discarded human placenta was supplied in 2004 from on a request from this department through the Director to the Obstetrics Department of the Cosmopolitan Hospital in Trivandrum in accordance with the ethical guidelines of that institution. Cultured (75% confluent P3 passage cells) rat cardiac fibroblasts from *Sprague-Dawley* strain were obtained from Department of Cellular and Molecular Cardiology of this institute. Blood samples for the isolation of monocytes were collected from healthy volunteers of this hospital staff with institutional ethical board approval and informed consent (SCT/IEC-442).

2. General methods

2.1. Protein estimation by Lowry's method

Reagents

a. 2% sodium potassium tartarate ; b. 1% copper sulfate

c. 2% sodium carbonate solution in 0.1 N sodium hydroxide.

d. Alkaline copper reagent: 1 ml of reagent 'a' and 'b' were mixed at the time of experiment and made up to 100 ml with reagent 'c'.

e. 1 N Folin Ciocalteau reagent.

Assay

0.5 ml of protein solution was mixed with 2.5 ml of alkaline copper reagent and incubated at 25 ºC for 10 minutes. This was followed by the addition of 0.25 ml of 1 N Folin's reagent and incubation at 25 ºC for 30 minutes. Absorbance was measured at 660 nm using bovine serum albumin as protein standard (Lowry et al., 1951).

2.2. Protein estimation by Bradford's method

Reagents

a. Coommassie brilliant blue G-250 dye

b. 3% perchloric acid.

60 mg dye (0.06%) was dissolved in 100 ml distilled water containing 2.5 ml perchloric acid. The reagent was filtered though Whatman No.1 filter paper before use and stored in a brown bottle.

Assay

The reagent and protein solution were mixed in the ratio 1:1 and the absorbance at 620 nm measured immediately (Bradford, 1976).

2.3. Carbohydrate estimation by phenol-sulphuric acid method

The total neutral sugar was estimated by phenol-sulphuric acid method of Dubois et al (1956).

Reagents

a. cold sulphuric acid

b. 5% phenol - prepared by diluting distilled phenol 1:20 with water.

0.5 ml sample solution was mixed with 1 ml of 5% phenol, to this was added 4 ml of chilled sulphuric acid quickly and the mixture vortexed thoroughly. After 15

minutes incubation at room temperature, absorbance was measured at 485 nm using glucose or galactose as standard.

2.4. Preparation of affinity matrices

(i) Cross-linked guar gum (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described earlier (Appukuttan et al., 1977). After breaking lumps by gentle pressure 20 g guar gum powder was mixed thoroughly with a finely dispersed emulsion of 4 ml epichlorohydrin and 50 ml 3 N NaOH at room temperature until the mixture became a solid cake. It was then left at 40 °C in a water bath for 24 h and mixed occasionally and then kept at 70 °C in a hot air oven for 10 h. The resulting gel was soaked in distilled water and repeatedly washed with water until washings became neutral. The gel was then equilibrated with 20 mM potassium phosphate buffer pH 7.4 containing 150 mM NaCl (PBS) and homogenized in a blender to obtain particles of about 300 μm size. Fine particles were discarded by repeated decantation. The rest was kept at 4 °C in the presence of 0.02% sodium azide or packed into a column of required size for chromatography.

(ii) Lactose-Sepharose 4B matrix

Lactose was covalently attached to Sepharose 4B using divinyl sulfone as the cross-linker as described by Dean et al. (1985). Briefly, Sepharose 4B (20 g, moist weight) was washed in distilled water under suction and suspended in 20 ml of 1 M Na_2CO_3 pH 11.0. After adding 4 ml divinyl sulfone, the suspension was stirred with a magnetic bar for 1h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water and its wet cake obtained by suction filtration over sintered glass funnel was added to 20 ml 0.5 M lactose in 1 M Na_2CO_3 and stirred

overnight at room temperature as above. The reacted beads were washed successively with 20 times the gel volume of

a. 1 M Na_2CO_3 pH 11.0

b. 0.2 M Glycine-HCl, pH 3.0, containing 1 M NaCl

(to block unreacted activated groups in the gel).

c. 1 M NaCl and

d. Distilled water.

This lactose-Sepharose 4B matrix was equilibrated in PBS and stored at 4 °C with 0.02% sodium azide or packed into a column of required size for chromatography.

(iii) Jacalin - Sepharose 4B matrix

Sepharose 4B was activated using CNBr as described earlier (Cuatrecasas and Anfinsen., 1971). Briefly, Sepharose 4B (20 g, moist weight) was washed in distilled water under suction and suspended in 40 ml 2 M Na_2CO_3. The gel suspension was kept at 9 °C with constant stirring and 800 mg CNBr dissolved in 1-2 ml dimethylformamide was added and stirring was continued for 5 min. Activated gel thus obtained was immediately washed with 20 times gel volume of cold 0.1 M $NaHCO_3$ buffer pH 8.5. The protein sample in 0.1 M $NaHCO_3$ was added to the activated gel (2 mg protein per ml gel) and stirred gently overnight at 4 °C followed by incubating the gel with 0.1 M ethanolamine hydrochloride at 4 °C for 1 h to block the unconjugated activated groups on the gel. The uncoupled protein was removed by washing the gel successively with 20 times the gel volume of

a. 0.1 M $NaHCO_3$

b. Distilled water

c. 50 mM Acetate buffer containing 1 M NaCl, pH 5.

d. Distilled water

The jacalin-Sepharose 4B affinity matrix was equilibrated in PBS and stored at 4 °C with 0.02% sodium azide or packed into a column of required size for chromatography.

2.5. Isolation of lectins

(i) Preparation of jacalin

Jacalin was isolated from the seeds of *Artocarpus integrifolia* by the procedure described earlier (Sureshkumar et al., 1982). Briefly, 30 g jack fruit seeds were dehusked and soaked in PBS 6.5 for 12 h. The seeds were then cut into small pieces, homogenized in 300 ml PBS 6.5 and stirred for 2 h at 4 ºC. The supernatant of homogenate obtained by centrifugation at 14,000 g for 20 min was subjected to 65% ammonium sulfate saturation and stirred for 30 min at 4 ºC. The precipitated proteins recovered by a similar centrifugation were dissolved in PBS 6.5 and dialysed against the same buffer before loading on to cross-linked guar galactomannan column. The column was washed with PBS 6.5 and eluted with 0.15 M galactose in PBS 6.5. Protein containing fractions were pooled, concentrated using CENTRIPLUS; 10,000 Da molecular weight cut-off and stored with 30% v/v glycerol at -20 ºC. Lectin activity was assayed by hemagglutination with human RBC.

(ii) Isolation of concanavalin A

Concanavalin A (Con A) was isolated from seeds of *Canavalia ensiformis* (jack beans) as described earlier (Surolia et al., 1973). Briefly, husk was removed from 850 g of jack beans after soaking in distilled water. These seeds were homogenised with 1700 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4. The homogenate

was stirred for 24 h at 4 ºC and filtered through glass wool. The mixture was again stirred for 6 h with 1200 ml of 1 M NaCl-0.01 M Tris HCl pH 7.4 and filtered through glass wool. The filtrate was made up to 1 M acetic acid concentration by adding concentrated acetic acid and stirred for 20 min at room temperature. The mixture was then centrifuged at 14, 500 g for 20 min. The supernatant was dialysed against 1 M NaCl-0.01 M Tris HCl pH 7.4, 1 mM Mg^{2+}, 1 mM Ca^{2+} and 1 mM Mn^{2+}. After dialysis the pH of the sample was made up to 7-7.4 with Tris and NaOH. The sample was centrifuged and the soluble part was passed through Sephadex G-50 column. The column was washed with 1 M NaCl-0.01 M Tris HCl pH 7.4 and bound proteins were eluted with 0.1 M dextrose in 1 M NaCl-0.01 M Tris HCl pH 7.4. Fractions having Con A activity were pooled together and dialysed successively against 1 M NaCl in distilled water containing Ca^{2+} , Mg^{2+} and Mn^{2+} and finally in distilled water. After dialysis insoluble particles were removed by centrifugation and soluble part was freeze-dried.

(iii) Isolation of galectin-1

Human placenta was washed extensively in running water to remove blood clots and debris followed by washing in ice cold PBS. After removing fat deposits tissue was cut into pieces (70 gm) and kept frozen at -20 ºC before use.

Galectin-1 from human placenta was isolated as described earlier (Sangeetha and Appukuttan, 2005). Briefly, the tissue was homogenized in cold PBS containing 5 mM 2-ME, 0.2 mM PMSF, 2 mM benzamidine hydrochloride and 50 mM lactose. The homogenate was centrifuged at 16,000 g for 20 min. The supernatant proteins were then precipitated with ammonium sulfate at 70% saturation, dialyzed against PBS containing 5 mM 2-ME, and passed through a lactose-Sepharose column. The

column was washed with 800 mM NaCl in PBS and bound galectin-1 was eluted with PBS containing 150 mM lactose and 50 mM iodoacetamide and dialyzed against PBS. All operations were carried at 4 ºC. After concentration, the lectins were subsequently, either, conjugated with HRP/biotin or stored with 30% v/v glycerol in the presence of 50 mM lactose at -20 ºC until use. Lectin activity was assayed by hemagglutination with human RBC.

2.6. Isolation of Lp(a)-rich fraction of plasma by conventional method

Lp(a)-enriched lipids were isolated as described earlier (Chellan et al., 2006). Briefly, after adjusting density to 1.05 g per ml by adding solid KBr 5 ml plasma was subjected to ultracentrifugation at 435, 000 g for 90 min at 15 ºC in 1 ml tubes. The top layer (200 µl) containing VLDL, IDL and LDL was removed and remaining bottom layer (800 µl) was pooled. L-proline was added to this to a final concentration of 0.2 M and the density was adjusted to 1.12 g per ml. After ultracentrifugation at 435, 000 g for 150 min at 15 ºC the floating lipid fraction (top 200 µl) consisting of Lp(a), LDL and HDL_2 was taken for further experiments.

2.7. Isolation of complexed lipoproteins from plasma by PEG-6000 precipitation

Complexed lipoproteins were precipitated by treating plasma with 2% PEG-6000 by a modification of the procedure by Hudson and Hay (1980)**.**

Reagents

Solution A: 20% PEG-6000 solution

Dissolved 20 g PEG-6000 (w/v) in 100 ml distilled water and stored in brown bottle.

Solution B: 0.2 M EDTA in 0.1 M NaOH, pH 7.6

Solution C: Veronal buffered saline (VBS), pH 7.4

2 mM sodium barbitone, 3 mM barbituric acid and 140 mM sodium chloride. Dilute 5 times in distilled water before use.

PEG working solution (12% PEG)

Solution A - 600 µl

Solution B - 300 µl

Solution C - 100 µl

Should be freshly prepared.

PEG washing solution (12% PEG)

Solution A - 100 µl

Solution B - 50 µl

Solution C - 200 µl

Distilled water - 650 µl

Should be freshly prepared.

Briefly 1 ml plasma was treated at 37 °C with 200 µl 12% PEG solution and solution was kept at 37 °C for 30 minutes and then for overnight at 4 °C. Precipitated lipoprotein complex was collected by centrifugation at 2000 g for 20 minutes. After decanting the supernatant precipitate was washed with 2 ml 12% PEG washing solution to remove unwanted sticking plasma proteins and centrifuged again at 2000 g for 20 minutes. The precipitate collected was redissolved in 200 µl PBS by keeping at 37 °C and protein content was assayed as described earlier.

2.8. Preparation of horse radish peroxidase (HRP)-labeled proteins

Antibodies or lectins were labeled with HRP as described earlier (Heyderman et al., 1989).

Reagents

a. Freshly prepared 0.3 M $NaHCO_3$
b. 1% FDNB in absolute ethanol
c. 0.06 M sodium periodate in distilled water
d. 0.32 M ethylene glycol in distilled water
e. 1% potassium borohydride in distilled water

Two hundred micro litre of HRP (2 mg per ml) in solution 'a' was incubated with 10 µl of solution 'b' for 1 h. To this mixture 200 µl solution 'c' was added and kept for 30 minutes followed by the addition of 200 µl solution'd' and kept for another 1 h. All steps were carried out at room temperature under dark. Activated HRP made up to 1 ml with 10 mM $NaHCO_3$ buffer pH 9.5 as well as antibodies or lectins (1 mg per ml) to be conjugated were dialysed separately in the above buffer for 10 h at 4 ºC. Protein solution was mixed with activated HRP in the ratio of 3:2 by mass and incubated for 2 h at room temperature under dark. Then solution 'e' was added to a final concentration of 0.1% and kept for 30 minutes at room temperature under dark. The mixture was then dialysed against PBS with one change overnight. The labeled lectin/antibody was stored with 30% v/v glycerol at -20 ºC until use.

2.9. Removal of jacalin binding proteins from HRP-labeled antibodies

Specified concentrations of HRP-labeled antibodies were treated with jacalin-Sepharose (2 mg jacalin per ml) in PBS-T (PBS in 0.05% Tween-20) in the ratio 4:1 (v/v) for 1 h at 4 ºC with intermittent mixing. The supernatant, devoid of jacalin binding proteins was used in further experiments.

2.10. Preparation of biotin-labeled proteins

Lectins and lipoproteins were labeled with biotin by incubating 1 mg per ml of protein solution with 2 mM sulpho NHS-LC biotin in 20 mM PBS at pH 8.0 for 2 h at 4 ° (Paul et al., 2011). In the case of lectins, protein solutions were pre-incubated with 25 mM of specific sugars for 2 h at 4 °C in order to block its active sites. Unconjugated biotin reagent and free sugars were separated from protein solution by dialysis against PBS. The biotin-labeled proteins were then stored with 30% glycerol (v/v) and 25 mM sugar (in the case of lectins) at - 20 °C until use.

2.11. Preparation of fluorochrome-labeled LDL

Flourescein isothiocyanate (FITC) was mixed with LDL (0.15 mg FITC per mg protein) solution in 0.25 M carbonate-bicarbonate buffer, pH 9 and kept at 4 °C for 24 h in dark (Harlow et al., 1988). The FITC-labeled LDL from the free flourochrome was separated by subjecting the mixture to gel filtration through Biogel P-4 in PBS. The protein fractions containing fluorescence were pooled concentrated and stored in a dark bottle.

2.12. Reduction of lipoproteins or glycoproteins by DTT

Specified concentrations of lipoproteins or glycoproteins were subjected to reduction with 10 mM DTT at 37 ºC for 15 minutes to break the disulfide bond (Fless et al., 1986). Soon after dialysis the protein solution was dialysed against specified buffer.

2.13. Copper induced oxidation of LDL

LDL was subjected to copper mediated oxidation (Galle et al., 1991). Briefly, 500 ng per ml LDL in PBS was incubated with 5 µM $CuSO_4$ for 24 h at 37 ºC and

dialysed thoroughly in PBS to remove copper ions. Along with this a control without $CuSO_4$ was also kept.

2.14. Preparation of asialofetuin

Fetuin (2 mg per ml) was dissolved in 0.1 N H_2SO_4 and kept at 80 ºC in a water bath for 1 h. The protein solution was then neutralised to pH 7.4 with 1N NaOH and dialysed against PBS. Protein was quantitated and kept at -20 ºC.

2.15. Electrophoresis

(i) Lipoprotein electrophoresis in 3.75% native PAGE using Tris-Borate EDTA (TBE) buffer

Lipoproteins were subjected to electrophoresis in 3.75% disc gels with the TBE chamber buffer as described earlier with some modifications (Chellan et al., 2006).

Reagents

Solution A: TBE buffer, pH 8.7

0.05 M Tris, 0.025 M boric acid, 0.003 M disodium salt of EDTA. Should be freshly prepared in deionized distilled water.

Solution B: TBE buffer with TEMED

1 ml Solution A was mixed with 2 ml deionized distilled water containing 14 µl TEMED.

Should be freshly prepared in deionized distilled water.

Solution C: 15% acrylamide/bisacrylamide (19:1, w/w)

Acrylamide - 14.25 g

Bis acrylamide - 0.75 g

Dissolved in 100 ml deionized distilled water, filtered through Whatmaan No.1 filter paper and stored in dark bottle at 4 ºC.

Solution D: 25% acrylamide/bisacrylamide (20:5, w/w)

Acrylamide - 20 g

Bis acrylamide - 5 g

Dissolved in 100 ml deionized distilled water, filtered through Whatmaan No.1 filter paper and stored in dark bottle at 4 ºC.

Solution E: Riboflavin, 0.004% solution.

Solution F: Ammonium persulfate (0.2%) in deionized distilled water.

Solution G: Tracking dye: Bromophenol blue, 0.005% solution.

Solution H: Fixative: 12.5% trichloroacetic acid in distilled water.

Solution I: Tube gel stain: 120 mg% Coommassie brilliant blue (CBB R-250) in a methanol: acetic acid: water mixture (11:3:11, v/v).

Solution J: Destaining solution: methanol: acetic acid: water mixture (1:1.5: 17.5, v/v).

Gel preparation

The gels were cast in 5 mm internal diameter (ID), 9 cm long glass tubes (BROVIGA DISC electrophoresis apparatus).

A. Separating gel (3.75% acrylamide)

Solution A - 2 ml

Solution C - 2.5 ml

Solution F - 5.5 ml

TEMED -10 µl

Polymerization was achieved at room temperature.

B. Spacer Gel

Solution B	- 1 ml
Solution D	- 1 ml
Solution E	- 1 ml
De ionized distilled water	- 5 ml

Polymerization was achieved under fluorescent light and gels were kept at 4 ºC in a wet condition for overnight.

Reservoir buffer

Pre-cooled (4 ºC) TBE buffer, pH 8.7 (0.05 M Tris, 0.025 M boric acid, 0.003 M disodium salt of EDTA).

Sample preparation

Lipoprotein samples were dialyzed against the reservoir buffer (1:9, v/v diluted chamber buffer) for 10 h. Fifty micro litre sample containing 50 µg protein was mixed with 10% glycerol and loaded over individual disc gels. A tiny speck of bromophenol blue was diluted in 100 µl of reservoir buffer and 3 to 5 µl was added to the prepared sample and loaded over one tube to track the protein.

Electrophoresis

Electrophoresis was run at 25 °C at 3 mA current per tube till the bromophenol blue ran out and then for 50% more time. After the run, one gel was fixed in 12.5% trichloroacetic acid for 45 minutes, stained with Coommassie brilliant blue R-250 and destained.

(ii) 3.5% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE on 3.5% gel was done according to the method described earlier (Laemmli, 1970).

Reagents

Solution A: 30 g of acrylamide

0.8 g of bis acrylamide

Dissolved in 100 ml deionized distilled water, filtered through Whatmaan No.1 filter paper and stored at 4 ºC in dark bottle.

Solution B: 0.614 M Tris/HCl buffer, pH 8.8.

For 100 ml of buffer, 164 mg of SDS was dissolved.

Solution C: 0.147 M Tris/HCl buffer, pH 6.8.

For 100 ml of buffer, 108 mg of SDS was dissolved.

Solution D: Ammonium per sulfate (15 mg per ml) in distilled water.

Solution E: Bromophenol blue (Tracking dye), 0.005% solution.

Solution F: Fixative: 50% methanol in distilled water

Solution G: (a) Tube gel stain: 120 mg% Coommassie brilliant blue R-250 in a methanol: acetic acid: water mixture (11:3:11, v/v).

(b) Slab gel stain: 60 mg% Coommassie brilliant blue R-250 in a methanol: acetic acid: water mixture (11:3:11, v/v).

Solution H: Destaining solution: methanol: acetic acid: water mixture (1: 1.5:17.5, v/v).

Gel preparation

For apo(a) size determination the gels were cast in 5 mm ID, 9 cm long glass tubes (BROVIGA DISC electrophoresis apparatus). For Western blotting gel was cast in the 'mini slab gel' apparatus (Hoefer Scientific 7.3 x 8.3 cm).

A. Separator gel (3.5% acrylamide)

Solution A - 1.575 ml

Solution B - 8.25 ml

Distilled water - 2.925 ml

Solution D - 0.675 ml

TEMED - 15 µl

B. Spacer gel (1% acrylamide)

Solution A - 0.5 ml

Solution C - 4.25 ml

Solution D - 0.25 ml

TEMED - 5 µl

Reservoir buffer

0.025 M Tris/0.192 M glycine pH 8.3 containing 0.1% SDS.

Sample preparation

Lipoprotein samples were dialyzed against the reservoir buffer (1:9, v/v diluted chamber buffer without SDS) for 10 h. To 50 µl of sample containing at least 50 µg proteins 1% SDS, 10% glycerol and 3 µl 2-ME were added and kept in a boiling water bath for 3 min. A tiny speck of bromophenol blue was diluted in 100 µl of reservoir buffer and 3 to 5 µl was added to the prepared sample after cooling. For Western blotting of lipoproteins the sample load was approximately 30 µg of lipoprotein per well. For the comparison of amount of LDL associated with Lp(a) 3.5% SDS-PAGE of plasma Lp(a)-containing lipid layer (PLL) isolated from small and large Lp(a) plasma by jacalin affinity precipitation was done under non-reducing condition (without 2-ME).

Electrophoresis

Electrophoresis was run at 25 °C at 18 mA current till the bromophenol blue dye ran out. After the run gel was fixed in 50% methanol for 45 minutes, stained with Coommassie brilliant blue R-250 and destained.

2.16. Electroelution of lipoproteins

Electroelution of lipoproteins from acrylamide gel was done according to the method described earlier (Ogden and Adams, 1987). Proceeding PAGE, the gels were taken out of the glass tubes and washed with distilled water. One of the gels was then stained for protein with Coommassie brilliant blue R-250 and destained. Using this gel as a marker, individual bands were sliced from the non stained gels and equilibrated in 1 to 2 ml Tris-acetate buffer (0.01 M, pH 8) for 15 min and homogenized using a Potter-Elvehjem homogenizer. Protein in suspensions were electroeluted into the buffer by immersing the bags containing the homogenized gels between opposite electrodes in a rectangular reservoir with 2 L of cold Tris-acetate buffer and applied a constant voltage of 100 V across the electrodes for 3 h. The bags were then dialyzed against PBS, containing 0.1% EDTA and 0.01% sodium azide and the gel pieces were pelleted by brief centrifugation at 3000 g. The protein concentration of the eluted lipoprotein was estimated as described earlier.

2.17. Western blotting of lipoproteins

Following SDS-PAGE in slab the separated lipoproteins were transferred on PVDF membrane as described earlier (Towbin et al., 1979). The PVDF membrane was pre-wetted in methanol and washed in cold transfer buffer consisting of 25 mM Tris, 192 mM glycine, 0.1% SDS and 5% methanol, pH 8.3 prior to sandwiching between the gel and the electrode and a constant current of 0.8 mA per cm^2 of gel

was applied for 3 h at 25 °C. After the transfer PVDF membrane was air dried and preserved closed in cold room. For detection PVDF membrane strips containing western blots of proteins were blocked with PBS-T for 2 h followed by overnight incubation at 4 ºC with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (15 µg per ml) in the above solution. After three washings with PBS-T at 4 ºC and once with PBS alone, protein bands were identified as bound HRP detected by dipping the strips for 5 min in HRP substrate (1 ml 0.3% 4-chloro-1-naphthol in anhydrous methanol mixed with 5 ml PBS and 3 µl 30% H_2O_2) at 25 ºC, followed by washing with PBS. Blots were washed again in PBS and photographed.

2.18. Cell-culture of monocytes isolated from human peripheral mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy volunteers by a density gradient centrifugation method using Ficoll Histopaque 1077 in 1:1 ratio (v/v) (Panda et al., 2012).

Reagents

a. Sterile RPMI -1640 medium (with L-Glutamine and 25 mm HEPES, without sodium bicarbonate)

The entire contents of powdered media in each package were dissolved in 900 ml sterile distilled water at 15-20 °C with gentle stirring under sterile condition. The pH of the solution was lowered to 4.0 with 1N HCl to completely dissolve the media. After rising the pH to 7.2 with 1N NaOH, 2 g sodium bicarbonate was added and stirred until dissolved. While stirring, the pH of the medium was adjusted to 0.1-0.3 pH units below the desired pH with either 1N

HCl or 1N NaOH since it may rise during filtration. The solution was then made up to 1000 ml with sterile distilled water. Media was then sterilized immediately by filtration using a membrane with a porosity of 0.22 microns and aseptically dispensed into sterile containers and stored at 4 °C.

b. Heat-inactivated donor-specific (autologous) human serum

Serum was subjected to heat inactivation by placing in a 55 °C water bath for 30 minutes. After keeping it at 37 °C for 1 h, it was filtered using a 25 mm Whatmann syringe filter and stored at -20 °C as aliquots till use.

c. Ficoll solution (density is 1.077 g per ml): or Histopaque 1077

Ficoll 9% (w/v) in distilled water. Sodium diatrizoate 33.9% in distilled water. For use 12 parts of Ficoll stock were mixed with 5 parts of sodium diatrizoate. Should be stored at 4 °C.

d. Sterile PBS

e. Pencillin-streptomycin solution

f. Gentamycin

g. Freshly collected heparinised blood

Human venous blood was collected in a heparinised tube and mixed well by gently inverting the tube several times. The blood (3 ml) was gently layered on the top of 3 ml Ficoll Histopaque in a 15 ml centrifuge tube in such a way that blood and Ficoll Histopaque stayed as two different layers. After centrifuging the tubes for 30 minutes at 100 g in a swing-out bucket, the whitish buffy coat (about 1 ml) (PBMCs) formed in the interphase between histopaque and medium was aspirated, washed and centrifuged twice in 100 g for 10 minutes with 10 ml of sterile PBS or sterile RPMI medium. The approximate yield of cells from 3 ml of blood varies from 10^5 to 10^6.

After resuspending the cells in 1.6 ml RPMI-1640 medium, cell viability was determined by trypan blue dye exclusion test and was found to be greater than 95%. The mononuclear cells were then seeded into sterile 4 well (400 µl per well) tissue culture plastic wells (17mm diameter) and kept in an atmosphere of 5% CO_2 at 37 °C for 3 h allowing monocytes to adhere and lymphocytes to remain in suspension. Non-adherent cells were removed by washing the wells three times with sterile PBS and the monocytes adhered to dishes were maintained in RPMI 1640 medium supplemented with 10% (v/v) autologous human serum, 100 IU per ml penicillin, 100 µg per ml streptomycin and 100 µg per ml gentamycin for 9 days to differentiate into monocyte derived macrophages (hMDM).

2.19. Oil red O staining

Reagents

a. Isopropanol

b. Oil red O solution - 0.5% oil red O dye in isopropyl alcohol.

A working solution was prepared with 3 parts of 0.5% oil red O in isopropyl alcohol mixed with 2 parts of water. The solution is then filtered to remove any particulates. Should be prepared freshly.

Oil red O is a lysochrome (fat-soluble dye) diazo dye and was used for staining of deposition of lipoproteins in monocytes-macrophage culture or rat cardiac fibroblast culture treated with Lp(a), LDL and adduct in serum free medium (Mat et al., 2011). After removing the media the cell culture was washed three times with PBS. Oil red O solution was layered over the culture for 10 minutes and then few

times in 60% ethyl alcohol to clear the background. Cultures are rinsed quickly twice with distilled water to remove excess stain. Staining was continued with Giemsa stain and then the wells were washed with distilled water and observed under light microscope. Lipoprotein components were stained deep red or deep orange, nuclei stained blue and other structures were unstained.

2.20. Enzyme-labeled immunosorbent assay (ELISA) and enzyme-labeled lectin assay (ELLA)

Lipoproteins, lectins, glycoproteins or other proteins were coated on polystyrene wells of 96 well ELISA plates by incubation of 200 µl of specified concentration of their PBS solution in the wells at 37 °C for 3 h, followed by washing with PBS containing PBS-T, blocking with PBS containing 0.5% Tween 20 for 30 min at 37 °C and finally washing with PBS-T. Wells were then incubated at 4 °C with the specified concentrations of HRP-labeled lectins, glycoproteins or antibodies in 200 µl PBS-T for 2 h and washed thrice with the same buffer. To assay the bound HRP, the washed wells were treated with 200 µl OPD (0.5 mg per ml) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.03% H_2O_2 for 30 minutes, followed by addition of 50 µl of 12.5% H_2SO_4 and absorbance measurement at 490 nm in a BIOTECH (USA) ELISA reader.

For quantitation of concanavalin A (Con A) binding, wells coated as above with proteins were treated with 200 µl of specified concentration of lectin solution in PBS-T for 2 h at 4 ºC followed by washing with PBS-T. Bound lectin was measured by further incubation with 200 µl of specified concentration of HRP solution in PBS-T for 1 h, washing and OPD treatment as above.

For quantitation of biotin-labeled lipoprotein binding, wells coated as above were treated with 200 µl the specified concentration of biotin-labeled protein in PBS-T for 2 h at 4 ºC followed by washing with PBS-T. Bound biotin-labeled protein was measured by further incubation with 200 µl of specified concentration of HRP-labeled avidin in PBS-T for 2 h, washing and OPD treatment as above.

To quantify HRP-labeled jacalin used as tracer to study distribution of jacalin in ultracentrifugation layers, 100 µl of an appropriate dilution of respective layer was treated with 100 µl OPD solution and absorbance measured as above.

2.21. Inhibition ELISA

Polystyrene wells were coated with glycoproteins or Lp(a) and blocked as described above. One twenty microlitre of specified concentration of HRP-labeled galectin-1 was pre-incubated for 1 h at 4 ºC with 120 µl of varying concentrations of inhibitors [lipid layer of plasma (U-20%), lactose or plasma glycopeptides] in PBS-T and 200 µl of this mixture was added to the blocked wells. Bound HRP was measured as above and reduction in HRP-labeled galectin-1 binding to glycoproteins or Lp(a) was taken as measure of inhibition.

2.22. Lp(a) assay on polystyrene wells by jacalin method (j-a method)

Lp(a) in plasma, electroeluted samples, 0.8% PEG [LDL-free Lp(a)] and PLL was assayed by a lectin-assisted immunoassay procedure recently reported by Anuradha et al. (2013). Briefly polystyrene wells were coated with jacalin (1 µg per well), After blocking dilutions of plasma or other Lp(a) samples in 200 µl PBS-T were added and wells incubated for 2 h at 4 ºC followed by three washings with PBS-T. Wells were then incubated with 200 µl of specified concentration of HRP-

labeled anti-human apo(a) antibody in PBS-T for 2 h at 4 ºC, washing and OPD treatment as above.

2.23. Enzyme treatment of lipoproteins on polystyrene wells

Polystyrene wells were coated with lipoproteins (100 ng per well) as described above. After washing and blocking, coated lipoproteins were desialylated by incubating with 200 µl of 5 mU neuraminidase solution in PBS-T at 37 ºC for 1h. Heat inactivated neuraminidase (+NH) was used in control experiment.

2.24. Immobilization of galectin-1

Galectin-1 was immobilized by a protocol described earlier (Paul et al., 2011). Briefly, biotin-labeled galectin-1 prepared (as described in 'Methods 2.10') was incubated with 25 mM lactose for 2 h at 4 °C. For immobilization, this biotin-labeled galectin-1 pre-incubated with sugar (5 µg per well) was added to blocked streptavidin coated (1 µg per well) polystyrene wells and kept at 4 ºC for 2 h. Immobilization of galectin-1 was confirmed by addition of 2 µg per well HRP-labeled fetuin in the presence and absence of 50 mM lactose.

2.25. Assay on polystyrene wells to check oxidation status of LDL

The extent of oxidation of LDL was checked by assaying the efficiency of copper oxidised LDL to excite a nonfluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein (DCF) as described by (Zhang et al., 2010). Briefly, LDL (100 ng per well) was coated on polystyrene wells as described above and after blocking, DCFH-DA (20 µg per well) in PBS was added and wells were incubated at 37 ºC for 2 h under dark. The oxidation status of LDL was checked by detecting its

fluorescence at excitation wavelength of 480 nm and emission wavelength of 520 nm in a fluorescent ELISA (BIOTECH, USA) reader. Oxidised LDL prepared as above was used as a control.

2.26. Statistical analysis

All results are expressed as mean ± SD. Student's paired t test and ANOVA were used to determine the significance of differences between the groups. A value of $P < 0.05$ was considered as statistically significant.

3. Isolation and characterization of Lp(a) isoforms

3.1. Isolation of Lp(a) isoforms by affinity precipitation from plasma by jacalin

Jacalin solution in PBS (4 mg in 5 ml) was added to a stirred solution of 5 ml plasma in proportions and the mixture was kept at 4 ºC overnight. The precipitate formed was collected by centrifuging at 64,000 g for 45 minutes and redissolved in 2 ml of 150 mM 1-O-methyl α-D-galactopyranoside in PBS for 2 h at 4 ºC. The mixture was adjusted to a density of 1.24 g per ml with KBr and subjected to ultracentrifugation at 535,000 g for 4 h in 1 ml tubes. The jacalin-precipitated lipids (JL1) which segregated to the upper 20% fraction (U-20%) was collected from each tube and pooled. Sub-lipid layers, middle 50% (JPM-50%) and bottom 30% (JPB-30%) were also collected in order to check the efficiency of sugar treatment in releasing Lp(a) from jacalin precipitate. Plasma without jacalin (OL1, as a control) and the supernatant of jacalin precipitate (JSL1) were also subjected to ultracentrifugation at 535,000 g for 4 h and lipid layer which floated in the upper 20% volume fraction was collected in order to check the efficiency of jacalin in precipitating Lp(a) isoforms from plasma.

To follow distribution of jacalin during the above isolation procedure HRP-labeled jacalin (0.5% of total) was included in some trials.

In one preparation JL1 was collected after washing jacalin precipitate with same concentration of jacalin solution to remove any non-specifically sticking plasma LDL to the Lp(a)-jacalin precipitate.

3.2. Tris-borate EDTA (TBE) disc gel electrophoresis of lipid layer of jacalin-precipitated lipids (JL1) followed by electroelution

JL1 which contained all the Lp(a) was subjected to 3.75% native PAGE using Tris-borate EDTA buffer, lipoprotein bands were cut and electroeluted as described in 'Methods 2.15 (i)'. To confirm the lipoprotein nature of the electroeluted bands, each of them was subjected to ultracentrifugation at 535,000 g for 4 h after adjusting density to 1.24 g per ml with KBr, and analyzing the presence of apo(a) containing bands in various layers by ELISA as described earlier.

Lp(a)-rich fraction of plasma isolated by conventional method (as described in 'Methods 2.6') was also subjected to 3.75% TBE electrophoresis and electroelution in order to compare the enrichment of Lp(a) in lipid layer after jacalin precipitation.

3.3. Immunoblotting of JL1 to characterize Lp(a) isoforms

JL1 was subjected to 3.5% SDS-PAGE under reducing conditions followed by western blotting on PVDF membrane as described earlier. Apo(a) and apoB bands were identified by probing with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (15 μg per ml) respectively as described above. The ratio of mobility

of apoB subunit [mB] to that of apo(a) [m(a)] was used as an index of molecular size of apo(a), since apoB size remains constant among Lp(a) isoforms.

4. Study of glycosylation difference in Lp(a) isoforms

4.1. Differential response of lectins to Lp(a) containing lipid layer of plasma

The lipid-rich upper 20% volume (U-20%) obtained after ultracentrifugation of 1 ml plasma at 535, 000 g for 4 h at 4 °C was coated on polystyrene wells (1 μg per well), blocked and washed as described earlier. To check the differential response of lectins, wells were probed with HRP-labeled anti-human apo(a) (300 ng per well), anti-human apoB (300 ng per well), galectin-1 (1.5 μg per well), jacalin (4.7 ng per well) and Con A (2 μg per well) as described earlier.

Response of galectin-1 to various Lp(a) samples was also checked by inhibition ELISA using Lp(a)-containing lipid layer of plasma (U-20%) as an inhibitor to decrease the binding of HRP-labeled galectin-1 to asialofetuin. HRP-labeled galectin-1 (900 ng in 120 μl) was pre-incubated with 120 μl of lipid layer containing varying amounts of Lp(a) assayed by j-a method (Methods 2.22) in PBS-T for 1 h at 4 °C and 200 μl of this mixture was added to asialofetuin coated polystyrene wells and bound HRP was quantitated as described earlier. The minimum concentration of Lp(a) in the lipid layer required to get 50% inhibition of galectin-1 binding (inhibitory concentration, IC 50) to asialofetuin was determined.

4.2. Determination of availability of antigenic and oligosaccharide epitopes in subunits of Lp(a) isoforms

Lp(a) isoforms were coated on polystyrene wells (100 ng per well). Wells were blocked and washed as described earlier and incubated at 4 °C with the specified concentrations of HRP-labeled anti-human apo(a), anti-human apoB, jacalin, galectin-1 and Con A. Bound HRP was quantitated as described above and lectin binding per unit anti-apo(a) response was determined.

To determine availability of antigenic epitopes on apoB subunit, Lp(a) isoforms diluted in PBS-T were captured on jacalin previously coated to polystyrene wells (1 μg per well) as described earlier. After incubation at 4 ºC for 2 h, followed by washing with PBS-T wells were treated with HRP-labeled anti-human apo(a) or anti-human apoB antibody (300 ng per well) and absorbance was measured as earlier. Extend of availability of apoB subunit is expressed as the ratio of absorbance for anti-apoB to that for anti-apo(a).

Differential accessibility of epitopes of apoB subunit in different Lp(a) isoforms in the presence or absence of apo(a) were also checked by reducing the electroeluted Lp(a) (500 ng per ml) with DTT as described earlier to break the disulfide bond between apo(a) and apoB in Lp(a) and to release an apo(a)-free lipoprotein [Lp(-a)] from Lp(a). After adjusting the reaction mixture to a density of 1.24 g per ml the lipoprotein solution was subjected to ultracentrifugation at 535, 000 g and Lp(-a) was collected in the upper 20% volume (U-20%). Five dilution of the lipid layer was directly coated on polystyrene wells and availability of apoB epitope for anti-apoB antibody as well as for lectin (Con A) in reduced and non-reduced

Lp(a) was checked by ELISA. Freshly isolated Lp(a) was used for all the above experiments.

5. Study of association between Lp(a) and LDL to form adduct

5.1. Isolation of free and complexed lipoproteins from plasma and comparison of their lipoprotein composition with respect to anti-apo(a) and anti-apoB response

Free lipoprotein layer was collected as upper 10% volume (U-10%) after subjecting 1 ml samples of plasma to ultracentrifugation at 535,000 g for 4 h at 4 °C. After discarding 10% - 20% layer from top, bottom 80% fraction was made up to 1 ml with PBS and again subjected to ultracentrifugation at 220, 000 g for 4 h at 4 °C and upper 70% (U-70%) volume containing lipoprotein complexes were collected. After dialysis complexed lipoproteins were precipitated using 2% PEG-6000 (700 µl lipid fraction containing complexed lipoproteins was treated with 140 µl of 12% PEG-6000 solution) in veronal buffer pH 7.4 as described in 'Methods 2.7'. After centrifugation precipitated lipoprotein complexes were collected and redissolved in 200 µl PBS. Soluble proteins in free and complexed lipoproteins were estimated as described above and 1 µg per well proteins were directly coated on polystyrene wells and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies as described earlier. The response of anti-apo(a) and anti-apoB reflects the amount of Lp(a) and LDL in complexed lipoproteins respectively.

5.2. Isolation of plasma Lp(a)-containing lipids (PLL) to study Lp(a):LDL adduct

Three different protocols were employed to isolate PLL from plasma;

(i) In the first protocol, lipid layer of jacalin precipitate of plasma (JL1) isolated by affinity precipitation with jacalin as described earlier was taken as PLL.

In order to rule out non-specific association as cause for Lp(a):LDL adduct formation, PLL was isolated using the same concentration of the lectin as described earlier (0.4 mg per ml), but using 5 times diluted plasma in PBS.

(ii) In the second protocol, PLL was isolated by jacalin affinity chromatography. Lipid layer of plasma (U-10%) obtained upon ultracentrifugation of 8 ml plasma at 535, 000 g for 4 h at 4 °C was diluted 5 times with PBS 6.5 and loaded on 2 ml jacalin-Sepharose column (0.2 mg jacalin per ml). After washing the column with PBS 6.5 bound proteins were eluted with 0.8 M galactose in PBS. Eluate was used as PLL.

PLL thus isolated were coated on polystyrene wells, blocked and probed separately with anti-human apo(a) and anti-human apoB antibodies and bound HRP was measured as described earlier. Extend of adduct formation was calculated from the ratio between response to anti-apoB ([apoB]) and anti-apo(a) ([apo(a)]) and the index of adduct was expressed as the increase in the ratio between [apoB] and [apo(a)] from the corresponding ratio for pure Lp(a) isolated from the same plasma.

(iii) For cell culture studies adduct [devoid of LDL-free Lp(a)] was isolated by jacalin affinity precipitation as described above from the dialysed supernatant after removal of 0.8% PEG precipitate from plasma.

5.3. De novo adduct formation with electroeluted Lp(a) and LDL

De novo adduct formation was demonstrated by two different protocols;

(i) In one polystyrene wells coated TBE-purified Lp(a) (100 ng per well) was incubated with 200 ng per well biotin-labeled LDL for 4 h at 4 °C and bound LDL was detected by HRP-labeled avidin (300 ng per well) as described earlier.

(ii) In the other, adduct was allowed to form between TBE-purified Lp(a) (100 ng) and LDL (200 ng) by incubating the two in 240 µl PBS at 4 °C for 4 h and to that 10 µl of biotin-labeled jacalin (125 µg per ml) was added and the mixture incubated for 2 h at 4 °C. Two hundred microlitre of this mixture in PBS-T was added to streptavidin-coated (1 µg per well) polystyrene wells to capture jacalin-Lp(a):LDL adduct complex and probed separately with HRP-labeled anti-human apo(a) and anti-human apoB antibodies and bound HRP was measured as described earlier. Extent of adduct formation was determined as described above.

5.4. Precipitation of Lp(a) from plasma with varying concentrations of PEG

Plasma (1 ml) was treated with varying percentage of PEG-6000 (0.2%, 0.4%, 0.6%, 0.8% and 1%) in veronal buffer as described in 'Methods 2.7' to fix an appropriate PEG percentage to selectively precipitate LDL-free Lp(a) form plasma. PEG precipitate collected was redissolved in 100 µl PBS. Out of the above mentioned PEG percentages, 0.6%, 0.8% and 1% PEG precipitate of plasma, were subjected to 3.75% TBE disc gel electrophoresis as described earlier and protein bands were identified by staining with Coommassie brilliant blue R-250.

5.5. Assay to check whether PEG-6000 can precipitate free LDL from plasma

Lipid layer of plasma (U-5%) collected after ultracentrifugation of 6 ml plasma in 1 ml tubes at 535,000 g for 4 h at 4 °C was made up to 6 ml with PBS and divided into three aliquots. Two ml aliquots were treated with 1 ml jacalin-Sepharose

gel (2 mg jacalin per ml gel), 1 ml Sepharose gel and 1 ml PBS respectively and kept at 4 °C for 2 h with intermittent mixing. The removal of Lp(a) or Lp(a):LDL adduct by jacalin-Sepharose in the gel supernatant was checked by assaying Lp(a) by j-a method. The gel treated and un-treated lipid layers were then subjected to 1% and 3% PEG treatment as described above and checked for the precipitate.

5.6. Effect of PEG-6000 on Lp(a):LDL adduct

Effect of PEG-6000 on Lp(a):LDL adduct was checked by assaying the efficiency of de novo adduct formation in the presence of 0.6%, 0.8% and 1% PEG solution. Two hundred microlitre of biotin-labeled LDL (200 ng per well) with 0.6%, 0.8% and 1% PEG was added to blocked, polystyrene well-coated TBE-purified Lp(a) (100 ng per well) and kept at 37 °C for 30 minutes and then for overnight at 4 °C. Bound LDL was detected by HRP-labeled avidin (300 ng per well) as described earlier.

5.7. Assay to check the role of lysine binding site of apo(a) in adduct formation

Two different protocols were employed; (i) Efficiency of polystyrene wells coated with TBE purified Lp(a) to form de novo adduct with biotin-labeled LDL in the presence of 100 mM lysine, ε–aminocaproic acid and proline was checked by ELISA as described in ‘Methods 5.3 (i)’. (ii) PLL isolated from plasma by jacalin precipitation with or without prior incubation with 0.1 M proline at 37 ºC for 15 minutes was subjected to 3.75% TBE disc gel electrophoresis.

5.8. Assay to check the role of sialic acid moiety of apo(a) in adduct formation

Polystyrene wells coated with neuraminidase treated and non-treated Lp(a) were checked for their efficiency in de novo adduct formation with biotin-labeled LDL using ELISA as described earlier.

5.9. Qualitative analysis of LDL in adduct and non-adduct (free) forms

Jacalin-precipitated plasma lipids (PLL) and lipid layer of supernatant after jacalin precipitation (JSL1) were used as sources of adduct LDL and free LDL respectively. Both PLL and JSL1 were subjected to 3.75% TBE disc gel electrophoresis as described earlier. One gel from each group was stained with Coommassie brilliant blue R-250 to compare the mobility of adduct-forming LDL and free LDL. Electroeluted lipoprotein bands were identified by ELISA as described earlier.

To check the difference in glycosylation between adduct and non-adduct LDL, TBE purified adduct and non-adduct LDL isolated as above were coated on polystyrene wells (100 ng per well), blocked and probed with HRP-labeled lectins wheat germ agglutinin (WGA), galectin-1 and Con A as described earlier.

Oxidation status of adduct and non-adduct LDL coated on polystyrene wells was checked as described ‘Methods 2.25’. Copper induced oxidized LDL prepared as described in ‘Methods 2.13’ was used as a control. Efficiency of de novo adduct formation of polystyrene well-coated TBE purified Lp(a) (100 ng per well) with adduct-forming LDL, free (non-adduct) LDL and copper oxidised LDL was compared as described in ‘Methods 5.3 (i).

5.10. Identification of Lp(a):LDL adduct with varying stoichiometry

Plasma (2.5 ml) was treated with varying percentage of PEG-6000 (0%, 1.5%, 2%, 2.5% and 3%) in veronal buffer as described in 'Methods 2.7' and the supernatant of PEG precipitate collected after centrifugation at 2000 g for 20 minutes was dialysed against PBS. Lp(a):LDL adduct remaining in the supernatant was isolated by jacalin affinity precipitation as described in 'Methods 5.2. PLL thus collected was coated on polystyrene wells (1 μg per well) and assayed for adduct as described earlier. The above mentioned PLL was also subjected to 3.75% TBE disc gel electrophoresis at pH 8.7 as described above and protein bands were stained with Coommassie brilliant blue.

6. Physiological significances of Lp(a):LDL adduct

6.1. Cell culture studies to check binding of Lp(a):LDL adduct to LDL-receptor

Nine days old human monocyte-macrophage culture as well as 75% confluent (P3 passage) rat cardiac fibroblast culture in serum deprived condition was washed twice with sterile PBS and incubated with 4 μg protein of TBE purified Lp(a), LDL or PLL devoid of LDL-free Lp(a) (source of adduct, isolated as described in 'Methods 3.1 and 5.2') in 400 μl RPMI medium in an atmosphere of 5% CO_2 at 37 ºC for 24 h. Control without cells was also kept. The cell supernatant was assayed for Lp(a), LDL and adduct as described in 'Methods 2.20, 2.22' and compared with that of cell-free control. Rat cardiac fibroblast cells treated with Lp(a), LDL or PLL devoid of LDL-free Lp(a) as described above was washed twice with sterile PBS and stained with oil red O as described in 'Methods 2.19' to check the uptake of Lp(a), LDL or Lp(a):LDL adduct by cells.

In order to rule out the dissociation of Lp(a) or adduct during cell treatment, 200 µl of cell supernatant in PBS-T was added to blocked, polystyrene wells-coated jacalin (1 µg per well) and probed separately with anti-human apo(a) and anti-human apoB antibodies freed as described earlier. Integrity of Lp(a) and adduct was checked by comparing the ratio between response to anti-apoB ([apoB]) and anti-apo(a) ([apo(a)]) with that for cell-free controls.

6.2. Binding of de novo adduct to immobilized galectin-1

De novo adduct was prepared from TBE-purified Lp(a) (100 ng) and FITC-labeled LDL (200 ng) by incubating the two in 240 µl PBS at 4 °C for 4 h. De novo adduct formed (200 µl) was added to immobilized galectin-1 captured on streptavidin-coated polystyrene wells (as described in 'Methods 2.24') and incubated for 2 h at 4 ºC under dark. The binding of de novo adduct to immobilized galectin-1 was checked by measuring the bound fluorescence at excitation wavelength of 492 nm and emission wavelength of 515 nm using fluorescence ELISA (BIOTECH, USA) reader. Control using LDL-FITC alone was also used to record non-specific binding of LDL.

6.3. Isolation and purification of glycopeptides from albumin-free fraction of plasma proteins

Albumin-free fraction of plasma proteins was collected by 45% ammonium sulfate precipitation of 5 ml plasma diluted in PBS in 1:2 ratio. The precipitated protein was collected by centrifugation at 14, 000 g for 20 minutes at 4 ºC and redissolved in 2 ml PBS (26.68 mg per ml average) and reduced by treating with 10 mM DTT for 25 minutes at 37 ºC. The protein solution was then dialysed against 10

mM Tris-HCl buffer, pH 7.9 and subjected to pronase digestion (2 mg pronase per 100 mg protein) in the presence of 2 mM Ca_2Cl and 2 mM sodium azide at 37 ºC for 2 days. The pronase digest of proteins was subjected to ion-exchange chromatography on 15 ml DEAE-Sephadex A-50 column packed in 10 mM Tris-HCl buffer, pH 7.9. Unbound proteins were washed out with the same buffer and bound protein was eluted with 250 mM NaCl in the same buffer. Fractions containing protein and neutral sugar were pooled and dialysed against distilled water. After lyophilisation glycopeptide was redissolved in 2 ml PBS, protein content as well as neutral sugar content was quantitated and used as an inhibitor of galectin-1 binding to Lp(a) as described in 'Methods 2.21'.

To check the enrichment of O-glycans in isolated plasma glycopeptide crude protein in 45% ammonium sulfate precipitate of plasma and isolated glycopeptide in PBS (1 µg per well) were coated on polystyrene wells, blocked and probed with specified concentrations of lectins like jacalin and Con A as described earlier.

In order to check whether pronase itself can bind to DEAE-Sephadex A-50, pronase digest (50 µl) obtained after self digestion under the same conditions as described above was treated separately with (100 µl) packed gel of DEAE-Sephadex A-50 or Sephadex A-50 in 10 mM Tris-HCl buffer, pH 7.9 in the ratio 1:10 and kept at 4 ºC for 2 h with intermittent mixing. Supernatant was assayed for protein.

To check whether there is any substantial loss of glycopeptides during dialysis (using 10 kDa molecular weights cut off membrane), DEAE-eluted fractions were pooled and subjected to ultrafiltration through PM-10 membrane under N_2 gas. The presence of any jacalin-inhibitory material in the filtrate was checked by comparing its ability to decrease the binding of HRP-labeled jacalin to polystyrene

well-coated asialofetuin with that of the concentrate using inhibition ELISA as described earlier.

6.4. Hemagglutination inhibition assay to check the presence of any blood group antigens in isolated glycopeptides

Five microlitre of glycopeptides (1 mg per ml) pre-incubated with 20 µl of anti-human blood group A antibody or anti-human blood group B antibody at 4 ºC for 30 minutes was added to blocked U-bottom polystyrene wells (Laxbro) and mixed with human erythrocytes (25 µl of 20% suspension in PBS) from A and B blood group respectively. Agglutination was checked after 1 h with mixing. Settling of erythrocytes within 2 minutes after the final mixing was marked as positive agglutination.

RESULTS

PART- I

Isolation and purification of Lp(a) isoforms by jacalin affinity precipitation followed by electrophoresis

Variations in kringle IV type 2 repeats of apo(a) gives rise to intra- and inter-individual size heterogeneity in Lp(a) making it a highly polymorphic molecule. Genetic studies showed that the presence of two Lp(a) isoforms in an individual is likely. For study of the role of size polymorphism in pathogenesis as well as the fate of Lp(a) in plasma it is imperative to obtain all Lp(a) isoforms in purified form. Conventional protocol employing sequential ultracentrifugation from serum or plasma (Kostner et al., 1999) is not effective in the complete separation of Lp(a) from LDL because of overlapping size distribution and the tendency of Lp(a) to aggregate with LDL (Gaubatz et al., 2001). Heterogeneity in apo(a) isoform size, lysine-binding capacity, extent of glycosylation and in apolipoprotein content makes it arduous and laborious to isolate Lp(a) from other plasma lipoproteins utilising density difference alone. Moreover it would be difficult to separate the two Lp(a) isoforms in an individual unless a significant difference exists in the size of the two apo(a)s. Another method used electrophoresis to purify Lp(a) after sequential ultracentrifugation (Chellan et al., 2006). While this protocol eliminated contaminating LDL, yield of less abundant isoforms was poor. Hence the need for a new protocol to isolate all Lp(a) isoforms in pure form regardless of abundance. In the present section affinity precipitation of plasma with the lectin, jacalin was employed to isolate Lp(a) isoforms differing in size and abundance.

1.1. Isolation of Lp(a) isoforms from plasma by affinity precipitation with jacalin

Jacalin, a lectin isolated from the seeds of jack fruit (*Artocarpus integrifolia*) possesses high affinity for O-linked oligosaccharides containing core-1 type T

antigen structure [Gal β 1→3 GalNAc] leading to precipitation of O-glycosylated proteins. Lp(a) is uniquely rich in T antigen containing O-glycans. This property of jacalin was exploited to precipitate all Lp(a) isoforms from twice diluted plasma (with PBS) by incubating overnight with jacalin (0.4 mg per ml) solution. The precipitate thus formed was treated with a solution of 1-O-methyl α-D-galactoside, a monosaccharide specific for jacalin, so as to dissociate the lattice of jacalin and Lp(a). The clear solution thus obtained contains a mixture of free jacalin, released Lp(a) and other O-glycans. Lp(a) was then separated from this mixture by ultracentrifugation and collected in the top 20% lipid-rich layer (JL1).

Results in Fig.1.A shows that 0.4 mg per ml jacalin could precipitate all Lp(a) from plasma since lipid layer of supernatant after jacalin precipitation (JSL1) contained no detectable amount of Lp(a). Fig.1.A also indicates that 150 mM 1-O-methyl α-D-galactopyranoside, a sugar specific to jacalin was effective in complete dissociation of Lp(a) from jacalin as the denser bottom layers of ultracentrifuged fractions of jacalin precipitate (JPM-50%, JPB-30%), where undissociated jacalin-Lp(a) complex if any could be found due to higher density, were free from appreciable amounts of Lp(a).

To confirm complete dissociation of jacalin from jacalin-precipitated plasma Lp(a) following its sugar treatment and to rule out the presence of jacalin in the lipid layer of jacalin precipitate, two different protocols were performed. In the first protocol HRP-labeled fetuin was used as a probe to detect jacalin (in Fig.1.B). Being an O-glycosylated protein fetuin is rich in core-1 type glycan structures and has a high affinity to jacalin. In the second protocol, HRP-labeled jacalin was added as

tracer at a concentration of 0.5% of total jacalin in the procedure described in Fig.1.C. Results (Fig.1.B and C) show that JL1 fraction containing the entire jacalin-precipitated Lp(a) was devoid of jacalin.

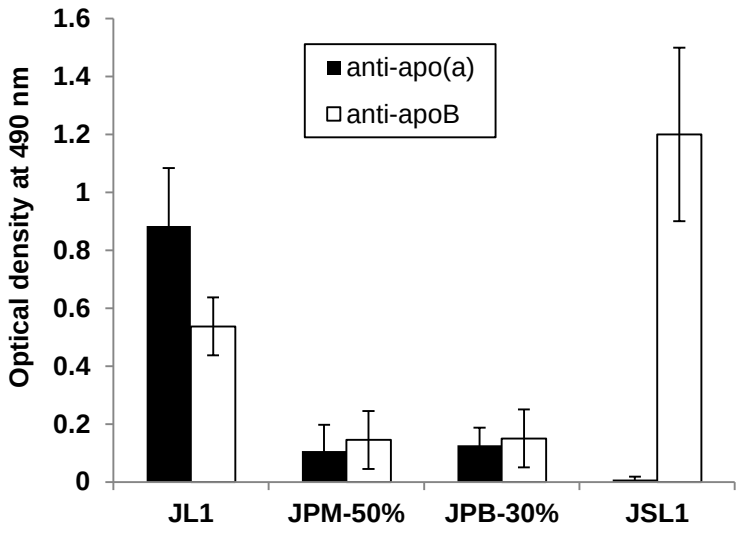


Fig.1.A. Complete precipitation of Lp(a) from plasma by jacalin and its recovery from jacalin precipitate: Jacalin-precipitated plasma proteins redissolved in jacalin-specific sugar (150 mM 1-O-methyl α-D-galactoside) and subjected to ultracentrifugation in 1 ml tubes (Methods 3.1) were collected in fractions as upper 20% containing lipoproteins (JL1), middle 50% (JPM-50%) and bottom 30% (JPB-30%) as non-lipoprotein fractions. Plasma lipoproteins not precipitated by jacalin (JSL1) were also collected as upper 20% after ultracentrifugation of supernatant of jacalin precipitate. Proteins were coated on polystyrene wells (1 μg per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well) and bound HRP was assayed as described in 'Methods 2.20'. Values are mean ± SD of six trials ($p < 0.0001$ for anti-apo(a) response in JL1 vs other samples).

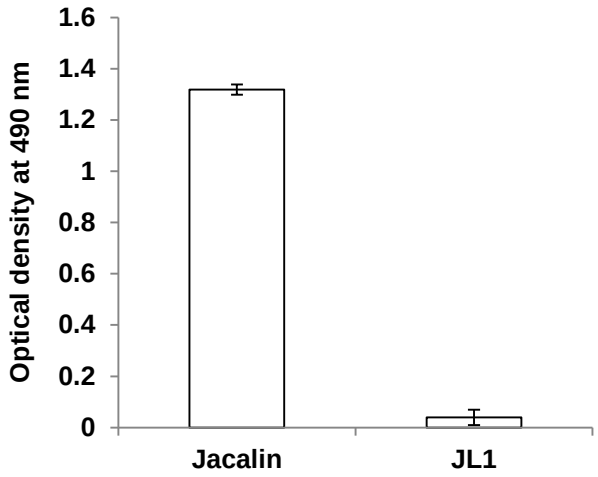


Fig.1.B. Assay to check the presence of jacalin in JL1: Jacalin and JL1 were coated (2 µg protein per well) on polystyrene wells, blocked and probed with 750 ng per well HRP-labeled fetuin and bound HRP was assayed as described in 'Methods 2.20'. Values are mean ± SD of twelve trials.

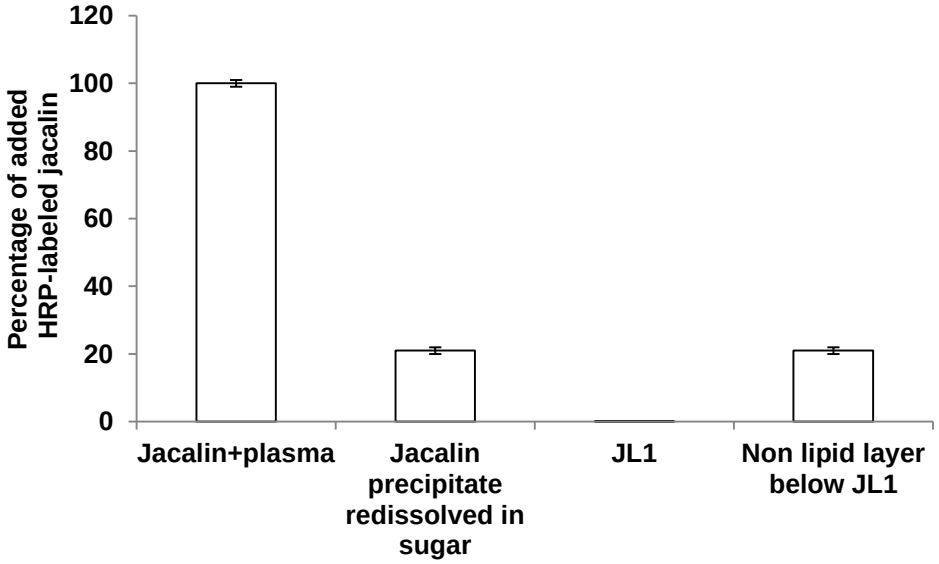


Fig.1.C. Jacalin distribution at various stages of Lp(a) isolation: The presence of jacalin in different stages of Lp(a) isolation was detected by assaying jacalin-HRP used as tracer (0.5% of total). For this 100 µl of an appropriate dilution of test solution in 0.1 M citrate phosphate buffer pH 5.0 was incubated at 4 °C for 30 min with 100 µl OPD (0.5 mg per ml) in the same buffer containing 0.03% H_2O_2, followed by addition of 50 µl of 12.5% H_2SO_4. Absorbance was measured at 490 nm and plotted as percentage of absorbance of total added HRP-labeled jacalin. Values are mean ± SD of three trials.

1.2. Purification of Lp(a) from lipid layer of jacalin precipitate by TBE disc gel electrophoresis followed by electroelution and identification of eluted bands

When jacalin-precipitated plasma lipoproteins (JL1) were subjected to TBE disc gel electrophoresis, two different electrophoretic patterns were observed (Fig.1.D). Some samples resolved as two bands while others resolved as three. After electroelution followed by polystyrene well coating, when these bands were probed with anti-apo(a) and anti-apoB all bands except the fast moving one indicated presence of both apo(a) and apoB subunits in them (Fig.1.E,F). The two gels in Fig.1.D represent two and three lipoprotein band phenotypes respectively in JL1 sample loaded. Both showed a fast moving band which contained apoB, but not apo(a). Rest of the bands contained both the subunits. Bands containing both apo(a) and apoB antigens could be either Lp(a) or apo(a)-apoB chain derived from Lp(a) while those containing only apoB could be either LDL or apoB derived from Lp(a) or LDL.

In order to verify the lipoprotein nature of electroeluted bands of Fig.1.D, they were subjected to ultracentrifugation and fractions were collected as lipid layer (upper 20%) and non-lipid layer (bottom 30%). When these fractions were directly coated on polystyrene wells and probed with HRP-labeled anti-apo(a) and anti-apoB, response was found only in the upper 20% layer (Fig.1.G) confirming that the electroeluted bands contained lipoproteins rather than apolipoproteins or subunits. Thus the fastest moving band in Fig.1.D was confirmed as LDL and those behind it as Lp(a). This result is in accordance with the findings of (Utermann et al., 1987)

who first demonstrated the existence of at least two different Lp(a) isoforms in an individual.

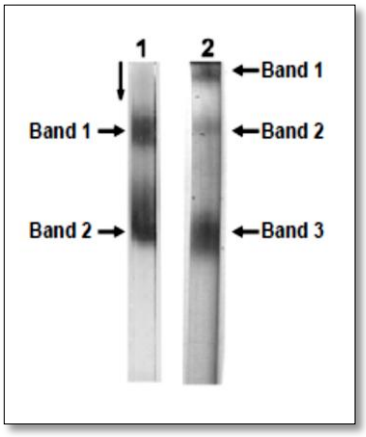


Fig.1.D. TBE disc gel electrophoresis of lipid layer of jacalin precipitate (JL1): Coommassie brilliant blue staining after 3.75% TBE disc gel electrophoresis at pH 8.7 of JL1 samples (50 µg proteins in each) containing two (1) and three (2) bands.

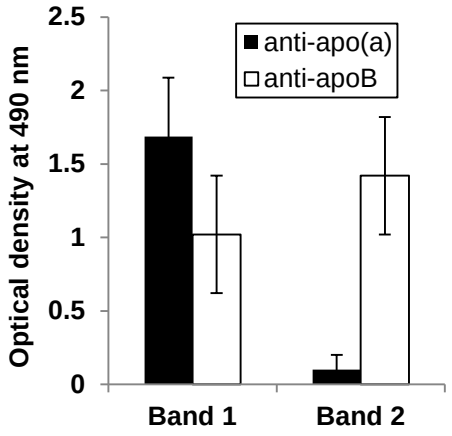


Fig.1.E

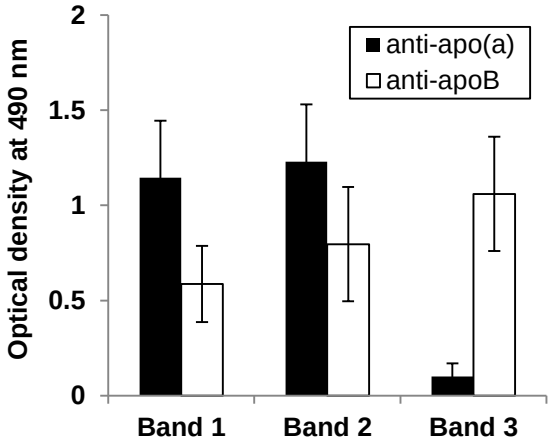


Fig.1.F

Fig.1.E,F. Identification of electroeluted bands of Fig.1.D by ELISA: Same amount of electroeluted bands (100 ng per well) were coated on polystyrene wells and probed with HRP-labeled anti-human apo(a) or anti-human apoB antibodies (300 ng antibody per well). Singe Lp(a) band and double Lp(a) band phenotypes of Fig.1.D are shown in E and F respectively. Values in each group in Fig.1.E and F are mean ± SD of fourteen trials.

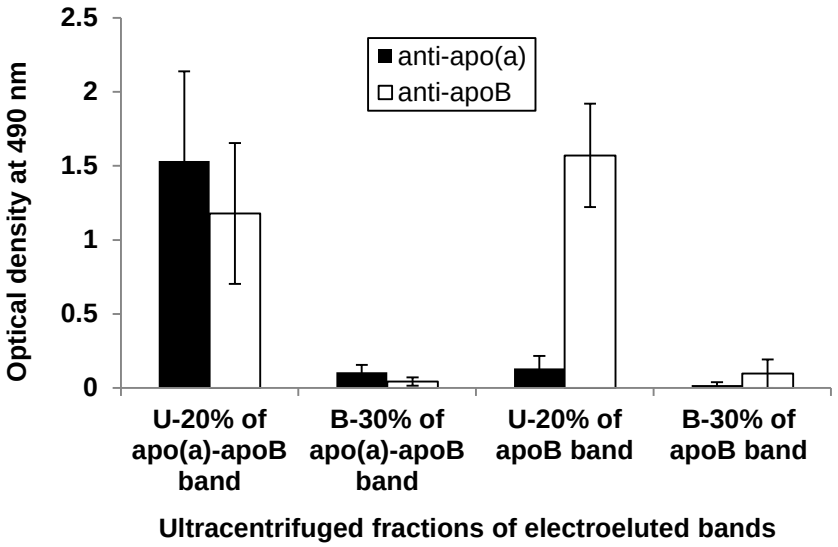
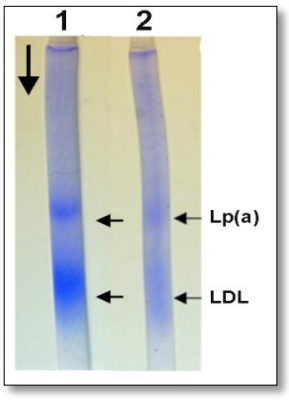


Fig.1.G. Assay to check the integrity of electroeluted bands: Electroeluted bands (100 ng per well) containing both apo(a) and apoB or apoB alone (Fig.1.E,F) were subjected to ultracentrifugation (as described in 'Methods 3.2'). Upper 20% (U-20%) and bottom 30% (B-30%) were diluted 5 times in PBS and coated on polystyrene wells and probed with 300 ng per well HRP-labeled anti-apo(a) or anti-apoB. Values are mean ± SD of six trials. $p < 0.0001$ for both anti-apo(a) and anti-apoB responses in U-20% vs B-30%.

Non-specific adherence of LDL to Lp(a) in JL1 was ruled out since further washing of jacalin precipitate with jacalin solution (0.4 mg per ml) did not result in removal of LDL from JL1 (Fig.1.H). LDL was found not to be recognized by jacalin (Fig.1.I) in agreement with the known oligosaccharide composition of this lipoprotein. Possibility of immune complex of LDL with the jacalin-binding immunoglobulin IgA1 getting included in JL1 is excluded since the latter has been found to be devoid of immunoglobulins (Fig.1.J). The only route therefore for LDL to be part of JL1 is through formation of Lp(a):LDL adduct which has been reported to be a major characteristic of Lp(a) (Yashiro et al., 1993, Trieu and McConathy, 1990). Structure and properties of Lp(a):LDL adduct was examined in detail in subsequent chapters (see Results part-III and IV).



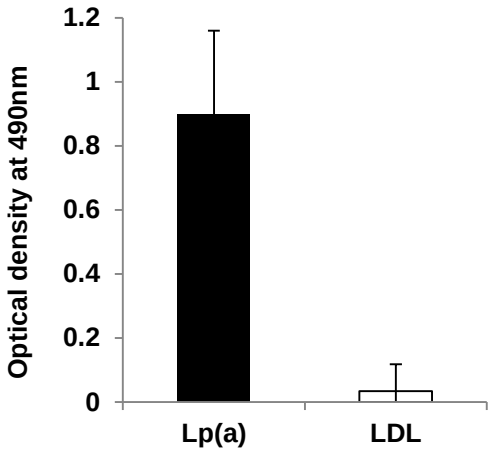


Fig.1.H **Fig.1.I**

Fig.1.H,I. LDL in JL1 is specifically interacted to Lp(a): (H) JL1 collected as described above (1) and after washing jacalin precipitate with same concentration of jacalin solution (2), to remove any non-specifically sticking plasma LDL to the Lp(a)-jacalin precipitate, were subjected to TBE electrophoresis (50 µg proteins in each). Lipoprotein bands were stained and electroeluted bands were identified as Lp(a) and LDL by ELISA as described earlier. Result was confirmed with three different set of experiments. **(I)** Electroeluted Lp(a) and LDL (100 ng per well) coated on polystyrene wells, blocked and probed with HRP-labeled jacalin (4.7 ng lectin per well). Bound HRP was assayed as described earlier. Values are mean ± SD of nine trials.

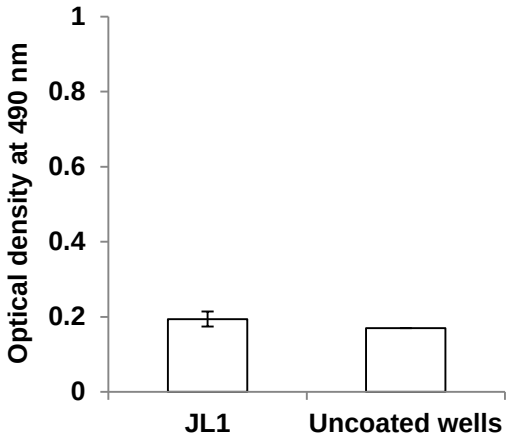


Fig.1.J. JL1 is devoid of antibodies: JL1 was coated (1 µg per well) on polystyrene wells, blocked and probed with mixture of HRP-labeled anti-human IgA, IgG and IgM antibodies (300 ng antibodies in each per well). Bound HRP was assayed as described earlier. Values are mean ± SD of seven trials.

1.3. Advantages of the new protocol for Lp(a) isolation

1.3.1. Enrichment of Lp(a) in lipid layer of jacalin precipitate (JL1)

Above results show that jacalin could precipitate all Lp(a) molecules from plasma (Fig.1.A). But the presence of LDL in JL1 indicate that Lp(a) is not the sole lipoprotein present in JL1. An important question that arose was stoichiometry of Lp(a) and LDL in JL1. For this purpose JL1 along with total plasma lipoproteins collected in upper 20% volume following ultracentrifugation at 535, 000 g of untreated plasma (OL1) were coated on polystyrene wells and amounts of Lp(a) and LDL were checked by probing with anti-apo(a) and anti-apoB antibodies respectively.

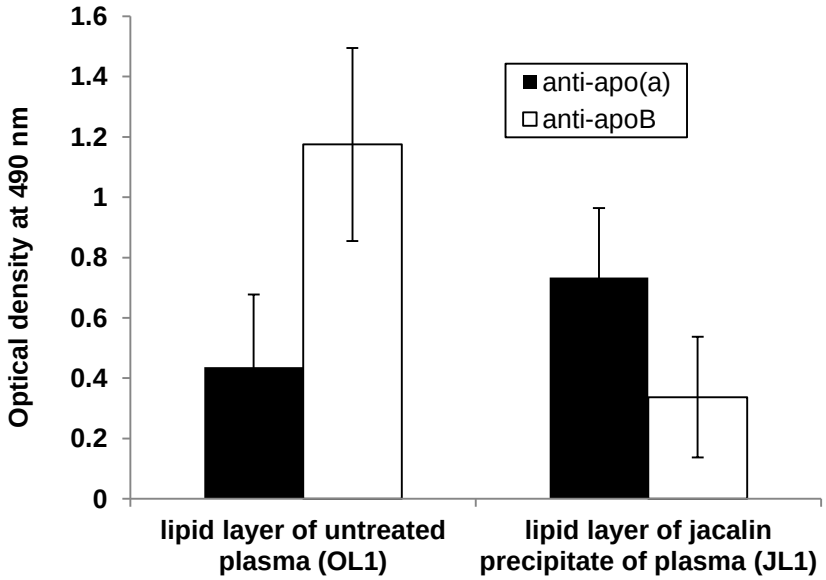
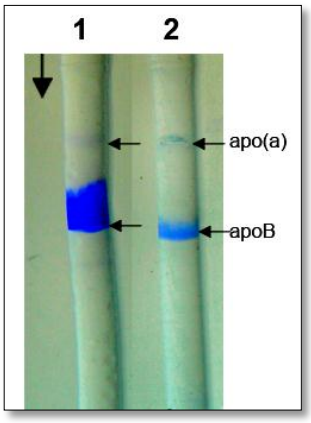


Fig.1.K. Enrichment of Lp(a) in lipid layer after jacalin precipitation: Lipid layer of untreated plasma (OL1) and lipid layer of jacalin precipitate of plasma (JL1) were collected as described in 'Methods 3.1'. Equal amount of JL1 and OL1 (200 ng per well) were coated on polystyrene wells, blocked and probed with anti-human apo(a) and anti-human apoB antibodies (300 ng per well). Bound HRP was assayed as described earlier. Values are mean ± SD of twenty two trails.

Fig.1.K shows that jacalin precipitation of plasma resulted in an enrichment of Lp(a) in the lipid layer of jacalin precipitate of plasma (JL1). As the concentration of LDL far exceeds that of Lp(a) in plasma, the response to anti-apoB in lipid layer of plasma (OL1) was higher than that to anti-apo(a). But in the lipid layer of jacalin precipitate of plasma (JL1) the ratio is reversed. i.e. anti-apo(a) response was higher than anti-apoB response showing the preferential precipitation of Lp(a) from plasma by jacalin. The result was confirmed by checking the relative intensity of Lp(a) and LDL band when OL1 and JL1 were subjected to 3.5% reducing SDS-PAGE and 3.75% TBE electrophoresis in tubes.



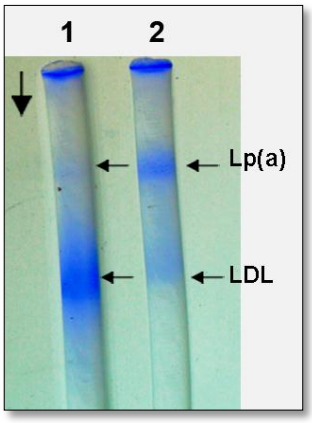


Fig.1.L **Fig.1.M**

Fig.1.L,M. Electrophoresis to check enrichment of Lp(a) in JL1: OL1 and JL1 were subjected to 3.5% SDS-PAGE under reducing condition **(L)** or 3.75% TBE disc gel electrophoresis **(M)** separately and stained with Coommassie brilliant blue. Fifty microgram protein was loaded in all gels. In SDS-PAGE bands were identified by immunoblotting with anti-apo(a) and anti-apoB antibodies. In TBE electrophoresis bands were identified after electroelution followed by ELISA as described earlier.

In SDS-PAGE, bands were separated as lipid free apo(a) and apoB. Fig.1.L shows the relative intensity of apo(a) band is higher in JL1 compared to that in OL1. Same pattern of intensity of bands was seen in TBE gels also. i.e, more Lp(a) than LDL (Fig.1.M). These results confirm the efficiency of jacalin to specifically precipitate all Lp(a) molecules from plasma.

Fold purification of Lp(a) represents the enrichment of Lp(a) after isolation from other plasma proteins and its further purification. So fold purification was calculated from Fig.1.E,F,K. Table 1.1 shows that fold purification of Lp(a) recovered in JL1 was 30.75 ± 8.9 times compared to Lp(a) in the respective plasma samples and fold purification of Lp(a) in electroeluted samples was 25.63 ± 3.2 times compared to Lp(a) in JL1. The overall fold purification of Lp(a) in electroeluted sample calculated from data above was about 788.12 ± 28.48 compared to plasma indicating near absence of impurities since in most plasma samples total protein concentrations are about 800 times more than that of Lp(a).

Table 1.1. Fold purification of Lp(a) by affinity precipitation with jacalin:

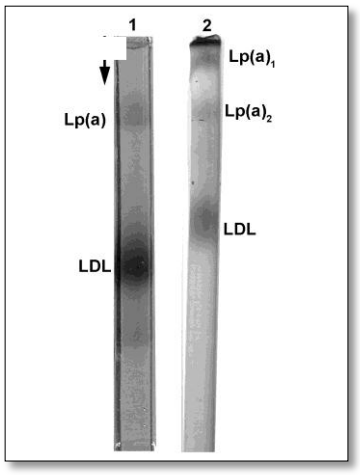
Sample	Fold purification of Lp(a) from plasma level*
Lipid layer of jacalin precipitate (JL1)	30.75 ± 8.9
Electroeluted Lp(a)	788.12 ± 28.48

* Data was derived from Fig.1.E,F,K by assaying the Lp(a) concentration per unit protein in plasma, JL1 and electroeluted Lp(a) by j-a method.

1.3.2. Isolation of all Lp(a) isoforms from plasma

The complete precipitation of all Lp(a) from plasma by jacalin (Fig.1.A) enabled isolation of all isoforms of Lp(a) by the present protocol. Fig.1.N shows that Lp(a) with high molecular weight and often low in plasma concentration were also isolated by the present method, unlike conventional method which could isolate only the dominant isoform from the same plasma. The lower mobility of the LDL band in JL1 (Fig.1.N) compared to the LDL in Lp(a)-containing sample prepared by conventional method could arise from a greater dragging of LDL by the high molecular weight Lp(a) present in the former sample since larger Lp(a) molecules bind more avidly to LDL (our unpublished observation).

Five different clusters of apo(a) size have been reported starting from F (smaller than apoB), through B (equal to apoB in size), S1, S2, S3, S4 to S5 (all larger than apoB) in the increasing order of size (Utermann et al., 1987, Marcovina et al., 1993). Detection of apo(a) and apoB bands in JL1 isolated from five different individuals, each representing a different apo(a) phenotype is shown in Fig.1.O. Apart from the detection of four different apo(a) isoforms larger than apoB a notable feature of the result was detection of B band apo(a) which is equal in size to apoB.



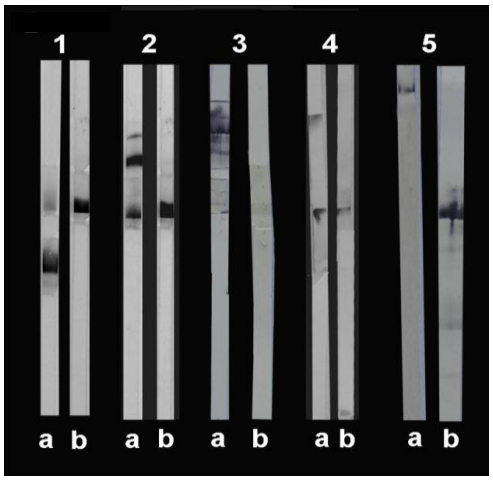


Fig.1.N **Fig.1.O**

Fig.1.N,O. Variation in Lp(a) and apo(a) isoforms in plasma: (N) TBE disc gel (3.75%) electrophoresis of Lp(a) fraction from same plasma isolated by conventional method (1) and new method (2). Protein (50 µg per tube) was run and stained with Coommassie brilliant blue. Bands were identified after electroelution followed by ELISA as described earlier. Result was confirmed with three different set of experiments. **(O)** JL1 isolated from five different plasma samples were subjected to 3.5% SDS-PAGE disc gel electrophoresis under reducing conditions and electroblotted to PVDF membrane. Bands were identified as apo(a) or apoB by incubating the transfer blot with 15 µg per ml HRP-labeled anti-apo(a) and anti-apoB antibodies (a and b respectively).

As a result of more efficient isolation of larger isoforms, two-Lp(a) phenotype has been detected in 48 out of 58 samples used in jacalin precipitation method of isolation of Lp(a) (result not shown). Given the chances of variation among populations this incidence (83%) is in agreement with the detection of two-Lp(a) phenotype in 79% of samples in the Western population, by immunoblotting of electrophoretically separated plasma rather than after isolation of Lp(a) (Marcovina et al., 1993).

Discussion

Lp(a) is the only known core-1 type O-glycan-containing lipoprotein in humans and exhibits considerable size polymorphism in any population due to multiple tandem repeats of encoding sequence for kringle IV type 2 repeats in its apo(a) subunit (McLean et al., 1987, Lackner et al., 1991, Marcovina et al., 1993). Depending on size upto 30% of apo(a) subunit mass could be carbohydrate largely due to O-glycosylation at interkringle linker regions. About 80% of the Ser/Thr-linked O-glycans present in all apo(a) isoforms are of the monosialylated core-1 type structure, NeuNAcα2-3Galβ1-3GalNAc (sialylated T antigen) the rest being disialylated and nonsialylated O-glycans (Garner et al., 2001). The pathogenicity of Lp(a) is mainly attributed to the structural similarity of apo(a) with plasminogen. But the presence of apo(a) in atherosclerotic plaques, kidney mesangial cells and placenta of pre-eclampsia patients suggest alternative avenues of its atherogenic potential. Some studies indicate that Lp(a) is retained more avidly in the vasculature than is LDL (Pepin et al., 1991) but the structural features and interactions of Lp(a) that might contribute to this behaviour have not been described. Investigations in this direction require isolation of all Lp(a) isoforms in pure form.

Even though 34 different apo(a) isoforms were identified to date in population (Marcovina et al., 1993) with apo(a) size ranging from 300 to 800 kDa (Kratzin et al., 1987), an efficient method to isolate corresponding Lp(a) isoforms intact was lacking. Attempts to isolate Lp(a) from very low Lp(a) plasma samples by ultracentrifugation have failed so far due to the overlapping of density of Lp(a) with that of LDL and HDL. For the same reasons it has been difficult to get all Lp(a)

isoforms from some individuals giving rise to overestimation of no-Lp(a) individuals as well as single-band Lp(a) phenotypes associated with high Lp(a) concentrations. Thus, size exclusion or ion exchange chromatography is usually required as complementary methods to ultracentrifugation along with a lysine-Sepharose affinity chromatography. But further purification of Lp(a) using immobilized lysine was also not satisfactory since Lp(a) populations with widely different lysine binding affinity are reported (Xia et al., 2000). In this context the unique kringle IV type 2 heterogeneity as well as unusually heavy O-glycosylation of apo(a) was exploited here for Lp(a) isolation (described in 'Methods 3.1'), involving affinity precipitation of Lp(a) with jacalin which has strong affinity and specificity for O-linked oligosaccharides of the core-1 type present in apo(a) regardless of sialylation. The isolated Lp(a) isoforms were then separated by Tris-Borate EDTA disc gel electrophoresis followed by electroelution.

Results suggest the efficiency of jacalin to precipitate all Lp(a) isoforms from plasma (Fig.1.A). Immunoblotting of Lp(a) isoforms isolated from plasma by jacalin affinity precipitation shows a high incidence of double Lp(a) phenotype (83%) as well as that of B form apo(a) indicating the efficiency of the present protocol in detecting and isolating Lp(a) isoforms of varying plasma concentrations (Fig.1.O). In addition to the two major apo(a) isoforms, some samples showed [sample (1) and (2) in Fig.1.O] more than two apo(a) bands, which was also observed in an earlier study (Kamboh et al., 1991). Though these authors attributed this result to partial proteolysis of apo(a) in stored Lp(a) samples Lp(a) used in the present work was freshly prepared suggesting need for further investigation into this phenomenon.

Conventional method of Lp(a) isolation which employs two successive ultracentrifugation at high density to liberate lipoproteins to the top is not effective in the isolation of all Lp(a) isoforms from plasma as a substantial amount of Lp(a) remains in the lower layers. So the lipid layer collected as source of Lp(a) by conventional method does not represent a total Lp(a) population (Fig.1.N). The new method of Lp(a) isolation is superior to the conventional method as it is cheap and simple while the latter is laborious and time consuming. Moreover the fold purification of Lp(a) isolated by the present protocol was about 788 compared to plasma indicating near absence of impurities since in most plasma samples total protein concentrations are about 800 times more than that of Lp(a) (Table 1.1).

This emphasises that the new protocol can also be used to isolate and characterize Lp(a) which is deposited on atherosclerotic plaques at various tissues like placenta, kidney and blood vessels. As circulating levels of Lp(a) are not significantly modified by traditional lipid lowering therapies alternative approaches to lower circulating Lp(a) is a long felt therapeutic necessity. In this regard, the affinity of jacalin for Lp(a) which is strong enough to cause precipitation of the lipoprotein from plasma can be exploited to target Lp(a) or apo(a).

PART - II

Differential accessibility of antigenic and oligosaccharide epitopes of apo(a) and apoB subunits in Lp(a) of varying molecular size

Lp(a) and LDL are atherogenic lipoproteins each containing an apolipoprotein, apoB. Lp(a) contains an additional protein subunit called apo(a) which is heavily glycosylated with core-1 type O-glycans. Apo(a) in Lp(a) appears as a belt extending around the core particle and joined to apoB by a single disulfide linkage (Xu et al., 1998). However, apo(a) might have more contact with the aqueous environment than with the lipid surface due to its numerous highly hydrophilic O-glycan chains (Fless et al., 1986) implying that, with the exception of its covalent attachment through disulfide bonds to apoB, apo(a) may be loosely bound to the rest of the lipoprotein thereby making its epitopes available for antibody or lectin binding and at the same time mask apoB epitopes. Moreover Lp(a) isoforms differ from each other in molecular size mainly due to the difference in number of kringle IV type 2 repeats. Varying extent of glycosylation resulting from differences in apo(a) size is also expected to affect response of antibodies or lectins towards Lp(a) isoforms. So this part deals with the availability of antigenic as well as glycan epitopes of apo(a) and apoB subunits of Lp(a) isoforms for recognition by respective antibodies or N- and O-glycan specific lectins.

2.1. Comparison of accessibility of antigenic and oligosaccharide epitopes of native Lp(a) and LDL for binding to antibodies or lectins

Lipoprotein mixture of Lp(a) and LDL isolated from plasma as upper 20% was checked for the response to antibodies (anti-human apo(a) and anti-human apoB) and lectins (jacalin, galectin-1 and Con A) using ELLA. Fig.2.A shows that among six plasma samples the variation in affinity of O-glycan specific lectins, jacalin and galectin-1 to the lipoprotein mixture is in accordance with anti-apo(a) response but

unrelated to the response of Con A or anti-apoB suggesting Lp(a) is the sole O-glycan-containing lipoprotein.

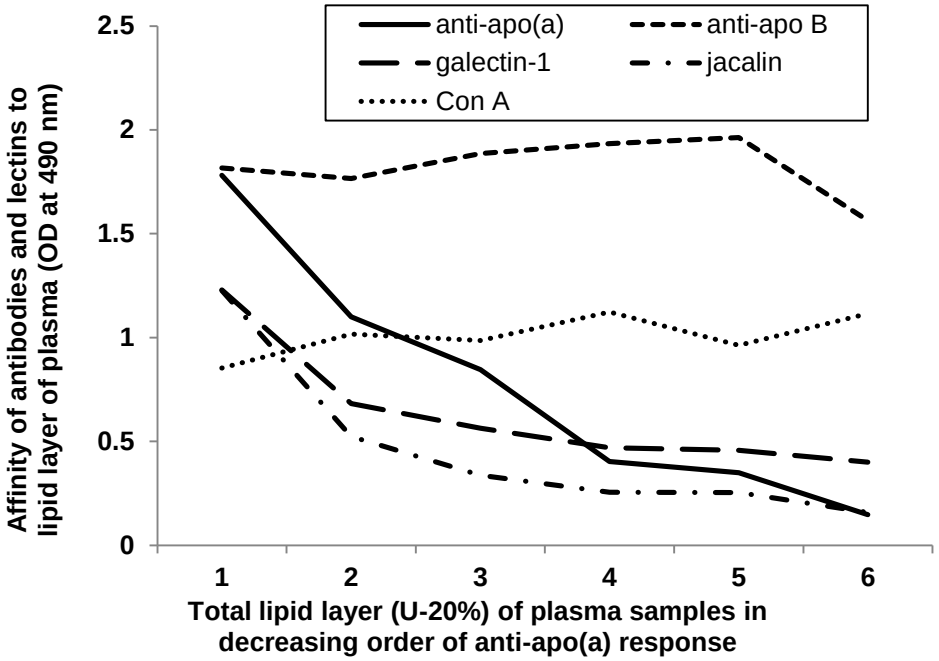


Fig.2.A. Differential response of lectins to lipid layer of plasma: Total lipid layer (U-20%) containing mixture of Lp(a) and LDL was collected from six plasma samples after ultracentrifugation at 535, 000 g for 4 h at 4 °C and coated (1 µg per well) on polystyrene wells, blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well), galectin-1 (1.5 µg per well), jacalin (4.7 ng per well) or Con A (2 µg per well). Bound HRP was measured as described earlier and antibody or lectin binding is expressed as absorbance at 490 nm.

This result was further confirmed by comparing the glycosylation patterns of native Lp(a) and LDL by checking the affinity of N-and O-glycan specific lectins. Fig.2.B shows that in contrast to LDL Lp(a) was rich in O-glycans specific for jacalin and galectin-1 located in the heavily glycosylated apo(a) subunit. Even though both apo(a) and apoB in Lp(a) contains N-glycans, Con A binding to Lp(a)

was lower than that of LDL. This result confirmed that the major N-glycoprotein in lipid layer was not Lp(a), but LDL.

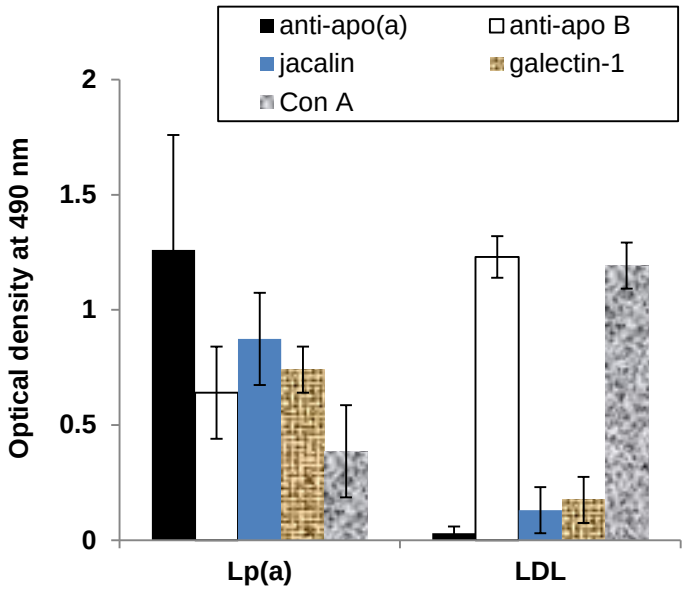


Fig.2.B. Affinity of N-and O-glycan specific lectins to native Lp(a) and LDL: Electroeluted Lp(a) and LDL (100 ng per well) were coated on polystyrene wells, blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well), galectin-1 (1.5 µg per well), jacalin (4.7 ng per well) or Con A (2 µg per well). Bound HRP was measured as described earlier. Values are mean ± SD of eight trials.

2.2. Affinity of N-and O-glycan specific lectins for Lp(a) isoforms

Lp(a) isoforms differ in number of kringle IV type 2 repeats. This also reflects in the extent of glycosylation as inter kringle regions are potent sites of glycosylation mostly with O-linked oligosaccharides resulting in the occurrence of two different Lp(a) glycoproteins in a single individual. To verify this assumption, Lp(a) isoforms were isolated from plasma having double-Lp(a) phenotype (Fig.1.F), coated on polystyrene wells and probed with anti-apo(a) and N-and O-glycan specific lectins.

Since antigenic epitopes in apo(a) are not reported to be polymorphic among Lp(a) molecules ratio of response (OD 490 nm) of lectin binding to that of anti-apo(a) binding was taken as index of affinity of lectin for the Lp(a) isoforms. In Fig.2.C, the two Lp(a) isoforms showed different lectin interaction per unit apo(a).

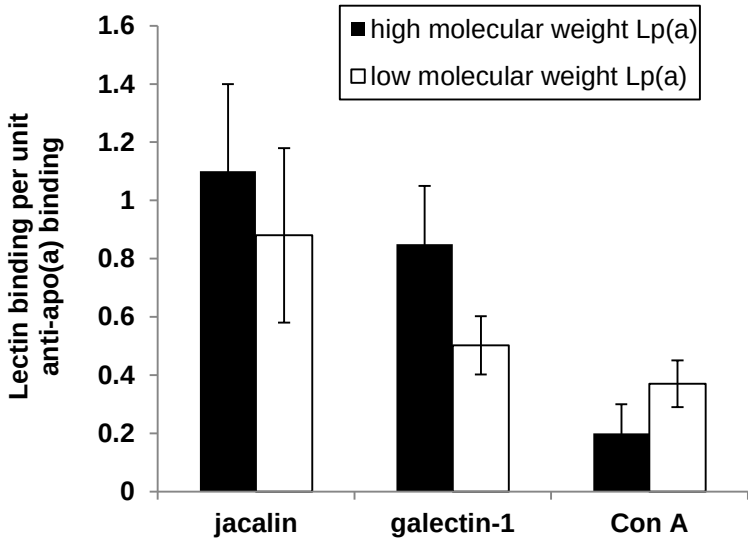


Fig.2.C. Differential lectin binding of Lp(a) isoforms: Electroeluted Lp(a) isoforms of high and low molecular weight (Fig.1.F) were coated on polystyrene wells (100 ng per well), blocked and probed with HRP-labeled anti-human apo(a) (300 ng per well), galectin-1 (1.5 µg per well), jacalin (4.7 ng per well) or Con A (2 µg per well). Glycosylation difference was expressed as lectin binding per unit anti-apo(a) binding obtained as ratio of response towards lectin to response towards anti-apo(a). Values are mean ± SD of five trials.

O-Glycan specific lectins like jacalin and galectin-1 showed stronger affinity towards the slow moving large Lp(a) than the fast moving small Lp(a) suggesting the presence of more O-glycans in large Lp(a) compared to the small one. In contrast to the greater binding of O-glycan specific lectins, the Con A binding to large Lp(a) isoforms was low. Even though Lp(a) contains two different N-glycosylated Con A-binding proteins (apo(a) and apoB), the lower affinity of large Lp(a) isoform to Con

A compared to the small Lp(a) isoform for a given amount of protein suggests masking of oligosaccharide epitopes of apoB subunit by the extended apo(a) chain.

2.3. Effect of apo(a) size on galectin-1 recognition of Lp(a) isoforms

The galactose binding galectin-1 is the most abundant and ubiquitous lectin in mammalian tissues, including endothelial and smooth muscle cells. Among common oligosaccharide sequences encountered in human tissues, the core-1 type O-glycan containing T antigen with or without sialic acid substitution has been found to be the most efficient ligand for galectin-1 (Sangeetha and Appukuttan, 2005). This conclusion was further supported by the observation *in vitro* that unlike the O-glycan free LDL, Lp(a) which is apparently the most densely O-glycosylated human glycoprotein in circulation, was recognized by human aortal endothelial cell galectin-1 (Chellan et al., 2007). The response of galectin-1 towards lipid layer of various plasma samples which differ with respect to the Lp(a) concentration (assayed by j-a method described in 'Methods 2.22') was checked by inhibition ELISA, in which lipid layer of plasma was used as an inhibitor to decrease the binding of HRP-labeled galectin-1 to polystyrene wells coated with asialofetuin. The minimum concentration of Lp(a) required to get 50% inhibition of the binding of galectin-1 to asialofetuin (IC50) was the measure of affinity of galectin-1 towards lipid layers with varying Lp(a) concentration. Fig.2.D shows that IC50 of Lp(a) in lipid layer increases steadily with increase in Lp(a) concentration (correlation co-efficient, $r = 0.515$, $p = 0.029$) suggesting Lp(a) in lipid layer of plasma with lower Lp(a) concentration is more efficient in binding to galectin-1 and thereby blocking its binding to asialofetuin. As there is an inverse relation between apo(a) size and plasma Lp(a)

concentration, the above result indicated that lipid layer of plasma with lower Lp(a) concentration and thereby large apo(a) with more O-glycosylation has higher affinity towards galectin-1.

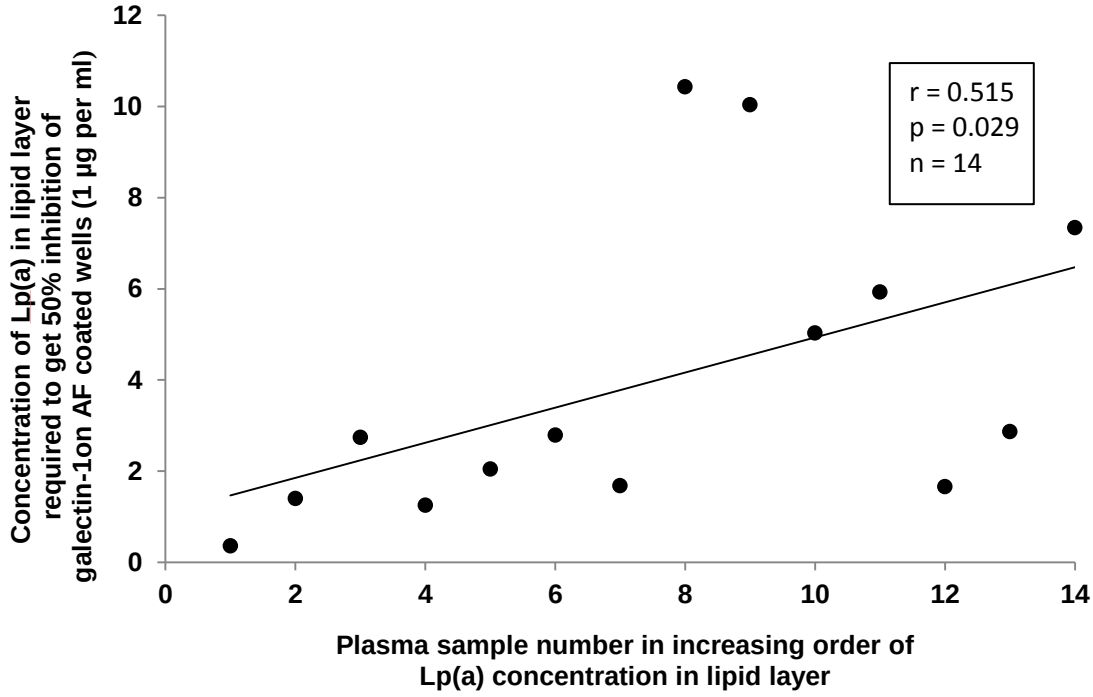


Fig.2.D. Assay to check the affinity of galectin-1 towards lipid layer of varying Lp(a) concentration using inhibition ELISA: The affinity of galectin-1 towards lipid layer of plasma (U-20%) was checked by assaying the inhibitory capacity of assayed Lp(a) in lipid layer containing varying Lp(a) concentration to decrease the binding of HRP-labeled galectin-1 (1.5 µg per well) on polystyrene wells coated with asialofetuin (1 µg per well) and expressed as the minimum inhibitory concentration required to get 50% inhibition of galectin-1 binding on asialofetuin (IC50) as described in 'Methods 2.21'. Plasma Lp(a) concentration was assayed by j-a method.

As the endothelial expressed lectin, galectin-1 plays a major role in atherosclerosis (Chellan et al., 2007, Nachtigal et al., 2008, Thijssen et al., 2008) the enhanced binding of galectin-1 to large Lp(a) is physiologically relevant. With Lp(a) of various sizes in pure form available by the new protocol (described in 'Results

part-I'), variation in galectin-1 binding as a function of Lp(a) size was examined. Since Lp(a) size is decided solely by apo(a) and apoB size is invariant, molecular size of each Lp(a) preparation could be represented by the ratio of mobility of apoB subunit [mB] to that of apo(a) subunit [m(a)] observed in SDS-PAGE of JL1 samples in 3.5% acrylamide gels under reducing conditions (Fig.2.E).

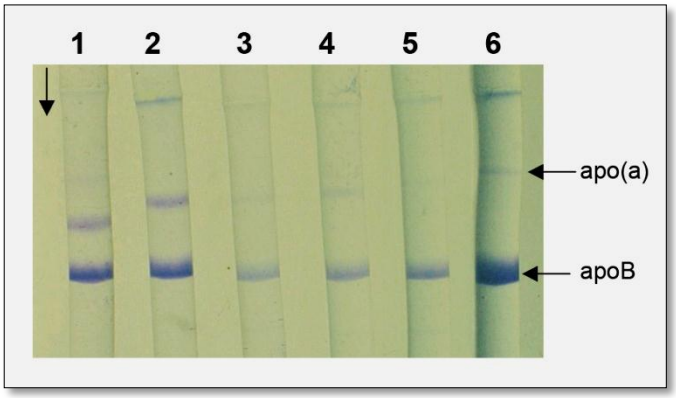


Fig.2.E. Molecular size determination of Lp(a) isoforms: Lipid layer of jacalin precipitate of plasma (JL1) (50 µg per tube) was subjected to 3.5% SDS-PAGE in tubes under reducing condition. Protein bands were stained with Coommassie brilliant blue and identified as apo(a) and apoB after immunoblotting with HRP-labeled anti-human apo(a) and anti-human apoB antibodies as described in 'Methods 2.17'. The ratio of mobility of apoB subunit [mB] to that of apo(a) subunit [m(a)] was used to express the size of apo(a).

Result (Fig.2.F) shows that binding of jacalin as well as of galectin-1 increases steadily with apo(a) size with correlation co-efficient (r) of 0.861 ($p = 0.0014$) and 0.844 ($p = 0.0021$) respectively. This result justified the use of the ratio of mobility of apoB to that of apo(a) in SDS-PAGE as an index of Lp(a) size and suggested large apo(a) subunits are stronger ligands for O-glycan specific lectins. So the higher the molecular weight of Lp(a) stronger it is as ligand for galectin-1 on endothelial cell surface.

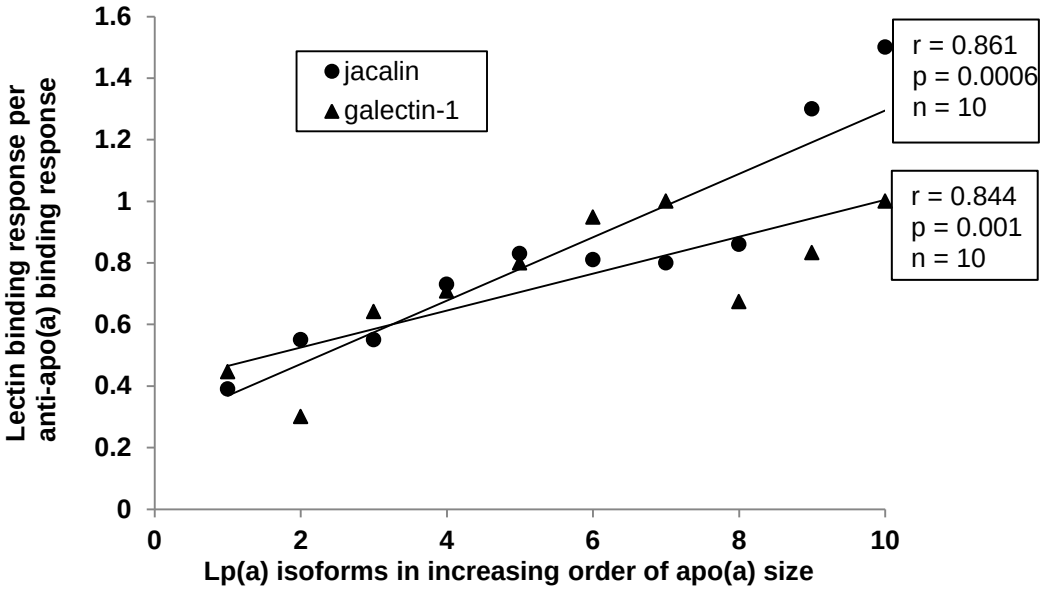


Fig.2.F. O-glycan specific lectin recognition of Lp(a) increases with size of apo(a): Electroeluted Lp(a) isoforms from different individuals were coated (100 ng per well) on polystyrene wells, blocked and probed with HRP-labeled anti-human apo(a) (300 ng per well), galectin-1 (750 ng per well) and jacalin (4.7 ng per well). Bound HRP was measured as described earlier. Lectin response per unit anti-apo(a) response was plotted against size of apo(a) subunit [mB/m(a)] determined from Fig.2.E.

2. 4. Effect of apo(a) size on availability of antigenic epitopes of apoB on Lp(a)

To quantitate the availability of apoB and apo(a) subunits of Lp(a) for recognition by respective antibodies, purified Lp(a) samples were immobilized by capturing them on plate-coated jacalin which recognizes only the O-linked oligosaccharides that occur in kringle IV type 2 repeat region of apo(a) but not in its antigenic site. Since Lp(a) content of Lp(a) samples of differing size in pure form were assayed by j-a method which measures the number of Lp(a) molecules regardless of size, given amount of Lp(a) from all samples contained equal number of Lp(a) molecules. After measuring the response to respective HRP-labeled antibodies, availability of apoB was expressed as ratio of response to anti-apoB

antibodies (OD 490 nm) to that to anti-apo(a) antibodies. Result (Fig.2.G) shows that among fourteen Lp(a) samples examined, as Lp(a) size increases availability of apoB decreases steadily (correlation coefficient, r = - 0.829, p = 0.0001).

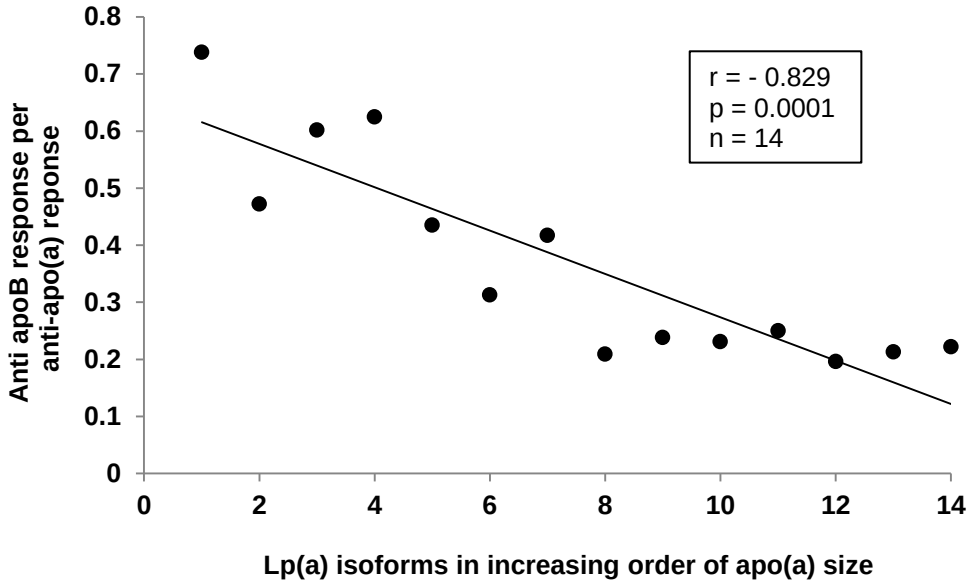


Fig.2.G. ApoB subunit availability in Lp(a) is inversely related to the molecular size of apo(a): Electroeluted Lp(a) isoforms from different individuals were added (100 ng per well by j-a assay) to jacalin coated polystyrene wells (1 µg per well), incubated at 4 ºC for 2 h, washed and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well) as described earlier. Index of availability of apoB was expressed as ratio of response (absorbance at 490 nm) of anti-apoB to response of anti-apo(a) and plotted against the size of apo(a) subunit [mB/m(a)] determined from Fig.2.E.

2.5. Increased accessibility of apoB subunit in Lp(a) after removal of apo(a)

To assess further the extent to which apo(a) affects availability of apoB epitopes two groups of Lp(a) samples, large Lp(a) group [mB/m(a) values > 2] and small Lp(a) group [mB/m(a) values < 2] were treated with the reducing agent DTT that cleaves the lone disulphide bond linking both subunits covalently. This treatment detaches only the apo(a) subunit from Lp(a), resulting in an apo(a)-free lipoprotein

particle with apoB-lipid association intact and resembling LDL closely. The latter lipoprotein termed Lp(-a) (Fless et al., 1986) in Fig.2.H,I was separated from the liberated apo(a) by ultracentrifugation at 535,000 g for 4 h and collected in the upper lipoprotein layer (U-20% volume) while apo(a) segregated along with other protein to the bottom of the tube. Lp(-a) was compared with its parent Lp(a) in plate-coated form in terms of recognition by anti-human apoB antibody and the lectin Con A. Results in Fig.2.H,I indicate that consequent to an almost complete disappearance of apo(a) chains by DTT treatment, apoB in Lp(-a) offered its antigenic epitopes as well as N-linked oligosaccharides recognized by Con A remarkably more than as part of intact Lp(a). DTT treatment of LDL did not increase its recognition by Con A or anti-apo B indicating that reduction of apoB per se was not responsible for the above effect (Fig.2.J).

N-Linked glycans are far fewer in apo(a) than in apoB and belong extensively to the complex type which are poor ligands for Con A in comparison with the high mannose N-linked oligosaccharides that occur frequently in the apoB subunit (Garner et al., 2001, Harazono et al., 2005). Increase in binding of anti-apoB antibody and Con A to apoB following removal of the associated apo(a) (Fig.2.H,I), despite the latter being itself a Con A-reactive moiety though poor, further confirms masking of apoB by apo(a) in Lp(a).

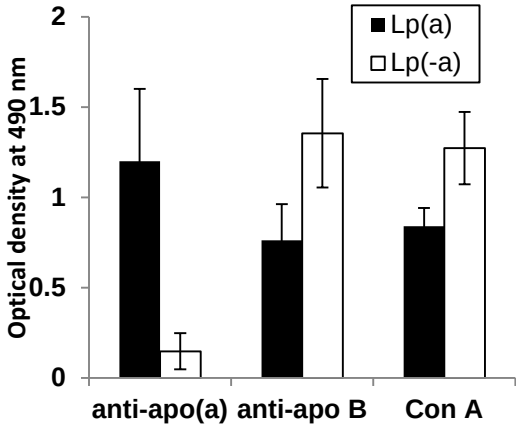


Fig.2.H

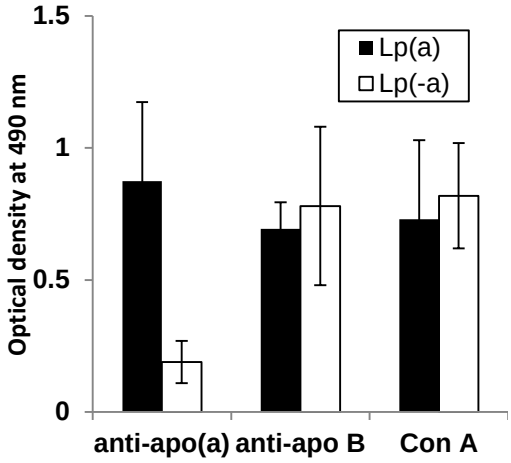


Fig.2.I

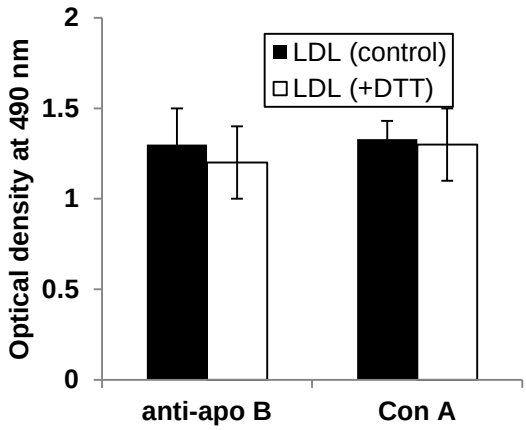


Fig.2.J

Fig.2.H,I,J. Increased masking of apoB and N-linked oligosaccharides in large Lp(a) isoform: Equal amounts of electroeluted Lp(a) isoforms and LDL (100 ng per well) were treated with 10 mM DTT at 37 °C for 15 min before ultracentrifugation at 535,000 g. Controls without DTT were also kept. Lipids in U-20% volume diluted 5 times with PBS was directly coated on polystyrene wells and blocked as described before. Wells were then probed with Con A (2 µg per well), HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well) as described earlier. Antibody or lectin binding was expressed as absorbance at 490 nm. Values are mean ± SD of six samples in either group. H. Large Lp(a) isoform group; I. Small Lp(a) isoform group. J. Electroeluted LDL. Lp(-a): lipoprotein part remaining after removal of apo(a) from Lp(a) by DTT treatment.

Further, Table 2.1 shows that this liberation of apoB epitopes of Lp(a) after removal of apo(a) subunit was much more pronounced in the group of six large Lp(a) molecules compared to the group of six small Lp(a) molecules (43 ± 18% and 13 ± 5% respectively, p = 0.0063) whereas as the increase in Con A binding of apoB after removal of apo(a) subunits were 33 ± 12% and 10 ± 6% for large and small Lp(a) groups respectively (p = 0.0248).

Table 2.1. Percentage of increase (%) in availability of antigenic or N-linked oligosaccharide epitopes of apoB subunits of Lp(a) isoforms after DTT treatment for anti-apoB antibody and Con A binding respectively:

***Increase (%) in apoB binding of**	**Large Lp(a) group (n = 6)**	**Small Lp(a) group (n = 6)**	**P value**
anti-apoB	43 ± 18	13 ± 5	P = 0.0063
Con A	33 ± 12	10 ± 6	P = 0.0248

* Data was derived from Fig.2.H,I.

Above results show that as the size of the Lp(a) molecule increases due to large apo(a) chains containing extended kringle IV type 2 repeat regions and proportionately more O-glycan chains, greater masking of apoB chain takes place making the latter less available for lectins or antibodies. This was further confirmed by plotting the percentage increase in anti-apoB and Con A binding to the non-apo(a) part of Lp(a) [Lp(-a); Fig.2.H,I] against apo(a) size determined from 3.5% SDS-PAGE (Fig.2.E). Fig.2.K shows a steady increase in anti-apoB and Con A binding with increase in apo(a) size (r = 0.700, p = 0.0053 and r = 0.688, p = 0.0065 respectively for anti-apoB and Con A). This result also underlined the increased

masking of apoB by large apo(a) chains. Earlier study had reported that on plastic-coated Lp(a) availability of antigenic epitopes of apoB for antibody recognition was not affected by the presence of apo(a) (Zawadzki et al., 1988). To determine availability of apoB antigenicity, the present work employed both direct coating of Lp(a) and Lp(-a) on polystyrene wells (Fig.2.H,I) and Lp(a) captured through its O-glycans on plate-coated O-glycan-specific lectin (Fig.2.G). Masking of apoB antigenic epitopes in both cases in good correlation with apo(a) size suggests that this effect could not be artifactual.

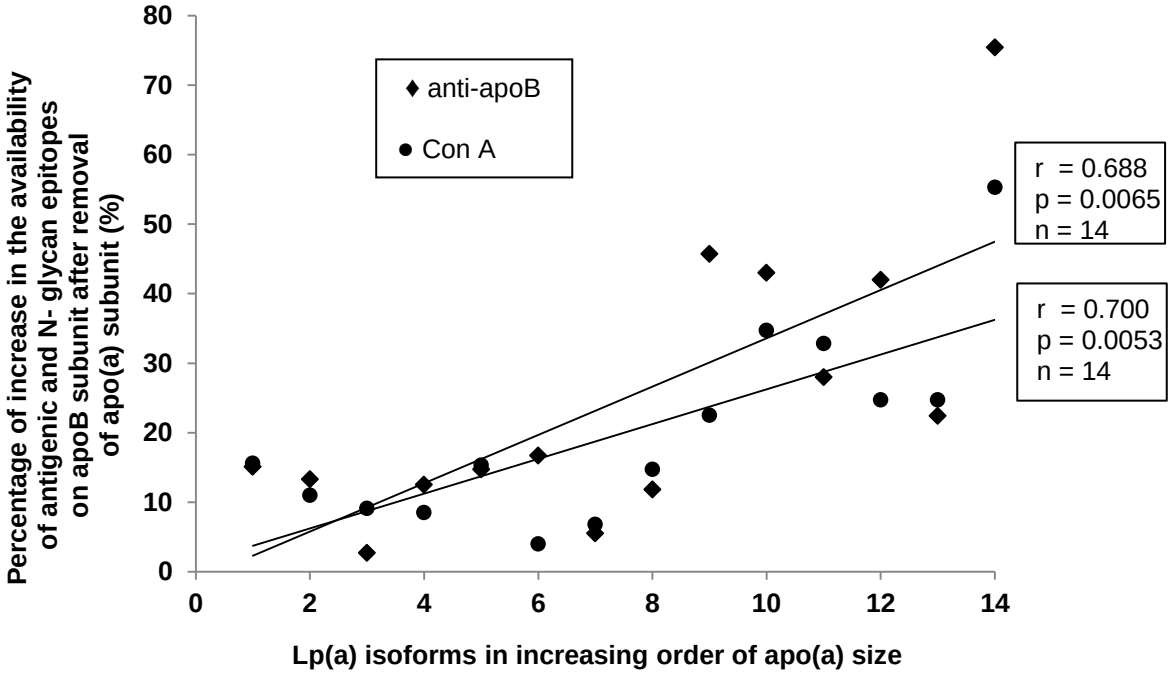


Fig.2.K. Percentage of increase in availability of antigenic and oligosaccharide epitopes of apoB subunit in Lp(a) isoforms after removal of apo(a) subunit: From Fig.2.H and I percentage of increase in availability of antigenic and oligosaccharide epitopes of apoB subunit after removal of apo(a) subunit in each Lp(a) isoforms was calculated and plotted against the size of apo(a) subunit [mB/m(a)] determined from Fig.2.E.

Discussion

In Lp(a) [apo(a)] is linked by a disulfide bond (Fless et al., 1986) to its apoB subunit, on a 1:1 ratio (Koschinsky et al., 1993). While apoB remains relatively constant in size among different Lp(a) samples, apo(a) varies in size due to polymorphism in the number of tandemly repeated kringle IV type 2 domains (Utermann., 1987). Total number of glycan chains varies according to the kringle IV type 2 repeats thereby giving rise to various Lp(a) glycoprotein phenotypes. In contrast to the heavily O-glycosylated apo(a), apoB is glycosylated exclusively with N-glycans. We compared the availability of glycan and antigenic epitopes of Lp(a) for being recognized by antibodies and lectins. For this purpose we used O-glycan specific lectins, jacalin and galectin-1 and N-glycan specific lectin, Concanavalin A.

Lp(a) minus its apo(a) component [Lp(-a)] is a lipoprotein that is chemically and immunologically similar to LDL (Gries et al., 1988). As molecular size increases, apo(a) chain is more extended with more O-glycans which might cause masking of apoB chain, making the latter less available for lectins or antibodies. This was confirmed from in Fig.2.H,I and K which show the greater increase in anti-apoB and Con A binding to the non-apo(a) part of Lp(a) [Lp(-a)] when the latter was freed from a large Lp(a) than from a small Lp(a) by reduction. This was also supported from the finding that after removal of apo(a), Lp(-a) was bound, internalized, and degraded through the LDL-receptor pathway with the same affinity and efficiency as normal LDL, whereas native, unreduced Lp(a) failed to bind (Armstrong et al., 1985). Possible reasons for this difference are (Gries et al., 1988): a) certain "domains" close to the binding domain of the antibody or lectin are covered by apo(a); b) apo(a) causes steric hindrance in the interaction of Lp(a) with the antibody

or lectin; c) apo(a) causes conformational changes in the anti-apoB binding region of apoB. In any case result indicates that apoB-mediated processes such as LDL receptor binding will be suppressed more in the large Lp(a) isoform. Zawadzki et al. (1988) had speculated based on results of antibody recognition of plate-coated Lp(a) that conformational changes induced in apoB by apo(a), rather than steric hindrance was cause for reduced availability of epitopes. Oligosaccharide chains of N-and O-linkage in apo(a) are hydrophilic and should be exposed to the aqueous environment regardless of protein conformation. Reduced availability of oligosaccharide moieties of apoB for lectin recognition observed in the present study therefore suggests that masking of apoB surface epitopes also accompanies apo(a) attachment to form Lp(a). This conclusion was further supported by the increase in masking of apoB epitopes with increase in size of apo(a).

Another important observation was that apo(a) size polymorphism may modulate a T antigen specific lectin, galectin-1 recognition of Lp(a). As increase in apo(a) size results in the extension of O-glycans, the large Lp(a) molecules are more efficient as ligands for galectin-1 (Fig.2.D and F). The higher accessibility of antigenic and glycan epitopes of apo(a) compared to that of apoB subunits of Lp(a) indicated the predominant role of apo(a) in various biological as well as pathological functions of Lp(a). Moreover Lp(a)-galectin-1 interaction is a possible route through which Lp(a) may get deposited on endothelial cell surface and in plaques (Chellan et al., 2007). So the present results indicate that in free form large Lp(a) isoforms might get deposited more on these cell surfaces.

PART - III

Study of association of Lp(a) with LDL to form adduct

Apo(a) is usually present in human plasma in several physical forms: (i) lipid-free, unbound protein, (ii) lipid-free protein linked by disulfide bond to apoB, (iii) disulfide-linked to apoB of LDL in an Lp(a) particle, (iv) associated with more than one LDL particles in Lp(a):LDL adduct (Trieu and Mcconathy et al., 1990), and (v) associated with plasma proteins to form Lp(a) immune complex (Lp(a)-IC) (Wang et al., 2003b, Wang et al., 2009). Thus in general, plasma Lp(a) exists in two different forms; free Lp(a) and complexed Lp(a). Since both Lp(a) and LDL might play crucial roles in the development of atherosclerosis and are reported to be present in plaque tissues, studies on interactions of Lp(a) with plasma LDL are pathologically relevant. However the role of structural features of Lp(a) or other factors influencing its biological fate remains unclear. Whether preferential accumulation of Lp(a) over LDL in plaques is a reflection of its ability to exist as complexed form is worth investigating.

3.1. Distribution of Lp(a) in free and complexed lipoprotein fraction

Distribution of Lp(a) in free and complexed lipoprotein fraction was checked by comparing the response of anti-apo(a) and anti-apoB antibodies towards polystyrene well-coated free and complexed lipoproteins isolated from same plasma as described in ‘Methods 5.1’. Here the water retaining ability of PEG-6000 polymer was exploited for the isolation of complexed lipoproteins from plasma (Methods 2.7) (Hudson and Hay, 1980). Fig.3.A shows that anti-apo(a) response is high in the case of complexed lipoprotein fraction suggesting the preferential tendency of Lp(a) to form complexes with other plasma proteins compared to that of LDL despite its much lower concentration in plasma than that of LDL.

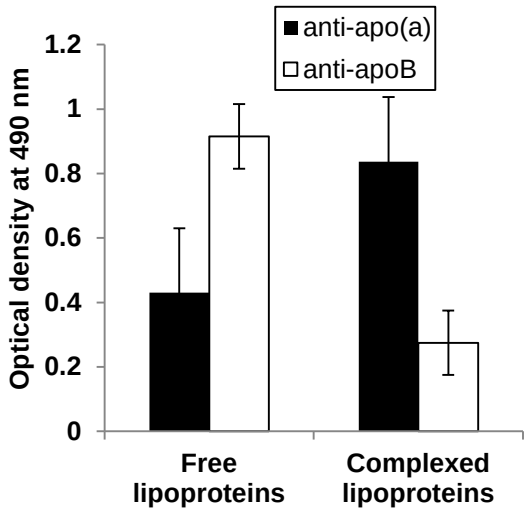


Fig.3.A. Assay to check the distribution of Lp(a) in plasma: Free and complexed lipoproteins were collected as described in 'Methods 5.1', coated on polystyrene wells (1 µg per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well). Bound HRP was measured as described in 'Methods 2.20'. Values are mean ± SD of seven trials.

To selectively isolate free (un-complexed) Lp(a) from plasma, a sequential precipitation of plasma with varying PEG concentration ranging from 0.2% to 1% was employed (Methods 2.7). Absence of any visible precipitate in the lower concentrations (0.2% and 0.4%) of PEG suggest the inefficiency of lower PEG concentrations to precipitate Lp(a) (data not shown). So only 0.6%, 0.8% and 1% PEG precipitate of plasma were subjected to 3.75% TBE disc gel electrophoresis and stained with Coommassie brilliant blue. Single band was obtained in 0.6% and 0.8% PEG precipitate (Fig.3.B) while on increasing the PEG concentration to 1% a fast moving band appeared. The slow and fast moving bands were identified as Lp(a) and LDL respectively as described in 'Fig 1.D'.

Non-specific attachment of LDL to Lp(a) as cause for precipitation of free LDL along with Lp(a) was ruled out, since the PEG precipitates collected were

washed with the corresponding concentration of PEG solution in veronal buffer pH 7.4. To check the possibility of independent precipitation of free LDL by PEG lipid layer of plasma (U-5%) was collected and an aliquot was treated with jacalin-Sepharose to make it Lp(a) as well Lp(a):LDL adduct free (Methods 5.5). The lipid layer with or without Lp(a) was then checked for precipitation with 1% and 3% PEG as described in 'Methods 2.7'. The absence of any PEG precipitate in the jacalin-Sepharose-treated lipid layer (Table 3.1) indicates that free LDL is not precipitated with PEG even at high concentration (3% PEG) and that the appearance of LDL band in 1% PEG precipitate is due to the precipitation of Lp(a)-associated LDL similar to that obtained in Fig.1.D and confirmed the existence of Lp(a):LDL adduct in circulation.

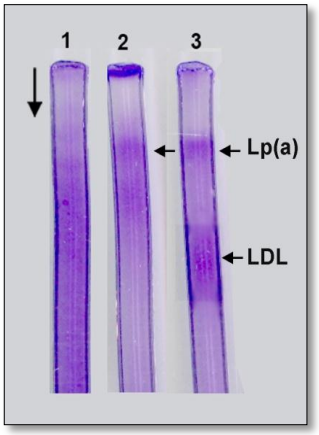


Fig.3.B. TBE disc gel electrophoresis of PEG precipitate of plasma to fix an appropriate percentage of PEG to selectively precipitate free Lp(a) from plasma: PEG precipitate of 1 ml plasma with varying percentage of [(1) 0.6%, (2) 0.8% and (3) 1%) PEG was collected as described in 'Methods 5.4'. The precipitate was re-dissolved in 100 µl TBE buffer, pH 8.7 and subjected to 3.75% TBE disc gel electrophoresis (50 µg proteins in each). Lipoprotein bands were stained and electroeluted bands were identified as Lp(a) and LDL by ELISA as described earlier. Result is reproducible in seven consecutive trials.

Table 3.1. Assay to check whether 1% and 3% PEG-6000 can precipitate free LDL from plasma:

Sample used for PEG precipitation*	Precipitation of Lp(a):LDL adduct	
	1% PEG	3% PEG
Lipid layer	+	+
Supernatant of lipid layer treated with Sepharose gel	+	+
Supernatant of lipid layer treated with jacalin-Sepharose gel	-	-

* Samples were prepared as described in 'Methods 5.5'. After treatment with 1% and 3% PEG (Methods 2.7) presence (+) or absence (-) of visible PEG precipitate of plasma was checked. Result is reproducible in three consecutive trials.

As per the above result (Fig.3.B) PEG concentration of 0.8% was found to selectively precipitate free Lp(a) from plasma. In order to examine whether qualitative features of Lp(a) like apo(a) size influence its propensity to form complexes, free (un-complexed) Lp(a) was isolated from plasma by 0.8% PEG precipitation and assayed along with total plasma Lp(a) by j-a method as described in 'Methods 2.22'. The content of free Lp(a) as percentage of total was plotted against size of apo(a) determined as described in 'Methods 3.3'. Fig.3.C shows a strong negative correlation between amount of free Lp(a) and apo(a) size with a correlation co-efficient of -0.887 ($p = 0.0001$) suggesting the role of apo(a) size in governing the fate of Lp(a), the large Lp(a) being more efficient in complex formation with other plasma proteins. Dissociation of Lp(a):LDL adduct upon PEG treatment leading to overestimation of free Lp(a) in plasma was ruled out since de novo adduct formation was not impaired in the presence of 0.6%, 0.8% and 1% PEG concentrations (Fig.3.D).

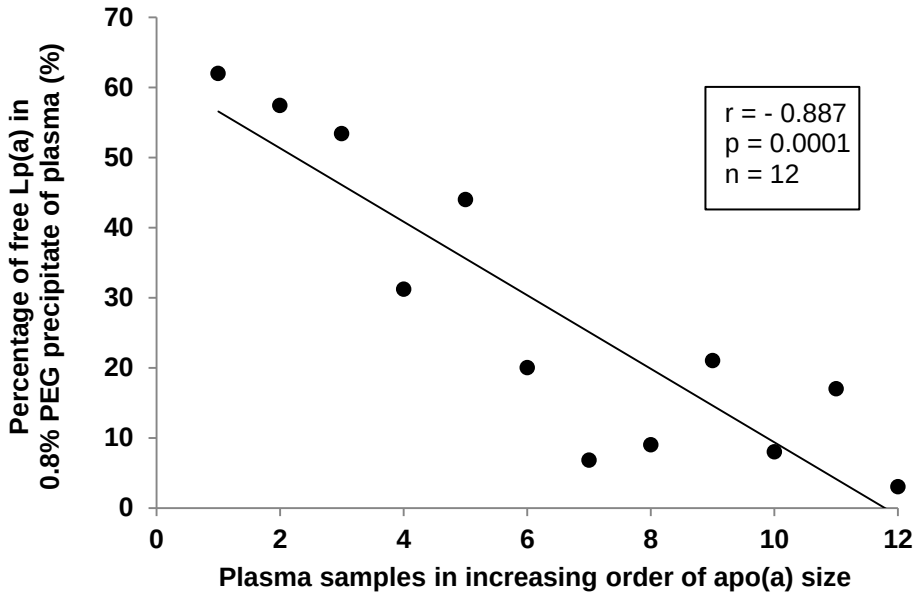


Fig.3.C. Relation between size of apo(a) and fraction of free Lp(a) in plasma: Free Lp(a) was isolated from plasma by 0.8% PEG precipitation and its Lp(a) content and also the total Lp(a) content of plasma was checked by j-a method as described in 'Methods 2.22'. The content of free Lp(a) as percentage of total Lp(a) was plotted against apo(a) size determined as described in 'Methods 3.3'.

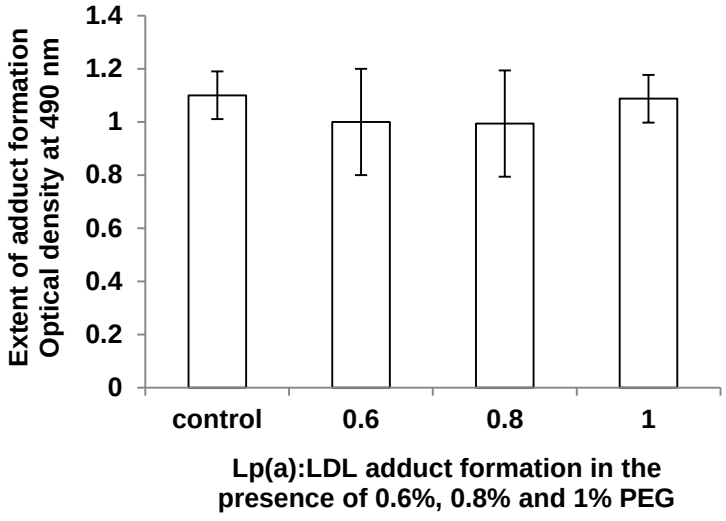


Fig.3.D. Effect of PEG-6000 on Lp(a):LDL adduct: Effect of PEG-6000 on Lp(a):LDL adduct was checked by comparing the efficiency of biotin-labeled LDL to bind polystyrene well coated-TBE purified Lp(a) in the presence of 0.6%, 0.8% and 1% PEG solution with that of control (without PEG) as described in 'Methods 5.6'. Values are mean ± SD of four trials.

3.2. Association between Lp(a) and LDL in Lp(a):LDL adduct is specific and mediated through the sialic acid moiety of apo(a) subunit

To study Lp(a):LDL adduct, plasma Lp(a)-containing lipoproteins (PLL) including free Lp(a) and Lp(a) involved in adduct formation with LDL were isolated by utilizing the affinity of Lp(a) and its complexes for jacalin. From jacalin precipitate of plasma, PLL isolated by ultracentrifugation at 535, 000 g was identical with JL1 described in 'Results part-I'. Alternatively total plasma lipoproteins isolated by similar ultracentrifugation was bound to jacalin-Sepharose and PLL was collected after elution with specific sugar (Methods 5.2). On polystyrene well-coated PLL, response to HRP-labeled anti-apoB ([apoB]) as well as anti-apo(a) ([apo(a)]) was measured and the index of adduct was expressed as the increase in the ratio between [apoB] and [apo(a)] compared to that of native Lp(a) isolated from the same plasma.

In order to check whether the LDL co-precipitated with Lp(a) during jacalin precipitation of plasma was specifically associated with Lp(a), we tried dissociation of the interaction between Lp(a) and LDL in adduct by diluting plasma with PBS. Undissociated adduct remaining in the lipid layer of jacalin precipitate (PLL) was isolated by affinity precipitation with jacalin as described earlier. The higher value of [apoB]/[apo(a)] in adduct isolated from control as well as diluted plasma compared to that in native Lp(a) isolated from the same plasma (Fig.3.E) showed the presence of adduct in PLL. Fig.3.E also shows that a large majority of adduct remains in the plasma as undissociated form even after five times dilution of plasma with PBS suggesting that the association between Lp(a) and LDL in adduct is a specific one. However a reduction in adduct upon dilution of plasma may be due to the presence

of some low affinity adducts in which Lp(a) and LDL are held together by water reversible non-covalent interactions. Both hydrophobic and ionic interaction have been suggested for the Lp(a):LDL adduct formation (Trieu et al., 1990, Yashiro et al., 1993).

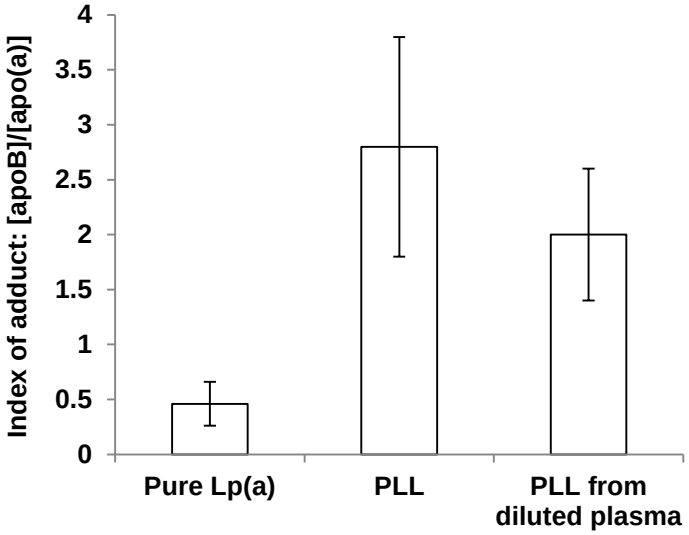
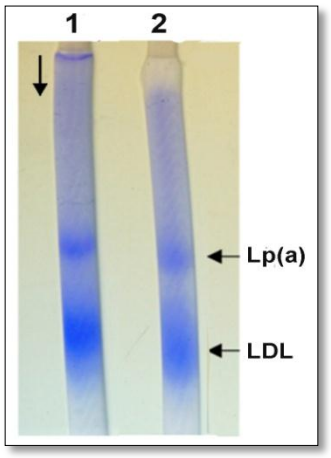


Fig.3.E. Lp(a) specifically interacted with LDL to form Lp(a):LDL adduct: PLL isolated from the lipid layer of jacalin precipitate of 5 times diluted and undiluted plasma were coated on polystyrene wells (100 ng per well), blocked and probed with HRP-labeled anti-human apo(a) and apoB antibodies (300 ng per well). Bound HRP was assayed as described earlier. Index of adduct was expressed as described earlier. Values are mean ± SD of six trials.

The apo(a) moiety of Lp(a) is responsible for its binding to other apoB-containing lipoproteins including LDL (Trieu and McConathy, 1990). Since the initial interaction of apo(a) with LDL in Lp(a) involves several non-covalent lysine binding sites of apo(a) and lysine residues of apoB of LDL (Becker L, 2004) the same interaction may also have a role in the Lp(a):LDL formation (Trieu and McConathy, 1990). So in order to check whether the Lp(a):LDL adduct co-

precipitated during Lp(a) isolation was lysine dependent two different protocols were employed (described in 'Methods 5.7').



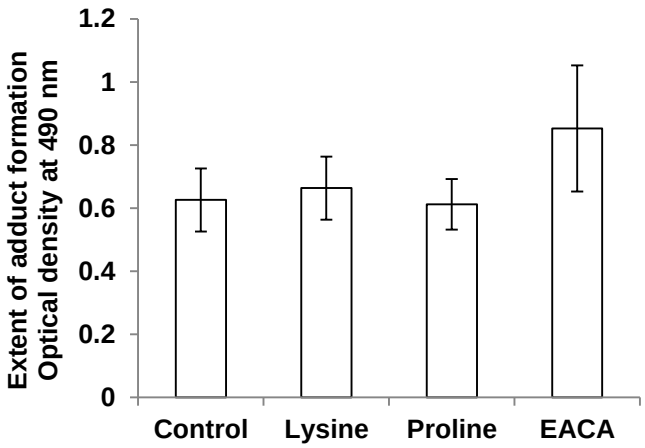


Fig.3.F **Fig.3.G**

Fig.3.F,G. The effect of amino acid treatment on adduct formation: (F) Lipid layer of jacalin precipitate collected from plasma pre-treated with 0.1M proline (2) was subjected to 3.75% TBE disc gel electrophoresis (50 µg proteins in each) and stained along with the control (1). Protein bands were stained and identified as Lp(a) and LDL as described earlier. **(G)** Effect of amino acid treatment on formation of de novo adduct was checked by adding biotin-labeled LDL to Lp(a) coated on polystyrene wells in the presence of 100 mM lysine, proline and ε-amino caproic acid (EACA) as described in 'Methods 5.7'. Values are mean ± SD of four trials.

The presence of LDL band in Fig.3.F shows that pre-treatment of plasma with proline (a structural analogue of lysine) does not remove LDL from Lp(a):LDL adduct. This result was again confirmed by checking the efficiency of Lp(a) to form de novo adduct with biotin-labeled LDL in the presence of lysine and lysine analogues [ε-amino caproic acid (EACA), proline]. Fig.3.G shows that reagents like lysine, proline or EACA couldn't prevent de novo adduct formation and suggested

that LDL attachment to apo(a) was not solely mediated by the lysine-binding site of the latter.

The basis for Lp(a):LDL adduct had been suggested to be Ca^{2+} cross-bridges between Lp(a) and LDL particles mediated by sialic acid residues in the heavily O-glycosylated kringle IV of apo(a) and the bulk positively charged lysine residues on apoB in LDL (Yashiro et al., 1993). In order to confirm the role of sialic acid of apo(a) in adduct formation, efficiency of native and desialylated Lp(a) to form de novo adduct with biotin-labeled LDL was checked by ELISA as described in 'Methods 5.8'. Fig.3.H shows that after desialylation of Lp(a) by neuraminidase treatment there was 42% reduction in de novo adduct formation suggesting the role of sialic acid moiety of apo(a) in mediating adduct formation by binding to the positive surface charge on lysine residue of apoB in LDL.

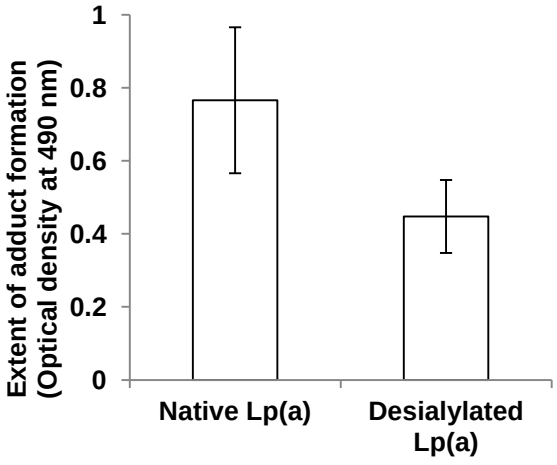


Fig.3.H. Role of sialic acid of apo(a) in Lp(a):LDL adduct formation: Sialic acids were removed from Lp(a) coated on polystyrene wells by treatment with neuraminidase (0.12 U per ml) for 1 h at 37 ºC. Heat inactivated neuraminidase was used in control experiment. Binding of biotin-labeled LDL to the desialylated Lp(a) and the efficiency of adduct formation was checked as described in 'Methods 5.2 (i)'. Percentage of reduction in adduct formation (%) upon desialylation of Lp(a) was calculated as 41.67 ± 8.5%. Values are mean ± SD of six trials. $p < 0.05$.

3.3. Qualitative analysis of LDL in adduct and non-adduct (free) forms

Though formation of Lp(a):LDL adduct had been reported (Trieu et al., 1990) very little is known about the composition of Lp(a):LDL complexes and about the factors governing this interaction. Both components in adduct may have equal contribution in adduct formation. In order check whether adduct formation is a function of the apoB subunit of LDL, LDL in adduct and non-adduct (free) forms were isolated from lipid layer of jacalin precipitate of plasma (PLL) and supernatant of jacalin precipitate (JSL1) respectively as described in 'Methods 5.9' were qualitatively analysed in terms of mobility in TBE disc gel electrophoresis, glycosylation pattern and oxidation status.

As the plasma LDL concentration far exceeds that of Lp(a) concentration, it is important to check whether the association of additional LDL with Lp(a) to form Lp(a):LDL adduct is a function of total plasma LDL concentration. For this index of adduct in jacalin-precipitated plasma lipids (PLL) was plotted against total plasma LDL concentration determined as anti-apoB response of total lipid layer (U-20%) of plasma coated on polystyrene wells (Fig.3.I). Result shows that association of additional LDL with Lp(a) to form adduct was independent of total plasma LDL concentration (correlation co-efficient, $r = - 0.123$, $p > 0.05$).

As the existence of two different LDL populations - small dense and large buoyant – in plasma have been suggested (Austin et al., 1988), mobility (as a function of LDL size) of LDL in adduct and non-adduct (free) forms isolated was compared by TBE disc gel electrophoresis as described earlier. Both adduct and free

LDL showed same pattern of mobility (Fig.3.J) suggesting the absence of any preference for LDL subpopulations to get associated with Lp(a).

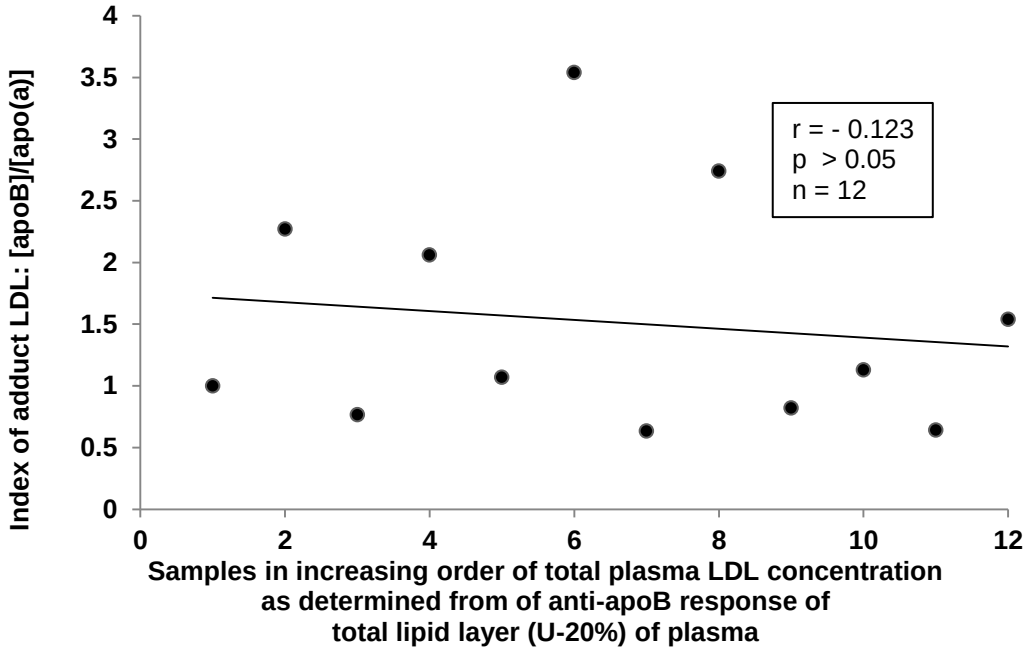


Fig.3.I. Lp(a):LDL adduct formation is independent of total plasma LDL concentration: PLL isolated by jacalin precipitation (source of LDL in adduct form) and total lipid layer of plasma (OL1, source of total LDL) were coated on polystyrene wells (200 ng per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies separately (PLL) or anti-apoB alone (OL1) (300 ng per well). Bound HRP was assayed as described earlier. Index of adduct was expressed as [apoB]/[apo(a)] ratio and was plotted against total LDL concentration determined as the anti-human apoB response of total lipid layer (OL1) of plasma. Correlation co-efficient, $r = -0.123$, $p > 0.05$.

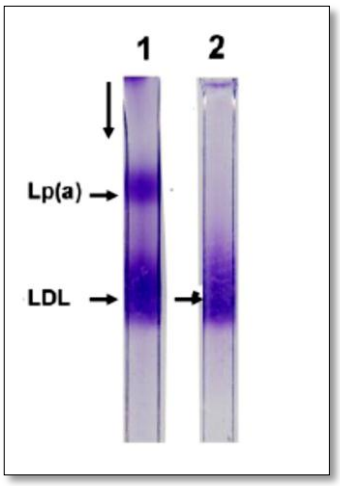
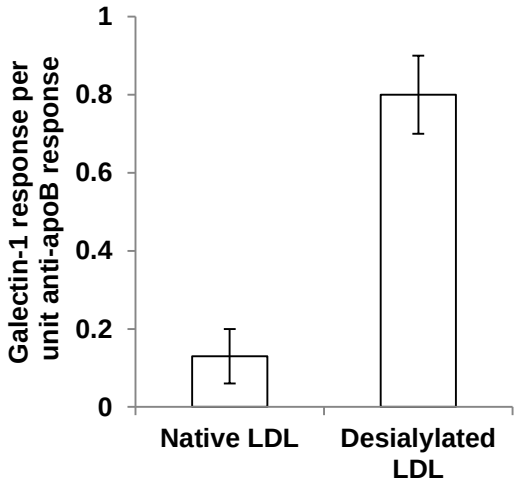


Fig.3.J. Coommassie brilliant blue staining to compare the mobility of LDL in adduct and non-adduct (free) form: (1) PLL isolated after jacalin precipitation of plasma and (2) lipid layer of supernatant of jacalin precipitate (JSL1) of same plasma (50 µg per tube) was subjected to TBE disc gel electrophoresis as described earlier. Protein bands were stained and identified as Lp(a) and LDL as described earlier. Result was confirmed with three different set of experiments.

As adduct formation between Lp(a) and LDL is mediated largely through sialic acid moieties in apo(a) which are integral components of O-glycans we examined whether N-glycans in apoB of LDL have any role in adduct formation. N-Glycan content and sialylation status of both adduct and free LDL were compared by ELLA (Fig.3.K,L). ApoB is enriched with complex N-glycans easily recognised by Con A. Lectins like galectin-1 and wheat germ agglutinin (WGA) were used to check the sialylation status of LDL. Even though native LDL is a poor ligand for galectin-1 (see Fig.2.B also), the enhanced lectin binding after desialylation of LDL by neuraminidase treatment (Fig.3.K) suggested the ability of galectin-1 to differentiate between native and desialylated LDL. The absence of significant difference in lectin binding between adduct and non-adduct (free) LDL in Fig.3.L shows that both LDL samples were identical with respect to their glycosylation pattern.



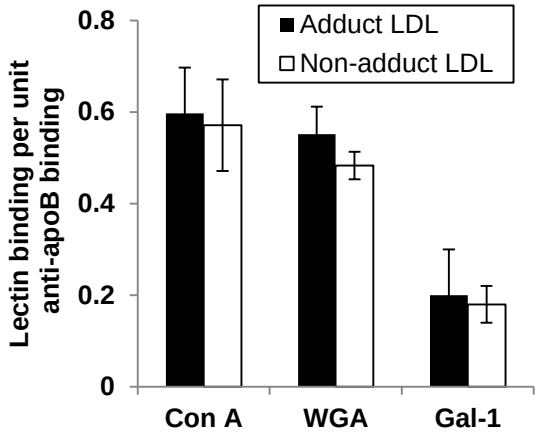
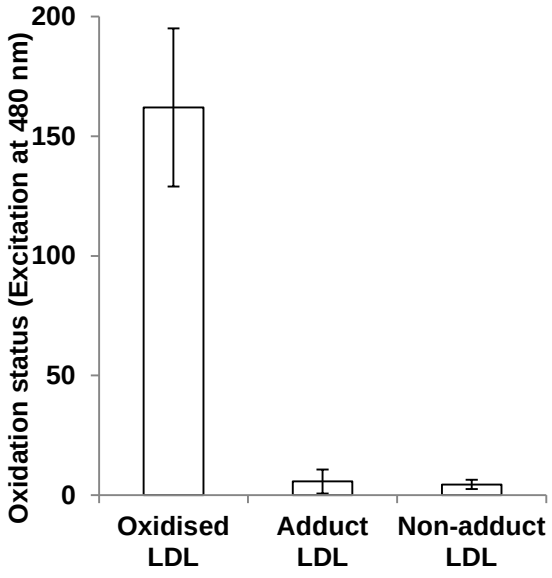


Fig.3.K **Fig.3.L**

Fig.3.K,L. Glycosylation and sialylation status of adduct and non-adduct LDL: (K) To check whether galectin-1 can be used as a probe for the identification of sialylation status of LDL, polystyrene wells coated with electroeluted LDL (100 ng per well) was incubated with neuraminidase (0.12 U per ml) for 1 h at 37 ºC and response to HRP-labeled galectin-1 was assayed as described earlier. Heat inactivated neuraminidase was used in control experiment. Values are mean ± SD of six trials. $p < 0.05$. **(L)** Adduct and non-adduct LDL were coated on polystyrene wells (100 ng per well), blocked and probed with HRP-labeled anti-human apoB antibody (300 ng per well), wheat germ agglutinin (WGA, 2 μg per well) and galectin-1 (1.5 μg per well). Con A (2 μg per well) was also used as described earlier. Bound HRP was measured as described earlier and lectin binding per unit anti-apoB binding plotted for each lectin. Values are mean ± SD of six trials.

The scavenging action of Lp(a) in which Lp(a) preferentially binds and removes oxidized LDL (oxLDL) from circulation (Bergmark et al., 2008) may be a possible reason for the existence of Lp(a):LDL adduct in circulation. So the oxidation status of LDL in adduct and non-adduct (free) form was checked by assaying the efficiency of oxLDL to excite a non-fluorescent dye, dichloro-fluoresceine diacetate (DCFH-DA) to highly fluorescent dichlorofluorescein (DCF) as described in 'Methods 2.25'. Copper oxidized LDL prepared as described in

'Methods 2.13' was used as a control. Fig.3.M shows that neither adduct LDL nor non-adduct (free) LDL was oxidized. This result was confirmed by comparing the efficiency of both native and copper oxidized LDL to form de novo adduct with native Lp(a) (Fig.3.N). Both LDL samples were found to be almost equal in their ability to form de novo adduct. This result shows that oxidation of LDL did not enhance its association with Lp(a) to form adduct.



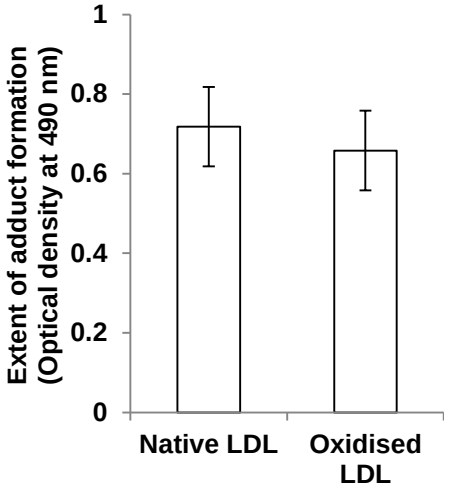
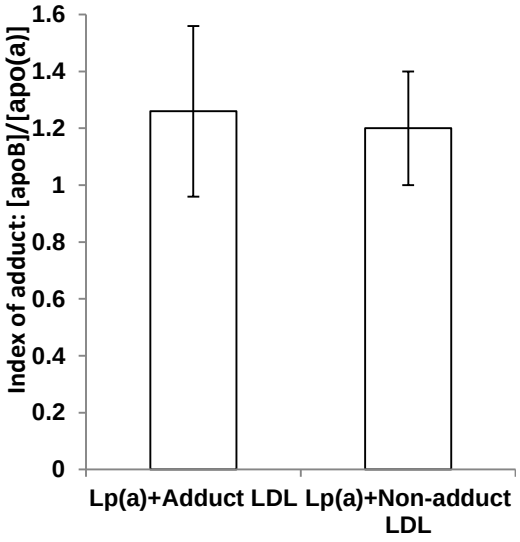


Fig.3.M **Fig.3.N**

Fig.3.M,N. Oxidation status of adduct and non-adduct LDL: (M) Polystyrene wells were coated with equal amounts of copper oxidized LDL, adduct LDL and non-adduct LDL (100 ng per well). After blocking, 2',7'-dichloro-fluoresceine diacetate (DCFH-DA, 20 µg per well) was added and mixture incubated at 37 ºC for 2 h in the dark. Nonfluorescent probe DCFH-diacetate, which is converted to highly fluorescent dichlorofluorescein (DCF) by oxLDL was checked by assaying the fluorescence at excitation wavelength of 480 nm and emission wavelength of 520 nm as described in 'Methods 2.25'. **(N)** Efficiency of copper oxidised LDL to form de novo adduct was compared by adding biotin-labeled native or oxidized LDL to native Lp(a) coated on polystyrene wells. Bound LDL was assayed by probing with HRP-labeled avidin (300 ng per well) as described in 'Methods 2.20'. Values are mean ± SD of six trials.

The above results were further confirmed by comparing the efficiency of adduct and non-adduct LDL to form de novo Lp(a):LDL by associating with native Lp(a) using two different protocols described in 'Methods 5.3'. Fig.3.O,P shows that the efficiency to form de novo adduct was same for both adduct-derived LDL and free LDL and indicated that the stoichiometry of adduct may be decided largely by the other component, viz. Lp(a).



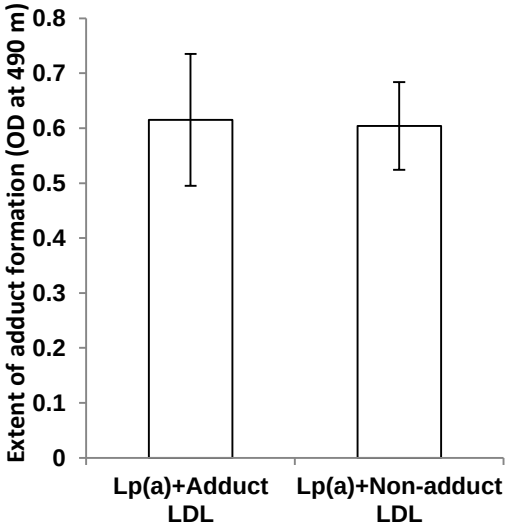


Fig.3.O **Fig.3.P**

Fig.3.O,P. Comparison of efficiency of adduct and non adduct LDL to form de novo adduct with Lp(a): Two protocols were employed as described in 'Methods 5.3'. **(O)** In protocol 1, de novo adduct was allowed to form between TBE-purified Lp(a) (100 ng) and adduct or non-adduct (free) LDL (200 ng) separately by incubating the two at 4 °C for 4 h. Biotin-labeled jacalin was added to probe the adduct formed and captured on streptavidin-coated (1 µg per well) polystyrene wells to capture jacalin-Lp(a):LDL adduct complex and probed separately with HRP-labeled anti-human apo(a) and anti-human apoB antibodies and bound HRP was measured as described earlier. Index of de novo adduct was expressed as described earlier. **(P)** In protocol 2, biotin-labeled adduct or non-adduct LDL (200 ng per well) was added separately to polystyrene wells coated with Lp(a) (100 ng per well) and incubated at 4 ºC for 2 h to form adduct. De novo adduct formed was assayed by quantitating bound biotin-labeled LDL by probing with HRP-labeled avidin (300 ng per well) as described above.

3.4. Role of apo(a) size on Lp(a):LDL adduct formation

Apo(a) is apparently the most polymorphic protein in human plasma so far identified. Since the role of variations in kringle IV type 2 repeats of apo(a) and of proportionate change in O-linked oligosaccharides in the function of circulating Lp(a) is unknown their effect on adduct formation was examined. For this PLL was isolated by affinity precipitation of plasma with jacalin as described in 'Methods 5.2'. Index of adduct defined as [apoB]/[apo(a)] was plotted against size of apo(a) [mB/m(a)] determined as described in 'Methods 3.3'. A strong positive correlation (r = 0.834, p = 0.001) between index of adduct and apo(a) size in Fig.3.Q indicates that large apo(a) can associate with proportionately more LDL.

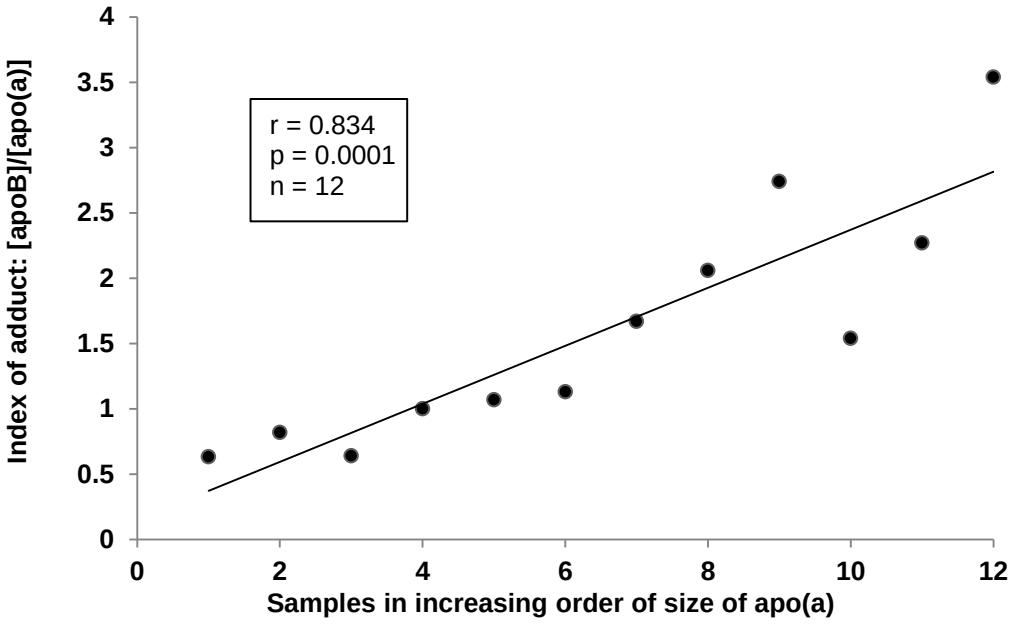


Fig.3.Q. Role of size of apo(a) in Lp(a):LDL formation: PLL was coated on polystyrene wells (200 ng per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well). Bound HRP was assayed as described earlier. Index of adduct expressed as [apoB]/[apo(a)] was plotted against apo(a) size, [mB/m(a)].

This result was confirmed by assessing the relative intensities of apolipoprotein bands obtained in non-reducing SDS-PAGE of PLL isolated by jacalin precipitation. The latter contains all Lp(a)-containing particles from plasma including free and adduct Lp(a). SDS-PAGE of PLL without reduction separated the apolipoprotein components of Lp(a) and LDL, viz apo(a)-apoB and apoB. Plasma samples were divided into two groups – large Lp(a) group (mB/m(a) > 2) and small Lp(a) group (mB/m(a) < 2). PLL from each plasma sample was subjected to 3.5% SDS-PAGE under non-reducing condition as described in 'Methods 2.15 (ii)', bands stained with Coommassie brilliant blue and identified as apo(a)-apoB and apoB by immunoblotting (Fig.3.R).

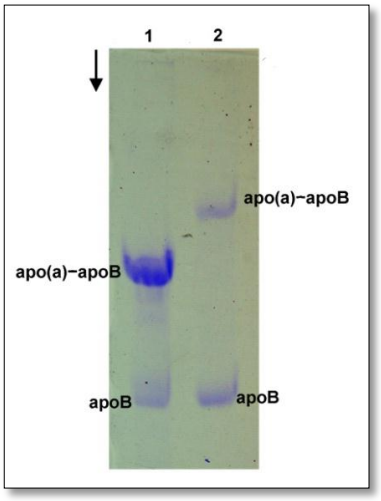


Fig.3.R. Non-reducing SDS-PAGE of PLL isolated by jacalin precipitation of plasma to confirm the higher potential of large Lp(a) to get associated with more LDL: PLL isolated by jacalin precipitation from one each of large and small Lp(a) plasma groups (50 µg proteins in each) was subjected to 3.5% SDS-PAGE under non-reducing conditions and stained with Coommassie brilliant blue. Bands were identified as apo(a)-apoB and apoB by immunoblotting as described in 'Methods 2.17'. (1) PLL from small Lp(a) plasma, (2) PLL from large Lp(a) plasma. Result was confirmed with three different set of experiments.

Intensities of apoB and apo(a)-apoB bands were measured using digital scanning. The mean ratio of intensity of apoB band to that of apo(a)-apoB band, for three samples in each group was calculated and was found to be 0.33 ± 0.06 and 1.5 ± 0.1 for small and large Lp(a) groups respectively suggesting the far greater tendency of large Lp(a) to get associated with more LDL as adduct. Fig.3.R shows a representative electrophoretic pattern from either group.

The amount of associated LDL in adduct was further assessed by comparing the [apoB]/[apo(a)] values of PLL, isolated from plasma using two different protocols – jacalin affinity precipitation of plasma and jacalin affinity chromatography of total plasma lipoproteins (Methods 5.2), with that of purified Lp(a) isolated from the same plasma samples. The three different preparations from each plasma were coated on polystyrene wells, blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies and the ratio [apoB]/[apo(a)] was compared. Since PLL contains Lp(a) in adduct form as well as in free form, the increase in its [apoB]/[apo(a)] ratio compared to that of respective pure Lp(a) measures the extent of adduct formation in plasma Lp(a). PLL isolated from large and small Lp(a) group plasma by two different protocols showed similar result (Fig.3.S,T). The increase in [apoB]/[apo(a)] value in PLL compared to that in pure Lp(a) was much higher for large Lp(a) group. De novo adduct prepared using purified large and small Lp(a) samples also showed the same result (Fig.3.U) with higher potential of large Lp(a) molecules to get associated with more LDL forming Lp(a):LDL adduct compared to the small Lp(a) molecules (Table 3.2). These results strongly support the direct influence of apo(a) size in Lp(a):LDL adduct formation.

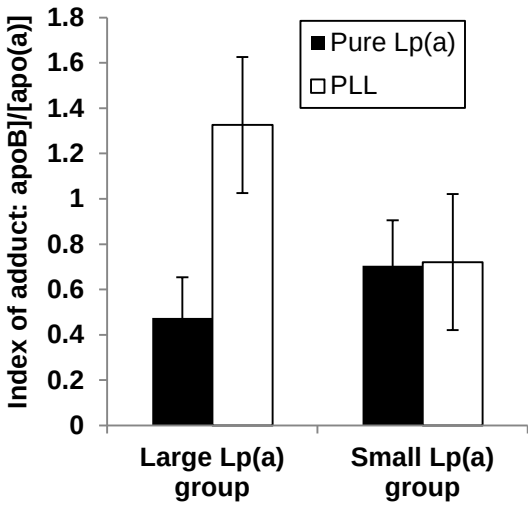


Fig.3.S

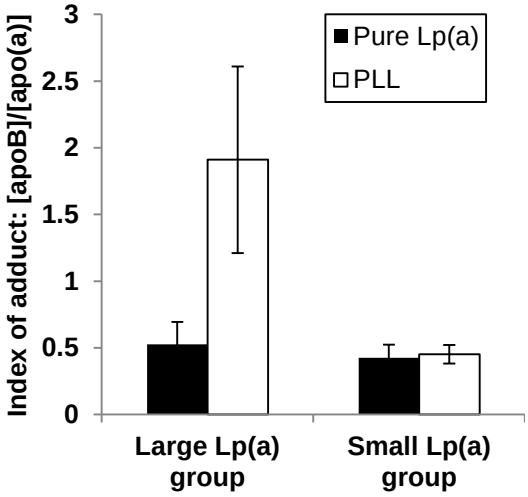


Fig.3.T

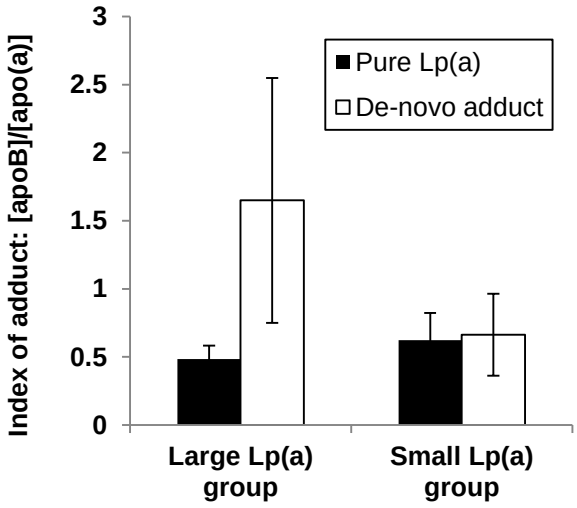


Fig. 3.U

Fig.3.S,T,U. Large apo(a) can associate with more LDL to form adduct: Plasma Lp(a)-containing lipids (PLL) was isolated from plasma using two different protocols as described in 'Methods 5.2'. Pure Lp(a) was also isolated as described in 'Methods 3.1'. All samples were coated on polystyrene wells (200 ng per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well). Bound HRP was assayed as described earlier. Index of adduct was expressed as [apoB]/[apo(a)] and was compared with that of purified Lp(a). **(S)** PLL isolated by jacalin affinity precipitation of plasma. Values are mean ± SD of nine trials. **(T)** PLL isolated by affinity chromatography of total plasma lipoproteins on jacalin-Sepharose. Values are mean ± SD of four trials. **(U)** De novo adduct was prepared as described in 'Methods 5.3 (ii)', and index of adduct was expressed as described above. Values are mean ± SD of four trials.

Table 3.2. Percentage increase in [apoB]/[apo(a)] value in PLL and de novo adduct compared to native Lp(a):

Method of isolation of PLL	Increase in [apoB]/[apo(a)] value (%) of PLL compared to that of pure Lp(a)*	
	Large Lp(a) group	Small Lp(a) group
Jacalin affinity precipitation (n = 9)	63 ± 14.4	10.56 ± 9.7
Jacalin affinity chromatography (n = 4)	68 ± 21.2	9.5 ± 19
De novo adduct (n = 4)	66 ± 10.4	5.4 ± 10.3

*Data was derived from results described in Fig.3.S,T,U.

The higher potential of larger Lp(a) to get associated with more LDL as well as sialic acid mediated interaction of Lp(a) and LDL in adduct shows the influence of O-glycans in the kringle IV type 2 repeats of apo(a) in adduct formation. Previous results (see Fig.2.F and Fig.3.Q) indicate that the degree of O-glycosylation as well as association of LDL with Lp(a) increases steadily with increase in apo(a) size. Result from Fig.3.V shows that the increase in association of LDL with Lp(a) is out of proportion to the increase in degree of O-glycosylation of apo(a).

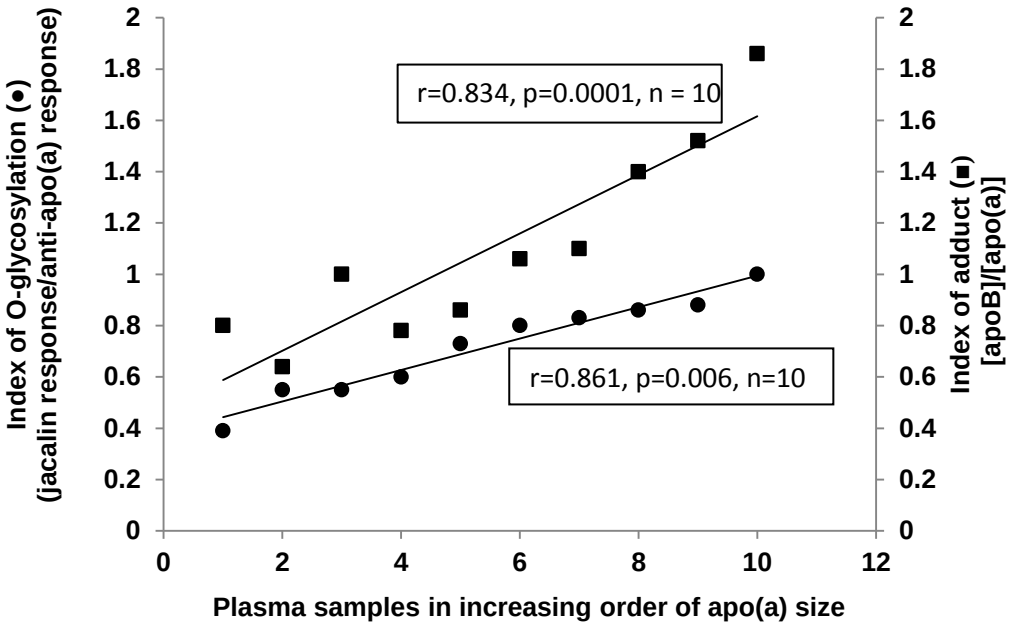
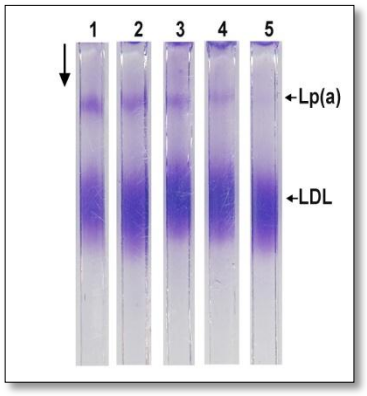


Fig.3.V. Rate of increase in adduct formation is out of proportion to increase in O-glycosylation of apo(a): Index of adduct was calculated and expressed as ratio of response of anti-apoB [apoB] to that of anti-apo(a) [apo(a)] as described in Fig.3.Q. Index of O-glycosylation of native Lp(a) was calculated from the jacalin binding per unit anti-apo(a) binding as described in Fig.2.F. Index of O-glycosylation or index of adduct was plotted against apo(a) size determined from 3.5% SDS-PAGE disc gel electrophoresis of JL1 as described in 'Methods 3.3'.

3.5. Identification of varying stoichiometry of Lp(a):LDL adduct in plasma

The higher potential of large Lp(a) to get associated with more LDL may result in the existence of very large particles of Lp(a):LDL adduct in circulation. To examine if stoichiometry of the adduct remains the same in a given individual we used different concentrations of PEG solution to selectively precipitate adduct with varying molar ratios of LDL. Above results have shown that at PEG concentration of 1% (Fig.3.B) adduct starts precipitating from plasma. To obtain adducts with differing compositions percentages of PEG ranging from 1.5 through 2, 2.5 to 3 were

employed to remove adduct from plasma by precipitation. Adduct not precipitated by PEG remained in the supernatant was collected by jacalin precipitation as described in 'Methods 5.10' and analyzed by TBE disc gel electrophoresis and ELISA. Control without PEG was also used.



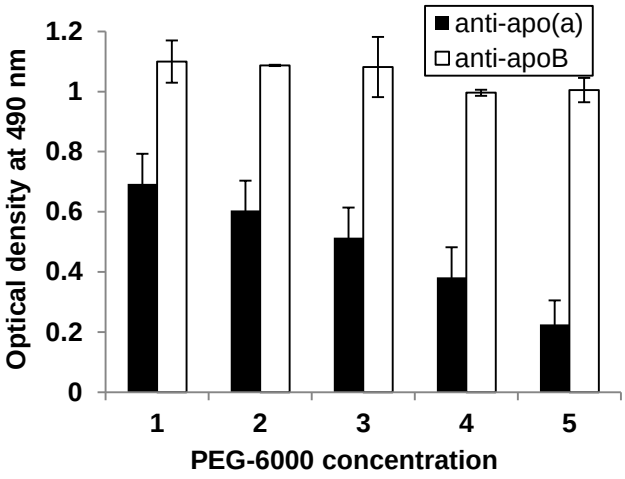


Fig.3.W **Fig.3.X**

Fig.3.W,X. Lp(a):LDL adduct remaining in the supernatant of PEG precipitate of plasma: Lp(a):LDL adduct was sequentially removed from plasma by varying PEG concentration [(1)0%, (2)1.5%, (3)2%, (4)2.5% and (5)3%]. The adduct remaining in the supernatant of PEG precipitate was collected as PLL by jacalin affinity precipitation followed by ultracentrifugation and subjected to 3.75% TBE disc gel electrophoresis (50 µg per tube) **(W)** or coated on polystyrene wells (200 ng per well), blocked and probed with HRP-labeled anti-human apo(a) and anti-human apoB antibodies (300 ng per well) **(X)**. Bound HRP was assayed as described above. Values are mean ± SD of three trials.

With increasing PEG concentration there was a decrease in anti-apo(a) response in ELISA and in Lp(a) band in TBE electrophoresis of the same amount of PLL while both anti-apoB response and LDL band in TBE electrophoresis remained largely unaffected (Fig.3.W,X). This indicated that LDL molecules are attached to Lp(a) in widely varying LDL to Lp(a) ratio to form adducts and that adducts with

higher LDL to Lp(a) ratio are proportionally more hydrophobic since PEG precipitates proteins and their complexes by water deprivation. This result suggests that Lp(a):LDL adduct formation makes LDL more hydrophilic and Lp(a) more hydrophobic. So it is interesting to examine how these changes affect the half life or fate of Lp(a) in circulation. Fig.6 shows the schematic representation of nascent Lp(a) transformation in circulation and attachment of more LDL molecules to form Lp(a):LDL adduct.

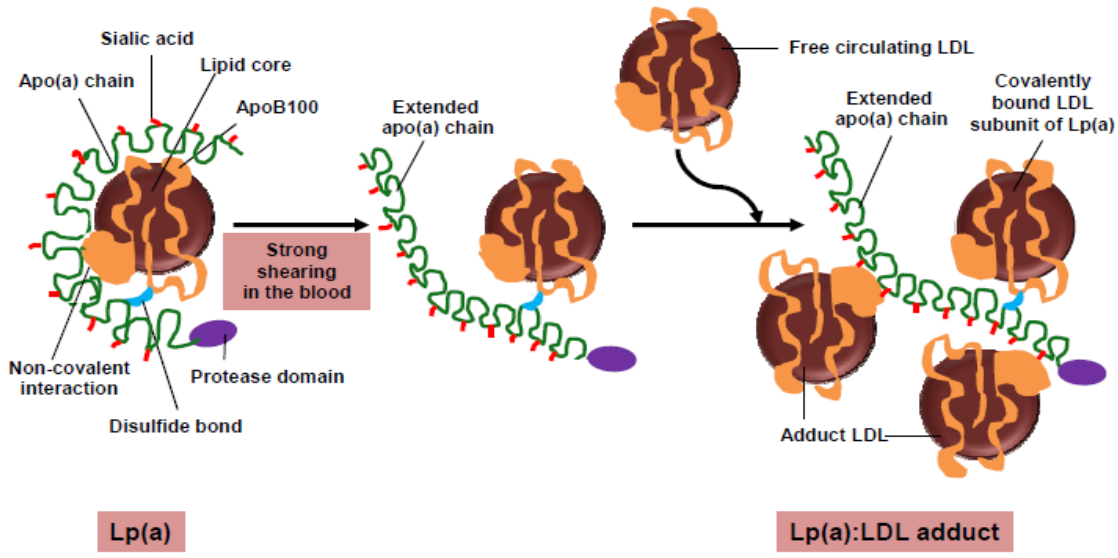


Fig.6. Schematic representation of nascent Lp(a) transformation in circulation and attachment of more LDL molecules to form Lp(a):LDL adduct

Discussion

In Lp(a), the apo(a) protein winds around the LDL particle, but after strong shearing in the blood (Weisel et al., 2001) the apo(a) protein undergoes a conformational change and the bulk of apo(a) extends out into solution away from

the lipoprotein surface though remaining linked to the LDL particle by the disulfide bond so that it may interact with other ligands (Phillips et al., 1993) to form complexes in plasma. Association of additional LDL molecules non-covalently to Lp(a) results in adduct formation (Trieu et al., 1990). Lp(a) immune complexes with autoantibodies have been reported to correlate with cardiovascular diseases (Wang et al., 2004). Recent work in this laboratory has shown that anti-α-galactoside antibody (anti-Gal) is chiefly involved in immune complex formation with Lp(a) in all plasma samples examined (Geetha et al., 2013). These observations warrant investigations on the contribution of structural differences in Lp(a) among individuals towards formation of complexes with LDL or antibodies. Preferential accumulation of Lp(a) compared to LDL in atherosclerotic lesions underline a significant role for the unique structural features of apo(a) in Lp(a) function and pathology. So we checked whether this scenario is reflected in complex formation of Lp(a) by studying the distribution of Lp(a) in plasma as free or complexed form in comparison to that of LDL. Higher anti-apo(a) response in complexed lipoprotein fraction compared to that in the free lipoprotein layer suggested the remarkable potential of Lp(a) for complex formation with plasma proteins (Fig.3.A) leaving only a minor fraction of plasma Lp(a) in free form (Fig.3.C). A strong negative correlation between amount of free Lp(a) and apo(a) size in Fig.3.C ($r = -0.887$, $p = 0.0001$) suggests the potential role of apo(a) size in governing the fate of Lp(a), the large Lp(a) being more efficient in complex formation with LDL or antibodies to form Lp(a):LDL adduct or Lp(a)-IC respectively. This is in accordance with Trieu et al. (1990) who showed that up to 72% of purified Lp(a) added to Lp(a)-negative hypertriglyceridemic plasma floated

with apoB-containing lipoproteins suggesting the higher potential of Lp(a) to get associated with apoB subunit of LDL and exist as Lp(a):LDL adduct.

Even though both lysine and sialic acid mediated interactions have been implicated in adduct formation (Trieu et al., 1990, Yashiro, 1993), our result favours the sialic acid mediated interaction in adduct since desialylation of Lp(a) by neuraminidase treatment resulted in 42% reduction in de novo adduct formation (Fig.3.H). An interesting observation during this study was that instead of inhibition, EACA (a structural analogue of lysine) enhanced the binding of LDL to Lp(a) (Fig.3.G). This was also observed in an earlier experiment in which Lp(a) appears to undergo a dramatic conformational change in the presence of 6-aminohexanoic acid (Fless et al., 1996). Another study has reported enhancement in Lp(a) assembly by EACA at low concentrations by altering the conformation of apo(a) (Becker et al., 2003).

Analysis of features of the two components in adduct revealed that the association of additional LDL with Lp(a) is independent of total plasma LDL concentration (Fig.3.I, correlation co-efficient, $r = - 0.123$, $p > 0.05$). Further results suggest that neither LDL sub-populations nor modifications in apoB protein or glycan structure has any role in adduct formation (Fig.3.J,L,M and N). Lack of correlation between size of LDL particles and the extent of their recognition by apo(a) towards Lp(a) formation had been reported (Becker et al., 2001). Above results along with the identical capacity of adduct-derived and non-adduct LDL to form de novo adduct (Fig.3.O,P) clearly established that adduct formation is independent of the quantitative or qualitative nature of plasma LDL. On the other

hand a strong positive correlation (r = 0.834, p = 0.001) between index of adduct formation and apo(a) size in Fig.3.Q indicated the higher potential of large Lp(a) to associate with more LDL. This was further confirmed from Fig.3.R,S and T. Extent of formation of de novo adduct by mixing LDL with large and small Lp(a) species separately also supported the above observations (Fig.3.U).

In all the above cases we expressed adduct formation as percentage increase of ratio between anti-apoB response [apoB] and anti-apo(a) response [apo(a)] from that of native Lp(a). In Fig.3.Q and 3.S,T,U observed response of anti-apoB or [apoB] in coated adduct samples includes that of apoB covalently attached to apo(a). Result in section 2.4 (Fig.2.G), however shows that as Lp(a) size increases antigenic epitopes of apoB in pure Lp(a) are increasingly less available leading to lower response to anti-apoB in large Lp(a) molecules. It follows that but for this pull in the opposite direction, the rate of increase in [apoB] to [apo(a)] ratio for adduct in Fig.3.Q,S,T and U would have been faster and that the actual increase in number of attached LDL molecules on each Lp(a) molecule with increase in apo(a) size is much larger than indicated by the observed correlation co-efficient. This was reflected in Fig.3.V in which the increase in association of LDL with Lp(a) is not parallel but out of proportion to the increase in glycosylation of apo(a). With graded increase in PEG concentration used for precipitation and analyzing the adduct in respective supernatants by jacalin precipitation and ELISA adducts with more LDL content were found more resistant to PEG precipitation (Fig.3.W,X). As additional LDL increases hydrophobicity of Lp(a):LDL adduct there is a possibility of increase in half life of Lp(a) in circulation and eventually to its attachment on various tissue sites.

Physiological consequence of the direct influence of apo(a) size on Lp(a):LDL adduct formation can be speculated as follows: (i) the higher potential of large Lp(a) to bind galectin-1 may have a prominent role in the binding of large Lp(a):LDL adduct to endogenously expressed galectin-1 leading to its deposition on tissue sites. (ii) the presence of extra LDL in adduct may help Lp(a), the relatively poor ligand for LDL-receptor, to bind to the receptor and eventually get removed from circulation, contributing to the low plasma concentration of large Lp(a) molecules compared to that of small Lp(a). (iii) on the other hand the higher potential of large Lp(a) to get associated with more LDL may compensate for its lower plasma concentration as Lp(a) being a quick source of cholesterol in wound sites and other areas of tissue repair. The next part of the results deals with the first two possibilities using cell culture experiments.

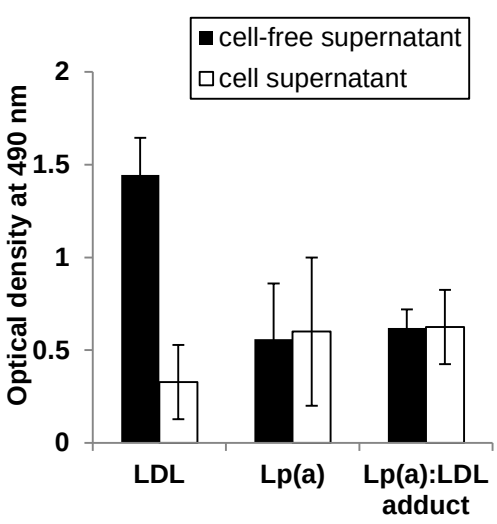
PART IV

Role of Lp(a):LDL adduct as a vehicle for lipid transport to cells: studies using cell-surface LDL receptor and immobilized galectin-1

Several studies have addressed the binding of Lp(a) to LDL receptor and found that Lp(a) is not a ligand for LDL-receptor (Reblin et al., 1997). The recognition domain for binding of the LDL particle to the receptor resides on apoB (Mahley et al., 1980). The ability of Lp(a) to bind to the receptor is strongly modulated by the presence of apo(a) glycoprotein as after removal of apo(a) from Lp(a) by reduction, the latter was bound, internalized, and degraded through the LDL-receptor pathway with the same affinity and efficiency as normal LDL (Armstrong et al., 1985). Since apoB of LDL in Lp(a) is unavailable for receptor binding, it is reasonable to examine if extra LDL molecules in Lp(a):LDL adduct can facilitate the entry of Lp(a) into cells through LDL receptors. Moreover previous results (Results part-II) show that large Lp(a) molecule with more O-glycans possess higher affinity towards galectin-1, an endogenous lectin expressed on various tissues. So it is also important to check the effect of association of LDL with Lp(a) in galectin-1 recognition. This part of thesis focuses on these two aspects.

4.1. Cell-culture studies to check binding of Lp(a):LDL adduct to LDL-receptor using human monocyte derived macrophages and rat cardiac fibroblasts

To evaluate the effect of association of LDL with Lp(a) in the interactions of the latter with the LDL receptor, two culture systems were used – 9 days old human monocyte derived macrophages and 75% confluent rat cardiac fibroblasts. The cell-cultures were subjected to 24 h serum deprivation by keeping in serum-free media as lipoprotein depletion of cells results in up-regulation of the LDL receptor (Ho et al., 1976) without altering the conformation or function of Lp(a) and LDL. This step is also essential to avoid the interference of binding of serum lipids to the receptor.



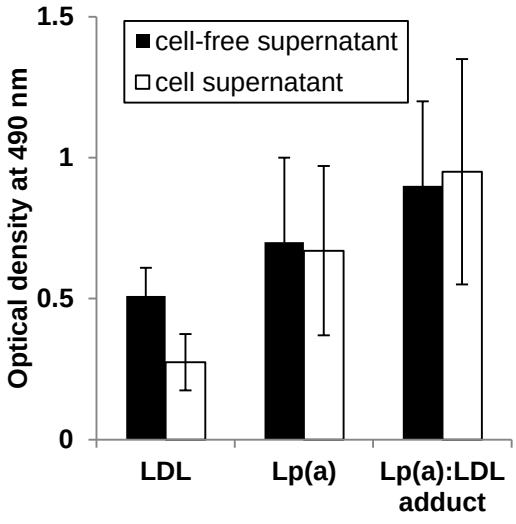


Fig.4.A **Fig.4.B**

Fig.4.A,B. Cell culture studies to check the binding of Lp(a):LDL adduct to LDL receptor: (A) Human monocyte-derived macrophages **(B)** rat cardiac fibroblasts. Serum-deprived (24 h) cells were treated with 4 µg lipoproteins (Lp(a) and LDL) and adduct in 400 µl media without serum by incubating in an atmosphere of 5% CO_2 at 37 °C for 24 h. Binding of lipoproteins to LDL receptor was checked by assaying cell supernatant for the decrease in LDL, Lp(a) and adduct by ELISA. LDL was assayed by adding the cell supernatant on anti-apoB coated polystyrene wells (1 µg per well) and bound LDL was assayed by probing with HRP-labeled anti-apoB antibody (300 ng per well). Lp(a) and adduct were assayed by j-a method. Native Lp(a) and LDL were isolated from JL1 and JSL1 respectively (see Section 1.1; results). Adduct was isolated from plasma after removal of free Lp(a) by 0.8% PEG precipitation as described in 'Methods 5.2 (iii)'. Values in each group in Fig.4.A and B are mean ± SD of six consecutive plasma samples with mB/m(a) >2.

Cells were treated with appropriate dilution of filtered lipoproteins and adduct in media without serum under sterile condition by incubating in an atmosphere of 5% CO_2 at 37 °C for 24 h. In order to check the binding of lipoproteins to LDL receptor, cell supernatants were assayed for the decrease in LDL, Lp(a) and adduct by ELISA. Control experiments were performed without

cells. Fig.4.A,B shows that there is no reduction of Lp(a) or adduct in the cell supernatant compared to the control suggesting that neither Lp(a) nor adduct binds to LDL receptor. The reduction in LDL content confirms the expression and function of LDL receptor on cell surface. This results indicate that the presence of extra LDL in adduct did not make the latter a ligand for LDL receptor probably due to LDL receptor binding domain of apoB of additional LDL molecules in the adduct being involved in adduct formation with Lp(a). Any degradation of Lp(a) or adduct during cell treatment was ruled out by comparing the integrity of Lp(a) and adduct determined from ratio of response to anti-apoB and anti-apo(a) [apoB]/[apo(a)] with that of control (Table 4.1).

Table 4.1. Integrity of Lp(a) and Lp(a):LDL adduct during cell treatment:

Cell culture used	[apoB]/[apo(a)] ratio*			
	Lp(a)		Lp(a):LDL adduct	
	cell-free supernatant	cell supernatant	cell-free supernatant	cell supernatant
Human monocyte derived macrophages	0.61 ± 0.25	0.612 ±0.25	1.95 ± 0.6	2.18 ± 0.58
Rat cardiac fibroblasts	0.49 ± 0.18	0.48 ± 0.17	1.19 ± 0.6	1.188 ± 0.6

*The [apoB]/[apo(a)] ratio of Lp(a) or adduct in supernatant after cell treatment for Fig.4.A,B was determined after capturing them on plate-coated jacalin as described in ‘Methods 6.1’. Cell-free supernatant was used as control. Vales in each group are mean ± SD of six trials.

Oil red O staining of lipoprotein- or adduct- treated rat cardiac fibroblasts (Fig.4.C,D,E,F) also demonstrated that the LDL receptor interaction of apoB was

impaired and suggested that the receptor binding domain of apoB may be masked either by lipids or by the extended apo(a) chain.

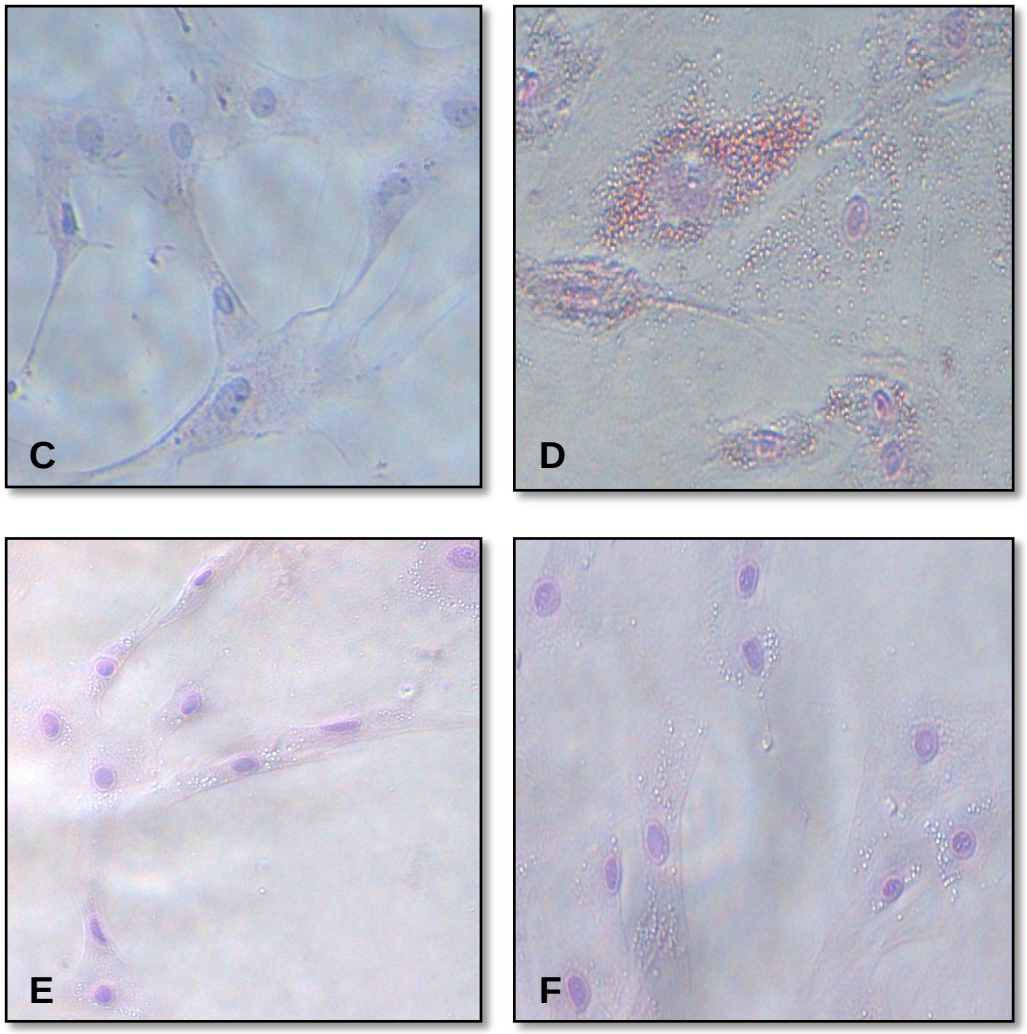
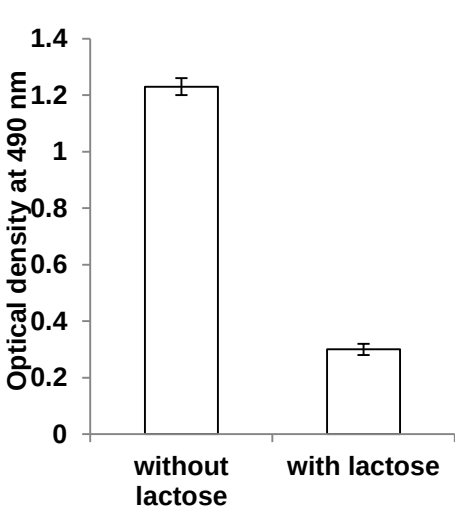


Fig.4.C,D,E,F. Oil Red O staining of lipoproteins- and de novo adduct- treated rat cardiac fibroblast cells: After removing the media the lipoproteins- and adduct-treated and un-treated rat cardiac fibroblast cells were washed three times with PBS and subjected to Oil red O staining as described in 'Methods 2.19' and observed under 40 X light microscope. Deep red or deep orange colour shows the presence of lipoprotein components and blue colour shows nuclei. Other structures remain unstained. (C) Control without lipoprotein (D) LDL treated (E) Lp(a) treated (F) PLL (adduct) treated.

4.2. Lp(a) mediated binding of de novo Lp(a):LDL adduct to immobilized galectin-1 on polystyrene wells

Lp(a) has been shown to be an efficient glycoconjugate ligand for galectin-1, an endogenous lectin expressed on most tissues (Chellan et al., 2007). Results in the previous parts suggest that apo(a) size polymorphism may modulate galectin-1 recognition of Lp(a) suggesting Lp(a)-galectin-1 interaction is a possible route through which Lp(a) may get deposited on these cell surfaces. As large Lp(a) has greater tendency to associate with more LDL molecules, effect of adduct formation by Lp(a) in its recognition by galectin-1 is physiologically relevant. The binding of Lp(a):LDL adduct to immobilized galectin-1 was demonstrated using biotin-labeled galectin-1 and FITC-labeled de novo adduct. Immobilization of galectin-1 was confirmed from the binding of HRP-labeled fetuin in the presence or absence of lactose (Fig.4.G). The binding of de novo adduct (prepared as described in 'Methods 6.2') to immobilized galectin-1 captured on streptavidin-coated polystyrene wells in Fig.4.H suggested a possible route through which Lp(a) and LDL may get deposited on tissue surfaces.



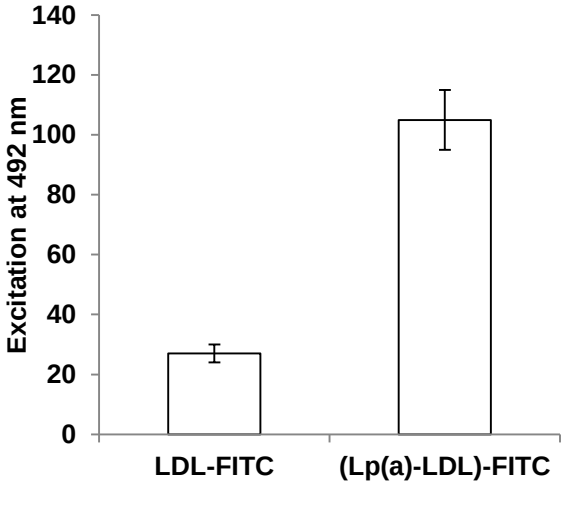


Fig.4.G **Fig.4.H**

Fig.4.G,H. Binding of de novo adduct to immobilized galectin-1: (G) Polystyrene wells were coated with streptavidin (1 µg per well) and blocked. Biotin-labeled galectin-1 (5 µg) in 200 µl PBS-T pre-incubated with 25 mM lactose was added to the wells and immobilized by incubating at 4 ºC for 2 h. Immobilization of galectin-1 was confirmed by addition of HRP-labeled fetuin (2 µg per well) to washed immobilized galectin-1 in the presence and absence of 50 mM lactose. Bound HRP was assayed as described earlier. Values are mean ± SD of three trials. **(H)** De novo adduct was prepared by mixing TBE purified Lp(a) and FITC-labeled LDL as described in 'Methods 6.2' and added to immobilized galectin-1 captured on streptavidin-coated polystyrene wells as described above and incubated for 2 h at 4 ºC under dark. The bound fluorescence of Lp(a):LDL-FITC was measured at excitation wavelength of 492 nm and emission wavelength of 515 nm as described in 'Methods 2.11'. Control without Lp(a) was also used to measure the non-specific binding of LDL-FITC alone. Values are mean ± SD of eight trials.

4.3. Human plasma-derived glycopeptides as inhibitors of galectin-1-Lp(a) interaction

As recognition of circulating Lp(a) by cell-surface galectin-1 is a very likely event which could eventually lead to the deposition of Lp(a) on atherosclerotic

plaques, search for an efficient inhibitor to block this interaction is relevant. Albumin-free fraction of human plasma proteins were obtained as 45% ammonium sulfate precipitated fraction. After pronase digestion of the latter, glycopeptides binding to DEAE-Sephadex A-50 were isolated as described under 'Methods 6.3'. Compared to the unmodified albumin-free plasma proteins, glycopeptides eluted from DEAE-Sephadex (with an average protein concentration of 1.2 mg per ml and neutral sugar concentration of 832 µg per ml) were remarkably enriched in O-glycosylated peptides and as much poor in N-glycans, judging from the recognition of polystyrene-coated glycopeptides by the N-glycan specific Con A and the O-glycan specific jacalin (Fig.4.I).

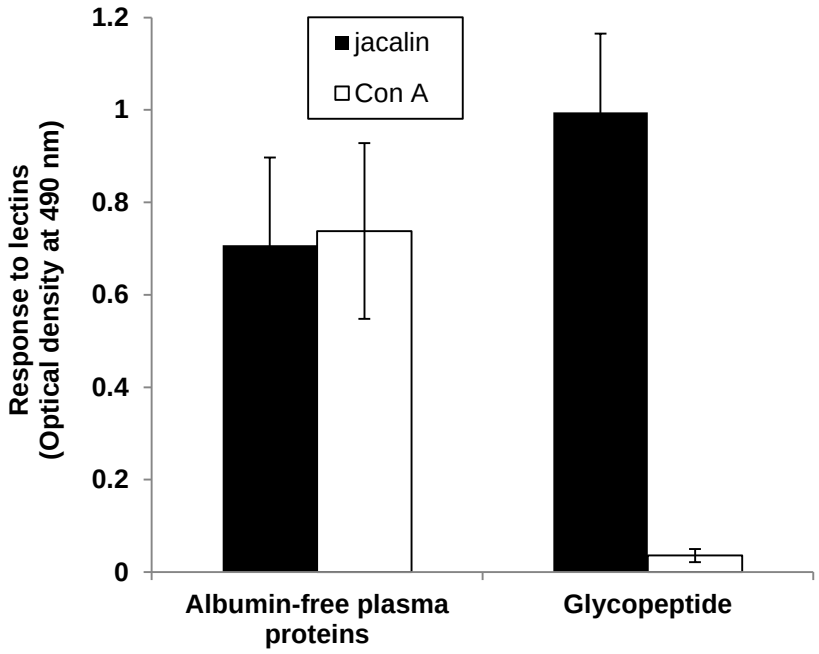


Fig.4.I. Assay to check the enrichment of O-glycans in isolated plasma glycopeptide: Albumin-free fraction of plasma proteins isolated by 45% ammonium sulfate precipitation of plasma and glycopeptides obtained as DEAE-binding fraction of pronase-digested albumin-free fraction were coated on polystyrene wells (1 µg per well), blocked and probed with specified concentrations of HRP-labeled jacalin and Con A as described in 'Methods 2.20'. Vales are mean ± SD of six trails.

As an inhibitor of human galectin-1 binding to either Lp(a) or to asialofetuin these glycopeptides were several fold more effective than lactose, which is perhaps the best known inhibitor of this lectin (Table 4.2). The glycopeptides were free from blood group antigens since pre-incubation of anti-blood group antibodies with the glycopeptides did not affect the agglutination of corresponding RBCs (Table 4.3). In separate experiments it was observed that a) self digestion product of pronase did not bind to DEAE-Sephadex and b) more than 90% of O-glycosylated peptides from digestion product were above 10, 000 kDa in size and retained in dialysis bag of this cut-off limit (data not shown).

Table 4.2. Inhibitory potential of DEAE-Sephadex eluted glycopeptides from albumin-free plasma proteins:

Inhibitor*	**Neutral sugar content (µg per ml) for 50% inhibition of galectin-1 binding to**	
	Asialofetuin	**Lp(a)**
Plasma glycopeptide	2.44 ± 0.77	4.62 ± 1.8
Lactose	45 ± 6	45 ± 8

* Serial dilutions of DEAE-Sephadex eluted glycopeptides or lactose were incubated with HRP-labeled galectin-1 (1.5 µg per well) for 1 h at 4 °C and free lectin conjugate was assayed using inhibition ELISA as described in 'Methods 2.21'. Values are mean ± SD of six trails.

Table 4.3. Hemagglutination inhibition assay to check the presence of blood group antigens in isolated glycopeptides:

Hemagglutination between	DEAE-Sephadex eluted glycopeptides from albumin-free fraction of plasma*					
	1	2	3	4	5	6
A group human RBC and anti-human blood group A antibody	+	+	+	+	+	+
B group human RBC and anti-human blood group B antibody	+	+	+	+	+	+

* DEAE-Sephadex eluted glycopeptides (20 µg) pre-incubated with 20 µl of anti-human blood group A antibody or anti-human blood group B antibody for 30 minutes at 4 ºC was mixed with 25 µl of 20% suspension of human erythrocytes from A and B blood group respectively as described in 'Methods 6.4'. Agglutination was checked after 1 h with mixing and settling of erythrocytes within 2 minutes after the final mixing was marked as positive agglutination (+). Result was reproducible in six consecutive trials.

Discussion

The rationale for choosing rat cardiac fibroblasts and human monocyte-macrophage cell culture to check if extra LDL in adduct helps the latter in binding to LDL receptor were (i) macrophage is an important player in atherosclerosis and gets converted to foam cells upon accumulation of lipoproteins thereby enhancing the pathogenicity of the disease. Even though fibroblasts are mainly engaged in supporting the architecture of blood vessels some recent studies have shown their role in atherosclerosis and sequestration of lipoproteins (Xu et al., 2007) and (ii) both macrophages and fibroblasts are involved in lipid accumulation because of the presence of specific lipoprotein binding receptors. LDL receptor is one of them and is expressed on smooth muscle cells (Goldstein and Brown, 1974), lymphocytes (Ho

et al., 1976), cell surface of human fibroblasts, endothelial cells and (Sanan et al., 1987) macrophages and mediates the catabolism of LDL. Results suggested that even in non-covalently attached LDL molecules the LDL receptor binding domains of apoB subunits were unavailable apparently due to their engagement by the extended apo(a) kringle IV type 2 domains (Fig.4.A,B,C,D,E,F). Results also underline the availability of receptors for free human LDL on rat fibroblasts as well in agreement with the report that human and rat LDL binding domain of LDL receptors share 76% homology (Law and Scott, 1990). Dissociation of adduct during experiment was ruled out by checking the integrity of Lp(a) and de novo adduct in terms of [apoB]/[apo(a)] ratio (Table 4.1).

Invitro evidence suggests that Lp(a) may be taken up and degraded by macrophage-derived foam cells and fibroblasts via a cellular receptor distinct from the LDL receptor, scavenger receptors, the LDL receptor-related protein or plasminogen receptors (Bottalico et al., 1993, Keesler et al., 1994). Moreover in kidney mesangium Lp(a) gets bound more tightly to extracellular matrix than does native LDL (Kramer-Guth et al., 1996) through an LDL-receptor independent route. One recent study shows that combined effect of elevated LDL and the presence of Lp(a) promote changes in the expression of a unique set of arterial proteins in apo(a) transgenic mice which may be an early indicator of the metabolic disturbances and structural remodelling in the arteries preceding atherosclerosis (Rodger et al., 2012). Our results in part – II clearly show that apoB in Lp(a) is no longer available due its masking by the extended O-glycan structure of apo(a) subunit. On the other hand the superior galectin-1 affinity of the unique component of Lp(a) viz apo(a) may dictate its capacity for homing to cell surfaces. In this context we hypothesised that Lp(a)

being an efficient glycoconjugate ligand for galectin-1, an endogenous lectin expressed on most tissues including endothelial cells of heart, placenta and kidney mesangial cells (Thijssen et al., 2007), Lp(a):LDL can also bind to this cell surface lectin through its apo(a) component. We used polystyrene well-immobilized galectin-1, which mimics the in vivo situation to check the binding of Lp(a):LDL adduct to galectin-1. The binding of fluorescently tagged de novo adduct (Lp(a):LDL-FITC) to immobilized galectin-1 (Fig.4.H) showed the availability of oligosaccharides of apo(a) subunit in adduct for binding to galectin-1. We propose that the tendency of Lp(a) to get associated with extra LDL as well as the binding of adduct to immobilized galectin-1 may be a possible route through which these atherogenic lipoproteins may get deposited in various tissues leading to pathological consequences like atherosclerosis acute atherosis with pre-eclampsia and glomerulosclerosis respectively. The preferential accumulation of Lp(a) in arterial intima could arise even if Lp(a):LDL adduct with many times more LDL than Lp(a) are vehicles of lipid transport, if we take into account the fact that degradation rate of Lp(a) is on average only 39% of that of LDL (Nielsen LB., 1996). Significance of results in this chapter is that while there are many studies on the pathological consequences of lipid deposition in various tissues studies dealing with the cause or the route through which they get deposited on tissues are few.

In this context search for an efficient inhibitor to block this interaction is relevant. Lactose derivative-based anti-galectin drugs have been tested for blocking the interaction between galectin-1 and cell surface or free glycoconjugates with limited success (Rabinovich et al., 2006). The protocol used for plasma glycopeptide preparation specifically separates and purifies O-linked glycopeptides from the

pronase digest by exploiting their abundant negatively charged sialic acid residues. As the reduction of albumin free fraction of human plasma with DTT disrupts the secondary structure of apo(a) by breaking the numerous disulfide bonds in the cysteine-rich kringles (Chiesa et al., 1992) the glycan epitopes become more available for inhibition of glycoprotein/Lp(a)-galectin-1 interaction. The glycopeptides isolated were enriched in O-glycans with poor or no N-glycan content (Fig.4.I). Inhibition assay showed that the minimum concentration of DEAE-Sephadex-purified glycopeptide required for 50% inhibition of galectin-1 binding to asialofetuin or Lp(a) was 2.44 ± 0.77 and 4.62 ± 1.8 respectively in contrast to the far higher value for lactose (Table 4.2). The absence of any blood group-interacting substances in the O-glycan enriched plasma glycopeptides as well as its several fold higher inhibitory efficiency towards carbohydrate-dependent recognition of Lp(a) by galectin-1 offers a possible way to prevent or reverse deposition of Lp(a) or other glycoconjugates to galectin-1 bearing tissues/cells. Moreover production from discarded human plasma enhances the therapeutic viability of these glycopeptides.

SUMMARY, CONCLUSION, SIGNIFICANCE OF THE WORK AND FUTURE DIRECTIONS

1.1. Summary and Conclusion

Lp(a) has gained a great clinical interest since many studies have shown a strong relationship between its plasma level and coronary vascular disease (Kamstrup et al., 2012). But it is not clear whether the manifestation of coronary heart disease is mainly associated with Lp(a) levels or with the apo(a) phenotypes. Results over the years from both in vitro and in vivo studies have shown the pro-atherosclerotic and pro-thrombotic functions (Kamstrup et al., 2012) of Lp(a) suggesting it as an elusive "link" between atherosclerosis and thrombosis. However the presence of Lp(a) deposits in atherosclerotic plaques (Rath et al., 1989) as well as in placenta (Salafia et al., 1998) and kidney mesangial cells (Sato et al., 1993) strongly suggests its prominent role in lipid deposition and so in atherogenicity. Moreover the association of Lp(a) with LDL in circulation may augment the atherogenic potential of Lp(a). LDL receptor-mediated uptake of Lp(a) or its adduct with LDL is excluded since the apoB chain in either case is masked by apo(a). The present study explores the role of Lp(a) size heterogeneity in its intra-molecular structure, its interaction with other LDL molecules and its recognition by the host protein, galectin-1.

As there was no suitable isolation method to get all Lp(a) isoforms from plasma the high affinity of the plant lectin jacalin for the heavily O-glycosylated apo(a) chain of Lp(a) was utilized for precipitation and isolation of Lp(a) isoforms. The new method enabled isolation of all categories of Lp(a) isoforms with near absence of other plasma impurities. The efficiency of the present protocol in isolating Lp(a) isoforms of varying plasma concentrations enabled demonstration of

availability of both apo(a) and apoB subunits of Lp(a) for lectin and antibody binding. The apo(a) size dependent masking of N-glycan and antigenic epitopes of the invariant apoB subunit of Lp(a) and recovery from masking upon removal of apo(a) subunit confirms the unavailability of apoB subunit of Lp(a) by the extended apo(a) structure. This may explain the lower binding of larger Lp(a) isoforms to LDL receptor. On the other hand the apo(a) size dependent binding of galectin-1 to microplate-coated Lp(a) isoforms showed the dominant role of apo(a) subunit of Lp(a) in its physiological and pathological function.

Circulating Lp(a) can be free or complexed with LDL to form Lp(a):LDL adduct or with antibodies to form Lp(a) immune complexes. As a result of adduct formation with the more hydrophobic LDL Lp(a) molecules in plasma become more hydrophobic and so less precipitable with PEG. Titration with PEG showed that all LDL free Lp(a) molecules precipitated with 0.8% PEG and that the fraction of free Lp(a) out of total decreases with apo(a) size suggesting greater tendency of larger Lp(a) to exist in complexed form. While adduct formation was independent of the quantitative or qualitative nature of plasma LDL the apo(a) size was the major determinant of the number of LDL molecules in adducts and the sialic acid moieties in O-glycans of apo(a) were crucial in stabilizing LDL attachment. Moreover the increase in rate of adduct formation with apo(a) size was faster than the rate of increase in O-glycan content of apo(a) indicating ability of a single Lp(a) molecule to carry more than one LDL molecules and exist as a huge lipoprotein complex in circulation. Also Lp(a) molecules with the same size could form Lp(a):LDL adduct of differing stoichiometry resulting in differential precipitability with PEG. Cell

culture studies showed that presence of extra LDL in Lp(a):LDL adduct does not make the latter a ligand for LDL receptor probably due to LDL receptor binding domain of apoB of additional LDL molecules in the adduct being involved in association with Lp(a). On the other hand the binding of Lp(a):LDL adduct to immobilized human galectin-1, a lectin ubiquitously expressed on various tissue sites, offers a possible route through which these lipoproteins may get deposited on tissue sites. Results suggest that by their increased capacity to get associated with LDL molecules and consequent increased delivery of LDL to tissue surfaces through carbohydrate-dependent uptake of Lp(a) mediated by galectin-1, larger Lp(a) molecules may be individually more atherogenic than smaller. In this context search for an efficient inhibitor to block this interaction is relevant. For this purpose, glycopeptides with O-linked sugars were prepared from discarded human plasma proteins by proteolysis followed by ion-exchange chromatography. The resulting glycopeptides were free of detectable N-glycans, rich in O-glycans and several fold more inhibitory towards carbohydrate-dependent recognition of Lp(a) by galectin-1, than was lactose, the best known inhibitor of galectin-1.

1.2. Significance of the work

As all Lp(a) isoforms could be isolated from plasma by the new protocol it can also be used to isolate and characterize Lp(a) which is deposited on atherosclerotic plaques at various tissues like placenta, kidney and blood vessels.

The higher accessibility of antigenic and glycan epitopes of apo(a) compared to that of apoB subunits of Lp(a) demonstrated in the present study indicates the

predominant role of apo(a) in various biological as well as pathological functions of Lp(a).

In all populations studied to date, plasma levels of Lp(a) vary over a much broader range than do plasma concentrations of LDL (1000-fold range vs. 3-fold range) (Albers et al., 1990). It has since been found that 12 to 40 identical kringle IV type 2 repeats are present in individuals giving rise to apo(a) chains of apparent molecular mass from; 250,000 to 800,000 (Kratzin et al., 1987, Lackner et al., 1993). The biological significance of polymorphisms of apo(a) is still unclear but a highly significant inverse correlation was found between the molecular weight of apo(a) and the plasma Lp(a) concentration. When considering the biological role of Lp(a) as a quick source of cholesterol in wound sites and other areas of tissue repair, we propose that the direct relation between apo(a) size and Lp(a):LDL adduct formation may compensate for lower plasma Lp(a) concentration. Moreover the association with Lp(a) makes LDL more hydrophilic and easy to circulate.

On the other hand the tendency of larger Lp(a) isoforms to associate with large molar excess of LDL makes it more hydrophobic and the inefficiency of LDL receptor to remove this huge complex increase its plasma half life facilitating its deposition on various tissue sites through galectin-1. Plasma glycopeptides that are non-antigenic in human and capable of inhibiting galectin-1 recognition of Lp(a) offers a way to prevent or reverse deposition of Lp(a) or other glycoconjugates to galectin-1 bearing tissues/cells. Production from discarded human plasma enhances the therapeutic viability of these glycopeptides.

1.3. Future Directions

(i) Population study to check the prevalence of Lp(a) isoforms in local population and its correlation to incidence of cardiovascular disease or stoke.

(ii) Isolation and characterization of tissue deposited Lp(a) isoform from plaques, kidney mesangial cells from glomerulosclerotic patients and placenta from pre-eclampsia patients using newly standardized protocol.

(iii) Investigation on protection of LDL from intracellular degradation by Lp(a):LDL adduct formation.

(iv) Demonstration of Lp(a):LDL adduct binding to surface galectin-1 on cultured cells.

(v) Use of plasma glycopeptides to inhibit Lp(a)-galectin-1 interaction in cell culture as well as primate animals.

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LIST OF PUBLICATIONS

1. **V Kalaivani**, P S Appukuttan (2015) Plasma Lipoprotein(a) Size Polymorphism and Function: Apo(a) Subunit Size Determines Galectin-1 Recognition and ApoB Subunit Epitope Masking. Open Journal of Biochemistry, 2(1).
2. **V Kalaivani**, P S Appukuttan (2014) Circulating Lp(a):LDL complexes contain LDL molecules proportionate to Lp(a) size and bind to galectin-1: A possible route for LDL entry into cells. Lipids 49: 1101-1113.
3. M Geetha, **V Kalaivani**, P S Sabarinath, P S Appukuttan (2014) Plasma anti-α-galactoside antibody binds to serine- and threonine-rich peptide sequence of apo(a) subunit in Lp(a). Glycoconjugate Journal, 31(4):289-298.
4. M Geetha, P S Sabarinath, **V Kalaivani**, P S Appukuttan (2013) Human plasma anti-α-galactoside antibody forms immune complex with autologous lipoprotein(a). Immunological Investigations, 42(4):324-40.