CARBOHYDRATE – DEPENDENT ANCHORING OF LIPOPROTEIN(a): RELEVANCE TO ATHEROGENESIS

A THESIS PRESENTED BY

BIJOY CHELLAN

TO

THE DEPARTMENT OF BIOCHEMISTRY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM – 695 012

The Thesis Entitled

CARBOHYDRATE – DEPENDENT ANCHORING OF LIPOPROTEIN(a): RELEVANCE TO ATHEROGENESIS

Submitted By

BIJOY CHELLAN

For the Degree of Doctor of Philosophy

of

Sree Chitra Tirunal Institute for
Medical Sciences and Technology
Thiruvananthapuram

Evaluated and Approved By

Name of Guide

Name of Thesis Examiners

DECLARATION

I, **Bijoy Chellan**, hereby declare that I had personally carried out the work depicted in the thesis entitled **"Carbohydrate-Dependent Anchoring of Lipoprotein(a): Relevance to Atherogenesis"** under the direct supervision of **Dr.N. Jayakumari**, Professor, Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, except where external help sought and acknowledged.

20/8/07

Bijoy Chellan

Dr. N. Jayakumari
Professor

Department of Biochemistry
Sree Chitra Tirunal Institute for Medical Sciences & Technology
Medical College P.O, Thiruvananthapuram

CERTIFICATE

This is to certify that **Mr. Bijoy Chellan**, in the Department of Biochemistry of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to his thesis entitled **"Carbohydrate-Dependent Anchoring of Lipoprotein(a): Relevance to Atherogenesis"** was carried out under my direct supervision.

20/08/2007

Dr. N. Jayakumari

Dr. N. Jayakumari
Professor

Department of Biochemistry
Sree Chitra Tirunal Institute for Medical Sciences & Technology
Medical College P.O, Thiruvananthapuram

CERTIFICATE

This is to certify that **Mr. Bijoy Chellan**, in the Department of Biochemistry of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to his thesis entitled **"Carbohydrate-Dependent Anchoring of Lipoprotein(a): Relevance to Atherogenesis"** was carried out under my direct supervision.

20/08/2007

Dr. N. Jayakumari

To my wife, Jyothi

ACKNOWLEDGEMENTS

It is a pleasure to thank the many people who made this thesis possible.

It is difficult to overstate my gratitude to my Ph.D. supervisor, Dr. N.Jayakumari, Professor, Dept. of Biochemistry, SCTIMST. I am greatly indebted to her constant encouragement, motivation, valuable inputs and enthusiastic guidance shown throughout the course of my study and supporting me with good wishes in completing this work

I express my sincere gratitude to the members of my Doctoral Advisory Committee, Prof. P.S. Appukuttan (Head, Dept. of Biochemistry, and Co-guide), Dr. Lissy K. Krishnan (Head, Thrombosis Research Unit), Dr. K. Shivakumar (Scientist E, Division of Cellular & Molecular Cardiology) and Dr. K. Subramonia Iyer (Retired Professor, Dept. of Biochemistry) of our institute for their valuable suggestions and constant encouragement. A special word of thanks to Dr. Lissy K. Krishnan, the staff and the research scholars of Thrombosis Research Unit, for helping me through with my immunocytochemical experiments.

I am grateful to the Director, SCTIMST, for providing me necessary facilities throughout the period of my study. I would also like to acknowledge the help offered by the Registrar and the Administrative Department, SCTIMST.

I would like to thank the Council of Scientific and Industrial Research (CSIR), New Delhi, for the financial assistance in the form of a Senior Research Fellowship (SRF NET). I would also like to thank Dr. Linda G. Baum, UCLA School of Medicine, Los Angeles, CA, USA, for the galectin-1 antibody and Dr.Guy H.Carpenter, Secretory and Soft Tissue Research Unit, The Rayne Institute, London, for the O-glycosidase enzyme.

I would like to thank Dr. K. Jayakumar (Professor and Head), Prof. Dr. M. Unnikrishna Menon, and the staff of the Department of Cardiovascular & Thoracic Surgery of our institute for taking a sustained interest in my efforts and providing me with surgical sections of human artery. I take this opportunity to thank to Dr. V.V.

Radhakrishnan (Professor and Head) and the staff of the Department of Pathology for helping me through with my immunohistochemistry experiments. A special thanks to all staff of the Blood bank and the Central Clinical Laboratory for providing me blood and plasma samples at any time, all day, all year round.

My sincere thanks are accorded to Annamma K.I, Thejasee Bai.G, Mary.M.T, Geetha.M, Sangeetha S.R, Anuradha, Anu Paul, Suboj Babykutty, Vinod.V, Priya. P.S and Dr.G. Srinivas, for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring they provided. My special thanks to all staff in the medical illustration department, and also like to thank the scientists, staffs and my friends in other departments of our institute for their sincere and timely help and good company.

I am indebted to my parents for the support they provided me through my entire life.

Lastly but most importunately, I must acknowledge my wife, Jyothy, without whose love, encouragement and constant support, I would not have finished this thesis.

Bijoy Chellan

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Apo B	Apolipoprotein B
Apo(a)	Apolipoprotein(a)
BHL	Bovine heart lectin (galectin-1)
BSA	Bovine serum albumin
CHD	Coronary heart disease
Con A	Concanavalin A
CRD	Carbohydrate recognition domain
d	Density
ECM	Extra cellular matrix
ELLA	Enzyme Linked Lectin Analysis
FITC	Fluorescein isothiocyanate
Gal	Galactose
Gal-1	Galectin-1
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcNAc	N-acetylglucosamine
HAA	*Helix Aspersa* (garden snail) agglutinin
HDL	High density lipoprotein
HDL_2	High density lipoprotein (density 1.063-1.125 g/ml)
HDL-C	High density lipoprotein- Cholesterol
HPL	Human placental lectin (galectin-1)
HRP	Horse radish peroxidase
HSPG	Heparan sulphate- containing proteoglycans
IDL	Intermediate density lipoproteins
JSA	Jack seed agglutinin
KDa	Kilo Daltons
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein- Cholesterol

Man	Mannose
MM- LDL	Minimally oxidized LDL
NAc	N-acetyl group
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Tween -20- Phosphate buffered saline
PNA	Pea nut agglutinin
PVDF	Polyvinylidene diflouride
RCA-1	*Ricinus Communis (castor bean)* agglutinin-1
SDS	Sodium dodecyl sulphate
SMC	Smooth muscle cells
T- antigen	Thomsen-Friedenreich antigen (Gal β1-3 GalNAc)
TBE	Tris- Borate- EDTA Buffer
TRITC	Tetra-methyl-rhodamine isothiocyanate
VLDL	Very low density lipoprotein

SYNOPSIS

Atherosclerotic cardiovascular diseases are one of the major causes of mortality in the world. Exactly how atherosclerosis begins or what causes it isn't known. Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of this disease from initiation through progression and, ultimately, the thrombotic complications of atherosclerosis. Subendothelial retention of atherogenic lipoproteins is recognized as a major pathogenic process leading to atherogenic inflammatory responses.

Lipoprotein(a) or Lp(a) is a type of plasma lipoprotein highly associated with atherosclerotic diseases, stroke and myocardial infarction. Lp(a) is composed of one copy of LDL linked via a disulphide bridge with an apo(a) glycoprotein. The mechanism of accumulation of these apo B- containing lipoproteins [Lp(a) and LDL] within the arterial wall is not very clear. Positively charged domains in apo B of LDL is known to bind with the negatively charged matrix components, including proteoglycans, collagen and fibronectin. However, considerable gaps still remain in our understanding of how Lp(a) accumulate in atherosclerotic vessels. The proteoglycan binding site in apo B is arguably masked in Lp(a) and apo(a) does not have stretches of positively charged residues that would predict interaction with the proteoglycans. Additionally, Lp(a) has been detected in the vessel wall, where it appears to be retained more avidly than LDL. All these suggests the involvement of some unexplored matrix components in the arterial accumulation of Lp(a), some molecules that may preferentially bind Lp(a) over LDL

Among galectins (galactose-binding mammalian lectins), galectin-1 is the most abundant in tissues and is present on endothelial and smooth muscle cell surfaces of the human artery. The T-antigen (Thomsen-Friedenreich antigen, Galβ1-3GalNAc) on O-linked sugars is one of the strong ligands for galectin-1. The role of the galectin-1 in the pathogenesis of malignant diseases including cancer is well documented; however, galectin-1 is least indicated in atherosclerosis research. The present study was an attempt to elucidate the role of tissue galectin-1, if any, in subendothelial lipoprotein retention. Briefly, glycosylation profiles in Lp(a) and LDL were analyzed using specific lectins and

the complementarities of these glycans with the human galectin-1 was analyzed in specific *in vitro* and *in situ* assays.

Human Lp(a) was isolated by sequential ultracentrifugation from serum. Highly purified Lp(a) was required to study its specific glycosylation properties *vis-à-vis* LDL. A new simple method was developed to purify Lp(a) after its isolation from serum. Lp(a) was selectively electroeluted after native PAGE of serum Lp(a) isolates using L-proline at 0.1 M concentration to prevent the aggregation of Lp(a) with LDL. The system described in text is simple and can be reproduced in any laboratory with basic instrumentation, while other methods for purification of Lp(a) such as gel filtration or affinity chromatography employs sophisticated detection and fractionating systems to accurately fractionate the closely eluting similar sized Lp(a) and LDL. Additionally, pure LDL can also be simultaneously prepared by this method.

Apo(a) and apo B, the glycoprotein components in Lp(a) were then isolated from Lp(a); both these glycoproteins were also used in the studies, since, in atherosclerotic plaques, it is these glycoproteins that are found relatively more than the intact Lp(a) molecule.

An understanding of the types of carbohydrate structures on Lp(a) *vis-à-vis* LDL is necessary to elucidate the role of galectin, if any, in lipoprotein retention. The types of sugars present on Lp(a), LDL, apo(a) and apo B were analyzed by enzyme linked lectin assay (ELLA) using HRP-conjugated lectins. N-linked sugar specific lectins, con A and WGA; O-linked sugar specific PNA, jacalin and HAA; and RCA-1 reactive to both O and N-linked sugars were used in the study. The findings of the study were that both Lp(a) and LDL [also apo(a) and apo B] possessed N-linked sugars. Lp(a) [also apo(a)] was rich in O-linked sugars, especially the T-antigens, and in comparison, LDL had a poor T-antigen profile. A significant finding of the assays was that LDL [also apo B] appeared to possess O-linked sugars which are hitherto unreported in literature. To support the ELLA data and to further confirm the T-antigen profiles in Lp(a) and LDL, dot blot assays were carried out. Lp(a) and LDL sugar chains were cleaved in sequential exoglycosidase digestions and also by alkaline β-elimination reaction. The O-linked sugars were then analyzed for T-antigen structures using HRP-conjugated PNA,

jacalin and HAA. These experiments confirmed that Lp(a) compared to LDL was rich in T-antigens.

Studies were then carried out to analyze the complementarities of the T-antigen structures on Lp(a) and LDL with galectin-1 and to investigate whether endogenous galectin-1 binds Lp(a) *insitu.* Galectin-1 was isolated from the human placenta. The differential response of human galectin-1 with Lp(a) and LDL, and the T-antigen specificity of the binding was analyzed by enzyme-linked lectin assay and dot blotting using HRP–conjugated galectin-1. Galectin-1 reactivity with Lp(a) and LDL was found to be exclusively T-antigen specific and galectin-1 showed a significantly increased sugar-specific response towards Lp(a) compared to LDL. More importantly, galectin-1 showed increased reactivity with the desialylated Lp(a) and LDL. The higher *invitro* reactivity of galectin-1 with Lp(a) compared to that with LDL may be relevant to atherogenesis if we are able to extent the results to the *invivo* scenario as well. Nevertheless, the observation is strong enough to support a galectin-1 mediated selective accumulation of Lp(a) *vis-à-vis* LDL in the arterial plaques. Additionally, desialylation of the lipoproteins increased galectin-1 binding which may be of added significance since the desialylated lipoprotein are reportedly more atherogenic than native ones. Another observation which is noteworthy in this context is that, galectin-1 response with apo(a) was more than that with the native Lp(a). It is to be read together with the fact that the structural components apo(a) and apo B are found in abundance in the arterial plaques rather than the native Lp(a) itself.

ApoB-containing lipoproteins reportedly bind weakly to proteoglycans in physiological ionic-strength environments suggesting the involvement of other matrix components in lipoprotein retention. Lectin binding is solely carbohydrate dependent, therefore, the present study investigated whether endogenous galectin-1 in human internal mammary artery (IMA) histological sections binds Lp(a) *in situ* . The smooth muscle tissue in IMA histological sections showed significant expression of galectin-1 which is indicative of the relative abundance of galectin-1 in the arterial tissues. Extensive Lp(a)-binding was observed when serial sections of the tissue were incubated with pure Lp(a) and immunoanalysed for Lp(a)-binding. More importantly, much of the Lp(a) failed to bind to the tissues when the sections were pretreated with lactose, a galectin-1 inhibitor, clearly demonstrating the involvement of tissue galectin-1 in the

Lp(a) binding. The extracellular matrix in and around the intima may be rich in galectin-1 since galectin-1 is also secreted to the extracellular matrix by the cells. Consequently, galectin-1, which is ubiquitous and abundant in the arterial histological organization, may possibly contribute to the accumulation or retention of Lp(a) within these tissues.

The endothelial layer forms the first barrier between arterial cells and the circulating lipoproteins. To investigate the possibility of a galectin-1 mediated Lp(a)-binding by human umbilical vein endothelial cells (HUVEC) in support for the glycohistochemical analysis data, localization of membrane bound galectin-1 in HUVEC and its sugar specific *in situ* binding of Lp(a) was analyzed by immunoflourescence. The expression of galectin was low in normal cells; however, galectin-1 expression was considerably elevated after cell activation with minimally oxidized LDL (MM-LDL).The staining pattern was very dispersive with almost the entire cell surface intensively stained indicating the abundance of membrane bound galectin-1. Substantial Lp(a) binding was observed when the tissue was incubated with pure Lp(a) and immunoanalysed for Lp(a)-binding which again was low after lactose pretreatment of the tissue as in the case of the IMA sections. Further more, colocalization of membrane bound galectin-1 and the membrane bound Lp(a) following incubation of the activated cells with Lp(a) was analyzed by double-staining immunocytochemistry and confocal microscopy. The results demonstrated significant colocalization of cell surface galectin-1 and the incubated Lp(a). These results demonstrate that HUVEC surface galectin-1 binds Lp(a) *in situ*. Significant Lp(a) binding was seen only in cells activated with MM-LDL. This is to be read with the significant surface expression of galectin-1 on cell activation and the fact that activated endothelium induces cellular inflammatory responses leading to atherogenesis

Lp(a) presumably traverses the endothelium through some receptor independent mechanisms. Galectin-1 in the endothelium may possibly bind Lp(a) and extensive galectin-1 expression in the endothelium possibly may lead to a concentration of Lp(a) and favor its transendothelial transport. Galectin-1 expression is shown to be up regulated after cell activation by inflammatory agents. Endothelial cell activation is a primary event in atherogenesis mediated by proinflammatory agents; increased galectin-1 expression, therefore, may be a consequence in atherogenesis that may augment the Lp(a) binding by these cells.

The extracellular matrix (ECM) is composed of a complex array of glycoproteins and glycosaminoglycans. Galactin-1 is secreted by the cells into the matrix where it may be associated with its natural ligands. Laminin and fibronectin are two ECM proteins proposed as the main receptors for galectin-1. The present study had reported the *in vitro* interaction of human galectin-1 and Lp(a) which is one of the few reports of an apo(a)-substrate interaction that is not lysine-dependent. The possibility of a galectin-1 mediated crosslinking of human Lp(a) with ECM proteins, laminin and fibronectin, which may likely be another mechanism for the subendothelial retention of Lp(a) in atherogenesis was investigated. Galectin-1 mediated cross-linking of Lp(a) with cellular fibronectin and laminin was determined by an ELISA based assay. Essentially,Lp(a) binding by laminin and fibronectin in the presence or absence of human galectin-1 was compared. Both laminin and fibronectin bound more of Lp(a) in the presence of galectin-1; however, Lp(a) binding by fibronectin was substantially more than that by laminin. A concentration dependent increase in Lp(a) binding was found only in the case of fibronectin and corresponding increase in Lp(a) binding by laminin was not very significant. Galectin-1 crosslinking Lp(a) with cellular fibronectin was further investigated by an immunoprecipitation based assay. The results showed that Lp(a) became cross-linked to fibronectin in the presence of galectin-1 in a concentration-dependent manner which were in agreement with the above ELISA data.

In tissues and cell extracts, galectin-1 presumably is not free but associated with its biological partners to form high mass complexes preventing the spontaneous inactivation of the native galectin-1 in the absence of a reducing environment. Cellular fibronectin is a major galectin-1 ligand and is abundant in the ECM and also elevated levels of fibronectin are reportedly found colocalized with Lp(a) in early atherogenesis. Galectin-1 may possibly crosslink Lp(a) with cellular fibronectin as also other major glycoproteins of the ECM such as laminin and vitronectin, and contribute to Lp(a) retention in atherogenesis.

Mechanisms for Lp(a) retention in the vascular extracellular matrix is lacking in literature. The present studies demonstrate compelling evidences for a T-antigen specific *in vitro* or *in situ* Lp(a)binding by human galectin-1. Based on these observations, it is reasonable to assume that specific carbohydrate determinants (T-antigens) on Lp(a) and LDL may initiate a pathogenic association with cell surface or extracellular matrix

galectin-1 in early atherogenesis. Additional investigation will be required to analyze in greater detail, the role of galectin-1 in Lp(a) accumulation and to delineate the biological significance of this interaction in atherogenesis.

Chapter 1

INTRODUCTION

1.1. Atherosclerosis

Cardiovascular diseases which include the CAD are one of the major causes of mortality in the world. During the past three decades, although the rates of CAD considerably decreased in the USA, Australia, Canada, France, Japan and Finland, it doubled in India during the same period (Ascherio et al., 1996; Enas and Mehta, 1995). It is fast becoming a major killer in India and is predicted to assume epidemic proportions by the year 2020 (Ascherio et al., 1996; Enas and Mehta, 1995).

Atherosclerosis in the coronary artery is generally termed coronary artery disease (CAD). Atherosclerosis is a type of arteriosclerosis (arteriosclerosis is a general term for the thickening and hardening of arteries). The name atherosclerosis comes from the Greek words athero (meaning gruel or paste) and sclerosis (hardness). It's the term for the process of fatty substances, cholesterol, cellular waste products, calcium and fibrin building up in the inner lining (intima) of an artery. This build up is called plaque (atheromas). Plaque can grow and can considerably narrow the artery, so the artery becomes constricted and the elasticity is reduced. The amount of blood able to travel through it is reduced. The number and thickness of plaques increases with age, which builds up over many years. The plaque may also encourage blood clots (thrombus) to form; these can block blood flow and may also travel to other parts of the body and block

smaller vessels. Such plaques in the coronary or the arteries of the brain may eventually block blood flow, partially or completely, leading to a heart attack or stroke respectively.

1.2. Pathology of atherosclerosis

Atherosclerosis is a complex, multi-factorial disease involving a number of different cell types and mechanisms. It affects large and medium-sized arteries. The type of artery and where the plaque develops varies with each person. It is a slow, progressive disease that may start in childhood. Exactly how atherosclerosis begins or what causes it isn't known; although controversial, it is generally agreed upon, that it begins with damage to the endothelium, the innermost lining of the artery, rendering a dysfunctional or functionally altered endothelium. Causes of damage to the endothelial wall include, elevated levels of cholesterol and triglyceride in the blood, hypertension, tobacco smoke, diabetes, and these are only a few among more than 300 risk factors attributed to the development of atherosclerosis (atherogenesis).

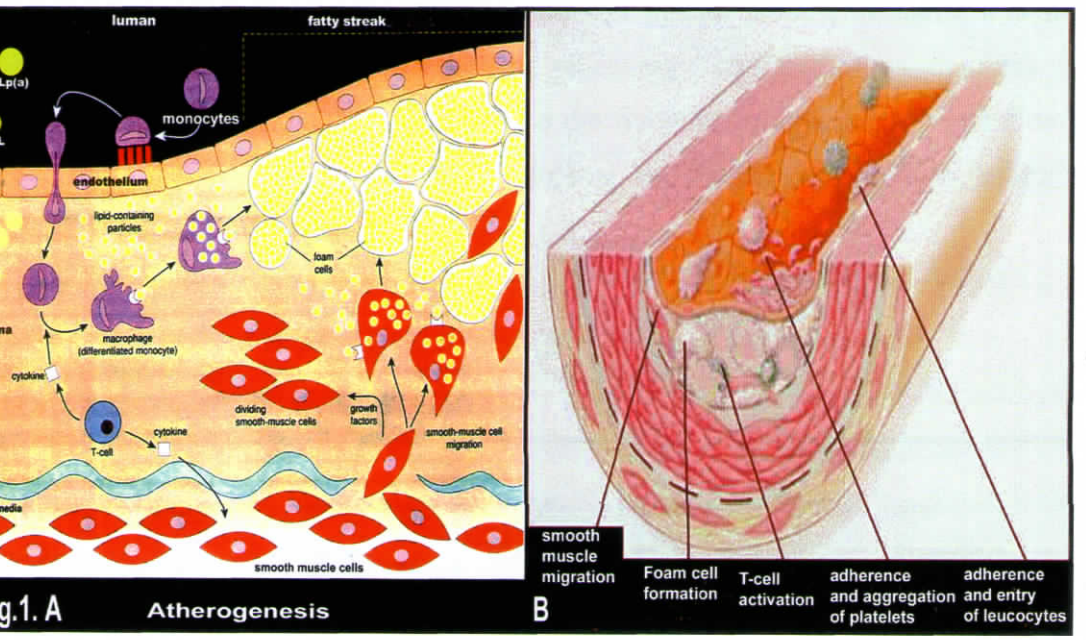
Cholesterol rich lipoproteins such as the low density lipoproteins (LDL) or lipoprotein(a) [Lp(a)], especially in hypercholesterolemic conditions and a dysfunctional endothelium, may find their way into the arterial intima, where their binding or prolonged residence may result in their oxidation by cellular free radicals. These oxidized lipids being cytotoxic, may induce inflammatory responses by the arterial wall which culminates in the recruitment of monocytes to the site of inflammation and their subsequent transformation into fat laden foam cells. The medial smooth muscle cell proliferation and migration to the site of inflammation is a characteristic feature in atherogenesis, and along with foam cells, forms the core of the lesion that finally develops into the atherosclerotic plaque (Fig. 1A & B).

1.3. Lipoprotein(a) [Lp(a)]

Although the importance of conventional risk factors such as cigarette smoking, diabetes, hyperlipidemia, and hypertension is well established, it is commonly suggested that more than 50% of patients with CHD lack any of the conventional risk factors

avazzi,1999; Futterman and Lemberg,1998). This implies that other factors play a nificant role in the development of this disease and, furthermore, that there is a stantial void in current understanding of the pathogenesis of CHD. This perceived d has led to considerable research on nontraditional risk factors and genetic causes of rt disease such as homocysteine, small dense LDL and Lp(a).

Lipoprotein(a) or Lp(a) is a type of plasma lipoprotein discovered by Kare Berg d his colleagues at the University of Oslo in 1963 as part of a search for variant rms of β-lipoproteins in the human population (Berg, 1963). Quantitative techniques



.1. A. Schematic model of the mechanism of atherogenesis- fatty streak formation. **B**. Cross tion of an atherosclerotic artery (schematic). Atherogenesis is a multifactorial, complex cess. Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) ether with T lymphocytes. Later they are joined by various numbers of smooth-muscle cells. steps involved in this process include smooth-muscle migration, which is stimulated by elet-derived growth factor, fibroblast growth factor 2, and transforming growth factor β; T- activation, which is mediated by tumor necrosis factor α, interleukin-2, and granulocyta- crophage colony-stimulating factor; foam-cell formation, which is mediated by oxidized LDL Lp(a),endothelial dysfunction inducing stimuli responsible for chemo attraction, adherence migration of leukocytes macrophage colony-stimulating factor, tumor necrosis factor α, and rleukin-1; and platelet adherence and aggregation, which are stimulated by integrins, P- ctin, fibrin, thromboxane A2 and tissuefactor.

subsequently showed that nearly all human subjects possess Lp(a) in their circulation, in widely varying amounts. The biomedical interest in Lp(a) increased in 1974 when Berg, Dahlen and Frick (Berg et al.,1974) reported an association between high plasma Lp(a) levels and coronary heart disease, an observation subsequently confirmed by a number of retrospective (Armstrong et al., 1986; Dahlen et al., 1986; Murai et al., 1986; Rhoads et al., 1986) and prospective (Bostom et al., 1994; Craig et al., 1998; Rosengren et al., 1990) studies. However, two prospective studies failed to see a significant association (Jauhiainen et al., 1991a ; Ridker et al., 1993). The reasons underlying the discrepancy in results among investigators are several, but they are likely to be related to genotypic and phenotypic variations among the patients selected, differences in ethnic background, effect of age and sex and lack of standardized methodology. Lp(a) is now considered as an independent risk factor for CAD with a desirable plasma level < 30 mg/dl. It is one of the most intriguing substances in human physiology and pathology that has been identified in the past three decades. Despite the overwhelming scientific attention this particle has received recently, relatively little is known about its role in health and disease.

Table.1. Composition of human LDL and Lp(a). TG: Triglycerides; P: Protein; PL : Phospholipids; EC: Ester cholesterol; FC: Free cholesterol.

Lipo-protein	Diameter (nm)	Density (g/ml)	Mol. Wt. ($\times 10^6$ Da)	Lipid Composition (%)	Apo-lipoproteins
LDL	18-25	1.019 -1.063	2.8	TG - 12 P - 22 PL - 20 EC - 36 FC - 10	B
Lp(a)	23-27	1.05 -1.11	3	TG - 10 P - 25 PL - 20 EC - 35 FC - 10	B, a

Lipoprotein(a) [Lp(a)] is a modified LDL molecule; Lp(a) closely resembles LDL in its content of cholesterol, phospholipid and apo B (apolipoprotein B-100) (Table 1; Fig. 2 A & B).What makes Lp(a) unique is the disulfide linkage of a specific glycoprotein apolipoprotein(a) or apo (a), varying in mass between 300 and 800 KDa to a single copy of apo B (approximately 500 KDa) (Fig.2 B) (Albers et al., 1996; Utermann and Weber, 1983.). Lp(a) thus exhibits size polymorphism which is a property of the size heterogeneity of its apo(a) glycoprotein. The heterogeneity of apo(a) is related to ultrastructural motifs in the molecule, strongly resembling the so-called kringles found in both plasminogen and other plasma proteins, such as proteases of the coagulation system (tissue type plasminogen activator and prothrombin). Kringles are looped structures stabilized by three disulfide bridges. Apo(a) Kringles have a high homology with the plasminogen kringles (Fig.2 C). Individual alleles of apo(a) contain a range of from 13 to 40 plasminogen- kringle 4-like units and a single plasminogen-kringle 5-homolog (McLean et al., 1987)

1.4. Lp(a) as cardiovascular pathogen

A high concentration of serum cholesterol is a major risk factor for atherosclerotic diseases (NCEP, 1994). Growing evidence indicates that this risk is mediated through the major cholesterol-carrying lipoprotein of serum, low-density lipoprotein (LDL). Lp(a) has also been implicated in cholesterol deposition. Lp(a) has been detected in the vessel wall, where it appears to be retained more avidly than LDL (Beisiegel et al., 1990; Dangas et al., 1998; Nielsen, 1999). In the most comprehensive studies assessing the role of Lp(a) in human vascular wall yet reported, it was found that the extracellular accumulation of Lp(a) closely correlated to the development of atherosclerotic plaques (Beisiegel et al., 1990; Rath et al.,1989). In several hundreds of histological cross sections from the human coronary arteries and the aorta, immunostaining for apo B without congruent staining for apo(a) was a rare event, indicating that the vascular wall deposition of LDL alone occurs rarely. In addition Lp(a) has been shown to be readily bound and internalized by macrophages, after oxidation or prior stimulation of the cells (Haberland et al., 1992; Bottalico et al., 1993). The structural homology of apo(a) with

plasminogen had inspired several hypothesis about the functions of Lp(a) and the speculation that apo(a) might serve as the link between the processes of thrombosis and atherosclerosis. As a molecular relative of plasminogen, apo(a) might compete with plasminogen for fibrin-binding sites and prove to be anti-thrombolytic since apo(a) lacks the clot-lysing functions of plasminogen (protease activity).

1.5. The response-to-retention hypothesis

Although it is well documented that elevated levels of LDL and Lp(a) cause increased atherosclerosis, the molecular and cellular mechanisms for the pathobiological changes that lead to the disease are still poorly understood. The initial molecular events in atherosclerosis are still debated, and currently three competing hypotheses have been articulated to explain the initiating events in atherogenesis. The 'response- to- injury' hypothesis proposes that the initial event in atherosclerosis is injury to endothelial and smooth muscles (Newby, 2000; Ross, 1993). The 'oxidation' hypothesis emphasizes the significance of the oxidative modification of LDL in inflammatory atherogenic responses, such as the recruitment of macrophages to lesion areas and foam cell formation (Podrez et al., 1999; Steinberg,1999; Steinberg et al., 1989). The third one, the 'response-to-retention' hypothesis, proposes that the initiating event in cholesterol-induced atherosclerosis is the retention and accumulation of LDL and other atherogenic lipoproteins in arteries; internalization by pro-inflammatory cells and generation of the respiratory burst becomes critical thereafter (Williams and Tabas, 1995,1998). Although these hypotheses are by no means mutually exclusive, and may even be considered mutually compatible with differences in emphasis, there is a growing body of recent evidence that supports the response-to-retention hypothesis (Boren et al.,2000;Camejo et al.,1998, 2002; Pentikainen et al., 2002). The results obtained from several key studies provide new clues as to the explanation for persistent CVD paradoxes. For example, normolipidaemic persons who develop coronary artery disease (CAD) may do so because of enhanced retention of particular lipoprotein phenotypes and not because of exaggerated arterial exposure.

disintegrates, releasing the lectin. Nothing is known about what triggers this process, its mechanisms or how the protein to be secreted gets specifically targeted to the blebs. The β-galactoside-binding site may constitute the primary targeting motif for galectin export

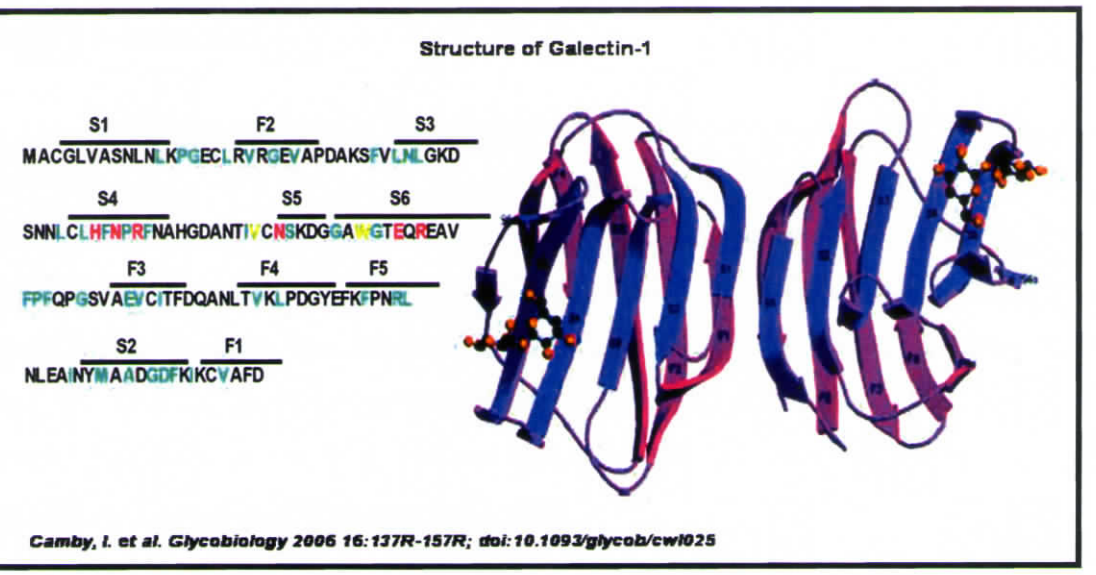


Fig.3. Structure of galectin-1. The overall folding of Gal-1 involves a β-sandwich consisting of two anti-parallel β-sheets of five (F1-F5) and six (Sl-S6a/b) strands respectively. The N and the C termini of each monomer are positioned at the dimer interface and the CRDs are located at the far ends of the same face, a configuration which constitutes a long, negatively charged cleft in the cavity (Lopez-Lucendo et al., 2004). The green amino acid symbols illustrate highly conserved residues. The key residues of the CRD, which is known to interact directly with bound carbohydrate by means of hydrogen bounds, are colored pink, while those interacting with carbohydrates via van der Waals interactions are orange and include His44, Asn46, Arg48, Val59, Asn61, Trp68, Glu71, and Arg73. The 3D ribbon diagram of the homodimeric human Gal-1 was designed with MOLSCRIPT by Lopez-Lucendo etal. (2004). A lactose (Gal β1-4 GlcNAc) is illustrated in the CRD.

machinery using β-galactoside-containing surface molecules as export receptors for intracellular galectin-1 (Nickel,2005). As a consequence there is a quality control mechanism present since the export machinery recognizes only properly folded galectin-1.

2.5.2.4. Galectin-1 binding partners

2.6.2.4.1. In the ECM

Galectin-1 binds to a number of ECM components in a dose-dependent and β-galactoside-dependent manner in the following order: laminin > cellular fibronectin >

and Wight, 2000; Khalil et al., 2004). However, considerable gaps still remain in our understanding of how lipoproteins such as Lp(a) accumulate in atherosclerotic vessels. The proteoglycan binding site in apo B is masked in Lp(a) as suggested by Khalil et al., (2004), and apo(a) does not have stretches of positively charged residues that would predict interaction with the proteoglycans (Chait and Wight, 2000). Additionally, what are the molecules or molecular mechanisms responsible for the binding avidity of Lp(a)[compared to LDL] for the arterial wall? All these suggests the involvement of some unexplored cellular or matrix components in the arterial accumulation of Lp(a) that may preferentially bind Lp(a) over LDL.

The diverse structural variations possible in oligosaccharide chains (glycans) attached to cell surface and extracellular proteins are known to mediate numerous biological roles and are implicated in events such as fertilization, development and differentiation, cancer metastasis, host pathogen interactions and apoptosis (Varki,1993). Apart from carbohydrate specific antibodies and enzymes, lectins are a major group of mammalian sugar-binding proteins. Galectin-1 is an endogenous lectin produced by arterial cells and may potentially be a molecule involved in the selective arterial accumulation of Lp(a).

1.7. Galectin-1

Galectins are a family of animal lectins with an affinity for β-linked galactosides. All galectins share a core sequence consisting of about 130 amino acids, many of which are highly conserved. The portion of the core sequence which represents the carbohydrate recognition domain (CRD) is contained between about residues 30 and 90, a segment generally encoded by a single exon (Cooper and Barondes, 1999). Fifteen mammalian galectins have been identified to date. Galectin-1 is the first protein discovered in the family, originally described by Teichberg and colleagues during studies on the possible presence of lectins in electric organs of the electric eel (Levi and Teichberg, 1981). Galectin-1 and -3 and to a lesser extent -7 are the most extensively studied members of the galectin family. Galectin-1 is differentially expressed by various normal and

pathological tissues and appears to be functionally polyvalent, with a wide range of biological activity. Galectins play a number of important roles in cancer in that they contribute to neoplastic transformation, to tumour cell survival, to angiogenesis and to tumour metastasis (Lahm et al., 2004). They can modulate immune and inflammatory responses and might play a key role in helping tumours to escape immune surveillance (Lahm et al., 2004).

1.8. Genesis of the thesis and results at a glance

Unlike LDL, the plasma levels of Lp(a) are not governed by food, diet or lifestyle modifications, but are solely determined by race, ethnicity and genetics(Brown et al., 1991; Maserei et al., 1984). An effective drug therapy to lower plasma Lp(a) levels are still nonexistent, although, niacin, in dosages of 3 to 4 g daily, has been shown to decrease the plasma levels of Lp(a) by about 30% (Carlson et al., 1989, Gurakar et al.,1985).This experience is not universal because of the potential side effects of high dosages of niacin. An understanding of the molecular mechanisms involved in Lp(a) binding and retention may facilitate the design and development of novel Lp(a) lowering therapies.

Among galectins, galectin-1 is the most abundant in tissues and is present on endothelial and smooth muscle cell surfaces of the human artery (Baum et al., 1995; Franklin et al., 1980). Both Lp(a) and LDL are highly glycosylated molecules. Galectin-1 may bind Lp(a) and LDL, or may be, bind Lp(a) preferentially over LDL *in vivo*. The possibility of a galectin-1 mediated binding and retaining of Lp(a) in the arterial wall with particular emphasis on the avidity of its binding when compared to LDL is a hitherto unexplored link in the search for novel molecules responsible for lipoprotein retention. This thesis details an attempt to elucidate the role of galectin-1, if any, in lipoprotein retention, which may contribute to the knowledge of the mechanisms involved in Lp(a) retention in atherosclerotic plaques.

Lp(a), compared to LDL, was found to bind significantly to galectin-1 in *in vitro*

assays. The binding was sugar specific and significantly increased after desialylation of the lipoproteins. Additionally, human arterial tissue galectin-1 and human umbilical vein endothelial cell galectin-1 was shown to bind Lp(a) *in situ*. These evidences, as detailed in the thesis, clearly suggests a potential role for arterial tissue galectin-1 in Lp(a) binding; a novel concept relevant to atherogenesis. Furthermore, *in vitro* studies demonstrate that galectin crosslinks Lp(a) with extracellular matrix (ECM) glycoproteins, laminin and fibronectin. This may be another potential mechanism by which galectin-1 retains Lp(a) in the arterial extracellular matrix.

Chapter 2

REVIEW OF LITERATURE

2.1. Atherosclerosis and coronary artery disease - Historical perspective

Atherosclerosis is not a new problem; using sophisticated histological techniques, paleo- pathologist, A.T.Sandison in 1967 confirmed that some Egyptian mummies had evidence of atherosclerosis (Sandison, 1967). Galen,the most influential physician of ancient Greece, described vascular aneurysms (Harris, 1973). Galen's teachings dominated medical theory and practice, such as they were, until the Renaissance. Several 16th century anatomists, including Andreas Vesalius and Gabriele Falloppio, described aneurysms of the aorta and peripheral arteries (Bing, 1992). By the beginning of the 17th century, it was recognized that the aorta and other major arteries degenerated with advancing age, but the pathophysiology of this process was unknown.

Because aneurysms of the peripheral arteries were visible and could be treated surgically, even before the advent of anesthesia or antisepsis, several articles and books appeared on this subject during the 18th century. An 1844 work by British surgeon John Erichsen provides valuable insight into early observations on arterial disease. This book includes nearly two dozen papers on aneurysms from antiquity to the late 18th century (Erichsen, 1844)

Although interest in vascular disease remained focused on aneurysms throughout much of the 19th century, some individuals turned their attention to the pathophysiology of atherosclerosis. London surgeon Joseph Hodgson published an important monograph on vascular disease in 1815. Hodgson claimed that inflammation was the underlying cause of atheromatous arteries. By this time, it was known that arteries consisted of three distinct layers. Hodgson identified atheromatous material between the intima and media and proposed that these changes could be traced an abnormality of the intima. Reflecting a growing appreciation of the value of chemistry in medicine, he also first reported the results of chemical analysis of atherosclerotic lesions. In an 1829 monograph on pathological anatomy, French pathologist, Jean Lobstein, introduced the term "arteriosclerosis" and, like Hodgson, published the results of a chemical analysis of calcified arterial plaques.

Several atlases of pathological anatomy were published in Europe during the 19th century, and some of them included illustrations of atherosclerotic arteries. Renaissance artist Leonardo Da Vinci was the first to describe and illustrate the coronary arteries accurately (Keele, 1952). Best known for his discovery of the circulation, reported in 1628 in his monumental book *De Motu tordis*, English physician and anatomist, William Harvey also first described the coronary circulation. In a 1649 book he described the anatomy of the coronary arteries and veins and proposed that the coronary circulation nourished the heart (Bedford, 1968). Viennese pathologist Carl Rokitansky's comprehensive monograph on pathological anatomy (1842-1846) included an extensive section on atherosclerosis although much of his theory regarding the cause of atherosclerotic lesions was later proved false.

German pathologist Rudolf Virchow, a great figure in the history of pathology, published his book *Cellular Pathology* in 1858 that signaled the end of humoralism and inaugurated a new era in the conceptualization of disease. Reflecting his enthusiasm for microscopy, Virchow used the instrument to study blood vessels. He concluded from these researches that atherosclerotic lesions were located within the intimal layer. He believed that these intimal deposits stimulated the proliferation of connective tissue,

which triggered further degenerative changes of the vessel wall. Virchow made pioneering observations on thrombosis and embolism. The first volume of his *Handbuch der Speciellen Pathologic und Therapie* published in 1854 contained important sections on thrombosis (a term he coined), embolism, and vascular obstruction (Virchow, 1854).

English physician William Heberden first described angina pectoris in a talk presented to members of the College of Physicians of London in July 1768. His paper, published 4 years later, is still recognized as one of the most classic clinical descriptions in medicine (Heberden, 1772). Edward Jenner, best remembered for his introduction of vaccination was the first to attribute angina to coronary artery disease. In a letter to Heberden, Jenner explicitly linked coronary artery disease to angina (Baron, 1827).

The clinical syndrome we term acute myocardial infarction was first clearly described in the 20th century. As to the relation between angina and myocardial infarction, several factors contributed to the delay between Heberden's 1772 paper on angina pectoris and James Herrick's classic description of acute myocardial infarction in 1912 (Fye,1986). In 1880, Carl Weigert, who worked with German pathologist Julius Cohnheim, published a paper that included a clear description of the pathophysiology of thrombotic occlusion of atherosclerotic coronary arteries leading to myocardial necrosis. His paper is a milestone in the description of the pathological aspects of coronary thrombosis and myocardial infarction. Weigert's clinical colleague Karl Huber extended these observations and claimed, in 1882, that angina pectoris and myocardial infarction were both manifestations of coronary artery disease. William Osler, the leading internist of his generation, had studied with Cohnheim and Weigert and appreciated the significance of their observations on coronary artery disease. As early as 1889, Osler observed in an editorial, "The local disturbances of nutrition caused by the blocking of a terminal branch of the coronary artery produces the condition known as infarct of the heart" (Osler, 1889).

During the final decades of the 19th century, several theories were advanced to attempt to explain the pathophysiology of the various forms of arterial disease that

pathologists had identified. The modern era of atherosclerosis research began in 1908, when Russian scientist Alexander Ignatovski showed that he could experimentally induce atherosclerosis in rabbits by feeding them a diet of milk and egg yolk. Extending these experiments, Nikolai Anitschkov showed that a diet rich in cholesterol caused atherosclerosis in experimental animals. In 1910, German chemist and Nobel Prize winner Adolf Windaus showed that cholesterol was present in atherosclerotic lesions in humans. Anitschkov published a valuable summary in English of the early experimental studies of atherosclerosis in 1933 (Anitschokow, 1933). Philadelphia pharmacologist Thomas H. F. Smith claimed in 1960 that "atherosclerosis had emerged by the end of the 1930s from the position of a medical curiosity. It was no longer considered a casual observation at autopsies but was now regarded as a major cause of death" (Smith, 1960).

2.2. Prevalence, incidence, and mortality of cardiovascular diseases

Cardiovascular diseases (CVD) are the number one cause of death globally: more people die annually from CVDs than from any other cause. An estimated 17.5 million people died from CVDs in 2005, representing 30% of all global deaths. Of these deaths, an estimated 7.6 million were due to coronary heart disease and 5.7 million were due to stroke (WHO- Feb, 2007). Over 80% of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women; by 2015, almost 20 million people will die from CVDs, mainly from heart disease and stroke (WHO- Feb, 2007).

Coronary artery disease prevalence in the Indian urban populations increased from 3.5% in 1960s to 9.5% in 1990s. In rural areas it increased from 2% in 1970s to 4% in 1990s (Gupta and Gupta, 1996). The rates appear to be higher in South India, with highest in Kerala (Enas et al., 1998). The risk of CAD in Indians is 3-4 times higher than White Americans, 6-times higher than Chinese, and 20-times higher than Japanese (Enas, 1998). Indians are prone as a community to CAD at a much younger age (Janus et al.,

1996). Premature CAD is defined as cardiac events occurring before the age of 55 in men and 65 in women. In its severe form it is defined as CAD occurring below the age of 40 years. CAD is affecting Indians 5-10 years earlier than other communities. Indians also show higher incidence of hospitalization, morbidity, and mortality than other ethnic groups (Enas et al., 1996). Conventional and non conventional risk factors for CAD such as hypertension, diabetes mellitus (DM), hypertriglyceridaemia, low levels of HDL-C, central obesity, high LDL-C, low levels of antioxidants (vitamin A, E, beta - carotene), rising affluence, rapid modernisation associated with sedentary but stressful life-style etc.. in summation, may be the underlying reason. They too do not fill all the blanks in information; new risk factors such as Lp(a) may be a pointer in the direction. Lipoprotein(a) [Lp(a)] is now recognized as an independent risk factor for CAD. It is a genetic risk factor. Lp(a) is ten-times more atherogenic than LDL-C (Lawn,1992). In Indians, both in India and abroad, the levels of Lp(a) are higher as compared to the whites in Great Britain, suggesting a genetic propensity (Bhatnagar et al.,1995)

2.3. Lipoprotein(a) [Lp(a)]

2.3.1. Structure and general properties

After its identification by Berg in 1963, lipoprotein (a), Lp(a), has proven to be one of the most intriguing plasma lipoproteins. Attention is now focused on the strong and independent relationship between the plasma concentrations of Lp(a) and the incidence of atherosclerotic vascular disease. Lp(a) levels might be powerful indicators of vascular disease, although the protein's role in atherogenesis is to be established

Lp(a) molecule is made up of an apo B and an apo(a) glycoprotein and is considered a modified LDL molecule (Fig.2B). Apo B, the characteristic glycoprotein in LDL has a carbohydrate composition varying from 4 to 10% by mass (La Belle and Krauss, 1990), while the apo(a) of Lp(a) contains 28.1% carbohydrate by mass (Fless et al.,1986). The binding of apo(a) to apo B is mediated by one disulfide bridge, endowing Lp(a) with specific chemical and physical properties. Apo(a) exhibits size heterogeneity

with isoforms varying from about 350 to 850 kDa. Generally, two of these isoforms are present in each human individual; some people (as far as presently known, 6% of the total) seem to have just one isoform in the circulation (Marcovina et al., 1993). Apo(a) has been sequenced at the protein and cDNA level; apo(a) has a high homology with plasminogen (McLean et al.,1987). Both apo(a) and plasminogen have kringles that share high degree of amino acid sequence homology. Plasminogen contains five types of kringles (kringle I to V), differing in amino acid composition and immunological properties. The function of the different types of kringles is to bind to substrates such as fibrin, tissue-type plasminogen activator (tPA) and cellular receptors.

Apo(a) contains one kringle-V domain that is highly similar to plasminogen kringle-V (95% homology). The other kringles of apo(a) resembles plasminogen kringle-IV (75-85% homology), constituting 10 different kringle types [apo(a) kringle $IV_{1 \text{ to } 10}$](Fig. 2C). All of these apo(a) kringle types (kringle $IV_{1 \text{ to } 10}$) are present in single copy except kringle IV_2, which is present in multiple copies (less than 10 to more than 50), giving rise to more than 30 different Lp(a) isoforms. The variability in number of kringles is genetically determined. Marcovina has described 34 different apo(a) isoforms differing in molecular mass by approximately 12.54 KDa, which closely corresponds to the predicted molecular mass of one kringle unit (Marcovina et al.,1993). The carboxy-terminal region in apo(a) closely resembles the protease domain in plasminogen with 94% overall nucleotide sequence identity (Guevara et al.,1992). The most important difference is the substitution of arginine by serine in the site responsible for proteolytic activity (position 4308) (Guevara et al., 1992). As a result, Lp(a) has no protease activity towards substrates for plasmin (Jauhiainen et al.,1991 b).

The human apo(a) structural gene has been mapped to the long arm of chromosome 6 at a locus that is genetically linked to the plasminogen gene with a LOD score for linkage of greater than 5.0 at 0% recombination (Lindahl et al., 1989). The evolutionary hypothesis is that the ancestral molecule of apo(a) was a plasminogen-type protein, having five kringles, that emerged by a duplication event from a protein with one kringle and one serine protease domain about 300 million years ago (Ikeo et al.,1991). It

is possible, in view of this evolutionary development, that the original function of Lp(a) in an era when cholesterol availability was low was to make cholesterol more easily available to cells and so promote wound-healing (based on the concept that Lp(a) may compete with plasminogen for fibrin binding) (Grinstead,1990). Next to humans, Lp(a) is also found in primates such as rhesus monkeys, baboons, chimpanzees and the hedgehog.

2.3.2. Concentration of Lp(a) in plasma of normal persons

The size of apo(a) is inversely related to plasma Lp(a) concentration. The inverse relationship between apo(a) size and concentration was first demonstrated in Caucasians by Uterman in 1988 (Utermann et al.,1988 a). Since the number of kringles in apo(a) is genetically determined, plasma levels of Lp(a) are to a large extent, but not fully, under genetic control (Boerwinkle et al., 1992;Gavish et al.,1989;Helmhold et al.,1992).

Racial differences in Lp(a) levels have been extensively documented in seven ethnic groups by Sandholzer et al.(1991) and in four different ethnic groups by Helmhold et al.(1992). Apo(a) size polymorphism affects Lp(a) levels in all groups, but the extent of this relationship differs in the diverse ethnic groups (Gaw et al. 1994). This was confirmed by studies conducted in Japanese (Abe and Noma, 1992), Koreans (Kim et al., 1992), Chinese (Cobbaert and Kesteroot, 1992), African blacks (Parra et al.,1987) and Eskimos (Gerdes et al.,1992). Chinese people have been reported to have the lowest average levels (0.070 g/L) (Helmhold et al., 1992), while Sudanese people show the highest levels (0.460 g/L).The distribution of Lp(a) concentrations in white populations is highly skewed, but does not differ significantly between males and females. The median Lp(a) value determined in a Belgian population sample is 0.140 g/L (Labeur et al., 1992), in agreement with values reported in other European and Caucasian American Populations (Helmhold et al., 1992). Mexican-Americans also show a highly skewed distribution; with mean and median lower than in non-Hispanic whites (Haffner et al., 1992). Population subsamples of American and African blacks, however, show a significantly different bell-shaped Lp(a) distribution (Haffner et al.,1992; Parra et al., 1987). The mean levels are twice those in Caucasians, indicating that the Lp(a)

distribution depends upon race (Guyton et al.,1985).

Around birth, Lp(a) concentrations are very low but increase rapidly between 0 and 7 days post-partum, followed by a continuous rise until 180 days (Schurnacher et al., 1994). According to Chandler and Loo, (1990), there is no circadian variation; but the distribution of Lp(a) over the different lipid fractions after a meal was found to differ from the distribution in fasting serum samples (Emancipator,1992). Heinrich, et al. (1991) observed remarkable differences between post- and pre-menopausal women, the first group having higher Lp(a) levels in plasma (mean 0.794 g/L against 0.515 g/L).

2.3.3. Biosynthesis of Lp(a)

Even though Lp(a) and LDL are very similar in structural respects, there is no evidence that the biosynthesis or catabolism of Lp(a) and LDL are coupled since there is no apparent correlation between the concentrations of Lp(a) and LDL in plasma (Krempler et al., 1980,1983).Lp(a) is synthesized mainly, probably only, in the liver. This is supported by the fact that patients with hepatic dysfunction due to cirrhosis and alcoholic hepatitis exhibit low plasma Lp(a) concentrations (Gregory et al.,1994). Patients subjected to liver transplantation manifest with a different apo(a) isoform, similar to that of the donor (Kraft et al.,1991).

The site of assembly of the Lp(a) particle, by covalent linkage of apo B to apo(a) is so far unknown. Lp(a) is not the result of VLDL catabolism, nor does Lp(a) assembly take place by linkage of apo(a) to circulating LDL (Fless et al., 1989) although a very minor portion of apo(a) in the circulation is not bound to Lp(a) (Gries et al.,1987). The observation that not all dietary and pharmacological interventions affecting serum LDL and apo B levels necessarily influence the plasma levels of Lp(a) and apo(a) supports the hypothesis that synthesis of Lp(a) is an independent process.

2.3.4. Catabolism and clearance of Lp(a)

The biological half-life in plasma of Lp(a) equals that of LDL (Krempler et al.

1983). However, LDL-receptor activity does not fully account for the main catabolic pathway of Lp(a) (Armstrong et al.,1985). Only a modest uptake, if any, of Lp(a) by the LDL receptors has been reported (Floren et al., 1981; Havekes et al., 1981). Armstrong et al. (1985) reported that removing apo(a) from Lp(a) leads to formation of a particle [Lp(-a)] that is taken up at the same rate as LDL by cells. Thus, it is possible that apo(a) interacts with apoB close to the LDL-receptor recognition site (Zawadski et al. 1988). Kostner investigated the affinity of Lp(a) to hepatic cells (HepG2 and HepSB) and detected an increased affinity of the LDL-receptor to LDL after incubation with apo(a) or Lp(a). Co-incubation with LDL caused a significant increase of Lp(a) degradation by HepG2 cells in "a hitch-hike like process"(Kostner,1993).

Another metabolic pathway is the uptake of Lp(a) by the scavenger receptor on tissue macrophages, leading to the formation of foam cells. According to Haberland et al. (1989) and Kostner et al.(1989), Lp(a) is only recognized by these scavenger cells after interaction of Lp(a) with sulfated glycosaminoglycans or proteoglycans, or after modification by malondialdehyde, or oxidation. Lp(a) forms complexes with these substances more strongly than LDL or the highly atherogenic acetylated LDL. Probucol, having antioxidant properties was shown to prevents enhanced uptake and degradation of Lp(a) by macrophages (Naruszewicz et al., 1992). Loscalzo, (1990), has reported another possible mechanism by which Lp(a) is catabolized, i.e., a non-receptor-mediated uptake by the endothelium.

2.3.5. Influence of environmental factors on Lp(a) concentration in plasma

Since there is a strong relationship between the concentration of apo(a) in blood and the incidence of atherosclerotic disease, many investigators have attempted to lower these levels.

The cholesterol content of the diet does not influence Lp(a) concentrations (Brown et al., 1991; Maserei et al., 1984). Cholesterol feeding is known to increase apoB

and LDL concentrations considerably apparently without changing Lp(a) and apo(a) levels. Only, diets enriched in fish oils have been reported to lower plasma Lp(a) concentrations (Dallongeville et al., 1992; Gries et al., 1990), probably as result of a reduced apo B synthesis and therefore reduced hepatic Lp(a) synthesis. Studies have shown that low-fat or high-polyunsaturated-fat diets have no substantial effect on Lp(a) levels (Mensink et al., 1992). There seems to be no relationship between weight or BMI and age and Lp(a) levels (Cobbaert and Kesteroot, 1992; Corsetti et al.,1991).

The synthesis of Lp(a) can be influenced by hormones, as is reported by Albers et al.(1984), who observed a reduction of Lp(a) concentrations in normolipidemic postmenopausal women treated during 6 weeks with the anabolic steroid stanazolol. The same effect was reported for norethisterone (Farish et al., 1991) and danazol (Crook et al., 1992). Exogenous estrogens, as used in treatment of postmenopausal complaints and in oral contraceptives, induce a decrease of Lp(a) (DePergola et al., 1993; Henriksson et al., 1992). Watanabe et al. (1993) investigated the influence of progestogen (alone) and detected a decrease of Lp(a) during treatment. There may be a significant negative correlation between endogenous testosterone and Lp(a) levels in man (Dionysosiou-Asteriou and Katimertzi, 1993), while, on the other hand, growth hormone treatment increases Lp(a) levels (Eden et al., 1993).

Reports about the influence of HMG-CoA-reductase inhibitors are conflicting (Fieseler et al., 1991; Hughes, 1989; Jurgens et al., 1989). In general, the effect is minimal or nonexistent. Fibrates and derivatives are reported to exert a lowering effect (Bimmermann et al., 1991; Farnier et al., 1994). Cholestyramin does not influence Lp(a) levels (Vessby et al., 1982). Neomycin/nicotinic acid combination therapy lowered Lp(a) markedly in some patients. Niacin, which, in dosages of 3 to 4 g daily, has been shown to decrease the plasma levels of Lp(a) by about 30% (Carlson et al., 1989, Gurakar et al.,1985). However, this experience is not universal and may depend on pretreatment plasma Lp(a) levels, apo (a) phenotype, dosage, and length of treatment. Because of the potential side effects of high dosages of niacin, the risk/ benefit relationships must be carefully evaluated with particular reference to impaired liver function, glucose

intolerance, hyperuricemia, and dermatological changes. Plasmapheresis induces impressive reduction of Lp(a) levels and seems a very effective procedure (Ritter et al., 1990).

2.3.6. Lipoprotein (a) and clinical manifestations of atherosclerosis

2.3.6.1. Lp(a) and coronary artery disease

The association between Lp(a) levels and CAD is well documented by large number of retrospective and prospective studies((Armstrong et al., 1986; Dahlen et al., 1986; Murai et al., 1986; Rhoads et al., 1986;Bostom et al., 1994; Craig et al., 1998; Rosengren et al., 1990). Dahlen et al.(1986) showed in their prospective analysis that Lp(a) is an independent risk factor for the clinical manifestations of coronary atherosclerosis, with a relative risk ranging from 1.6 to 3.6. Plasma Lp(a) levels above 30 mg/dl predicted CAD risk.. Similar results were seen with Hawaiian men of Japanese ancestry (Rhoads et al., 1986). Studies in Japan itself and in Chinese patients corroborated these findings and showed the generalizability of the association (Sandholzer et al. 1992; Sano et al., 1990). However, in blacks, despite mean Lp(a) levels twice as high as in whites, the incidence of cardiovascular disease was apparently identical (Guyton et al.,1985; Sorrentino et al., 1992). The role of Lp(a) in atherosclerotic vascular disease in blacks therefore remains to be established.

The significance of Lp(a) as a risk factor with high predictive potential has also become widely accepted in clinical medicine. Hearn et al. (1990) concluded from their study that the measurement of Lp(a) level provided the best predictive test for the incidence of CAD in their study population. They concluded that measurement of Lp(a) might become an important screening test, which was supported by Genest et al.(1992), when they provided evidence for the notion that the offspring of patients with premature coronary heart disease should be assessed for Lp(a) levels in order to predict future coronary atherosclerosis.

Seventy percent of the variation in Lp(a) concentrations can be accounted for by

genetic factors; accordingly, parental history of premature coronary heart disease is linked to elevated Lp(a) levels (Durrington et al.,1988; Utermann et al., 1988 b). When Rosengren et al (1990) showed in their prospective analysis that men with myocardial infarction had significantly higher levels of Lp(a), it was firmly established that Lp(a) is an independent risk factor for CAD.

2.3.6.2. Lp(a) and cerebrovascular and peripheral vascular disease

In addition to coronary sclerosis, evidence is accumulating that high Lp(a) levels may be important in the development of cerebrovascular and peripheral arterial disease, as well (Jovicic et al. 1993; Tyrrell et al., 1992). Lp(a) levels not only correlated well with clinical endpoints such as transient ischemic attack and cerebral infarction, but also were associated with the extent and severity of carotid atherosclerosis, as assessed by bidirectional Doppler ultrasound (Murai et al., 1986; Zenker et al., 1986).These reports strongly support a universal role for Lp(a) in the development and clinical manifestations of atherosclerotic disease in general.

Two studies seem to further validate the preceding hypothesis, insofar that in the first study, Lp(a) levels in women with peripheral vascular disease were significantly higher than in controls; in the second study, there was a trend toward higher levels in men that did not reach statistical significance(Norrgard et al., 1991). The same trend was reported in another study from Spain, in which patient numbers were small (Nogues et al., 1991). More and larger additional studies are required before the link between Lp(a) and peripheral vascular disease can be firmly established.

2.3.7. Lipoprotein(a), thrombogenesis, and fibrinolysis

To explain the relationship between Lp(a) concentrations and risk of atherosclerosis, several hypothesis could be brought forward: first, Lp(a) affects the metabolism of cholesterol and LDL; secondly, Lp(a) plays a role in foam-cell and plaque formation; thirdly, Lp(a) interacts with the activation of plasminogen to plasmin, the key

step in the fibrinolytic system (Miles et al., 1989).

2.3.7.1. Lp(a) and cholesterol metabolism

Lp(a) binds to the LDL receptor on cultured fibroblasts, although with a lower affinity than LDL itself. Once bound, Lp(a) has been shown to inhibits 3HMG-CoA reductase, indicating that it is taken up by the cells and by releasing its cholesterol moiety, regulates the de novo synthesis of cholesterol (Floren et al.,1981). High plasma concentrations of Lp(a) can, by this mechanism, influence cholesterol metabolism. However, since the LDL/Lp(a) ratio in plasma is about 50-100/1, this influence is marginal.

Lp(a) will also be targeted to uptake by macrophages by the scavenger receptor pathway. Macrophages, turning into foam cells, play an important role in plaque formation. Lp(a) can associate with LDL particles via the lysine rich domains in apo B (Ye et al., 1988). Since the putative receptor binding domain of apo B is lysine rich, the interaction of Lp(a) with this domain of apo B could significantly diminish the binding of apo B to high affinity LDL receptors and, as such, alter the intake of LDL by the apo B:E receptor pathway (LDL receptor pathway) indirectly influencing LDL and cholesterol metabolism.

2.3.7.2. Lp(a) and plaque formation

Loscalzo,(1990), Cushing et al.(1989) and Rath et al.(1989) reported a colocalization of undegraded Lp(a) and apoB in the extracellular space of the arterial wall. In contrast to LDL, Lp(a) is a substrate for tissue transglutaminase and Factor Xllla and can be altered to products that readily interact with cell surface structures (Borth et al.,1991).Localization of Lp(a) on the arterial wall can lead to oxidative changes in the lipid moiety of Lp(a) and induce the formation of oxidatively modified cholesterol esters. Since Lp(a) binds to fibrin, it can be directed to sites of fibrin deposition (vascular injury), providing a high concentration of cholesterol-rich lipoprotein that then can be taken up by macrophages via their scavenger receptors. This process could underlie the

formation of macrophage-derived foam cells, characteristic of atherosclerotic plaques. The LDL receptor has a much lower affinity for Lp(a) than for LDL. Therefore, the suggestion has been made (Haberland et al., 1989) that it is taken up by the scavenger pathway, preferentially after lipid peroxide products are formed by oxidation. Smith and Crosbie,(1991),did not detect a relationship between Lp(a) and plasminogen in eluates from human atherosclerotic lesions and thrombi. They concluded that the thrombogenicity of Lp(a) is caused by an accumulation of Lp(a) rather than a displacement of plasminogen by Lp(a).

Grainger et al.(1993) reported that Lp(a) promotes proliferation of human smooth muscle cells by inhibition of the activation of plasminogen and activating effect of plasmin on latent transforming growth factor-β. As Lp(a) also binds to glycoprotein-II b on platelets (Malle et al.. 1994), its role in formation of fibrous plaque lesions could also exist by this route. Thus, Lp(a) plays a very complex role in the interaction of cells and extracellular substrates, which all take part in the atherosclerotic process .

2.3.7.3. Lp(a) and fibrinolysis

The effects of Lp(a) on the fibrinolytic system are based on the homology between plasminogen and Lp(a) (Edelberg and Pizzo, 1992; Karadi et al. 1988). As the binding capacity of plasminogen [and most certainly also Lp(a)] is, at least for a part, to be attributed to the affinity for lysine, the possibility exists that the interaction of Lp(a) in the fibrinolytic process is at least partly influenced by the lysine-binding capacity of Lp(a). Plasminogen can bind to fibrin and fibrinogen. This process is markedly increased by partial proteolysis of fibrin, by which more lysine residues are exposed. During this process plasminogen is activated to plasmin by tissue type plasminogen activator (tPA) and also by the bacterial protein streptokinase (Edelberg et al. 1989, Edelberg and Pizzo, 1991). As apo(a) differs from plasminogen on sites 560 (Ser instead of Arg) and 561 (Ile instead of Val) in the protease part, it cannot be activated in the same way as plasminogen, as on these sites tPA acts as activator. There is no plasmin-like activity of Lp(a) after incubation with tPA.

Scanu,(1988) confirmed that Lp(a) binds to fibrin and competes with plasminogen and tPA. Thereby, inhibiting the activation of plasminogen, a process involving the ternary complex of tPA, plasminogen, and fibrinogen resulting in diminished clot lysing *in vivo*. Moreover, as plasmin degrades fibrin to smaller fragments, revealing more lysine binding sites, it enhances the binding of Lp(a) to immobilized fibrin and fibrinogen (Harpel et al.,1989;Leerink et al.,1991). This process is influenced by homocysteine and other sulfhydryl compounds (Harpel et al. 1992). The hypothesis at present, therefore, is that Lp(a) inhibits binding of plasminogen to plasmin-modified immobilized fibrinogen, thus inhibiting further thrombus degradation (Liu et al., 1994). All these theories are based on *in vitro* observations. As far as known, no *in vivo* experiment has proven these hypotheses.

2.4. The response-to-retention theory of early atherogenesis

Many processes have been implicated in early atherogenesis. These include endothelial denudation, injury, or activation, including shear stress-related events; local adherence of platelets; lipoprotein oxidation; lipoprotein aggregation; macrophage chemotaxis and foam cell formation; and smooth muscle cell alterations. It is not clear, which process, if any, could be regarded as the key event in early atherogenesis, ie, absolutely required, yet also sufficient as the sole pathological stimulus in an otherwise normal artery to provoke a cascade of events leading to lesion formation? The work of many investigators strongly supports the subendothelial retention of atherogenic lipoproteins as the central pathogenic process in atherogenesis originally proposed as the response-to-retention hypothesis of early atherogenesis by Williams and Tabas,(1995). According to the hypothesis, other contributory processes are either not individually necessary or are not sufficient and most often, they are merely normal, expected responses of otherwise-healthy tissue to the presence of retained lipoproteins.

2.4.1. Competing hypotheses

Other processes also have been argued to be central to the initiation of

atherogenesis such as endothelial denudation, injury, or activation, as outlined in the "response-to-injury" hypothesis of Ross, Glomset, and coworkers (Ross et al. 1977; Ross, 1993). Although this important hypothesis had stimulated huge interest in atherosclerosis research, there is no definitive evidence *in vivo* that endothelial injury is either necessary or sufficient for lesion formation.

The response-to-injury hypothesis originally presupposed endothelial desquamation as the key event in atherogenesis. It is now clear, however, that developing atheromata are covered by an intact endothelial layer throughout most stages of lesion progression: lipoprotein retention, fatty streak formation, and formation of advanced lesions (DiCorleto and Soyombo, 1993; Stary et al., 1994; Taylor et al., 1989; Wissler, 1991). In humans, only the most complicated, ulcerated lesions lose their endothelial layer. Furthermore, in some experimental models, an intact endothelium is required for lesion initiation and development, which do not occur in adjacent areas of denudation (Minick et al., 1977). Gross endothelial denudation, though presumably important in restenosis after balloon injury (Jackson et al, 1993) and in very advanced complicated plaques, does not appear to be central to early atherogenesis(Wissler,1991).

A refinement of the response-to-injury hypothesis states that endothelial injuries that are insufficient to cause gross denudation but severe enough to cause functional modifications are key to atherogenesis (DiCorleto and Soyombo, 1993; Ross, 1993). A major hypothesized change in endothelial function was increased permeability, particularly to atherogenic lipoproteins (Thubrikar et al., 1992). This idea is related to the lipid infiltration hypothesis which originated with Anichkov and Khalatov (Capron, 1989). Alterations in permeability or even microscopic losses of endothelial cells in excess of those due to normal cell turnover are not mechanistically required for atherogenesis, however, because normal, healthy endothelium transports or leaks many molecules, including lipoproteins (Nordestgaard and Nielsen, 1994;Vasile et al.,1983). In fact, the rate of LDL entry into the normal, healthy arterial wall vastly exceeds the LDL accumulation rate (Carew et al., 1984). Studies by Schwenke and Carew (Schwenke and Carew,1989 a, b) showed *in vivo* that the early prelesional accumulation of atherogenic

lipoproteins within the arterial wall is focally concentrated in sites that are known to be prone to the later development of atheromata, but that the rates of lipoprotein entry into prelesional susceptible versus resistant sites were not different These studies indicate that retention, not enhanced endothelial permeability to lipoprotein influx, is the key pathological event in this experimental model. Subsequent studies in several other animal models have demonstrated either increased (Thubrikar et al.,1992) or decreased (Schwenke and StGlair,1993) rates of lipoprotein entry into atherosclerosis-susceptible sites, suggesting a nonessential role for alterations in endothelial permeability. All studies agree, however, that prelesional susceptible arterial sites show enhanced retention of apo B- rich, atherogenic lipoproteins (Schwenke and Carew,1989a, b). Alterations in endothelial permeability, though apparently not essential to lesion development, may play a contributory role, eg, in smoking (Lin et al., 1992), dyslipidemias (Nordestgaard and Nielsen, 1994; Schwenke and Carew,1989 a,b), and possibly hypertension (Nordestgaard and Nielsen,1994).

Several other functional modifications have been documented in the endothelial layer *in vivo* during atherogenesis, but these occur comparatively late. For example, in rabbits, cell adhesion molecules, the earliest known being vascular cell adhesion molecule-1 (VCAM-1), are expressed by endothelial cells that overlie lesions, but only after more than 4 days of severe hypercholesterolemia and resultant foam cell formation (Johnson et al., 1994; Li et al., 1993). In contrast, lipoprotein retention and aggregation are detectable within minutes to hours after the onset of hypercholesterolemia (Nievelstein et al.,1991; Schwenke and Carew,1989b;Vasile et al.1983). Furthermore, atherogenic lipoproteins and their components have been shown to regulate endothelial expression of cell-adhesion molecules (Chisolm, 1993; DiCorleto and Soyombo, 1993). The most straightforward conclusion is that the earliest known endothelial changes during atherogenesis *in vivo*, such as VCAM-1 expression, cannot be a cause, and are likely to be a consequence, of the initial retention of lipoproteins within the arterial wall.

Referring to the effect of turbulent blood flow on the arterial wall - arterial segments that are subject to turbulent blood flow, such as those at branch points or during

hypertension were shown to have a predisposition to lesion development, though the precise relationship *in vivo* may be complicated (Zand et al., 1991). Because of the response-to-injury hypothesis, the connection between blood flow and atherogenesis has led to many *in vitro* studies on the effects of shear stress on the endothelium in cell culture experiments. Many alterations have been reported, such as intracellular calcium mobilization, ion channel activation, cytoskeletal changes, altered cellular alignment (DiCorleto and Soyombo, 1993), increased endothelial cell division (DePaola et al. 1992), and production of potentially atherogenic molecules, such as adhesive and growth factors (Nagel et al,1994; DiCorleto and Soyombo, 1993). Somewhat different results were obtained when shear was low instead of high, constant instead of pulsatile, laminar instead of turbulent, or spatially uniform instead of graded (DePaola et al., 1992; DiCorleto and Soyombo,1993) but the overall findings *in vitro* strongly support a contributory role for shear stress-induced alterations of the endothelium during atherogenesis.

In vivo, however, it is clear that sheer stress-induced endothelial alterations are neither necessary nor sufficient for atherogenesis. *In vivo* lesion development at sites of turbulent flow shows an absolute requirement for high plasma concentrations of atherogenic lipoproteins relative to those that occur naturally in nonhuman, nonatherosclerotic mammals: the plasma concentration of LDL cholesterol must exceed 2 mmol/L (80 mg/dl) for atherogenesis, even at sites of high shear stress (Brown and Goldstein,1986; Mills and Taylaur, 1971). Furthermore, at sufficiently high plasma lipoprotein concentrations, lesions develop even at sites of low shear stress, such as at non-branch points or within the pulmonary arteries (Ishibashi et al., 1994). Although stress-induced endothelial changes can play a contributory role in atherogenesis, the most directly relevant functional changes that have been documented at prelesional sites that are susceptible to atherogenesis, including those that are subject to turbulent blood flow, are altered proteoglycan structure (Hoff and Wagner, 1986;Wight et al., 1983) and increased lipoprotein retention (Nievelstein et al., 1991; Schwenke and Carew, 1989 b).

The response-to-retention hypothesis, therefore, stresses that atherogenic effects

of sheer stress *in vivo* are entirely dependent on lipoprotein retention within the arterial wall and are limited to increased local vulnerability to lipoprotein retention and the consequences thereof. Specifically, the role of shear stress in early atherogenesis is mediated primarily through the stimulation of intramural synthesis of molecules, such as proteoglycans, that promote lipoprotein retention (Camejo et al.,1990,1993a; Cardoso and Mourao,1994; Hoff and Wagner,1986; Wight et al. 1983).

The second process that has been proposed to be central to atherogenesis is lipoprotein oxidation (Chisolm,1991; Witztum and Steinberg,1991). Current evidence indicates, however, that pathophysiologically important oxidation can occur only after the retention of lipoproteins within the sequestered microenvironment of the arterial wall. Lipoprotein oxidation by cells or transition metals *in vitro* is blocked by small concentrations of plasma or plasma proteins, such as albumin (Halliwell, 1988; Kalant and McCormick, 1992), and any oxidized lipoproteins that might appear in the plasma *in vivo* would be rapidly removed by the liver (deRijke et al., 1992; vanBerkel et al., 1991) rather than be deposited into developing lesions within the arterial wall (Chang et al., 1993). In fact, oxidation maybe regarded as a normal, expected consequence of lipoprotein trapping: studies *in vitro* indicate that once lipoproteins are sequestered from the protective elements of plasma, nearby healthy arterial cells will cause oxidation,(Kalant and McCormick, 1992) apparently through their efficient generation of thiols (Sparrow and Olszewski, 1993). *In vivo*, myeloperoxidases may be involved; myeloperoxidase, a catalyst for lipoprotein oxidation is expressed in human atherosclerotic lesions (Daugherty et al. 1994) Adherence of LDL to arterial proteoglycans was shown to increase LDL's susceptibility to oxidation *in vitro* (Camejo et al.,1993 a), but prior oxidation of LDL does not enhance its retention in arteries(Chang et al.,1993). Therefore, according to the retention theory, oxidation is a normal, expected consequence of intramural sequestration of sufficient quantities of atherogenic lipoproteins within an otherwise healthy artery.

2.4.2. Molecular mechanisms involved in lipoprotein retention

Neither apo B lipoproteins nor their lipids accumulate with time in the normal intima (Small, 1988; Smith and Staples, 1980). Therefore, it seems that in normal intima most particles return to plasma from the fluid phase and those that are associated with the matrix are taken up by cells and degraded *in situ*. The situation in progressing lesions is entirely different. Lesions show accretion of apo B lipoproteins in the extracellular matrix and accumulation of their lipids in different types of intracellular inclusions and extracellular lipid aggregates (Gayton and Klemp,1996; Small,1988). The major determinants of LDL or Lp(a) accumulation in progressing lesions is the increased entry or permeability and their retention in the intima extracellular matrix, measured as increased residence time, increased distribution volume or decreased fractional loss (Collins and Carew, 1997; Schwenke,1995).

Several lines of evidence indicate that intramural retention of atherogenic lipoproteins involves extracellular matrix, chiefly proteoglycans (Camejo et al., 1990, 1993 a) and perhaps other structural elements such as fibrin and fibronectin (Ehnholm et al., 1990; Harpel et al., 1989) and lipolytic enzymes, chiefly lipoprotein lipase (LpL) (Williams et al.,1992) and sphingomyelinase (SMase)(Schissel et al., 1994).

2.4.2.1. Proteoglycans and the arterial wall

Proteoglycans are macromolecules composed of a core protein and complex, linear, long-chain carbohydrates, called glycosaminoglycans (GAGs). GAGs consist of repeating disaccharide units bearing negatively charged sulfate and carboxy groups. GAGs are covalently bound to the core protein via a tetrasaccharide linkage [GlcA-Gal-Gal-Xyl]. There are distinct types of GAGs: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin, keratan sulfate, and hyaluronan. Several classes of CS and DS containing proteoglycans are found in the artery. These include versican, biglycan and decorin. HS containing proteoglycans, the syndecans and glypicans, are associated with the cell membrane of smooth muscle cells (SMCs) and endothelial cells.

Perlecan, an important component of the artery wall, is the major HS in subendothelial matrix.

The extracellular matrix comprising collagens, elastin, proteoglycans and proteins like fibronectin, laminin and tenascin extends from the basement membrane of endothelial cells to the internal elastic lamina and forms a continuous space in contact with the pericellular region of SMCs and macrophages. Within the extracellular matrix, proteoglycans are distributed in 2 different locations. The proteoglycans of the pericellular environment are anchored to the cell plasma membrane, whereas those secreted by the cells are part of the extracellular space and usually do not physically connect with cells. The proteoglycans of the extracellular space are versican, decorin and biglycan. The pericellular environment includes the subendothelial matrix perlecan and the cell surface proteoglycans syndecan and glypican. Plasma lipoproteins, therefore, encounter a variety of types of proteoglycans throughout the artery wall.

The normal human intima is rich in versican and the content increases with lesion progression (Evanko et al., 1998). In lesions, CS chains are longer than those in proteoglycans from normal regions (Wagner et al., 1986). Vascular SMCs, endothelial cells, and macrophages are responsible for the synthesis of arterial wall proteoglycans. Pathological states such as atherosclerosis, hypertension, and diabetes are characterized by altered patterns of proteoglycan synthesis by these cells. The composition of proteoglycans synthesized by vascular cells fluctuates with the physiological state of the cells.

2.4.2.2. LDL apo B- proteoglycan interaction

LDL association with proteoglycans was documented in the early 1960s by Gero and Bihari Varga (Bihari Varga and Vegh, 1967, Gero et al., 1960); they showed that mucopolysaccharides of arteries bound to beta lipoproteins. Later several studies supported the association (Camejo et al, 1993 a; Iverius, 1972; Pentikainen et al., 1997). Purified arterial proteoglycans, particularly those from lesion-prone sites (Cardoso and

Mourao, 1994; Ismail et al., 1994) was shown to bind LDL *in vitro*, particularly LDL from patients with coronary artery disease.(Kostner and Bihari Varga,1990; Linden et al., 1989). LDL showed affinities in the 10^{-7} M range for versican-like proteoglycans secreted by human arterial SMCs and affinities in the 10^{-8} to 10^{-9} M range for decorin from fibroblasts at physiological ionic strength and calcium concentrations (Camejo et al.,1993 a; Pentikainen et al., 1997). The stoichiometry of these interactions was difficult to study because of the size heterogeneity of different proteoglycan preparations.

The LDL- proteoglycan interactions were interpreted as an ionic binding of positively charged amino groups on apo B to negatively charged groups on the GAGs. Boren et al.(1998) identified site B (residues 3359 to 3369) that lies in the carboxyl-terminal half of apo B as the proteoglycan binding site of apo B and showed that positively charged arginine and lysine residues of site B are critical for binding to CS/DS proteoglycans. OlinLewis et al. (2002) found that the small dense LDL bound biglycan better than large, buoyant sub-fractions.

2.4.2.3. LDL and non-proteoglycan associations

Lipoprotein lipase (LpL) was shown to enhance adherence of LDL *in vitro* to extra cellular matrix (Eisenberg et al., 1992) and to normal cell-surface proteoglycans (Williams et al., 1992). This adherence was independent of LpL enzymatic activity (Williams et al.,1992) and appeared to occur in vessels enriched *in situ* with LpL. A genetic absence of LpL in humans has long been known to cause hyperlipidemia without increased atherosclerosis (ZiIversmit, 1979) presumably because of poor generation of small, cholesteryl ester-rich particles (Zambon, et al.,1993) that are able to enter the arterial wall (Nordestgaard and Nielsen,1994) and loss of LpL-facilitated binding to arterial proteoglycans (Eisenberg et al., 1992; Tabas et al., 1993; Williams et al.,1992)

Sphingomyelinase {SMase) causes the formation of LDL microaggregates (Schissel et al., 1994) and LpL and SMase synergistically interact to cause massive retention and aggregation of LDL and Lp(a) *in vitro* to arterial cell proteoglycans and

matrix (Tabas et al., 1993)

2.4.3. Consequences of LDL retention

Following its retention by proteoglycans, LDL has been shown *in vitro* to undergo several modifications with important biological consequences. Minimally oxidized LDL induces endothelial and smooth muscle cells to express monocyte chemotactic activity (Cushing et al., 1990), and more extensively oxidized LDL is directly chemoattractive to monocytes (McMurray et al.,1993; Quinn et al.,1987), smooth muscle cells (Autio et al.,1990) and T-lymphocytes (McMurray et al.,1993), largely because of its lysophosphatidylcholine content (McMurray et al., 1993). Retained LDL was shown to be acted upon by arterial wall SMases (Schissel et al.,1994; Tabas et al.,1993) generating choline phosphate and ceramides. Ceramides are well documented to have many biological effects such as induction of NF-(kappa) B and stimulation of apoptosis or mitogenesis (Hannun, 1994).

LDL that has been aggregated or otherwise modified by arterial proteoglycans under a variety of conditions *in vitro* , is avidly taken up by normal cultured macrophages and smooth muscle cells,(Ismail et al., 1994) leading to foam cell formation (Kostner and Bihari Varga, 1990). Retained, altered lipoproteins (Autio et al.,1990) and nearby macrophages (Rennick et al ,1988) can stimulate chemotaxis and transformation of smooth muscle cells from the contractile to the proliferative state, which in smooth muscle cells causes increased synthesis of proteoglycans (Hoff and Wagner, 1986; Merrilees et al.,1990). Thus, retained lipoproteins can directly or indirectly provoke all known features of early lesions and, by stimulating local synthesis of proteoglycans, can accelerate further lipoprotein retention and aggregation.

Lesion development may be altered by local cellular expression of apo E within the arterial wall. As macrophages become foam cells *in situ*, they appear to increase their synthesis of apo E (Rosenfeld et al.,1993), a molecule that has been shown *in vitro* to release lipoproteins from the extracellular matrix (Saxena et al.,1993).

2.4.4. Proteoglycans and subendothelial retention of Lp(a): facts and gaps

The atherogenic nature of Lp(a) may originate from its extensive propensity for intramural retention (Dahlen et al.,1978; Kostner and Bihari Varga,1990; Scanu and Fless,1990). Despite plasma concentrations of Lp(a) that are far lower than those of other apoB-rich lipoproteins, Lp(a) may account for most of the apo B in human lesions (Beisiegel,1991; Smith and Cochran,1990). The amount of Lp(a) in the atherosclerotic plaques correlates with serum Lp(a) levels, and when corrected for plasma levels, the deposition of Lp(a) in the arteries were found to exceeds that of LDL (Bihari Varga et al.,1988; Krcuzer et al.,1994; Pepin et al.,1991; Rath et al.,1989). Lp(a), thus, appears to be preferentially deposited in atherosclerotic plaques where it appears to be retained more avidly than LDL (Beisiegel,1991; Dangas et al., 1998; Krcuzer et al., 1994; Nielsen, 1999). All these suggests that there should be an efficient trapping mechanism in the arterial intima for Lp(a).

In vitro, Lp(a) has been shown to bind to arterial proteoglycans with greater affinity and capacity than does LDL (Bihari Varga et al.,1988; Dahlen et al.,1978). After Lp(a) retention, many other biological effects occur, including enhanced LDL retention (Yashiro et al., 1993), stimulation of smooth muscle cell proliferation (Grainger et al.,1993), and, possibly, local inhibition of lysis of microthrombi (Etingin et al.,1991; Hajjar et al.,1989; Loscalzo et al., 1990; Miles et al.,1989).

The proteoglycan contribution to sub-endothelial retention of Lp(a) is, however, a much debated issue. According to Bihari Varga et al.(1988), Lp(a) may complex with arterial GAGs by the interaction of its apo B moiety and not by means of its apo(a). This conclusion is supported by the fact that apo(a)does not have GAG-binding consensus sequences such as those present in apo B or apo E (Khalil et al.,2004; McLean et al.,1987). However, the proteoglycan binding site in apo B is masked in Lp(a) as suggested by Khalil et al (2004), and much of the retention of Lp(a) within arteries appears to be due to its apo(a) component differing from the LDL retention which is

thought to involve apo B (Pillarisetti et al.,1997). LDL and other apo B containing lipoproteins were shown to bind weakly to heparin and other proteoglycans in physiologic ionic strength environments (Camejo et al., 1998); weather proteoglycans can contribute to Lp(a) retention *in vivo* is not clear and needs further study.

The factors responsible for focal retention of lipoproteins and subsequent lesion development are not clear Proteoglycan variations alone may not explain the focal development of early lesions, because potentially atherogenic proteoglycans are abundant and ubiquitous throughout the arterial tree (Camejo et al.,1990), though focal alterations in proteoglycans have been documented in prelesional (Cardoso and Mourao,1994; Hoff and Wagner,1986; Wight et al.,1983)and lesional sites (Wagner et al.,1986;YlaHerttuala et al.,1986). Nevertheless, the status of proteoglycans and possibly other apo B/ apo(a) - retentive molecules in prelesional susceptible versus prelesional resistant sites remains an important area for study.

Lp(a) has been shown to bind *in vitro* to purified matrix proteins such as fibrinogen, fibrin, fibronectin and, laminin (Loscalzo et al.,1990, van der Hoek, et al.,1994). This may be another route for Lp(a) retention within the arterial intima. Are other matrix components required for efficient arterial retention of Lp(a)? Additionally, what are the molecules or molecular mechanisms responsible for the binding avidity of Lp(a)[compared to LDL] for the arterial wall? All these suggests the involvement of some unexplored matrix components in the arterial accumulation of Lp(a) - some molecules that may preferentially bind Lp(a) over LDL. At the end of the day, considerable gaps still remain in our understanding of how Lp(a) accumulate in atherosclerotic vessels.

2.5. Galectins

Galectins, formerly known as S-type lectins or galaptins, are a family of β-galactosyl-binding lectins characterized by a typical motif of conserved amino acids in

their carbohydrate recognition domain(s) (CRDs) (Hirabayashi and Kasai, 1993; Barondes et al., 1994). Most galectins require a reducing environment for their binding activity. This reducing environment is to prevent oxidation of a particular -SH group that is crucial for their sugar-binding activity. Although initially described in vertebrate taxa, their presence has since been documented in prochordates, invertebrates, and fungi (Hirabayashi et al. 1996; Pfeifer. et al.,1993; Cooper et al., 1997) suggesting that they are widely distributed in nature. Almost all galectins discovered so far are small (14-36 KDa) soluble proteins with one or two CRDs.

2.5.1. The galectin family and specific features

The first galectins were discovered as part of a quest to find proteins recognizing cell surface carbohydrates thought to be involved in cell adhesion. Tissue or cell extracts which tested positive for agglutinating activities were subjected to affinity chromatography on immobilized β-galactosides and bound proteins eluted with lactose(a β-galactoside that resembled known structures on cell surface). In this way a 14 KDa protein, now known as galectin-1 was first isolated from electric eel by Teichberg in 1975 (Teichberg et al.,1975). After this investigation a number of β-galactoside binding lectins were identified in tissues from rat, chick, bovine, human, etc and their biochemical properties and histological distributions were studied in relation to development and differentiation.

In 1994, it was proposed that mammalian galectins be numbered in the order of discovery (Barondes, 1994). This numbering system cannot be applied directly to non-mammalian galectins because it is difficult to correspond non-mammalian galectins to mammalian ones and *vice versa*. Fourteen galectins have been identified in mammals and some organisms such as *Caenorhabditis elegans* may have many more (Cooper and Barondes,1999). The evolutionary conservation of galectins likely reflects the essential roles of galectins in development and function of multicellular organisms including cell adhesion, migration, differentiation and death (Perillo et al.,1998).

Galectins are predominantly, cytoplasmic proteins and lack a trans-membrane

segment (Ohyama et al.,1986). They also hold many features of cytoplasmic proteins ie, have no disulphide bridges, no sugar chains, no signal sequences, biosynthesis occurs on free ribosomes and in most cases their N-terminal amino acids are acetylated. However, their histological localization is diverse and they occur not only in cytoplasm but also in nuclei, on cell surfaces and in extra cellular spaces depending on galectin species.

The structure of most galectins have been determined using crystallography .The portion of the core sequence which represents the carbohydrate recognition domain (CRD) is contained between about residues 30 and 90, a segment generally encoded by a single exon. Of the original mammalian members, galectins 1 to 3 include just one CRD, whereas galectin-4 is composed of two nonidentical tandem core structures with two separate CRDs. Galectins-5 through -11 share the basic structural features and galactoside-binding of the first four. Galectin-5, 7, 10 and 11 has one CRD (Leonidas et al.,1995; Gitt et al.,1995; Magnaldo et al., 1995; Ogden etal.,1998) and the other three have two tandem CRDs like galectin-4 (Hadari et al., 1995; Wada and Kanwar,1997; Gitt et al.,1998).

Immunochemical studies with conventional antisera and amino acid analysis have suggested that the β-galactoside binding lectins among phylogenetically related species are antigenically and structurally related [Childs and Feizi,1979; Levi and Teichberg,1982]. The molecular properties of vertebrate galactose-binding lectins are strikingly similar from chicken to cow to man. The amino acid identity in the carbohydrate-binding domains among different known galectins from one mammalian species ranges from about 20 to 40% (Oda et al.,1993). The amino acid sequence homology of the same galectin from different mammalian species is 80-90%. Among the amino acid residues that are substantially conserved among various galectins, His44, Asn46, Arg48, Asn61, Trp68, Glu71, and Arg73 (residue numbers are those of bovine spleen) are recognized as critical for sugar binding (Lobsanov et al.,1993), whereas Ser29, Phe30, Asn33, His44, Asn4h, Arg48, and Asn61 interact with each other to provide the architecture of the CRD.

Unlike the original members of this family, which were discovered on the basis of

their lectin activity, the new galectins have primarily been identified in other ways, even in multiple contexts. Only when sequenced were they found to be members of the galectin family. For one of these galectins, an apparent lack of carbohydrate binding activity resulted in its initial exclusion from the galectin family. The Charcot-Leyden crystal protein, an abundant lyso-phospholipase of eosinophils, could only be named galectin-10 after it was shown to weakly bind to the same galactoside affinity columns that avidly bound other galectins (Leonidas et al.,1995).

A galectin-like protein apparently specific to the lens of the eye, GR1FIN (galectin-related interfiber protein), was recently discovered, but this candidate can not be numbered as an official galectin, because it does not have detectable lectin activity in assays standardly used for other galectins (Ogden et al., 1998).

The galectin composition varies between different cell types, but all cells appear to express at least one galectin. Galectin-1 is abundant in adult muscle and other cells of mesodermal origin; galectin-3 is abundant in various epithelial cells and in macrophages; galectin-4 is confined to epithelial cells of the alimentary tract and galectin-7 is found in epidermis (Colnot et al.,1996). Much less is known about the distribution of galectin-2, which is expressed in hepatomas. Galectin-3 surface expression has been shown in normal human monocytes and its level increases as monocytes differentiate into macrophages (Liu et al., 1995). High expression is seen in a few selected cell types and stages for each galectin during development. Galectin expression can also be induced by various stimuli. For example, galectin-9 that acts as a potent chemoattractant selectively for eosinophils is induced by allergic stimulation in monocytes (Matsumoto et al., 1998).

2.5.2. Galectin-1

Galectin-1 is the first protein discovered in the family (Teichberg, 1975) and also the most extensively studied galectin. Galectin-1 is abundant in adult muscle and other cells of mesodermal origin

2.5.2.1. Galectin-1: molecular structure

As reported by the MapViewer program and the Entrez genome database on the NCBI website (http://www.ncbi.nlm.nih.gov), galectin-1 is encoded by the LSGALS1 gene located on chromosome 22q 12. The 0.6 kb transcript (GenBank: NM_002305) is the result of the splicing of four exons encoding a protein with 135 amino acids (GenPept : NP_002296, SwissProt: P09382). Galectin-1 occurs as a monomer as well as a non-covalent homodimer consisting of subunits of one CRD (Barondes et al.,1994; Cho and Cummings, 1995).

The overall folding of galectin-1 involves a β-sandwich consisting of two anti-parallel β-sheets. This jellyroll topology of the CRD constitutes the typical folding patterns of galectins (Fig.3). Human galectin-1 exists as a dimer in solution (Lopez-Lucendo et al.,2004). The integrity of this dimer is maintained principally by interactions between the monomers at the interface and through the well-conserved hydrophobic core, a factor which explains the observed stability of the dimer in molecular terms (Lopez-Lucendo et al.,2004). Nevertheless, one of the main characteristics of the homodimeric galectin-1 protein is that it spontaneously dissociates at low concentrations (K_d ~ 7 μM) into a monomeric form that is still able to bind to carbohydrates (Cho and Cummings,1995), but with a lower level of affinity (Leppanen et al., 2005). Galectin-1 may, hence be dimeric and cross-link ligands if present at a high enough concentration, which is well within the range found in nature. But it may also act as a monomer at lower concentrations as suggested (Blaser et al.,1998). Galectin-1 can also exist in an oxidized form, that is, a form that lacks lectin activity (Outenreath and Jones,1992).

2.5.2.2. Galectin-1: Carbohydrate binding specificity

Most galectins have a core-binding site for lactose and related disaccharides. The galactose residue in lactose is most tightly bound and interacts with the protein along one side via hydrogen bonds involving OH on C4 and C6, the ring O, and van der Waals interaction between the hydrophobic patch formed by CH 3-5 and a Trp or Tyr in the protein(Camby et al.,2006) (Fig.3). The glucose residue also interacts, mainly via OH3

and is significant, since to most galectins lactose binds with about 100 fold higher affinities (K_d 0.5 mM) than galactose alone (Leffler and Barondes,1986). However there is some room for variation of the Glc residue. If the C-2 hydroxyl of the latter is replaced by acetamido group to form GlcNAc, the affinity goes up by a factor of about ten for some galectins but not others, due to interactions involving the NAc group. Gal bound β l-3 to GlcNAc also binds well because here OH4 of GlcNAc takes the steric place of OH3 of GlcNAc in LacNAc (Gal βl-4 GlcNAc) (Loris, 2002).

Although galectin-1 binds preferentially to glycoconjugates containing the ubiquitous disaccharide N-acetyllactosamine (Gal βl- 4 GlcNAc, also known as LacNAc), its binding to individual lactosamine units is characterized by relatively low levels of affinity (K_d ~ 50 μM)(Ahmad et al.,2004). It is the arrangement of lactosamine disaccharides in multiantennary repeating chains (up to three branches) that increases the binding avidity (K_d ~ 4 μM) and in contrast, there is no increase in avidity when the recognition unit is repeated in a string (poly-N-lactosamine) (Ahmad et al., 2004). In polysaccharides, galectin-1 does not bind glycans that lack a terminal non-reducing unmodified N-acetyllactosamine (Stowell et al.,2004).

2.5.2.3. The non-classical secretion of galectin-1

So far, the secretory pathway of galectins remains unknown. One of the reasons is that all known galectins lack signal peptides necessary for their insertion into the endoplasmic reticulum membrane and its subsequent secretion via the classical pathway (Hirabayashi and Kasai,1993). However, it is well-known that galectin-1 is secreted and can be found on the extracellular side of all cell membranes as well as in the extracellular matrices of various normal and neoplastic tissues (Cooper and Barondes,1990; van den Brule et al.,2003). An alternative pathway for secretion being considered is membrane blebbing (Cooper and Barondes,1990; Nickel,2005). When galectin-1 secretion starts during muscle cell develop-ment, the galectin is first accumulated underneath the plasma membrane, then in small membrane evaginations, the blebs, which are about 2 μM in diameter. These are subsequently released from the cell and their membrane

disintegrates, releasing the lectin. Nothing is known about what triggers this process, its mechanisms or how the protein to be secreted gets specifically targeted to the blebs. The β-galactoside-binding site may constitute the primary targeting motif for galectin export

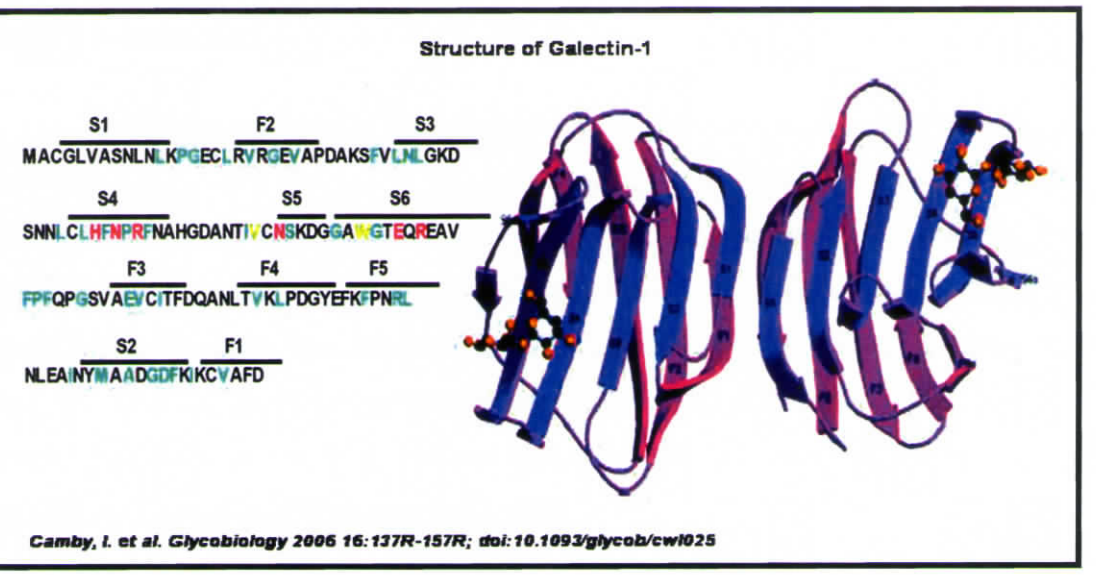


Fig.3. Structure of galectin-1. The overall folding of Gal-1 involves a β-sandwich consisting of two anti-parallel β-sheets of five (F1-F5) and six (Sl-S6a/b) strands respectively. The N and the C termini of each monomer are positioned at the dimer interface and the CRDs are located at the far ends of the same face, a configuration which constitutes a long, negatively charged cleft in the cavity (Lopez-Lucendo et al., 2004). The green amino acid symbols illustrate highly conserved residues. The key residues of the CRD, which is known to interact directly with bound carbohydrate by means of hydrogen bounds, are colored pink, while those interacting with carbohydrates via van der Waals interactions are orange and include His44, Asn46, Arg48, Val59, Asn61, Trp68, Glu71, and Arg73. The 3D ribbon diagram of the homodimeric human Gal-1 was designed with MOLSCRIPT by Lopez-Lucendo etal. (2004). A lactose (Gal β1-4 GlcNAc) is illustrated in the CRD.

machinery using β-galactoside-containing surface molecules as export receptors for intracellular galectin-1 (Nickel,2005). As a consequence there is a quality control mechanism present since the export machinery recognizes only properly folded galectin-1.

2.5.2.4. Galectin-1 binding partners

2.6.2.4.1. In the ECM

Galectin-1 binds to a number of ECM components in a dose-dependent and β-galactoside-dependent manner in the following order: laminin > cellular fibronectin >

2.6.2. The T and Tn –antigen

Core 1, O-glycan or T antigen [Gal (βl-3) GalNAc] (Fig.4 B) was discovered about 70 years ago by Thomsen and Friedenreich as a laboratory curiosity. The phenomenon of panagglutinability acquired by bacterially contaminated human red blood cells led to the discovery of T-antigen. The immunodeterminant group GalNAc, linked to the hydroxyl group of Serine or Threonine in the amino terminal region of glycoproteins is called Tn- antigen. T and Tn are usually covered by covalently linked carbohydrates holding high negative charge density in the case of sialic acid substitution (Springer and Desai, 1982) or are physically separated from the immune system (Pierce-Cretel et al., 1981). Unmasked T and Tn specific epitopes are unique carcinoma markers.

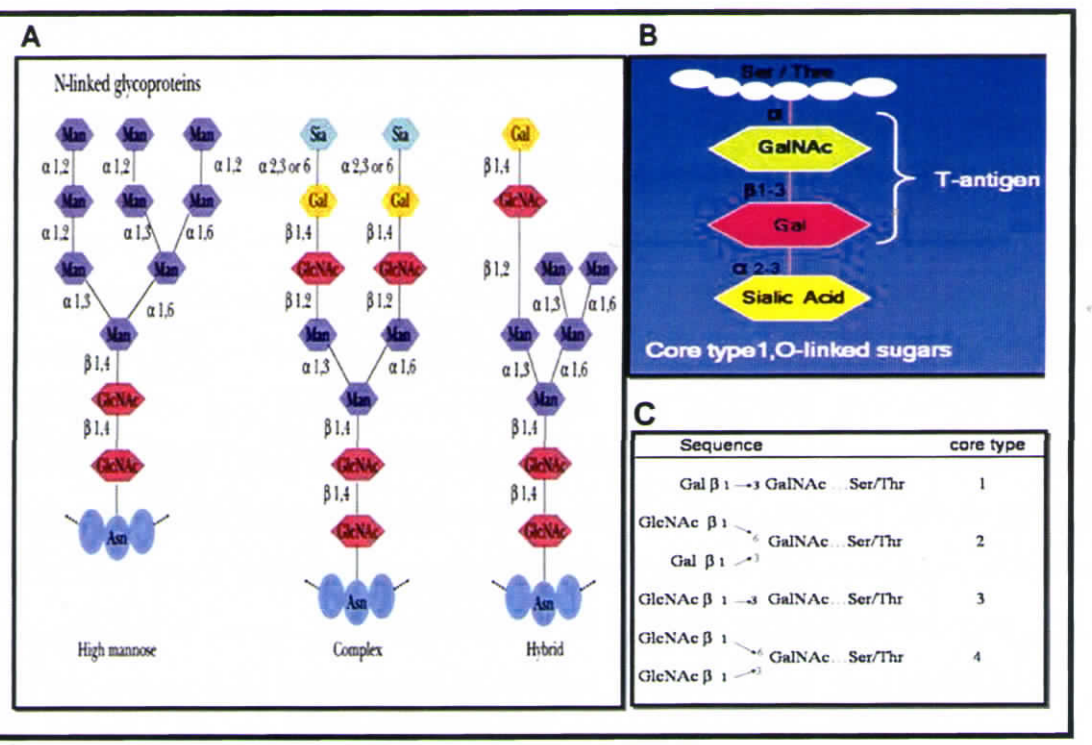


Fig. 4 A. Types of N-glycosylation, **B**: O-glycosylation in glycoproteins, **C**: Types of O-glycosylation ; Sia -sialic acid; Man –mannose; GlcNAc -N-acetylglucosamine; Asn – asparagine; Gal –galactose; GalNAc -N-acetyl galactosamine.

thrombospondin > plasma fibronectin > vitronectin > osteopontin (Moiseeva et al., 2000,2003). Laminin and cellular fibronectin are glyco-proteins which are highly N-glycosylated with bi- and tetra-antennary poly- N-lactosamines (Carsons et al.,1987: Fujiwara et al.,1988)

2.5.2.4.2. Integrins

Numerous variants of integrin glycoforms have been described in many normal and pathological cell types. Via its direct binding to β1 integrin sugars (without cross-linking them) galectin-1 increases the amounts of partly activated β1 integrins, but does not induce dimerization with α subunits (Moiseeva et al., 2003). In the case of vascular smooth muscle cells this interaction of galectin-1 with the α1β1 integrin has been reported both as transiently phosphorylating the focal adhesion kinase (FAK) and as modulating the attachment of cells and their spreading and migration on laminin, but not on cellular fibronectin (Moiseeva et al.,1999, 2003).

2.5.2.4.3. GM1 ganglioside

Galectin-1 is a major receptor for the carbohydrate portion of the ganglioside GM1 exposed on the surface of human neuroblastoma cells (Kopitz et al.,1998).

2.5.2.5. The effect of galectin-1 on cell signaling pathways

Galectin-1 is mitogenic for various types of normal or pathological murine and human cells, (Symons et al.,2000; Moiseeva et al., 2000). Galectin-1 inhibits the growth of other cell types such as neuroblastoma (Kopitz et al.,2001) and stromal bone marrow cells (Andersen et al, 2003). Furthermore, galectin-1 can also regulate cell cycle progression in human mammary tumor cells (Wells et al., 1999). The seemingly paradoxical positive and negative effects of galectin-1 on cell growth are highly dependent on cell type and cell activation status, and might also be influenced by the relative distribution of monomeric versus dimeric, or intracellular versus extracellular, forms.

2.5.2.6. Galectin-1: Regulation of cell migration processes

Cell migration is the net result of adhesion, motility, and invasion. Galectin-1 modifies each of these three cell migration-related processes.Galectin-1 has been shown to increase the adhesion of various normal and cancer cells to the ECM via the cross-linking of glycoproteins (integrins) exposed on the cell surfaces with carbohydrate moieties of ECM components such as laminin and fibronectin (Moiseeva et al.,1999; van den Brule et al., 2003). Galectin-1 causes the increased motility of glioma cells and the reorganization of the actin cytoskeleton associated with an increased expression of RhoA, a protein that modulates actin polymerization and depolymerization (Camby et al., 2002). Using a proteomic approach based on the comparison of highly and poorly invasive mammary carcinoma cell lines, Harvey et al.(2001) identified the membrane expression of galectin-1 as a signature of cell invasiveness.

2.5.2.7. Galectin-1 in tissue development and differentiation

Galectin-1 expression has been reported in male and female gonads (Wollina et al.,1999). In the uterus galectin-1 expression is restricted to the endometrium and varies during the menstrual cycle and the early phases of gestation (von Wolff et al., 2005). Galectin-1 is widely distributed in the central and peripheral nervous systems of rodents during their development (Akazawa et al., 2004;Egnaczyk et al.,2003). Numerous studies have demonstrated that galectin-1 may be a key element in the course of hematopoietic cell differentiation (Wang et al., 2004; Vas et al., 2005). Galectin-1 is externalized during adipocyte differentiation (Wang et al.,2004) and is able to modulate osteoblastic differentiation (Andersen et al., 2003) as well as the proliferation and death of hematopoietic stem and progenitor cells (Vas et al., 2005).

2.5.2.8. Galectin-1 and the immune system

Galectins in general and galectin-1 in particular, are known to be deeply involved in the initiation, amplification, and resolution of inflammatory responses

2.5.2.8.1. T-cell homeostasis and survival

A growing body of evidence indicates that galectin-1 functions as a homeostatic agent by modulating innate and adaptative immune responses. galectin-1 induces the inhibition of cell growth and cell-cycle arrest (Blaser et al.,1998) and promotes the apoptosis of activated, but not resting, immune cells (Perillo et al., 1995; Chung et al., 2000). Galectin-1 also suppresses the secretion of the pro-inflammatory cytokine interleukin-2 (IL-2) (Rabinovich et al.,1999) and favors the secretion of the anti-inflammatory cytokine IL-10 (van der Leij et al.,2004). All of these activities have been demonstrated by adding a relatively high concentration (μM range) of exogenous galectin-1 to T cells *in vitro*. One concern regarding the proapoptotic activity of galectin-1 is whether high levels of soluble protein can be achieved *in vivo*. Recent evidence indicates that the amount of galectin-1 secreted by different cell types in the ECM is sufficient to kill T cells (Perillo et al.,1995;Chung et al, 2000).The effects of galectin-1 on immune and inflammatory cells are likely to be due to the binding and cross-linking of cell-surface glycoproteins on these cells (Galvan et al., 2000).

2.5.2.8.2. T-cell immune disorders and chronic inflammation

In vivo, galectin-1 has powerful immunoregulatory effects through its ability to inhibit T-cell effector functions (van der Leij et al.,2004).Galectin-1 treatment has resulted in improvements and even in cases of prevention in a number of experimental models of autoimmune diseases (Offner et al.,1990; Wang et al.,2000). In addition to its role in adaptative immune responses and chronic inflammation, galectin-1 also participates in innate immunity and acute and allergic inflammation (Liu, 2000).

2.5.2.8.3. Host-pathogen (bacteria-virus-parasites) interactions

In vitro experiments had shown that galectin influences the ability of macrophages to control intracellular infections by inhibiting microbicidal activity, by promoting parasite replication, or by inducing host-cell apoptosis and the expression of galectin-1 is markedly upregulated after parasite or virus infection (Zuniga et al., 2001).

2.5.2.9. Galectin-1 involvement in tumor progression and tumor immune-escape

Galectin-1 could be involved in tumor angiogenesis because both vascular smooth muscle and endothelial cells express it (Moiseeva et al., 2000,2003). A number of studies have highlighted the expression of galectin-1 in the stromal tissue around tumors (Gillenwater et al.,1996; van den Brule et al.,2003) or in the endothelial cells from capillaries infiltrating them rather than in those in the adjacent non-tumoral stroma (Clausse et al.,1999) suggesting that galectin-1 might trigger the death of infiltrating T cells and protect these sites from the tissue damage induced by T-cell-derived proinflammatory cytokines. The correlation between galectin-1 expression in cancer cells and their aggressiveness suggest the hypothesis that tumor cells may impair T-cell effector functions through the secretion of galectin-1, and that this mechanism may contribute toward tilting the balance in favor of an immunosuppressive environment at a tumor site. The blockade of the biological activity of galectin-1 in melanoma tissue resulted in a reduced tumor mass and stimulated the *in vivo* generation of a tumor-specific T-cell response (Rubinstein et al., 2004).

2.5.2.10. Galectin-1 in the nervous system

Galectin-1 in its oxidized form- a form that lacks lectin activity- promotes neurite outgrowth (Outenreath and Jones, 1992) and enhances axonal regeneration in peripheral (Horie et al., 1999), and central (Rubinstein et al., 2004) nerves even at relatively low concentrations (pico M range) (Horie and Kadoya,2000). Various reports suggest a relation between galectin-1 expression (or altered expression) and neurological diseases. Galectin-1 expression by neuronal and glial cells is closely correlated with regenerative success after injury (Wada et al., 2003) and the level of autoantibodies to galectin-1 is significantly higher in patients with neurological disorders than in healthy controls (Lutomski et al.,1997). Galectin-1 induces astrocyte differentiation, with the subsequently differentiated astrocytes greatly enhancing their production of the brain-derived neurotrophic factor (BDNF) that, in turn, plays an important role in the survival, differentiation, and synaptic plasticity of neurons (Sasaki et al., 2004). In contrast to the effect of oxidized galectin-1 on axonal regeneration reported above, the effects of

galectin-1 on astrocyte differentiation and BDNF production depend on carbohydrate-binding activity and are astrocyte-specific since no effects on neurons have been observed (Sasaki et al., 2004). In this context galectin-1 may thus be considered as a means for the prevention of neuronal loss in cases of injury to the central nervous system (Egnaczyk et al., 2003).

2.5.2.11. Galectin-1 and atherosclerosis

The role of galectin-1 in the pathogenesis of malignant diseases including cancer is well documented; however, galectin-1 is least heard-of in atherosclerosis research. Nevertheless, two reports had highlighted the role of galectin-1 in smooth muscle cell (SMC) proliferation, an important step in atherogenesis (Moiseeva et al.,1999,2000). Recently galectin-2 was shown to bind to lymphotoxin-α (LTA) and regulate LTA secretion *in vitro* (Ozaki et al.,2004). LTA is an inflammation-mediating molecule involved in atherogenesis. Functional variations in LTA were shown to be associated with susceptibility to myocardial infarction (Ozaki et al., 2002).The role of galectin-1 and other galectins in atherogenesis remains unknown and is a novel approach to the study of the pathogenesis of atherosclerosis

2.6. Glycoproteins

Glycoproteins are proteins to which carbohydrates are covalently linked through glycosidic bonds and occur in all organisms. Apparently most of the proteins in nature are glycoproteins. Glycoproteins are found in soluble form in the extracellular fluids and in insoluble form in membrane and intercellular matrix components. This class of compounds includes enzymes, hormones, immunoglobulins, lectins, toxins, carriers and structural proteins. Proteoglycans are a diverse group of proteins containing a large number of glycosaminoglycan side chains and is distinct from glycoproteins in that they contain repeating disaccharide units characteristic of the glycosaminoglycan chains.

Although nearly 200 different monosaccharides are found in nature, only 11 are known to occur in glycoproteins (Sharon and Lis,1981). The carbohydrate content of glycoproteins varies from less than 1% to over 90% of the total weight and the carbohydrate moieties vary in size from mono- or disaccharides to oligosaccharides and they are distributed unevenly along the polypeptide back bone. The numbers of oligosaccharide chains attached to the glycoprotein are also varied. Derivatization to sulphate or phosphate is also observed occasionally

2.6.1. Glycosylation types in glycoproteins

The oligosaccharide chains of most glycoproteins are linked to the polypeptide backbone by either of two types of carbohydrate-peptide linkages: The N-glycosidic and the O-glycosidic; they differ markedly in their chemical properties, in particular their stability to acid and base.

2.6.1.1. N-glycosylation

The N-glycosidic linkage is between the anomeric carbon atom of N-acetyl-D-glucosamine and the amide nitrogen of asparagine in the polypeptide chain. The oligosaccharide chain is attached (via N-acetyl-D-glucosamine residue) by oligosaccharyl-transferases to asparagine occurring in the tripeptide sequence Asn-X-Ser or Asn-X-Thr, where X could be any amino acid except Proline. This sequence is known as a glycosylation *sequon.* Only about one third of the potential Asn-X-Ser/Thr sites in proteins are actually glycosylated. It has been suggested that rapid folding of the nascent polypeptide is responsible for the lack of glycan chains at potential glycosylation sites (Marshall, 1972). Since glycosylation occurs co-translationally, once the protein has folded, potential glycosylation sites are no longer accessible to the glycosyltransferases.

The carbohydrate units linked to the asparagine contain a common pentasaccharide core: Man α l-6 (Man α l-3) Man β(1-4) GlcNAc β (1-4) GlcNAc (Fig.4 A). Depending on the type of additional saccharide units attached to the pentasaccharide

core the N-linked oligosaccharides are categorized as high mannose, complex and hybrid types. The high mannose oligosaccharides have additional mannose residues attached to the core. The total number of mannose residues in this type ranges from 6 to 12 and the chains are often branched (Fig.4 A). The complex type contains the disaccharide N-acetyl lactosamine (Gal β(1-4) GlcNAc) attached to the core. Sialic acid residues may or may not be linked to the Gal. The third class of N-linked oligosaccharides is the hybrid type, which contains the features of both high mannose and complex types (Fig.4 A). Most hybrid molecules contain a "bisecting" N-acetyl glucosamine linked β(1-4) to the β-linked mannose residue, although some exceptions exist [Hunt et al., 1983].

2.6.1.2. O-glycosylation

The second major type of saccharide-peptide linkage occurring in glycoproteins is the O-glycosidic linkage, which is found in mucins, blood group active glycoproteins, certain plasma glycoproteins and membrane glycoproteins. In O-glycans, carbohydrate is attached to hydroxyl groups of amino acids, serine or threonine. *O*-linked glycosylation occurs during protein processing, most commonly by the addition of an N-acetyl galactosamine residue α-linked to the hydroxyl oxygen of serine or threonine residues by the enzyme *UDP-N-acetyl-D-galactosamine* (Fig 4 B). However, O-linkages are also found with N-acetylglucosamine in intracellular glycoproteins and xylose in proteoglycans.

Depending on which saccharide groups are subsequently attached to this first GalNAc residue, mucin O-glycans are divided into four major subtypes (Schachter and Brockhausen, 1992) (Fig.4 C). Other modifications to the core GalNAc structure have also been found but appear to be uncommon. Of the four main core O-glycan structures, the core 1 and 2 structures are widely distributed while the core 3 and core 4 structures are less common and expression has been mostly associated with mucin producing tissue of the digestive tract. Commonly the core 2 and the core 4 branches are elongated with one or multiple lactosamine structures (Gal β 1-4 GlcNAc) (Lowe, 2001).

2.6.2. The T and Tn –antigen

Core 1, O-glycan or T antigen [Gal (βl-3) GalNAc] (Fig.4 B) was discovered about 70 years ago by Thomsen and Friedenreich as a laboratory curiosity. The phenomenon of panagglutinability acquired by bacterially contaminated human red blood cells led to the discovery of T-antigen. The immunodeterminant group GalNAc, linked to the hydroxyl group of Serine or Threonine in the amino terminal region of glycoproteins is called Tn- antigen. T and Tn are usually covered by covalently linked carbohydrates holding high negative charge density in the case of sialic acid substitution (Springer and Desai, 1982) or are physically separated from the immune system (Pierce-Cretel et al., 1981). Unmasked T and Tn specific epitopes are unique carcinoma markers.

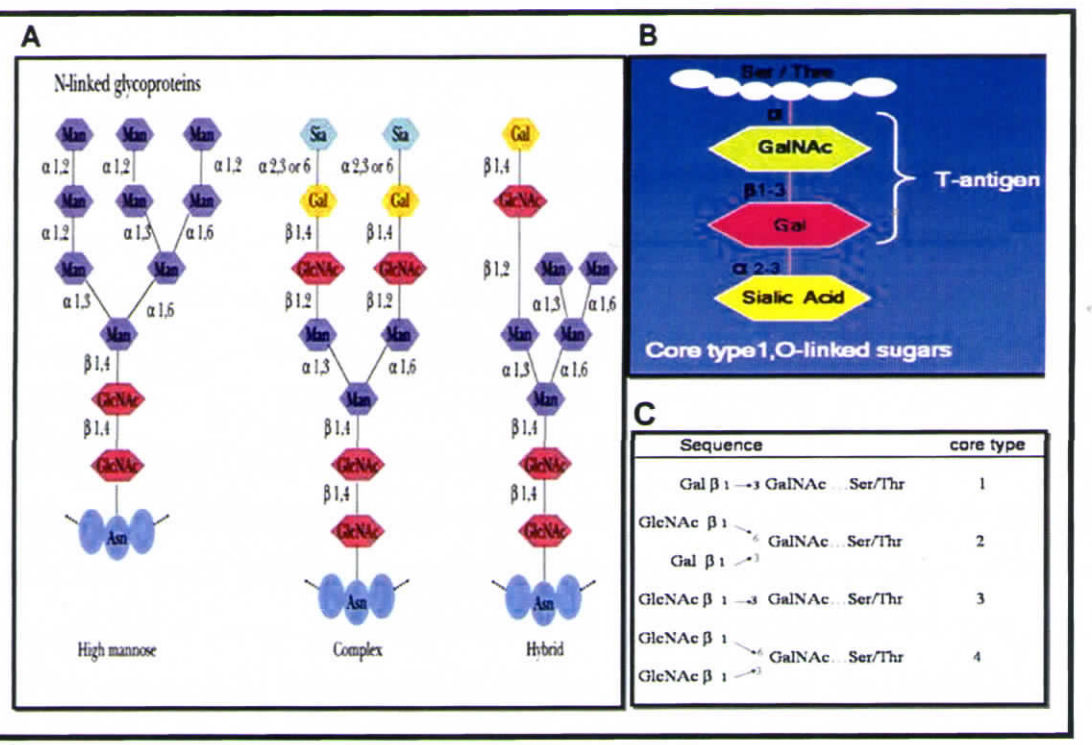


Fig. 4 A. Types of N-glycosylation, **B**: O-glycosylation in glycoproteins, **C**: Types of O-glycosylation ; Sia -sialic acid; Man –mannose; GlcNAc -N-acetylglucosamine; Asn – asparagine; Gal –galactose; GalNAc -N-acetyl galactosamine.

2.6.3. Biological significance of glycosylation

Glycosylation is one of the most frequent and important post-translational modifications. The glycosylation of a protein is not directly specified in the genome but is determined by levels of glycosyltransferase expression, accessibility of glycan attachment sites, concentrations of nucleotide sugar donors and other environmental factors. Though variable, the glycosylation of a protein is often characteristic of the cell type where it was synthesised. The glycosylation profile and the glycosyltransferase expression vary widely between different cell types and can change significantly during development or stimulation of a cell.

The carbohydrates of glycoproteins modify the physicochemical properties of proteins by changing their hydrophobicity, electrical charge, mass and size. The attachment of sugars to proteins is known to increase the solubility of the latter. Sialic acids in salivary glycoproteins are responsible for the high viscosity of the mucous solutions (Gottschalk and Thomas, 1961). Negatively charged acylneuraminic acid residues impart physical strength to cell membranes because of their mutual repulsion and influence the mutual adhesion of cells in organ structure. The antifreeze glycoprotein of antarctic fish depends on the integrity of the disaccharide (Gal βl-3 GalNAc) units for their activity. Glycoproteins rich in sugar are relatively resistant to proteolysis (Gottschalk and Thomas, 1961). The protection against proteolytic degradation is attributed to steric hindrance by the carbohydrate as well as the more stable conformation adorned by the glycosylated protein. The sugar chains of glycoproteins are involved in the initiation of correct polypeptide folding in the rough endoplasmic reticulum (ER) and in the subsequent maintenance of protein solubility and conformation. However glycosylation is not obligatory for protein export or translocation. For many proteins glycosylation is not a prerequisite for biological activity.

The carbohydrate units of membrane glycoproteins are always located on the outer surface of the cell and the coating of glycoconjugates covering a whole cell can present a 'glycocalyx' of substantial proportions. All most all cells carry carbohydrates on

their surfaces in the form of glycoproteins, glycolipids and polysaccharides (Cook, 1986). The greatest variation in glycosylation pattern tends to be found among the outermost (non-reducing terminal) regions of glycans on cell surfaces and extracellular molecules (Lis and Sharon,1993). These regions of sugar chains are best positioned to mediate recognition by carbohydrate binding proteins (lectins) (Baenziger, 1985).

Carbohydrates also serve as important recognition markers. Cell surface glycoproteins are the immunodeterminant structures of blood group A, B, H and M/N specificities (Watkins and Morgan, 1952). Certain oligosaccharides can act as highly specific receptors for a variety of viruses, bacteria and parasites.

Carbohydrates play important structural and functional role in various disease states. Changes in the sugar moieties on cell surface occur when normal cells are transformed into malignant ones. Such transformation also results in loss of contact inhibition suggesting the involvement of sugars in cellular recognition and intercellular communication. Cell surface glycoproteins may be regarded as principal candidates for involvement in tumor cell spread since they are generally oriented towards the exterior of the cells and thus ideally suited to mediate the interaction of metastatic cells with their environment. Alterations in cell surface glycoconjugates are considered to be relevant to the abnormal properties of cancer cells, such as uncontrolled cell growth, altered cell expression, avoidance of immunological destruction, invasiveness and metastatic spread (Bhavanandan, 1991).The biological roles of oligosaccharides therefore, appear to span the spectrum from those that are trivial, to those that are crucial for the development, growth, function or survival of an organism

2.7. Vascular extracellular matrix proteins

The principal glycoproteins in vascular tissue synthesized by vascular cells include fibronectin, laminin, thrombospondin, tenascin, and osteopontin. These proteins have similar modular structures and contain sequences that allow them to self-associate,

interact with other ECM components, and bind to cells through specific cell surface receptors. Through multiple interactions, these macromolecules regulate vascular ECM integrity and provide a variety of substrates for vascular cells

2.7.1. Fibronectins

Fibronectin is a high-molecular-weight glycoprotein containing about 5% carbohydrate. Two forms of fibronectin are met with; the soluble form found in plasma, and the insoluble form in the extracellular matrix. This glycoprotein consists of two similar peptide chains of -220 KDa held together at one end by disulfide bonds (Yamada,1991). Each chain consists of repeated copies of three distinct types of polypeptide domains (types I, II, III). There are 12 types I repeats, two type II repeats, and 15 to 17 type III repeats in human fibronectin. Approximately 20 different fibronectin chains have been identified in humans, all of which are generated by alternative splicing of the RNA transcript of a single fibronectin gene (Schwarzbauer, 1991).

Fibronectin is the principal attachment protein for vascular cells binding to receptor proteins that span the cell's membrane, called integrins. Fibronectin contains Arg-Gly-Asp sequences (RGD sequence or the integrin binding motif).In addition to integrins, they also bind extracellular matrix components such as collagen, fibrin and heparin and serves as a substrate for the migration of vascular cells during development and remodeling (Madri and Basson, 1992)

Fibronectin is present throughout all layers of the vascular ECM and in elevated amounts during development and in the neointima in response to injury and hypertension (Risau and Lemmon, 1988). Deposits of this glycoprotein in altered vascular tissue may influence the retention of lipoproteins in the vascular ECM because fibronectin interacts with lipoproteins, possibly through its heparin-binding domain (van der Hoek et al., 1994).

2.7.2. Laminin

Laminin is an ~800 KDa trimeric glycoprotein present in vascular basement membranes of endothelial and smooth muscle cells. Laminin also exists as multiple isoforms. Each laminin molecule is a heterotrimer assembled from alpha-, beta-, and gamma-chains (Colognato and Yurchenco,2000). There are five forms of alpha-chains: LAMA1, LAMA2, LAMA3, LAMA4, LAMA5, four beta-chains: LAMB1, LAMB2, LAMB3, LAMB4 and three of gamma-chains: LAMC1, LAMC2, and LAMC3. Fifteen laminin trimers have been identified to date (Colognato and Yurchenco, 2000).

Laminin interacts with key basement membrane components such as type IV collagen, nidogen, and heparan sulfate proteoglycans (HSPG) to form the fabric of the basement membrane during embryonic vasculogenesis and vascular wall maturation. Because laminin peptides contain multiple cell-binding sites (RGD sequences), this glycoprotein serves as a substrate for vascular cells. Laminin also interacts with arterial smooth muscle cells through more than one receptor (Hedin et al., 1988). This interaction in part maintains these cells in a nonproliferative and contractile phenotype, unlike fibronectin, which promotes modulation of arterial smooth muscle cells to a proliferative and secretory phenotype.

2.7.3. Thrombospondin

Thrombospondin (TSP) is a 450 KDa trimeric glycoprotein that consists of three identical 150 KDa chains joined together by disulfide linkages. The glycoprotein exists in more than one form and may constitute a family of related proteins generated by alternative splicing, although more than one TSP gene exists (Bornstein, 1992). Thrombospondin is synthesized by a variety of cells including vascular endothelial and smooth muscle cells (Majack et al., 1988). Thrombospondin possesses multiple binding sites and interacts with a variety of ECM components such as fibronectin, a number of different collagens, laminin, and HSPGs. Thrombospondin is present in different vascular layers but is elevated in the thickened intima in human vascular disease (Wight et al., 1985).

2.7.4. Tenascin

Tenascin is a glycoprotein that is transiently present in the vascular ECM. This glycoprotein is a large hexameric protein with disulfide-linked multidomain subunits of 190-240 KDa organized into a six-armed structure. As is true of other ECM glycoproteins, more than one form of tenascin exists, generated by alternative splicing involving various exons that code for the type III repeats in the molecule (Erickson. 1993). Tenascin is an ECM glycoprotein with a spatially and~ temporally restricted distribution. Tenascin is synthesized by both vascular smooth muscle and endothelial cells and is regulated in part by factors such as PDGF, TGF-β1, angiotensin II, and is present in increased levels in the neointima in hypertension (Mackie et al.,1992).Tenascin contains RGD sequences and influences vascular cell adhesion

2.7.5. Osteopontin

Osteopontin is an acidic, highly phosphorylated glycoprotein first identified in bone but subsequently found in a variety of tissues including blood vessels (Denhardt and Guo, 1993). Osteopontin is small compared to most ECM molecules and has an average molecular mass ranging from 44 to 85 KDa. The protein contains an RGD cell-binding domain, two potential Ca2+ binding sites, and a HSPG binding domain as well. Osteopontin is not present in the ECM of normal blood vessels but appears in the neointima following experimental balloon angioplasty and is present in human atherosclerotic plaques (Denhardt and Guo, 1993). There is a spatial and temporal expression of osteopontin that is coincident with the proliferation and migration of arterial smooth muscle cells during the invasion of the intima following vascular injury and in remodeling, and also osteopontin is chemotactic for arterial smooth muscle cells (Giachelli et al., 1995). These observations suggest a role for osteopontin in the early events associated with vascular disease. Because it contains an RGD cell-binding motif, osteopontin could serve as an adhesive ligand for vascular cells during the early phases of vascular remodeling (Giachelli et al.,1995). The finding of osteopontin in advanced atherosclerotic lesions also suggests a role for this molecule in late events of vascular

disease. Osteopontin tends to localize around areas of calcification in advanced human atherosclerotic plaques (Giachelli et al., 1995). These findings, coupled with the fact that osteopontin protein contains specific domains for Ca2+ binding, suggest a role for osteopontin in the calcification process involved in human disease. Whether this protein regulates arterial calcification remains to be determined.

Chapter 3

MATERIALS AND METHODS

3.1. Materials

Fetuin, neuraminidase from *Clostridium perfringens*, human IgG, ortho phenylene diamine (OPD), concanavalin A (Con A), *Helix aspersa* lectin (HAA), soluble guar gum, agarose(low EEO), molecular weight markers, Sepharose 4B, horse radish peroxidase, bovine serum albumin, Coomassie brilliant blue G 250 and R 250, Sudan black B, divinyl sulphone, cyanogen bromide, Tween 20, 4-chloro-l-naphthol, sodium dodecyl sulphate, acrylamide, N, N'-methylene bisacrylamide, TEMED, mannose, galactose, 2-mercapto ethanol,dithiothreitol, Iodoacetamide, potassium thiocyanate, sodium bromide, potassium borohydride, sodium borohydride, L-proline, gelatin, human placental laminin, human plasma fibronectin, β-galactosidase from bovine testis, lactose, phenylmethanesulfonyl fluoride (PMSF), anti-mouse IgG-HRP, anti-human β-lipoprotein (LDL), anti-sheep IgG-HRP, anti-goat IgG-HRP, anti-rabbit IgG- HRP, anti-rabbit IgG- FITC, anti-goat IgG-TRITC and polyvinylydene diflouride (PVDF) membranes were purchased from Sigma Chemical Company, U.S.A. O-glycosidase (from *Diplococcus pneumoniae*) was from Boehringer Mannheim. Mouse anti-human apo(a) was from ICN Biomedicals (USA). Sheep anti-human apo B was from Serotec(UK). Rabbit anti-human Lp(a) was from Dako Cytomation (Denmark). Protein A- Sepharose was from Pharmacia and Lp(a) assay kit [ELITEST-Lp(a)] was from Hyphen BioMed (France). All other chemicals used in

solvents and buffers were from Merck India. Ltd., and were of analytical grade. The seeds of Jack fruit (*Artocarpus integrifolia*), castor beans (*Ricinus communis) and* pea nuts (*Arachis hypogea)* were obtained locally.

Human placenta and umbilical chord, immediately after delivery, was collected from the Obstetrics Department, Cosmopolitan Hospital, Thiruvananthapuram. The tissue was washed extensively in running water to remove blood clots and debris and then washed in ice cold PBS 7.4. Placenta was then cut into pieces (50 gm) and kept frozen at -20° C before use. Human internal mammary artery (IMA) sections were obtained from patients undergoing coronary artery bypass surgery at the Department of Cardio-Thoracic Surgery, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. Fasting serum and blood samples were collected from the Central Clinical Laboratory and Blood Bank, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The collection of all biological samples had the prior approval of the institute.

3.2. Methods

3.2.1. Isolation and purification of Lp(a)

3.2.1.1. Isolation of serum Lp(a) fraction (density 1.05-1.12g/ml) by ultra centrifugation

Approximately 10 ml serum was collected from each volunteer after a 12h overnight fast. EDTA and sodium azide at final concentrations of 0.1% and 0.01% w/v, respectively, in addition to 0.001M of phenylmethanesulfonyl fluoride (PMSF) and benzamidine hydrochloride were immediately added. Ultra centrifugation was carried out in a Beckman Optima TLX preparative ultracentrifuge with fixed angle, type 120.2 rotor. Lp(a) was isolated from serum between density(*d*) 1.05 - 1.12 g/ml on the basis of the method described by Kostener et al.(1999) with some modifications. Briefly, the density of 1 ml of serum was adjusted to 1.05 g/ml by adding solid sodium bromide (NaBr).

After centrifugation at 435000 × g for 90 min at 15°C, the top layer (200µl) containing VLDL, IDL and LDL (*d* <1.05 g/ml) was discarded. L-proline was then added to the bottom fraction to a final concentration of 0.2 M and the density adjusted to 1.12 g/ml with solid NaBr followed by ultracentrifugation (435000 × g, 150min at 15°C). The floating Lp(a) fraction (top 200µl; *d* 1.05-1.12 g/ml) consisted of Lp(a), LDL (*d* >1.05 g/ml) and HDL_2

3.2.1.2. Determination of the optimum concentration of L-proline for the resolution of Lp(a) in native PAGE

3.2.1.2.1. Preparation of native 3.75% polyacrylamide disc gels

Native 3.75% polyacrylamide disc gels were cast in glass tubes (5mm ID,9 cm *l*). Ten ml of the gel solution contained 2 ml of TBE buffer, pH 8.7 (0.05 M Tris, 0.025 M boric acid, 0.003 M EDTA), 2.5 ml of 15% acrylamide/bisacrylamide (19:1), 5.5 ml of 0.2% ammonium persulfate and 10µl TEMED

3.2.1.2.2. PAGE of isolated Lp(a) fraction with varying concentrations of L-proline

The isolated Lp(a) fraction was dialyzed against the sample buffer (1: 9 diluted chamber buffer). TBE buffer, pH 8.7 was used as the chamber buffer. The sample contained 180 µl of the dialyzed Lp(a) fraction from serum (*d* 1.05-1.12 g/ml). L-Proline was added to each sample and incubated for 15 min at room temperature. The final concentration of proline in individual samples was 0.02, 0.04, 0.06, 0.08, 0.1, 0.3, 0.5, 0.8, 1, 2 and 3 M, respectively and a control was maintained without adding proline. All samples were then prestained for lipids with 20µl of Sudan black saturated ethylene glycol and incubated for 15 min. The lipoprotein load was approximately 300µg/tube. Each sample was then loaded over individual disc gels, layered with chamber buffer (TBE) and subjected to electrophoresis for 2h at a constant current of 2 mA/tube. The chamber buffer was pre-cooled to 15°C prior to electrophoresis. The glass tubes with the gels were then photographed and the resolutions of individual lipoproteins in the samples

were visually analyzed for the optimum concentration of L-proline inducing maximum resolution of Lp(a) on electrophoresis.

3.2.1.3. PAGE of the isolated Lp(a)fraction and the electroelution of lipoproteins

3.2.1.3.1. PAGE of the isolated Lp(a) fraction

To the dialyzed Lp(a) fraction was added L-proline (0.1M final concentration) and incubated for 15 min at room temperature before adding ethylene glycol (10% v/v). It was then subjected to native PAGE in 3.75% disc gels as detailed above for the determination of the optimum concentration of L-proline for Lp(a) electrophoresis. Sample volume was 200µl containing about 300µg of lipoprotein. Both PAGE and the subsequent electroelution of the Lp(a) were done at room temperature (25°C) to avoid cryoprecipitation of Lp(a).

3.2.1.3.2. Electroelution of lipoproteins

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 0.4 g/L Coomassie Brilliant Blue R-250 in 35 ml/L perchloric acid and the bands appeared in about 30 min. Three bands were visible: the far migrating HDL_2, the β- lipoprotein (LDL) and the pre-β lipoprotein [Lp(a)]. Using this gel as a marker, individual bands were sliced from the nonstained gels and equilibrated in 2 to 4 ml Tris-acetate buffer (0.05 M, pH.8) for 15 min. Pre-staining the marker gel with Sudan black was avoided as the dye may interfere with the actual mobility of the lipoproteins. The gel suspensions were then taken into individual dialysis bags and placed immersed in between opposite electrodes in a rectangular reservoir 20×20×7 (cm) with 2 L of cold (15°C) Tris- acetate buffer (0.05 M, pH.8). A constant voltage of 50 V was applied across the electrodes for 6 h with a buffer change after 3 h. The lipoproteins were then dialyzed against 0.01 M phosphate buffer, pH 7, 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 supernatant lipoprotein was estimated according to Lowry et al.(1951) using BSA as standard and stored at –20°C with 50% v/v ethylene glycol. Alternatively, the lipoproteins may be eluted in a single step with the same native electrophoretic buffer using any commercial electroeluter.

3.2.1.4. Electrophoretic analysis of purified lipoproteins

3.2.1.4.1. Native PAGE analysis of the purity of lipoproteins

The electroeluted Lp(a) and LDL (*d* >1.05 g/ml) were checked for purity by native PAGE in 3.75% disc gels essentially as described before.

3.2.1.4.2. Agarose gel analysis of the purity of lipoproteins

The purified lipoproteins were also analyzed in agarose gels as described earlier (Papadopoulos, 1979) with slight modifications. Briefly, the lipoproteins were loaded on to wells in an agarose gel cast within a submarine agarose gel electrophoretic apparatus. The agarose (0.6% in electrophoretic buffer) was heated over a boiling water bath until completely melted. The samples were prestained for lipids with Sudan black (ethylene glycol saturated with Sudan black) prior to loading on to the gel and electrophoresed in 0.045 M Barbital-HCl buffer, pH 8.5, for 45 min at a constant current of 20mA. Approxi mately 5µg of lipoprotein was applied per lane.

3.2.1.5. Dot blot- immuno analysis of purified Lp(a) and LDL

Isolated serum Lp(a) fraction, purified Lp(a) and LDL were coated on individual 5 × 5 mm squares of polyvinylidene difluride (PVDF) membranes. The sample load was approximately 1µg lipoprotein in 3µl of phosphate buffer (0.01M, pH7.4). The membranes were pre-wetted in methanol and rinsed with distilled water prior to application of the sample. It was allowed to air dry for 20 min and blocked with 0.5% Tween 20-PBS for 1 h at 37°C. One set of the blots were then incubated with mouse anti-

human apo(a)[ICN Biomedicals,USA] diluted 1: 40 with 0.5% Tween 20-PBS, while a second set was incubated with goat anti-human β-lipoprotein (LDL) [Sigma, USA] diluted 1:100 with 0.5% Tween 20-PBS for 1h at 37°C.After washing with 0.05% Tween 20-PBS, the first set of dots were incubated with anti-mouse IgG-HRP [Sigma, USA] diluted 1:2000 with 0.2% Tween 20-PBS while the second set was incubated with anti goat IgG-HRP [Sigma, USA] diluted 1:2000 with 0.2% Tween 20-PBS for 1h at 37°C. The dots were then washed three times with 0.05% Tween 20-PBS and incubated in substrate solution (1 ml of 0.1% 4-Chloro-1-naphthol in anhydrous methanol mixed with 5 ml PBS and 3µl of 30% hydrogen peroxide) at room temperature to develop color.

3.2.1.6. Immunoblotting of isolated serum Lp(a) fraction to demonstrate pre - β mobility of Lp(a) in PAGE

The serum Lp(a) fraction was subjected to native PAGE in 3.75% slab gels. The gel, sample preparation and the buffer system was similar to that detailed for PAGE in disc gels for the electroelution of Lp(a). The sample load was approximately 25µg of lipoprotein/well. The lipoproteins were western blotted to PVDF membranes as described earlier (Towbin et al., 1979; refer general methods). The PVDF membrane was pre-wetted with methanol prior to the transfer, and methanol and SDS were avoided in the transfer buffer. Transfer was done at 100 V constant for 1h. The membrane was then blocked with 0.5% Tween 20-PBS for 1h at 37°C. After washing with 0.05% Tween 20-PBS, the membrane was cut into two equal halves along the direction of the migration of the transferred proteins and one half was incubated with rabbit anti-human Lp(a) (Dako Cytomation,Denmark) diluted 1:100 with 0.5% Tween 20-PBS for 1 h at 37°C followed by washing with 0.05% Tween 20-PBS and incubation with anti rabbit IgG-HRP (Sigma,USA) diluted 1:300 with 0.25% Tween 20-PBS for 1 h at 37°C. For the detection of LDL, the second half of the membrane was incubated with sheep anti-human apo B (Serotec,UK) diluted 1:50 with 0.5% Tween 20- PBS for 1h at 37°C. The membrane after washing with 0.05% Tween 20-PBS was incubated with anti sheep IgG-HRP (Sigma,USA) for 1h at 37°C. Both the membranes were then washed three times with 0.05% Tween 20-PBS and incubated in substrate solution (4-Chloro-1-naphthol) at room

temperature to develop color.

3.2.2. Isolation of apo(a) and apo B

3.2.2.1. Isolation apo(a)

Apo(a) was isolated from Lp(a) by reduction with dithiothreitol. The procedure was a method modified from that described by Armstrong et al.(1985). Lp(a) (*d* 1.05-1.12 g/ml) isolated from fasting serum was made 0.01M with respect to dithiothreitol (DTT) and incubated in dark for 2 h at 37°C.The density was adjusted to 1.2 g/ml with NaBr and ultracentrifuged for 4 h at 435000×g in the Beckman Optima TLX ultra centrifuge. The sedimented apo(a) (about 100µl bottom fraction) was collected, dialyzed against 50mM TBS, pH 7.5 and stored at - 20°C with ethylene glycol (50% v/v).

3.2.2.2. Isolation of apo B from LDL

Apo B was isolated from the serum LDL fraction (*d* 1.02-1.04 g/ml). The LDL fraction was isolated by a method modified from that described by Camejo et al.(1993b). Briefly, the density of 1ml of fasting serum was adjusted to 1.02 g/ml by adding NaBr and after centrifugation at 435000 × g for 60 min at 15°C, the top layer (200µl) containing VLDL and IDL was discarded. L-proline was then added to the bottom fraction to a final concentration of 0.2 M and the density adjusted to 1.04 g/ml with solid NaBr. It was then subjected to ultracentrifugation (435000 × g, 150 min at 15°C) and the floating LDL fraction (top 200 µl, *d* 1.02-1.04 g/ml) was collected. Apo B was obtained by delipidising LDL with sodium deoxycholate. The protocol was one that was modified from the method described by Lundberg and Suominen (1984). Briefly, the isolated LDL fraction was dialyzed against 0.05 M Na_2CO_3, 0.05 M NaCl, pH 10, and sodium deoxycholate was added to a final concentration of 0.01 M and vortex mixed for 2 minutes. The delipidised LDL (apo B) was then purified from the lipids and contaminating delipidised Lp(a) particles by electroelution and stored at -20°C with ethylene glycol (50% v/v).

3.2.3. Elucidation of the carbohydrate structures on Lp(a), LDL, apo(a) and apo B

3.2.3.1. Isolation of jacalin

Jacalin (jack fruit seed agglutinin, JSA) was isolated from the seeds of *Artocarpus integrifolia* (jack fruit) by the procedure described by kumar et al.(1982). Thirty g of 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°C. The supernatant of homogenate obtained by centrifugation at 14500 × g for 20 min was subjected to 70% ammonium sulphate saturation and stirred for 30 min at 4°C. 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 gaur galactomannan (CLGG) column (refer 'general methods' for CLGG preparation). The column was washed with PBS 6.5 and eluted with 0.15 M galactose in PBS 6.5. Fractions containing proteins were pooled and dialysed against PBS 7.4.

3.2.3.2. Isolation of pea nut agglutinin (PNA)

Pea nut agglutinin was isolated from pea nuts (*Arachis hypogea*) as described by Chacko and Appukuttan (2001). All operations were at 4°C. Fifty gram dehusked peanut soaked for 24 h in 20 mM phosphate buffer, pH 6.5 containing 150 mM NaCl was homogenized in the same buffer and stirred for 1 h. After a 15,000 × g centrifugation, the lipid slab on top was removed, and the proteins from the supernatant precipitated by 70% ammonium sulphate saturation was dissolved in and dialyzed against PBS 6.5. The supernatant after a 15,000 × g centrifugation of the dialysate was passed through a 40 ml CLGG column equilibrated in the same buffer. After washing out unbound proteins with PBS 6.5, bound proteins were eluted using 0.15M lactose in the same buffer and concentrated using AMICON PM10 ultra-filtration membrane.

3.2.3.3. Isolation of Ricinus communis agglutinin (RCA-1)

Ricinus communis agglutinin (RCA-1) was isolated from the seeds of castor (*Ricinus communis*). This was done as described by Appukuttan et al.(1977). *Ricinus*

communis seeds (50 g) were peeled and soaked overnight in potassium phosphate buffer, pH 7.2 containing 50 mM NaCl. Then the seeds were homogenized in a blender. The supernatant of homogenate obtained by centrifugation at 12000×g for 30 min was subjected to 70% ammonium sulphate saturation and the precipitated proteins were redissolved in PBS and dialysed against the same buffer. The supernatant obtained by centrifugation at 10000 × g was loaded on to a CLGG column equilibrated with PBS 7.4. After washing away unbound proteins with PBS, bound proteins were eluted with 0.2 M lactose in PBS.

3.2.3.4. Isolation of galectin-1

Galectin-1 from both bovine heart (BHL) and human placenta (HPL) was isolated as described by Sangeetha and Appukuttan (2005). Briefly, the tissue was homogenized in cold PBS (phosphate buffered saline, pH.7.4) containing 2-mercaptoethanol (5 mM), phenylmethylsulfonylflouride (PMSF; 0.2 mM), benzamidine hydrochloride (2 mM) and lactose (50mM).The homogenate was centrifuged at 16000×g for 20min. The supernatant proteins were then precipitated with ammonium sulfate at 70% saturation, dialyzed against PBS containing 2-mercaptoethanol, and passed through a lactose-Sepharose column. The bound galectin-1 was eluted with PBS containing 150mM lactose and 50 mM iodoacetamide and dialyzed against PBS. All operations were at 4°C. The lectins were subsequently, either, conjugated with HRP (refer general methods) or stored with 30% v/v glycerol at –20°C until use. Lectin activity was assayed by hemagglutination with trypsinized human RBC (refer general methods). The isolated galectin was also immunoanlysed with anti galectin-1.

3.2.3.4.1. Immunodetection of galectin-1 in western blots of galectin-1 isolates

Isolated galectin-1 preparations were analysed by SDS PAGE (12%). After western blotting to PVDF, immunodetection of galectin-1 was done using rabbit anti-galectin-1 (kind gift of Dr. Linda G. Baum, UCLA School of Medicine, Los Angeles, CA 90095) diluted 1: 500 with PBS containing 1% BSA. The second anti body conjugate, anti- rabbit IgG-HRP (Sigma), was diluted 1 in 2000 in blocking buffer. Blocking,

washing and the color development were done same as described before for the immunoblotting of isolated serum Lp(a) fraction to demonstrate pre -β mobility of Lp(a) in PAGE

3.2.3.5. Enzyme linked lectin analysis (ELLA) of the carbohydrate structures on Lp(a), LDL, apo(a) and apo B

Purified Lp(a), LDL, apo(a) and apo B were coated on individual microtiter wells overnight (1μg protein/ 200μl /well),followed by washing and blocking with 0.05% Tween 20-PBS and 0.5% Tween 20-PBS respectively. The wells were then incubated for 2 h at 4°C with the HRP-conjugated lectins (7.5μg lectin/ml in blocking buffer). When PNA was used, desialylation of the coated proteins was done by incubating with 0.12 U/ml neuraminidase (from *Clostridium perfringens*) in blocking buffer at 37°C for 1h after blocking. The bound lectin conjugate was then detected with orthophenylene diamine, OPD (0.5 mg/ml, in 0.1 M citrate-phosphate buffer pH 5 containing 0.03% H_2O_2. Following the addition of 50μl 12.5% H_2SO_4, the absorbance at 490 nm was determined. All trials were in triplicate and the values were expressed as optical density (OD) units at 490 nm.

3.2.3.6. Dot blot analysis of T-antigen in Lp(a) and LDL

The native, β-eliminated and sequentially glycosidase digested Lp(a) and LDL (refer general methods) were dot blotted washed and blocked essentially as described before for the dot blot- immuno analysis of purified Lp(a) and LDL. The dots were then incubated for 2 h at 4°C with HRP conjugated T-antigen-specific lectins, namely, jacalin, PNA and HAA (lectin concentration- 70 μg/ml).The bound lectin conjugate was detected with 4-chloro-1-naphthol as described for the dot blot- immuno analysis of purified Lp(a) and LDL . The dot blots were digitalized using high performance scanner and the densitometric analysis of the dot blots was carried out using the free-domain package Image J 1.37 v (NIH IMAGE).

3.2.4. Elucidation of the galectin-1 recognition of Lp(a) and LDL

3.2.4.1 Enzyme linked lectin analysis (ELLA) of galectin-1 interaction with Lp(a), LDL, apo(a) and apo B

Lp(a), LDL, apo(a) and apoB coated to microtiter wells were analyzed by enzyme linked lectin analysis (ELLA) using HRP-conjugated galectin-1 (both from bovine heart and human placenta). The experimental procedures were same as that detailed before for the enzyme linked lectin analysis of carbohydrate structures on Lp(a), LDL, apo(a) and apo B. Desialylation of the coated proteins with neuraminidase prior to incubation with HRP conjugated galectin-1 was also done. Additionally, sugar inhibition tests was carried out, wherein, lactose at a final concentration of 50mM was incubated with galectin-1-HRP (for 1h at 4°C in dark) prior to incubation with the coated lipoproteins /apoproteins. All trials were in triplicate and the values were expressed as optical density (OD) units at 490 nm.

3.2.4.2. Dot blot analysis of galectin-1 interaction with Lp(a) & LDL

Native, β-eliminated and sequentially glycosidase digested Lp(a) and LDL dots were analyzed with HRP conjugated galectin-1 (both BHL and HPL).The experimental procedures were same as that detailed befrore for the dot -blot analysis of T-antigen in Lp(a) and LDL. The dot blots were digitalized using high performance scanner and the densitometric analysis of the dot blots was carried out using the free-domain package Image J 1.37 v (NIH IMAGE).

3.2.4.3. Glyco histochemical analysis of galectin-1 binding to Lp(a).

3.2.4.3.1. Tissue fixation

Trimmed internal mammary artery segments were fixed in 4% paraformaldehyde (refer general methods) for 24 h at 4°C.

3.2.4.3.2. Paraffin embedding, sectioning and drying of tissue

Dehydration of the tissue with graded alcohols and paraffin embedding at 58°C was carried out with fully automated tissue processor. Five µm transverse sections were cut from the paraffin embedded tissue using a rotary microtome. Sections were spread on to albumin-coated slides and dried in an oven at 37°C for 1h followed by incubating for 1 h at 60- 61°C (slightly above the melting point of wax; just enough to melt the wax, overheating may inactivate tissue galectin-1).

3.2.4.3.3. Deparaffinisation, rehydration, and the inhibition of endogenous peroxidase activity in tissue sections

The sections were deparaffinised in xylene with 3 changes (5min each), followed by rehydration in serial -graded alcohol solutions (100 % absolute alcohol to 90%, 80%, 70%, 50%, water, and then to PBS; 5 min in each successive solution). The sections were then incubated with 0.3% hydrogen peroxide in PBS for 10 min to inhibit endogenous peroxidase activity. The sections were finally transferred to PBS.

3.2.4.3.4. Localization of galectin-1

Sections were incubated for 1h at room temperature with rabbit antiserum raised against human recombinant galectin-1 (kind gift of Dr. Linda G. Baum, UCLA School of Medicine, Los Angeles, CA 90095) diluted 1:200 with PBS containing 1%BSA (1% BSA - PBS). Sections were then washed with PBS and incubated with anti rabbit IgG -HRP (Sigma; diluted 1:200 in 1%BSA-PBS) for 30 min at room temperature. After washing the sections with PBS, the HRP reaction was performed by incubating for 10 min in 3,3'-diaminobenzidine 4-HCl (DAB) - H_2O_2 mixture [25mg DAB in 100 ml PBS containing 0.03% H_2O_2]. Following the DAB treatment, sections were rinsed briefly under running tap water and incubated with 0.5% copper sulfate pentahydrate in PBS for 5 min to enhance signal. Sections were then counterstained with Mayer's hematoxylin, mounted in glycerol jelly and examined by light microscopy.

3.2.4.3.5. Localization of tissue galectin-1 binding Lp(a)

For detection of Lp(a) binding to tissue galectin-1 *in situ*, serial sections were cut from the same paraffin-embedded tissue from which sections were cut for determining the localization of galectin-1. Sections were incubated with Lp(a) in 1% BSA-PBS for 30 min at room temperature. The concentration of Lp(a) ranged from 20 to 100 mg/dl. To ascertain the sugar specificity of Lp(a) binding, sections were pre incubated for 30 min at room temperature with 50mM lactose in PBS (maltose was used as the control sugar). Incubation was then performed for 30 min with Lp(a) of the same concentration as above containing the corresponding sugar (50mM) as competitive inhibitor. All sections were then washed with PBS (3changes; 5 min each) and incubated with rabbit polyclonal anti-human Lp(a) [Dako Cytomation,Denmark] diluted 1:1000 in 1% BSA-PBS for 30 min at room temperature followed by incubation for 30 min with the HRP-conjugated second antibody diluted 1:200. The DAB reaction, counter staining and mounting was performed as described before for the localization of galectin-1.

3.2.4.4. Immunofluorescence analysis of the *in situ* binding of Lp(a) by human umbilical vein endothelial cell (HUVEC) membrane expressed galectin-1

3.2.4.4.1. Isolation, culture and characterization of human umbilical vein endothelial cells (HUVEC).

Isolation and culture of human umbilical vein endothelial cells are done routinely at the Thrombosis research unit in the BMT wing of our institution. The HUVECs used in my studies were courtesy of Dr. Lissy K Krishnan (Head, Thrombosis Research Unit). The immunofluorescence analysis studies were performed at the Thrombosis research unit.

3.2.4.4.1.1 ***Isolation of HUVEC.*** Human umbilical cord was collected immediately proceeding delivery in ice cold Ca^{2+} and Mg^{2+} -free Hank's balanced salt solution (HBSS) containing antibiotics (1000 U/ml of benzyl penicillin and 1000 µg/ml of strepto mycin; Sigma). Both distal and proximal ends of the vein were tied with sterile silk just

before removing the cord from the placenta to ensure sterility within the lumen.

Endothelial cells were isolated from the umbilical vein as described by Gimbrone et al. (1974) with slight modifications. The umbilical cord collected was processed within 4h of collection under the laminar flow hood. The cord was washed with fresh ice cold HBSS and damaged area were discarded. A small piece was cut off at one end of the cord so that a fresh non retracted vein end was obtained, and the vein was cannulated with a custom made stainless steel cannula and secured with a 3-way stopcock. The lumen of the vein was washed thoroughly with ample quantity of ice cold HBSS using a syringe via the 3-way stopcock, to remove blood. A small piece was cut off from the other end and a stainless steel cannula was inserted and secured with a 3-way stopcock. Subsequently the vein was filled with 0.2% type I collagenase (Sigma) in Medium 199(Sigma) and the 3-way was closed. After 15 min incubation at 37°C in a petri plate containing HBSS, the surface of the cord was gently massaged to dislodge the digested cells. The content of the lumen were collected into a 50 ml sterile centrifuge tube, using a syringe through the 3-way. The lumen of the vein was then flushed with 20 ml of M 199 to collect loosely attached cells and was pooled with the initial suspension. Cell suspension was centrifuged at 200 ×g for 5 min at 4°C in a tabletop refrigerated centrifuge, and the cell pellet was washed twice with M 199 by repeat centrifugation. The final cell pellet was re-suspended in complete MCDB 131 medium (Sigma) containing 20% FBS, 200 µg/ml growth factor mixture (Sigma), streptomycin sulfate (100µg/ml) and benzyl penicillin (100 U/ml). The cell suspension was then seeded on 25 cm^2 culture flask (NUNC), pre-coated with gelatin. The culture flask was incubated under 5% CO_2 at 37°C in an incubator. The culture was fed with fresh medium every alternate day until confluence.

3.2.4.4.1.2. ***Serial propagation of HUVEC.*** When the cells attain confluence, the cells were sub cultured. For harvesting, cells were washed with fresh, warm (37°C) serum free M 199 (two times), and the monolayer was incubated with 0.25% trypsin-EDTA (Sigma) solution until the cells start to detach (usually 3-5 min), followed by immediate dilution in ice cold complete medium to inhibit further trypsin activity. The cell suspension was mixed gently with a sterile pipette and transferred into a 10ml centrifuge tube. The cell

suspension was then centrifuged at 200 ×g for 5 min at 4°C, re-suspended in warm complete medium and seeded in fresh pre-coated culture dishes at a split ratio 1:3.

3.2.4.4.1.3. ***Morphology analysis.*** Routine evaluation of the quality and growth pattern of the cultured cells was done using an inverted phase contrast microscope at 10×, 20× and 40× magnifications. The cells were identified by their typical cobblestone morphology on attaining confluence.

3.2.4.4.1.4. ***Uptake of fluorescent labeled acetylated-LDL.*** Acetylated LDL is taken up by macrophage and endothelial cells that possess scavenger receptors specific for the modified LDL. Endothelial cells in primary cell culture can be identified by their ability to take up fluorescent AcLDL. Cells grown on pre-coated cover slip bottom tissue culture plates up to near confluent stage was used for Dil AcLDL uptake studies. In the near confluent stage, the medium was replaced with fresh MCDB 131 containing 10% FBS and 10 µg/ml of 1,1-dioctadecyl-3,3,3,3-tetramethyl Indocarbocyanine perchlorate (Dil) labeled acetylated low density lipoprotein (Dil AcLDL;Sigma) and replaced in the CO_2 incubator. After 4h, the culture plate was removed and washed three times with warm serum free M 199 and viewed under inverted fluorescent microscope using Rhodamine Filter.

3.2.4.4.1.5. ***Immunofluorescence analysis for von Willebrand Factor (vWF).*** The monolayer of endothelial cells grown on pre-coated 4-well cover slip (glass) bottom culture plates was fixed with 3.7% paraformaldehyde in PBS for 30 min. The fixed monolayer was washed with PBS three times and quenched for 20 min with 0.27% NH_4Cl / 0.38% glycine in PBS, washed again with PBS and permeabilized with 0.5% Triton X-100 in PBS. After washing thoroughly with PBS, incubated with monoclonal mouse anti-human von Willebrand Factor (vWF) antibodies (Dako,Denmark; diluted 1:200 in 1%BSA -PBS) for 30 min. Following this, wells were washed with PBS three times, and then incubated with 50µl polyclonal rabbit anti-mouse IgG-FITC at a dilution of 1:100 in 1% BSA-PBS for 20 min. Finally the monolayer was washed thoroughly with PBS, and the cells were viewed with a fluorescence microscope using FITC filter.

3.2.4.4.2. Localization of membrane expressed galectin-1 in HUVECs

HUVECs from the third passage were activated for increased membrane expression of galectin-1 with 100 µg/ml minimally oxidized LDL (MM-LDL) diluted in serum free medium for 4 h at 37°C. After washing twice with PBS (2 min each), the cells were fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The cells were now washed thrice with PBS and incubated with goat anti-galectin-1 (Santacruz, USA; diluted 1:10 in 1% BSA- PBS) for 30 min at room temperature. After washing thrice with PBS the cells were now incubated with anti goat IgG- TRITC (diluted 1: 50 with 1% BSA- PBS) for 30 min at room temperature. The cells were finally washed thrice with PBS, viewed with a fluorescence microscope using the TRITC filter and photographed. Cells without activation (MM-LDL induced activation) were also screened for membrane expression of galectin-1.

3.2.4.4.3. Localization of bound Lp(a) in HUVECs after incubation with Lp(a)

MM-LDL activated cells were incubated with Lp(a) in complete medium for 30 min at room temperature. The concentration of Lp(a) ranged from 20 to 100 mg/dl. To ascertain the sugar specificity of Lp(a) binding, cells were pre incubated for 30 min at room temperature with 50 mM lactose in medium. Incubation was then performed for 30 min with Lp(a) of the same concentration as above containing lactose (50mM) as competitive inhibitor. Cells were then fixed as described above; and after washing, were incubated with rabbit anti-human Lp(a) (Dako,Denmark: diluted 100 µg/ml in1% BSA-PBS) for 30 min,followed by anti rabbit IgG- FITC (diluted 1: 50 with 1% BSA- PBS) for 30 min at room temperature. The cells were finally washed thrice with PBS, viewed with a fluorescence microscope using the FITC filter, and photographed.

3.2.4.4.4. Colocalization of membrane expressed galectin-1 and bound Lp(a) in HUVECs after incubation with Lp(a)

MM-LDL activated cells were incubated with Lp(a) [50mg/dl; optimum

concentration] in complete medium for 30 min at room temperature. Cells were then fixed; and after washing, were incubated a mixture of rabbit anti-human Lp(a) (Dako,Denmark;100 µg/ml final dilution in1% BSA- PBS) and goat anti galectin-1 (Santa Cruz, USA; 1:10 final dilution in1% BSA- PBS) for 30 min , followed by a mixture of anti rabbit IgG- FITC and anti goat IgG-TRITC (both antibodies- final dilution 1: 50 in 1% BSA- PBS) for 30 min at room temperature. The cells were finally washed thrice with PBS and analyzed by confocal microscopy.

3.2.5. Galectin-1 cross links extracellular matrix (ECM) glycoproteins laminin and fibronectin with Lp(a)

3.2.5.1. Isolation of fibronectin by gelatin-sepharose affinity chromatography

Fibronectin was isolated from both human plasma and placenta. To isolate placental fibronectin, thawed and minced placental tissue (70g) was homogenized in PBS containing 2-mercaptoethanol (5 mM) and protease inhibitors (EDTA- 0.1%; PMSF- 0.2 mM and benzamidine hydrochloride-2 mM) and extracted for 1h at 4°C. The extract was separated by centrifugation at 17000 ×g .The supernatant was made lipid free using loose cotton and passed through a 5 ml gelatin-Sepharose column(refer general methods). For the isolation of plasma fibronectin, fresh fasting plasma (10ml) was first passed through a 20 ml Sepharose 4B column prior to passing it through the gelatin- Sepharose so as to remove sepharose binding plasma proteins (mainly IgG). The gelatin-Sepharose column is then washed with PBS followed by 10 ml of 1M NaCl and again with PBS. Elution is carried out using 4M urea in 0.05M Tris-Hcl buffer pH 7.5. PMSF is added to all the buffers. The isolated fibronectin is then stored at –20°C with glycerol 40% v/v.

3.2.5.2. Galectin-1 recognition of fibronectin and laminin (Enzyme linked lectin analysis)

Human placental laminin(Sigma) and fibronectin (both plasma and placental) were coated to microtiter wells and analyzed by enzyme linked lectin analysis (ELLA) using HRP conjugated galectin-1 as detailed before for the enzyme linked lectin analysis (ELLA) of galectin-1 interaction with Lp(a), LDL, apo(a) and apo B. Human placental

galectin-1 (HPL) was used in the assay.

3.2.5.3. Lp(a) binding by cellular fibronectin and laminin [ELISA]

Lp(a) binding to human placental laminin and cellular (placental) fibronectin coated to microtiter wells was analyzed by an ELISA based assay modified from the method described by van der Hoek et al.(1994). Briefly, Lp(a) [250 µg/ml in PBS] was incubated with the coated proteins (1µg/well) for 2h at 37°C, followed by first incubating with rabbit anti-human Lp(a) [Dako,Denmark; diluted 50µg/ml in blocking buffer with 1%BSA] for 30 min at room temperature, and finally with anti rabbit IgG-HRP (diluted 1: 10000 in PBS) for 30 min at room temperature. The blocking and washing solutions were same as that used in the enzyme linked lectin assays described before. Color was developed using OPD as substrate and the absorbance at 490 nm was determined.

3.2.5.4. Galectin-1 mediated cross-linking of Lp(a) with fibronectin and laminin: determination by ELISA based assay

Galectin-1 mediated cross-linking of Lp(a) with fibronectin and laminin was determined by an ELISA based assay modified from the method described by Romanic et al.(1998). Laminin and placental fibronectin coated to microtiter wells (1µg/well) were incubated with a mixture of increasing final concentrations of Lp(a) [1 to 500 µg/ml in PBS] and galectin-1 (HPL; at a constant final concentration of 600 µg/ml in PBS) for 2h at room temperature and the bound Lp(a) was assayed with rabbit anti human-Lp(a) and HRP conjugated second antibody as described before for the Lp(a) binding by cellular fibronectin and laminin. Control wells were maintained where the coated proteins were incubated with Lp(a) alone (without galectin-1).

3.2.5.5. Galectin-1 mediated cross-linking of Lp(a) with fibronectin: determination by immunoprecipitation with anti-fibronectin antibody followed by western blotting with anti-Lp(a)

Placental fibronectin at a constant final concentration [100 µg/ml] was incubated

with increasing final concentrations of Lp(a) [100 to 400 μg/ml] in the presence or absence of galectin-1(HPL; at a constant final concentration of 600μg/ml). The reactions were conducted at 4°C for 4 h in a buffer consisting of 40 mM Tris, 0.15 M NaCl, 5mM 2-mercaptoethanol, pH 7.5. Complexes formed between Lp(a) and fibronectin were by retrieved by immunoprecipitation with anti-fibronectin antibody. Briefly, anti-fibronectin (kind gift of Dr.Lissy K Krishnan, Thrombosis Research Unit, BMT Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram) at a final concentration of 20 μg/ml was added to the reaction mixture and incubated for 18 h at 4°C while being gently rocked on shaker. Protein A-Sepharose beads (20mg/ml; Pharmacia) were then added to the samples and incubated for an additional 4 h at 4°C while being rocked end to end gently. The Lp(a)- fibronectin-immunocomplexes were washed several times with 0.05% Triton X-100 in PBS. The beads were pelleted between each wash by centrifugation at 14000 ×g and the final pellet was resuspended in Laemli buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 0.7M 2-mercaptoethanol and 0.025% bromophenol blue and then heated at 4°C for 3 min. To identify the presence of Lp(a) within the immunoprecipitated complexes, samples were analyzed by SDS PAGE (3.75%) and Western blotting with the use of an antibody to Lp(a) [Dako, Denmark] as described before. The Western blots were digitalized using high performance scanner and the densitometric analysis of the bands were carried out using the free-domain package Image J 1.37 v (NIH IMAGE).

3.3. General Methods

3.3.1. Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 3.75%, 5% and 12% gels were done according to the method of Laemmli (1970).

3.3.1.1. Reagents

A. Gel Solution: 15 g of acrylamide and 0.4 g of bis acrylamide were dissolved in

distilled water and the volume made up to 50 ml. The solution was filtered and stored at 4°C in amber colored bottle.

B1. Buffer1: 0.614 M Tris/HCl pH 8.8. For 100 ml of buffer, 164 mg of SDS was dissolved.

B2. Buffer1: 1.5 M Tris/HCl pH 8.8. For 100 ml of buffer, 164 mg of SDS was dissolved.

C1. Buffer 2: 0.147 M Tris/HCl pH 6.8 .For 100 ml of the buffer, 108 mg of SDS was dissolved.

C2. Buffer2: 1 M Tris/HCl pH 6.8. For 100 ml of buffer, 108 mg of SDS was dissolved.

Table.2. Quantity of individual components in the making of polyacrylamide gels. **I.** 3.75% gel, **II.** 5% gel, **III.** 12% gel. (letters in parentheses represents the component solution)

I		**II**		**III**	
Separator (3.75%) ml	Spacer (3%) ml	Separator (5%) ml	Spacer 3% ml	Separator 12% ml	Spacer 5% ml
1.125 (A)	0.3 (A)	1.125 (A)	0.3 (A)	4 (A)	0.85 (A)
6.875 (B1)	2.45 (C1)	4.875 (B1)	2.45 (C1)	2.5 (B2)	0.625 (C2)
1 (D)	0.25 (E)	0.75 (D)	0.25 (E)	0.1 (D)	0.05 (D)
0.01 (H)	0.004 (H)	0.007 (H)	0.004 (H)	0.004 (H)	0.005 (H)
				3.4 (G)	3.45 (G)

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

E. Ammonium per sulfate (40 mg/ml) in distilled water.

F. Chamber buffer: 0.25 M Tris/0.192 M glycine pH 8.3 containing 0.1% SDS.

G. Double distilled water

H. TEMED (TEMED and freshly prepared ammonium persulfate were added only at

the time of casting the gel).

3.3.1.2. Sample preparation

To 50µl of sample containing at least 25µg protein was added 10µl of 10% SDS, 6µl glycerol and 3µl 2-mercaptoethanol and heated for 3min at 100°C. A tiny speck of bromophenol blue was diluted in 100µl of distilled water and 3 to 5 µl was added to the prepared sample after cooling.

3.3.1.3. Electrophoresis

Twenty micro liter of sample was applied to each well and electrophoresis was run in the 'mini slab gel' apparatus (Hoefer Scientific 7.3x 8.3 cm) at 15 mA /1mm gel until the bromophenol blue dye reached the bottom of the gel. The proteins in the gels were fixed using 50% methanol, stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water mixture(1: 1.5: 17.5,v/v).

3.3.2. Western Blottting

Following SDS PAGE the separated proteins were transferred to polyvinyl difluoride (PVDF) membrane using cold transfer buffer consisting of 25 mM Tris, 192 mM glycine, 0.1% SDS and 15% methanol, pH 8.3 and applying a constant current of 1 mA/cm^2 membrane for 2 h at 16°C as described by Towbin et al. [1979]. The PVDF membrane was prewetted in methanol and washed in transfer buffer prior to sandwiching between the gel and the electrode. Western blotting of native gels was done following the same procedures except that the transfer buffer was without SDS and methanol. In single well transfers, longitudinal strips 3 - 4 mm wide were cut out from the transferred membrane; each strip a representative of the resolved proteins, while in multi well transfers the whole blot was used. The blots were blocked with PBS containing 0.5% Tween 20 and probed with corresponding primary and second antibody conjugates (2 h at 4° C for each step with washing between each step using 0.05% Tween 20 - PBS). The blots were washed twice with 0.05% PBS-T and once with PBS alone before developing color with 4-chloronaphthol solution as described before. After staining for 2-5 minutes with 4-chloronaphthol

solution, blots were washed again in PBS and photographed.

3.3.3. Lipoprotein electrophoresis in 3.75% polyacrylamide disk gels (discontinues buffer system).

Conventional disk gel electrophoresis of lipoproteins employs a discontinuous buffer system on 3.75% polyacrylamide gels originally described by Frings et al.(1971).

3.3.3.1. Reagents

A. Dissolve 36.6 g of Tris and 0.23 ml of TEMED in about 100 ml of water. Add 48 ml of 1N HC1 and dilute to 200 ml with water. The pH should be 8.9. Stable for three months at 4°C in a brown bottle (as are solutions B through F)

B. Dissolve 5.98 g of Tris and 0.46 ml of TEMED in water. Add 48 ml of 1N HC1, and dilute to 100 ml with water. The pH should be 6.7.

C. Dissolve 15.0 g of acrylamide and 0.4 g of bisacrylmide in water, and dilute to 100 ml with water

D. Dissolve 20.0 g of acrylamide and 5.0 g of bisacrylamide in water and dilute to 200ml

E. Dissolve 8.0 mg of riboflavin in 100 ml of water.

F. Dissolve 200 g of sucrose in water and dilute to 500 ml with water.

G. Dissolve 0.14 g of ammonium persulfate in 100 ml water (prepare fresh)

1. Dye solution. Add 250 mg of Sudan Black B to 30 ml of absolute ethanol, mix well, and dilute to 100 ml with water (prepare fresh).

2. Separating gel solution. Combine solutions A, C, and G in the ratio of 1:1:2, by volume. The separating gel solution should be prepared just before use.

3. Spacer gel solution. Mix solutions B, D, E, and F in the ratio 1:2:1:4, respectively, by volume.

4. Sample gel solution. Just before use add 8 parts of spacer gel solution to 1 part of dye solution and mix by gentle inversion.

5. Reservoir buffer. Dissolve 6.0 g of Tris and 28.8 g of glycine in about 850 ml of water. If necessary, adjust the pH to 8.3 with Tris or glycine. Dilute to 1 liter with water.

3.3.3.2. Procedure

Add 1.0 ml of freshly prepared separating gel solution to each tube, carefully overlay with water. The separating gel solution polymerizes in about 30 min at room temperature. After polymerization is complete, invert the tubes and blot on absorbent paper. Add 0.1 ml of spacer gel solution to each tube. Layer the gel solution with water as described above and allow the tubes to remain undisturbed for 10 min under intense white light. After photopolymerization is complete, invert the tubes and blot on absorbent paper. Add 20µl of serum or plasma and 0.2 ml of sample gel solution to each tube. Mix each tube by inversion and layer each tube with reservoir buffer. Allow the tubes to remain undisturbed for 30 min under intense light. After photopolymerization is complete, the gel tubes are ready to be inserted into the electrophoretic cell. About 200 ml and 800 ml of reservoir buffer should be in the upper and lower parts of the electrophoretic cell, respectively. The electrophoresis is for 35min at 5 mA per gel tube (7 X 75-mm glass tubes).

3.3.4. Glycosidase digestions of lipoproteins

3.3.4.1. De-sialylation

Fifty microgram of lipoprotein [Lp(a) or LDL] was incubated with 0.2 U sialidase from *Clostridium perfringens* (Sigma) in a total volume of 50µl (pH.5) at 37°C for 18h

3.3.4.2. De-galactosylation

Fifty microgram of lipoprotein [Lp(a) or LDL] was incubated with 0.2 U sialidase and 0.1 U β-galactosidase from bovine testis (Sigma) in a total volume of 50µl

(pH.5) at 37°C for 18 h

3.3.4.3. De-O-glycosylation (selective removal of O-linked sugars enzymatic cally)

Twenty microgram of lipoprotein was incubated with 0.1 U of sialidase and 3 mU of O-glycosidase from *D.pneumoniae* (Boehringer mannheim) in a total volume of 100µl (pH. 6) at 37°C for 18 h

3.3.5. Alkaline β-elimination with borohydride (selective removal of O-linked sugars chemically).

Lipoproteins dot blotted on to PVDF membranes were subjected to alkaline β-elimination with sodium borohydride as described by Zhu and Bhavanandan (1995). Briefly, the lipoprotein dot-blots (1µg/dot) were incubated in β-elimination buffer (1M $NaBH_4$ in 0.1M NaOH) in tightly stoppered glass tubes at 37°C for 48 h. Dot-blots were subsequently washed in PBS.

3.3.5.1. Specificity of alkaline β- elimination: determination by dot blotting

Lp(a) was dotblotted onto 5 × 5 mm squares of polyvinylidine difluride (PVDF) membranes (1µg protein/dot). O-linked sugars in the dot blotted Lp(a) was selectively removed by incubating for 48 h in β-elimination buffer (1M $NaBH_4$ in 0.1M NaOH) at 37°C in tightly stoppered glass tubes. The native (control maintained at 37°C in PBS) and the β-eliminated lipoprotein dots were then washed and blocked with 0.05% and 0.2% Tween 20-PBS respectively, followed by incubating for 2h at 4°C with the HRP-conjugated lectins (70 µg lectin/ml). HRP conjugates of jacalin and con A were used and the bound lectin conjugate was detected with 4-chloro-1-naphthol as described before. The dot-blots were digitalized using high performance scanner and the densitometric analysis of the dot-blots was carried out using the free-domain package Image J 1.37 v (NIH IMAGE).

3.3.6. Conjugation of horse radish peroxidase (HRP) to lectins/antibodies

3.3.6.1. Periodate activation of HRP

Dissolve 10mg HRP in 1ml freshly prepared 0.3M $NaHCO_3$. Add 50µl of flourodinitrobenzene (FDNB; 1% in absolute ethanol) and mix gently at room temperature for 1h (to prevent self coupling). Add 1ml sodium meta periodate (0.06 M in water), mix gently for 30min at room temperature (to create reactive aldehyde groups on HRP). Add 1ml of ethylene glycol (0.32 M in water; make up 320µl to17.8 ml with water) and mix gently for 1hr at room temperature (to remove excess periodic acid). Dialyze activated HRP and also the protein to be coupled (separately) overnight in carbonate buffer 0.01M, pH.9.5. This will yield an activated HRP solution with a final concentration of 2 mg/ml. Store activated HRP in ice in dark

3.36.2. Conjugation of activated HRP to proteins

Conjugation of HRP to proteins was done according to Heyderman et al.(1989). Briefly,1 mg of lectin or antibody in 1ml of 10mM sodium bicarbonate buffer, pH 9.5, was mixed with 0.67 mg periodate activated HRP in the same buffer and incubated at 25°C for 2 h in the dark. 1% Potassium borohydride solution in distilled water was then added to a final concentration of 0.1% (to block unconjugated aldehyde groups).After 30 min,the mixture was dialyzed against PBS with one change overnight. The labeled lectin/antibody was stored in ice or preferably at -20°C with 30 % glycerol v/v.

3.3.7. Protein estimation (Lowry's method)

Protein was estimated by a modified version of the original method (Lowry et al., 1951).

3.3.7.1. Reagents

A. 2% Na_2CO_3 anhydrous in 0.1 N NaOH

B. 0.5% $CuSo_4.5H_2O$ in 1% tri-sodium citrate

C. 1 ml reagent B mixed with 50ml reagent A

D. Folin ciocaltue reagent 1N

3.3.7.2. Procedure

1 ml reagent C mixed with 50µl sample (containing 10-100µg protein) is allowed to stand for 10 min at room temperature. 50µl of reagent D is pipetted rapidly, mixed , and read at 750 nm after 30 min at room temperature.

3.3.8. Trypsinisation of human erythrocytes

Blood was collected in 5% tri-sodium citrate-dextrose solution (anticoagulant). The cells were washed thrice using cold PBS and settled by centrifugation at 400 × g for 10 min. 100µl of the packed cell was then added to 1.9 ml of 0.1% trypsin solution in PBS, mixed gently and incubated at 37° C for 1h with occasional mixing. The cells were then washed three times using cold PBS with centrifugation. The final volume of the suspension was made to 2 ml with PBS so as to make the suspension 5% v/v.

3.3.9. Hemagglutination assay

Agglutination titers of protein (galectin-1) samples were determined using U bottom 96-well polystyrene microtitre plates (Laxbro, New Delhi). Wells were first blocked with 0.05% Tween 20 - PBS for 1h. The blocking solution was drained of, and serial double dilutions of protein samples were made in a volume of 100µl PBS in the wells. Trypsinised human erythrocytes were then added (25µl of 5% suspension in PBS 7.4) to each well and mixed. Agglutination titer was scored after 1h; positive agglutination was scored by observation under the microscope. Serial dilutions starting with 4µg was optimum for hemagglutination assay of galectin-1 samples. Lectin activity was expressed as the minimum agglutinating concentration (MAC)

3.3.10. Preparation of cross-linked guar gum (CLGG)

Soluble guar galactomannan was cross-linked to form an insoluble gel by a modification of the procedure described by Appukuttan et al.(1977). Ten gram guar gum powder was mixed thoroughly with a finely dispersed emulsion of 2 ml epichlorohydrin

and 25 ml 3N NaOH until the mixture became a solid cake. It was then left at 40°C in a water bath for 24 h and then at 70°C for 10h. The resulting gel was soaked in distilled water and repeatedly washed with water until the washouts were neutral. The gel was then equilibrated with PBS and homogenized in a blender to obtain particles of about 300µm size. Fine particles were discarded by repeated decantation.

3.3.11. Preparation of lactose- Sepharose 4B matrix

Lactose was covalently attached to Sepharose using divinyl sulfone as the cross-linker. Lactose-divinyl sulfone-Sepharose 4B was prepared as described by Dean et al.(1985). Briefly, Sepharose 4B (20 g, wet weight) was washed in distilled water under suction and suspended in 20 ml of 1M Na_2CO_3, pH 11. After adding 4 ml divinyl sulfone, the suspension was gently stirred with a magnetic bar for 1h at room temperature. Activated gel thus obtained was washed thoroughly in distilled water. To the wet gel cake obtained after suction filtration over sintered glass funnel was added 30 ml saturated solution of lactose in (1M Na_2CO_3) and stirred over night at room temperature as above. The reacted beads were washed successively with the following.

1) 20ml of 1M Na_2CO_3, pH.11
2) 500ml of 0.2M glycine-HCl, pH.3, containing 1M NaCl (to block unreacted activated groups in the gel).
3) 500ml of 1M NaCl and
4) 500ml of distilled water.

Lactosyl-Sepharose 4B matrix was then equilibrated with PBS 7.4 and packed into chromatographic column of required dimension.

3.3.12. Preparation of gelatin- Sepharose 4B matrix

3.3.12.1. Cyanogen bromide activation of Sepharose 4B

Sepahrose 4 B is washed several times with distilled water by mild suction using a G-2 sintered glass funnel (Borosil,India). Washed gel (20g) was taken into a 100ml beaker and 40ml of 2M Na2CO 3 followed by 20 ml distilled water was added to the

gel. The gel suspension is kept stirring in ice bath and the temperature was maintained at 9°C. Cyanogen bromide (800 mg) dissolved in 1-2 ml dimethyl formamide is added to the gel while stirring in ice bath. Activation is continued for exactly 5 min and the gel is then transferred to a G-2 sintered funnel and washed instantly with 400 ml of cold 0.1 M NaHCO3. The gel is now activated and ready for coupling to proteins.

3.3.12.2. Preparation of Gelatin-Sepharose

Fifty milligrams of gelatin (Sigma, type I from swine skin) are dissolved in 25ml of coupling buffer (0.5M NaCl, 0.1M $NaHCO_3$) by heating the solution. The gelatin solution is cooled to 20-30°C and then mixed with 5g of cyanogen bromide-activated Sepharose. The gel is gently stirred using a magnetic stirrer overnight at + 4° C or for 4h at room temperature. The unbound protein is removed by washing the gel with the coupling buffer on a sintered-glass filter. The Sepharose is then incubated for 1hr at room temperature in 1M ethanolamine-HCl, pH 8.0, to block remaining active groups in the gel. After washing with PBS, several gel volumes of 8 M urea, and finally again with PBS, the adsorbent is ready for use. It is stored at 4°C in PBS containing 0.02% NaN3.

3.3.13. Preparation of 4% paraformaldehyde

Ten gram of paraformaldehyde in 200ml 0.1M Tris -HCl, pH7.4 (buffer preheated to 45°C), was mixed thoroughly using a magnetic stirrer. Heat gently to 58°C and remove from heat, the temperature may move up to 60- 61°C (desirable). Never overheat; above 60°C, the paraformaldehyde will be destroyed. To the cloudy solution, carefully add 5 to 10 drops of 10N NaOH until the solution just clears. Allow to cool; carefully adjust pH to 7.4 and make up to 250 ml with 0.1 M Tris-HCl buffer, pH 7.4. Always prepare fresh; potency of the buffer is only for 48 h.

3.3.14. Preparation of Glycerol jelly

Sprinkle 7g gelatin over 20 ml water mixed with 12 ml glycerin, and leave it to soak for at least five minutes. Melt it over a low heat, gelatin melts around 40°C, gently

stir. Leave the flask in warm water for a while and in a few minutes the bubbles formed will disappear. Pour the medium into a wide mouthed bottle and store at 4°C. While melting the jelly for mounting purposes, take care to warm the jelly slowly, do not overheat, otherwise air bubbles may form and the heat may digest the tissue.

3.3.15. Mayer's Hematoxylin Solution

Dissolve 50g alum (aluminum potassium sulphate) in distilled water. When alum is completely dissolved, add 1g hematoxylin. When hematoxylin is completely dissolved, add 0.2 g sodium iodate and 20 ml glacial acetic acid. Bring to boil and cool. Filter if necessary.

3.3.16. Preparation of minimally oxidized LDL (MM-LDL)

Minimally oxidized LDL (MM-LDL) can be prepared by storing LDL at 4°C for 3 to 6 months. Alternatively MM- LDL can be prepared by the method described by Kosugi et al.(1987). Briefly, EDTA free LDL at a concentration of 2mg/ml is dialyzed against against 9μM $FeSO_4$ in phosphate buffer, pH.7.2, for 72 h at 4°C. The MM- LDL is then dialyzed extensively against PBS and used fresh. The extent of lipoprotein oxidation was measured by thiobarbiturate-reactive substances(TBARS; malondi aldehyde equivalents) assay

3.3.17. Thiobarbituric acid -reactive substances (TBARS) assay for lipid peroxidation

Lipid peroxidation or the extent of lipoprotein oxidation was measured by thiobarbiturate-reactive substances (TBARS; malondialdehyde equivalents) assay as described by Agil et al.(1995). Briefly, 20μl plasma or serum was added to 4ml N/12 H_2SO_4, mixed gently, and added to 0.5ml of 10% phosphotungstic acid in water and centrifuged at 5000 ×g for 10min. The sediment was then mixed with 2ml N/12 H_2SO_4 and added to 0.3 ml of 10% phosphotungstic acid, mixed, and centrifuged at 5000 ×g for 10min. The supernatant was discarded and the sediment was mixed with 4ml of water

and added to 1 ml of thiobarbituric acid (TBA) reagent (prepared freshly a mixture of 0.67% TBA in water and glacial acetic acid in equal volumes),mixed well, and heated for 60 min at 95°C. After cooling, 5 ml butanol was added; the mixture was then shaken vigorously and centrifuged at 5000×g for 15min. The butanol layer was then separated for spectroflourometric analysis (excitation and emission wavelengths at 515 and 553 nm respectively).Freshly prepared malondialdehyde (MDA; tetramethoxy propane) was used as standard. Stock solution of standard was prepared by making up 8.3µl of MDA in 100 ml double distilled water. Dilute the stock solution 1:1000 with double distilled water to make a working standard (1 ml contains 0.5 nmol of MDA). The amount of TBARS or lipid peroxides was expressed in terms of malondialdehyde equivalents (µmol /L of plasma).

TBARS= absorbance of test /absorbance of standard × 25 (µmol/L MDA equivalents)

3.3.18. MM-LDL cytotoxicity assay with MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]

Cytotoxicity was determined by the MTT dye-reduction assay. The methodology described is a modification of the original MTT colorimetric assay developed by Mosmann(1983). In brief, HUVECs were cultured in 96-well microtiter plates. On confluence, the cells were incubated with MM-LDL (100 µg/ml in complete medium) for 4 h at 37°C (to control wells, only culture medium was added). Medium in each well was then aspirated and replaced with 110µl of MTT working solution (MTT stock solution mixed with medium to attain a final concentration of 0.5 mg/ml). The cells were incubated at 37°C for 4h, and then the medium was aspirated and replaced with 100 µl DMSO to dissolve the formazan crystals formed. The culture plates were shaken for 5 min and the absorbance of each well was read at 570 nm with 750 nm as the reference wavelength. The relative viability of the treated cells as compared to the control cells was expressed as the % cytoviability using the following formula:

% cytoviability = [A_{570} of treated cells] × 100 / [A_{570} of control cell]

3.3.19. Hank's balanced salt solution (HBSS; Ca^{2+} and Mg^{2+} free)

5.4 mM KCL, 4.4 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM Na_2HPO_4 , pH 7.2, solution in double distilled water is autoclaved for 30 min at 121°C . Glucose solution autoclaved separately was added to the buffer (1% final concentration) and stored at 2-8°C and used within one week.

3.3.20. Statistical analysis

3.3.20.1. F-test (variance ratio test): F-test is a statistical method for comparing the spreads or variabilities of two sets of figures to determine whether the two sets of figures were drawn from the same population. The F-test is designed to test if two population variances are equal. It does this by comparing the ratio of the variances.

The repeatability of the new method developed for Lp(a) purification was analyzed by a two tailed F-test using Microsoft Excel software. For this purpose Lp(a) was isolated and purified from five different individuals with a serum Lp(a) concentration that ranged from 4 to 71 mg/dl. The amount of Lp(a) in the isolated serum fraction and the purified Lp(a) was estimated using an Lp(a) assay kit, ELITEST- Lp(a), according to the manufacturers instructions. The variance in the amount of Lp(a) in the isolated serum fraction and the purified Lp(a) was then analyzed by F-test at 95% confidence levels.

3.3.20.2. Student's *t* -test : Student's *t*-test is a statistic for measuring the significance of a difference of means between two distributions. Student's *t*-test deals with "small" samples. In simple terms, the *t*-test compares the actual difference between two means in relation to the variation in the data (expressed as the standard deviation of the difference between the means). In my study, unpired students *t* test was used to compare different variables appropriately mentioned in the text. Microsoft Excel programme was used for the same.

Chapter 4

RESULTS AND DISCUSSION

4.1. Isolation and purification of Lp(a) and its structural glycoproteins apo(a) and apo B

4.1.1. Isolation and purification of Lp(a)

With every different Lp(a) measurement assay involving different isoforms of Lp(a), the universal acceptance of Lp(a) measurements is fraught with several problems. Standardization of Lp(a) measurements globally have reached no where; purified Lp(a) is increasingly needed as a standard to overcome most of the problems in the standardization of Lp(a) measurements and for *in vitro* biological studies(Albers et al.,1990). Human lipoprotein(a) or Lp(a) is isolated by sequential ultracentrifugation from serum or plasma. Lp(a) floats in the density range from 1.05 to 1.12 g/ml which overlaps that of LDL (1.02- 1.063) and HDL (1.063- 1.24), making it impossible to isolate Lp(a) without including isopycnic LDL and HDL particles.

Lp(a) isolated by ultracentrifugation is to be purified from contaminating LDL and HDL. Its lysine binding properties has been made use in its purification using lysine-sepharose affinity columns (Fless and Snyder, 1996). How ever, all Lp(a) in plasma does not bind to lysine-sepharose (Armstrong et al.,1990). Studies by different workers have reported varying amounts of plasma Lp(a) [17 to 91%] binding to lysine and the rest was

incompetent .This differential binding did not depend on specific apo(a) isoforms but was donor dependent (Xia et al.,2000). Several other methods have been proposed for the purification of Lp(a) but none of them appear completely satisfactory in terms of purity of Lp(a) and practicability (Albers and Hazzard,1974; Armstrong et al.,1985).The relatively low levels of plasma Lp(a) when compared to LDL and HDL is another problem in the purification of Lp(a).

Conventional Lp(a) purification techniques uses agarose matrixes (such as lysine affinity or size exclusion chromatography) which involves sophisticated instrumentation to purify Lp(a) from its overlapping contaminants LDL and HDL. Besides, Lp(a) cannot be completely separated from LDL because of overlapping size distribution and the tendency of Lp(a) to aggregate with LDL (Gaubatz et al.,2001).

Highly purified Lp(a) was required to study its specific glycosylation properties *vis-à-vis* LDL. In this perspective, considerable time and effort was invested in the quest for a new methodology which finally culminated in the development of a simple method to purify Lp(a) after its isolation from serum (Chellan et al.,2006).The method involves no sophisticated instrumentation and is applicable in any laboratory. Essentially, Lp(a) LDL and HDL exhibits pre-beta, beta and alpha migration respectively in native polyacrylamide gel electrophoresis (PAGE) from where Lp(a) may be selectively electroeluted using L-proline at 0.1 M concentration to prevent the aggregation of Lp(a) with LDL. The experimental approaches describing the development of the method are as follows:

4.1.1.1. The TBE buffer for lipoprotein electrophoresis

The choice of the suitable buffer system was the first obstacle in the objective to electroelute Lp(a) from native polyacrylamide gels after subjecting serum Lp(a) isolates to native PAGE. The serum Lp(a) isolate invariably contained LDL and HDL as contaminants. The size difference between Lp(a) and LDL being very narrow and the fact that Lp(a) easily aggregates and forms complexes with LDL especially on concentration necessitated the choice of an appropriate buffer system to ensure proper

separation of the two molecules on electrophoresis. A continuous buffer system (TBE; 0.05 M Tris, 0.025 M boric acid, 0.003 M EDTA, pH 8.7) was chosen over the conventional Tris-glycine discontinuous buffer system for lipoprotein electrophoresis. The discontinuous system was found to concentrate the lipoproteins [Lp(a), LDL and HDL] at the spacer-separator gel interface to such an extent that there is irreversible aggregation between the lipoproteins leading to cross contamination. The disposal of the spacer gel was also beneficial, saving time and effort. TBE buffer (pH 8.7) worked exceptionally well when compared to Tris-glycine buffer of similar ionic strength and pH. The bands appeared distinct and uniform relative to the much-diffused bands in the Tris-glycine system (Fig.5).

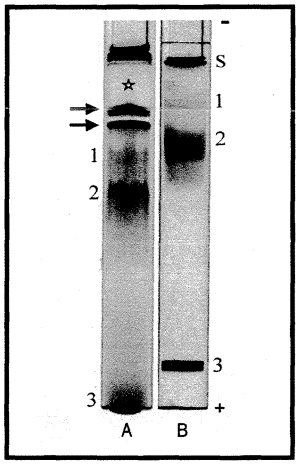


Figure 5. Native PAGE of isolated serum Lp(a) fraction (*d* 1.05-1.12 g/ml) in 3.75% disc gels with discontinuous and continuous buffer systems. **A**: PAGE with Tris-glycine buffer (0.05 M, pH 8.7). **B**: PAGE with Tris-borate EDTA buffer (TBE; 0.05 M, pH 8.7). **1**:Lp(a); **2**: LDL; **3**:HDL. The star indicates the spacer gel and the arrows indicate aggregated lipoproteins on either side of the spacer-separator gel interface. **S**: Start; Approx. 200µg of lipoprotein was applied per gel, pre-stained with Sudan black.

Tris and borate are only partly charged at the desired pH, which reduces their electrophoretic mobility. The reduced mobility of buffer ions leads to increased mobility of protein molecules as they act as carriers of charge, and allows for high concentration of the buffer solution to be employed with consequent benefit of buffering capacity and sample stability without producing unacceptably high conductivity and resulting heat production in the gel. Borate forms charged complexes with carbohydrates, thereby, borate buffers were used as elutants to purify glycoproteins in affinity chromatography systems (Gupta and Lowe, 2004). Borate ions forms negatively charged complexes with cis diols (hydroxyl groups) on glycoproteins, especially at pH 9, and these readily reversible complexes dissociate by a change in pH to around neutral pH or by incubating with polyhydroxy alcohols like ethylene glycol or glycerol. The increased mobility and

distinct band formation of Lp(a) LDL and HDL in alkaline electrophoresis using TBE buffer may be a function of the borate in the electrophoresis buffer.

4.1.1.2. Determination of the optimum concentration of L-proline for the resolution of Lp(a) in native PAGE

The second major problem faced while electroeluting Lp(a) was its aggregation with LDL. Pure Lp(a) not only self aggregates (particularly in the cold), but it also forms complexes with LDL and with many other proteins. Such complex formation can be inhibited with other compounds including proline [proline > ε-aminocaproic acid (EACA) > arginine > lysine] (Gaubatz et al., 2001). Proline, lysine and its analogue, the ε-aminocaproic acid (EACA) are known to bind Lp(a) (Fless and Snyder,1996;Ye et al.,2001). It was observed that, lipoproteins when purified by electroelution in the absence of proline were often cross contaminated and proline with an isoelectric point of 6.3 was best suited for the system. The isoelectric point of lysine and arginine is 9.59 and 11.15, respectively, and as such, neither is suited for the purpose (since buffer pH is 8.7).

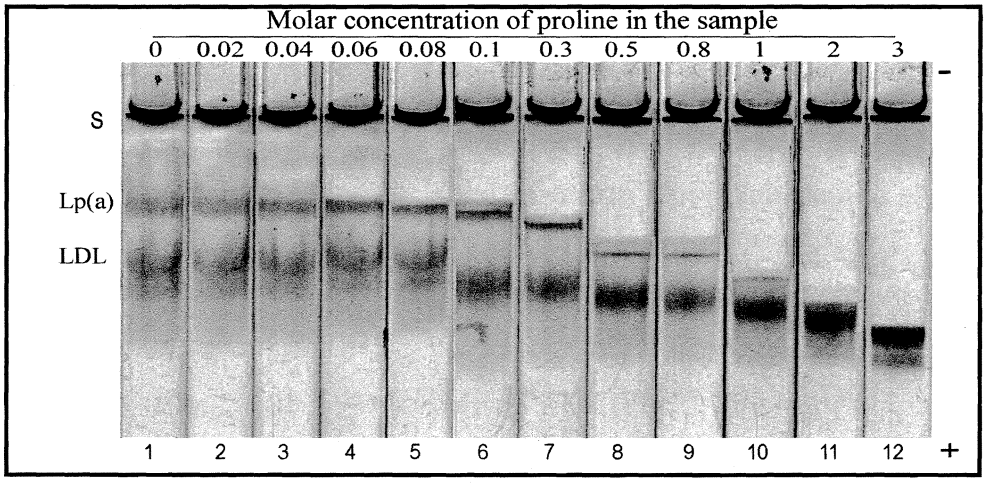


Figure 6. Native PAGE of isolated serum Lp(a) fraction (*d* 1.05-1.12 g/ml) in 3.75% disc gels with increasing concentration of proline in the sample. **1**: Control without proline; **2-12**: Proline in the sample at a final concentration of 0.02, 0.04, 0.06, 0.08, 0.1, 0.3, 0.5, 0.8, 1, 2 and 3 M, respectively; **S**: Start. Approx. 200 µg of lipoprotein was applied per gel, pre-stained with Sudan black.

Proline is known to bind to kringle IV type 7 of apo(a) similar to lysine (Ye et al., 2001). The experiment to determine the optimum concentration of L-proline for Lp(a) electrophoresis did show that the pre-β lipoprotein, Lp(a), appeared diffused and not clearly separated from the β-lipoprotein (LDL) in the control gel, while there is a progressive increase in the clarity of the Lp(a) band and a clear separation from the LDL band in the gels loaded with increasing concentration of proline in the sample (Fig.6). The lipoprotein bands in the gel with 0.1 M proline in the sample appear best resolved. The migration of Lp(a) relative to LDL is considerably increased with increasing concentration of proline. It is almost merged with LDL at 2M and it seems to have overtaken the LDL band at 3M. Proline is fully ionized at pH 8.7 and at higher concentrations proline may associate with Lp(a) giving it a net higher charge relative to that of LDL (Fig. 6).

With proline in the sample contributing to the migratory characteristics of Lp(a), acrylamide concentration of the gel and the gel strength determined by the acrylamide/ bisacrylamide ratio is also crucial for the distinct resolution of the constituent lipoproteins. Evaluation of 3, 3.25, 3.75 and 4% gels variously with 19:1, 27:1 and 37:1 acrylamide/bisacrylamide formulations were done and after long cumbersome experiments, it was concluded that 3.75% polyacrylamide gels with 19:1 acrylamide/bisacrylamide constitution performed optimally (data not shown).

4.1.1.3. Immunoblotting of Isolated serum Lp(a) fraction

Isolated serum Lp(a) fraction (*d* 1.05-1.12 g/ml) invariably contains Lp(a), LDL (*d* 1.02-1.063 g/ml) and HDL_2 (*d* 1.063-1.125 g/ml) which exhibits pre-beta, beta and alpha migration, respectively in native PAGE (Fig.7). The study objective was to achieve satisfactory resolution between the components from where Lp(a) may be selectively electroeluted. Immunoanalysis of the Lp(a) fraction electrophoretically transferred to PVDF (Fig.7) was done primarily as a proof for the migratory behavior of the component lipoproteins. Figure.7A shows the characteristic migratory pattern of Lp(a), LDL and HDL in native PAGE. L-proline had been added to the sample to a final concentration of 0.1 M to prevent the aggregation of Lp(a) with LDL. The pre-beta migration of Lp(a) is

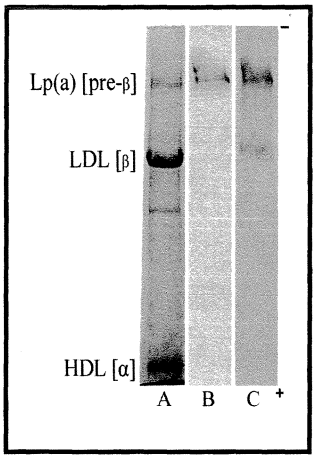


Figure 7. Immunoblotting of isolated serum Lp(a) fraction (d 1.05-1.12 g/ml). **A**: Native PAGE of isolated serum Lp(a) fraction in 3.75% slab gel stained with Coomassie Brilliant Blue, **B**: A electro blotted to PVDF and probed with rabbit anti human Lp(a) and detected with anti rabbit IgG-HRP, **C**: A electroblotted to PVDF and probed with sheep anti-human apoB and detected with anti-sheep IgG-HRP.

evident in Fig.7B as the antibody recognizes only Lp(a).The antibody to apo B on the other hand recognizes both Lp(a) as well as the beta lipoprotein, LDL (since apo B is common to both Lp(a) and LDL) (Fig.7C). The far migrating HDL was not analyzed since our concern was to resolve Lp(a) distinct from that of the closely migrating LDL.

4.1.1.4. Characterization of purified Lp(a)

Purified Lp(a) preparation was virtually homogenous. The absence of free LDL or HDL was verified by 3.75% native PAGE and agarose gel electrophoresis (Fig.8). Dot blot assays using mouse monoclonal antibody against human apo(a) [ICN, USA]

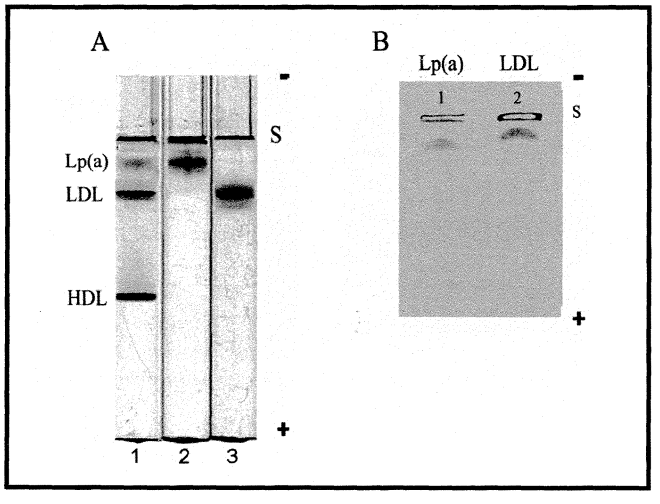


Figure 8. A: Native PAGE of electroeluted (purified) lipo proteins in 3.75% disc gels.
1: Original Lp(a) fraction isolated from serum; **2**:Electro eluted Lp(a); **3**: Electroeluted LDL. Approxi mately 200µg of lipoprotein was applied per gel, pre-stained with Sudan black.
B: Agarose gel electrophoresis of electroeluted lipoproteins.
1: Electroeluted Lp(a);
2:Electroeluted LDL. **S**:Start. Approx. 5µg of lipoprotein was applied per lane pre-stained with Sudan black

recognized electroeluted Lp(a) but not electroeluted LDL, while goat anti-human beta lipoprotein(LDL) [Sigma, USA] recognized only electroeluted LDL (Fig.9A&B), indicating non overlapping of bands during electrophoresis and the homogeneity of Lp(a) and LDL preparations. Purified Lp(a) and LDL preparations were not assayed for the presence of HDL since HDL does not associate with any of the component lipoproteins and migrating farthest, does not overlap with LDL or Lp(a) in native PAGE.

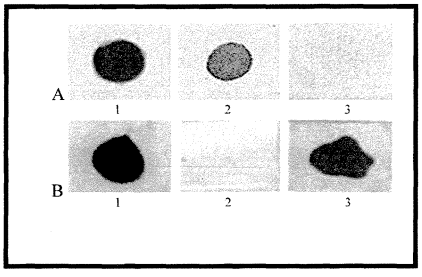


Figure 9. Dot blot analysis of electroeluted (purified) lipoproteins. **A**: Coated lipoproteins are probed with mouse anti-human apo(a) and detected with anti-mouse IgG-HRP. **B**: Coated lipoproteins are probed with goat anti-human beta lipoprotein (LDL) and detected with anti-goat IgG-HRP. **1**: Original Lp(a) fraction isolated from serum; **2**: Electroeluted Lp(a); **3**: Electroeluted LDL. Approx. 1µg of lipoprotein was applied per dot.

4.1.1.5. Statistics

In quantitation studies to analyze the repeatability of the method, serum from 5 individuals with a Lp(a) concentration that ranged from 4 to 71 mg/dl was used.

Donor.	Concentration of Lp(a) (mg/dl)				Electroeluted Lp(a)(%) $X = \frac{B}{A} \times 100$
	Serum	Lp(a) Fraction		Purified Lp(a)	
		A		**B**	
1	4	1.8	(45)	1 (25)	55
2	50.4	21.2	(42.1)	8.8 (17.5)	41.6
3	59.3	19.4	(32.7)	4.9 (8.3)	25.3
4	56	22	(39.3)	15.8 (28.2)	71.8
5	71	21.1	(29.7)	12.2 (17.2)	57.9
		Mean **A** (37.8) SD = 6.4		Mean **B** (19.2) SD = 7.7	Mean **X** = 50.4

Table.3: Yield data of serum Lp(a), isolated Lp(a) fraction(d 1.05-1.12 g/ml) and electroeluted (purified)Lp(a): Figures in parentheses (A&B) are net yields of Lp(a) (percentage). Variance ratio = 1.464; two tailed variance ratio (F test) between A and B at 95 % confidence levels (P = 0.721)

Amount of Lp(a) in the isolated serum fraction and the purified Lp(a) was estimated using an Lp(a) assay kit, ELITEST-Lp(a), according to the manufacturers instructions. The average yield of Lp(a) in the isolated serum fraction was approximately 38% of total Lp(a) in the serum sample and for the electroeluted, purified Lp(a), it was 19% with approximately 50% recovery of the proteins after electroelution (Table.3). The variance in the yield of the isolated serum Lp(a) fraction and that for the electroeluted Lp(a) was similar as analyzed by two tailed variance ratio test (F test) at 95% confidence levels (P = 0.721). This indicates a high degree of repeatability for the method.

4.1.1.6. Simplified description of the method and its applications

The serum Lp(a) fraction (*d* 1.05-1.12 g/ml), isolated by sequential ultra centrifugation was dialyzed against 1:9 diluted TBE buffer and subjected to continuous PAGE in 3.75% tube gels with TBE buffer (lipoprotein load approximately 300 μg). Prior to loading over the gel, the sample was incubated with L-proline at a final concentration of 0.1M for 15 min at room temperature followed by adding ethylene glycol (10% v/v). Post electrophoresis, one of the gels was stained for protein with Coomassie brilliant blue serving as a marker and the pre beta and beta lipoprotein, Lp(a) and LDL, respectively, were sliced out from the non stained gels. The gel slices were gently mashed, equilibrated with the electroelution buffer (0.05 M Tris-acetate,pH 8) and subjected to electroelution in dialysis bags that were placed in between opposite electrodes in a rectangular chamber. The lipoproteins were then dialyzed against 0.01 M phosphate buffer, pH 7, containing 0.1% EDTA and 0.01% sodium azide. The gel pieces were pelleted by brief centrifugation at 3000 ×g and the supernatant lipoproteins were stored at –20°C with 50% v/v ethylene glycol.

The system described here is simple and can be reproduced in any laboratory with basic instrumentation, while other methods for purification of Lp(a) such as gel filtration or affinity chromatography employs sophisticated detection and fractionating systems to accurately fractionate closely eluting similar sized Lp(a) and LDL. Pure LDL can also be simultaneously prepared by this method since LDL preparations in the density range

1.05-1.063 g/ml consistently contains Lp(a) as contaminant. Proline in the sample can be easily removed by dialysis in comparison to the rather difficult to remove ε-aminocaproic acid used as elutants in lysine affinity purification of Lp(a). The entire process may be simplified with the use of any commercial electroeluter. The proteins can be collected concentrated in about 400 µl in a single step and may be lyophilized using a volatile elution buffer such as 0.05 M ammonium bicarbonate

4.1.2. Isolation apo(a) and apo B [the glycoprotein components of Lp(a)]

Apo B and apo(a), the structural glycoproteins of Lp(a) were also isolated and purified to study their individual responses since it is often these glycoproteins (rather than the intact Lp(a) or LDL molecules) that are found in the atherosclerotic plaques.

4.1.2.1. Isolation of apo(a).

Lp(a) molecule is made up of an apo B and an apo(a) glycoprotein (Fig.2B). Since apo B is the glycoprotein component giving structural integrity to LDL, Lp(a) is considered as a modified LDL molecule. The binding of apo(a) to apo B is mediated by one disulfide bridge. Apo(a) was isolated from the serum Lp(a) fraction (*d* 1.05-1.12 g/ml) by reduction with dithiothreitol. Apo(a) glycoproteins were then separated in a pure form from the buoyant LDL like particles by ultracentrifugation (details in methods).

4.1.2.2. Isolation of apo B

Apo B was isolated not from Lp(a) but from the LDL fraction (*d* 1.02 -1.04 g/ml), preferably to reduce contamination from apo(a) particles. LDL on delipidation yields apo B. The conventional alcohol-ether delipidation methods for LDL worked well; but even after repeated attempts to remove the organic contamination with a stream of nitrogen, trace contamination always persisted in the sample. The organic contamination in the sample inhibited the protein binding to plastic surfaces and interfered with protein quantitation. Subsequently detergent delipidation of LDL was attempted; SDS, the most popular delipidisation agent was fount wanting as it was

difficult to remove it from apo B and also interfered in protein quantitation and binding to plastic surfaces. LDL was then delipidised using sodium deoxycholate (details in methods). Sodium deoxycholate was found to not interfere with apo B binding to microtiter wells, PVDF, or other plastic surfaces, nor does it interfere with the Lowry's method for protein estimation.

4.1.2.3. Purity analysis of isolated apo(a) and apo B

The purity of the isolated apo(a) and apo B was verified by 3.75% SDS PAGE(Fig.10). In agreement with Utermann and Weber(1983), apo(a) was found Coomassie-resistant and appeared faintly stained which is probably linked to its high carbohydrate content, and in comparison, apo B was highly responsive to Coomassie. The relative amount of apo(a) isolated *vis-à-vis* apo B was always low

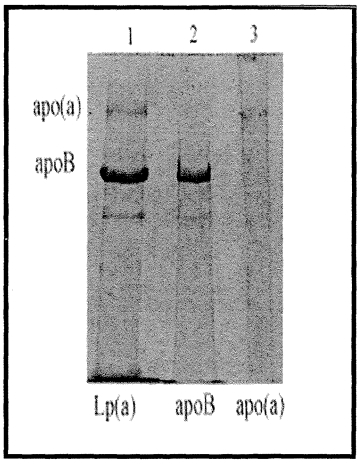


Figure.10. 3.75% SDS PAGE showing the purity of isolated apo(a) and apo B. **1**. Delipidised and reduced Lp(a), **2**. Apo B, **3**. Apo(a). Protein load is 25 µg/well approximately.

which reflects their relative composition in plasma. Nevertheless, apo(a) was isolated in pure form devoid of contamination from apo B or LDL like particles as is evident from the electrophoretogram (Fig. 10). Like wise, isolated apo B also appeared pure. The purity of the glycoproteins is an important prerequisite for further analysis of glycosylation-dependent properties of these glycoproteins and its relevance in atherogenesis.

4.2. Elucidation of the carbohydrate structures on Lp(a),LDL, apo(a) and apo B

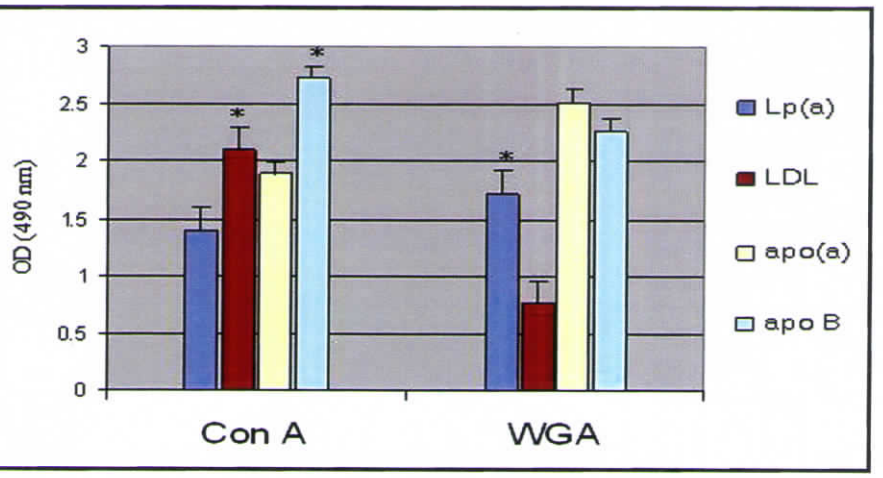
The importance of glycosylation in biological events and the role it plays in glycoprotein function and structure is an area in which there is growing interest. The primary objective of this Ph.D programme was to delineate the role of galectin-1 in lipoprotein retention (especially Lp(a) retention) which may be of relevance to atherogenesis. Galectin-1, as already mentioned before, binds to polylactosamine structures or T-antigen, and is present in arterial cells. Other than that reported for sialic acids (Tertov et al.,1993), the role of carbohydrate structures on Lp(a) and LDL, particularly their glycoprotein components apo(a) and apo B, in the development of atherosclerotic plaques is lacking in literature. An understanding of the types carbohydrate structures on Lp(a) *vis-à-vis* LDL is necessary to elucidate the role of galectin, if any, in lipoprotein retention. N- and O- linked sugar-specific lectins were used to analyze the glycosylation profiles in Lp(a), LDL, apo(a) and apo B.

4.2.1. Lectins

N-linked sugar-specific lectins used in the lectin analysis of glycosylation were concanavalin A (Con A; from jack bean, *Canavalia ensiformis*) and wheat germ agglutinin (WGA). The O-glycosylation-specific lectins used were, jack seed (*Artocarpus integrifolia*) lectin (jacalin), peanut (*Arachis hypogaea*)agglutinin (PNA), and *Helix aspersa* (garden snail) agglutinin (HAA). *Ricinus communis* (castor bean) agglutinin (RCA1) which binds terminal β-linked galactose (common to both N- and O-linked sugars) was also used. Jacalin, PNA and RCA-1 were isolated in our laboratory while Con A,WGA and HAA were from Sigma, USA.

4.2.2. Enzyme linked lectin analysis (ELLA)

The types of sugars present on Lp(a), LDL, apo(a) and apo B were analyzed by enzyme linked lectin analysis (ELLA) using HRP-conjugated lectins as detailed in 'methods'.



gure 11. Enzyme linked lectin analysis of Lp(a), LDL, apo(a) and apo B with Con A and GA. Microtiter wells coated with purified Lp(a), LDL, apo(a) or apo B (1µg protein/well) were obed with the HRP-conjugated lectin (7.5 µg lectin /ml) followed by detection of the bound ctin conjugate with OPD.The values are expressed as optical density (OD) units at 490 nm. ean + S.D of triplicate trials is shown. Lectin reactivity with Lp(a) vis-à-vis LDL and also o(a) vis-à-vis apo B was compared by unpaired t-test , *P < 0.05.

ConA binds α-mannose residues typically found in N-linked sugars on ycoproteins. Con A reactivity with LDL was significantly higher than that with Lp(a); kewise, apo B [compared to apo(a)] showed a significantly increased response to con A ig.11).On the other hand WGA reactivity was significantly higher with Lp(a) when mpared to LDL, while the lectin reactivity between apo(a) and apo b was more or less ual(Fig.11).WGA specifically binds N-acetyl glucosamine (GlcNAc), the characteristic gar that is a part of the pentasaccharide core structure of N-linked sugars. The con A d WGA reactivity with Lp(a),LDL, apo(a) and apo B suggest that, all of them possess -linked sugars.

When Lp(a), LDL, apo(a) and apo B were probed with RCA-1, a lectin specific r terminal galactose residues, Lp(a) and apo(a) showed a significantly higher response mpared to LDL and apo B respectively (Fig.12). Since galactose is a sugar that is und both in N- and O- linked sugars, it is an additional support to the suggestion that o(a), LDL, apo(a) and apo B possess N-linked sugars.

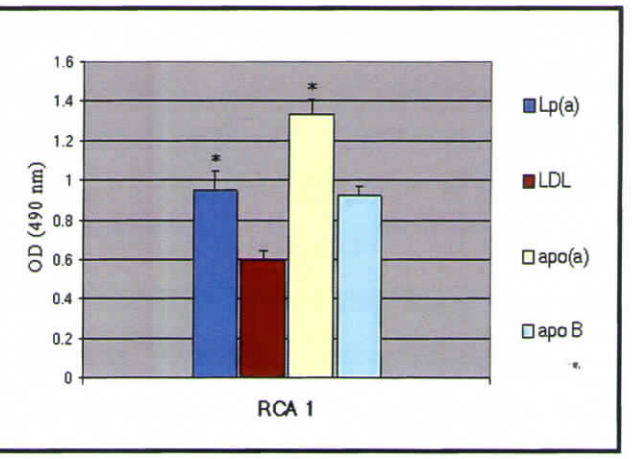


Figure12. Enzyme linked lectin analysis of Lp(a), LDL, apo(a) and apo B with RCA-1. Microtiter wells coated with purified Lp(a), LDL, apo(a) or apo B (1µg protein/well) were probed with the HRP-conjugated lectin (7.5 µg lectin /ml) followed by detection of the bound lectin conjugate with OPD. The values are expressed as optical density (OD) units at 490 nm. Mean + S.D of triplicate trials is shown. Lectin reactivity with Lp(a) vis-à-vis LDL and also apo(a) vis-à-vis apo B was compared by unpaired t-test , *P < 0.05.

In the enzyme linked lectin analysis with O-glycosylation specific lectins, PNA reactivity with Lp(a) was significantly higher than LDL or apo B (Fig.13); however, PNA did not show a similar response with apo(a) (Fig.13). Since apo(a) was isolated from

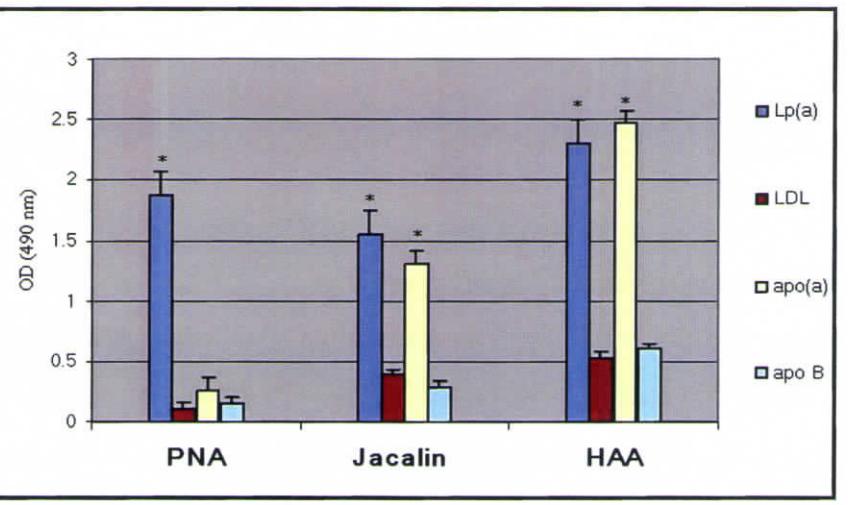


Figure 13. Enzyme linked lectin analysis of Lp(a) ,LDL, apo(a) and apo B with PNA, jacalin, and HAA. Microtiter wells coated with purified Lp(a), LDL, apo(a) or apo B (1µg protein/well) were probed with the HRP-conjugated lectin (7.5 µg lectin /ml) followed by detection of the bound lectin conjugate with OPD. In the case of PNA, desialylation of the coated proteins was done in separate experiments by incubating with neuraminidase (from Clostridium perfringens) prior to incubation with the lectin-HRP solution. The values are expressed as optical density (OD) units at 490 nm. Mean + S.D of triplicate trials is shown. Lectin reactivity with Lp(a) vis-à-vis LDL and also apo(a) vis-à-vis apo B was compared by unpaired t-test *P < 0.05.

Lp(a) after reduction with dithiothreitol, the chemical treatment may be interfering with the lectin binding in the case of PNA. PNA recognizes T-antigen (Gal β1-3

GalNAc) only after desialylation of the glycoproteins. Jacalin and HAA on the other hand showed a significant reactivity with both Lp(a) and apo(a) when compared with LDL and apo B (Fig.13). Jacalin recognizes T- antigen and also Tn antigen (α –GalNAc) (Jeyaprakash et al., 2003: Wu et al., 2003), while HAA binds terminal GalNAc residues on glycoproteins (Smith et al., 2006). The T- and Tn- antigen are part of core type 1 O-linked carbohydrate structures on glycoproteins. The ELLA results using PNA, jacalin and HAA shows that Lp(a) as also apo(a) is rich in T-antigen or T-antigen containing O-linked sugars, while LDL as also apo B does contain T-antigen but comparatively on a much lesser scale than that by Lp(a) or apo(a).

Lectin binding assays (ELLA) using O-glycosylation specific lectins, PNA, jacalin and HAA, revealed novel insights. O-glycosylation was observed, surprisingly, in LDL and also apo B (Fig.13). To best of our knowledge, O-glycosylation in LDL or apo B is not reported in literature so far.

4.2.3. Dot blot analysis of T-antigen in Lp(a) & LDL

Dot blot experiments were carried out to support the enzyme linked lectin analysis data. Lp(a) and LDL sugar chains were cleaved in sequential exoglycosidase digestions and also by alkaline β-elimination reaction. The O-linked sugars were then analyzed for T-antigen structures (T-antigen is part of core type1 O-linked sugars) using T-antigen specific lectins, PNA, jacalin and HAA (Fig 14 A). Individual responses or the blot intensity were quantified with image analysis software (NIH IMAGE) (Fig.14 B)

Terminal sialic residues were cleaved using sialidase (neuraminidase) from *Clostridium perfringens* (Sigma); the penultimate galactose residues were cleaved using β-galactosidase from bovine testis (Sigma); and the unsubstituted, Ser/Thr-linked GalNAc (α-GalNAc) was cleaved with endo-alpha-N-acetylgalactosaminidase (O-glycosidase) from *Streptococcus pneumonia* (Boehringer Mannheim). The entire O-linked structure was cleaved also chemically by alkaline β-elimination reaction using sodium borohydride.

Helix aspersa agglutinin (HAA) bound well to native Lp(a) (Fig.14A) and the nding was found to be approximately twice increased after degalactosylation following esialylation (Fig 14.B). HAA did not show any response with O-glycanase treated or β-iminated Lp(a) [both treatments results in the complete removal of O-linked sugars]. AA did show a low response with LDL which was more or less visible only after esialylation and degalactosylation (Fig 14 A). HAA binds to terminal GalNAc, and high nding of this lectin to native Lp(a) indicates a low degree of galactosylation and alylation of the Lp(a) O-glycan moieties.

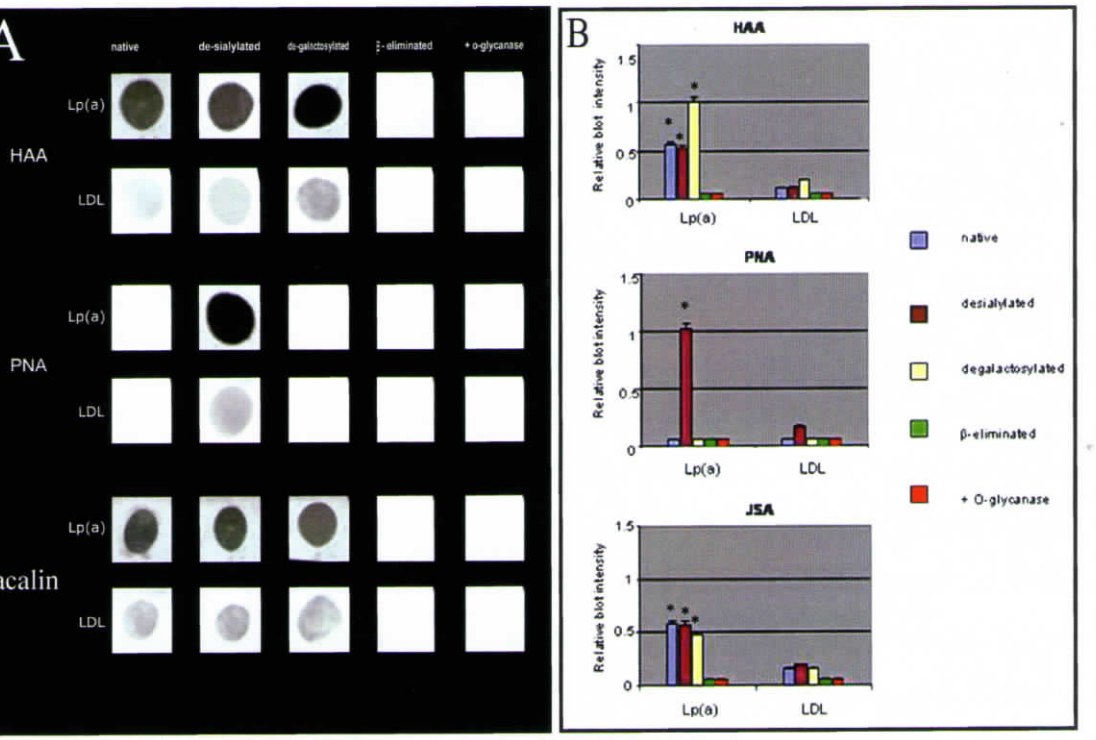


Figure 14. Dot blot analysis of T-antigen in Lp(a) & LDL. A: Native and variously treated Lp(a) nd LDL were dot-blotted on PVDF membranes (1 µg protein/dot) and probed with HRP-onjugated lectins (70 µg lectin/ml) followed by detection of the bound lectin conjugate with 4-hloro-1-naphthol. Each panel shows native, desialylated, degalactosylated, β-eliminated and de-glycosylated lipoprotein dots sequentially from the left and treated with the lectin conjugates. he response of each lectin HAA, PNA and jacalin with the variously treated Lp(a) and LDL are own in parallel, doublet panels B: Densitometric analysis of the dot blot experiments carried ut using Image J 1.37 v software (NIH IMAGE). Each intensity value represents the mean + S.D f four intensity measurements of a defined area adjusted for background intensity. The lectin activity with Lp(a) and LDL was compared by unpaired t-test. *P < 0.05.

PNA binds to the core 1 disaccharide Gal β1-3 GalNAc (T-antigen) and is extremely inhibited by the presence of sialic acid. PNA reactivity was seen only after desialylation of Lp(a) and LDL; although reactivity with LDL was weak and low, PNA binding to LDL was clearly evident (Fig 14 A & B). PNA response with the O-glycanase treated and β-eliminated lipoproteins were same as that for HAA.

Jacalin response with the lipoproteins were more less similar to that by HAA; however, desialylation and degalactosylation did not have much of an impact on jacalin binding to Lp(a), and also jacalin binding to native LDL was quite visible and evident even without desialylation and degalactosylation of LDL (Fig. 14 A & B)

Although protein O-glycosylation is a major post-translational modification, it is poorly understood compared with N-glycosylation. Mucin-type O-glycosylation starts with the attachment of alpha N-acetylgalactosamine (α-GalNAc) to a Ser/Thr residue and then proceeds through the transfer of various sugars. The addition of GalNAc to a Ser/Thr residue is catalyzed by uridine diphosphate (UDP)-GalNAc: polypeptide N-acetyl-galactosaminyltransferases (pp-GalNAc-Ts). Although each pp-GalNAc-T may have some preference for the amino acid sequences of the acceptor peptides and glycosylated peptides, there is no reliable method to predict actual α-GalNAc-attachment sites. In fact, there are more than 15 pp-GalNAc-Ts in humans, and probably they cooperate in various aspects of protein O-glycosylation in a cell (Cheng et al., 2004). O-glycosylation may occur in more than half of human proteins, but there are only about 200 O-glycosylation sites which were experimentally identified and recorded in protein database. Thus, unlike N-glycosylation sites, potential O-glycosylation sites are hardly predictable from the amino acid sequences of the proteins.

Poor understanding of protein O-glycosylation, in comparison with N-glycosylation, is largely attributed to technical difficulties in (1) prediction of O-glycan attachment sites *in silico* -based solely on amino acid sequences, (2) preparation of O-glycosylated proteins in a homogeneous manner, and (3) structural analysis of O-linked glycans and glycopeptides in a sensitive and high-throughput manner, which are due to

(a) obvious heterogeneity about carbohydrate structures as well as glycosylation occupancies, (b) substantial inaccessibility to protease digestion because of the presence of O-glycan barriers, and (c) the presence of many O-glycosylation sites often forming a mucin cluster. Because of these basic problems, both structural and functional approaches to O-glycosylated proteins have been considerably retarded (Tachibana et al., 2006)

This study used lectin binding to assess the composition of the N and O-glycan chains of Lp(a) and LDL. This is a somewhat imprecise method of glycosylation analysis, as the specificity of the lectins is not absolute and their binding may be affected by physicochemical factors other than the target sugar moiety. In the present study, lectin analysis was primarily used to delineate the O-glycosylation profiles in Lp(a) and LDL. Lectin binding cannot provide detail about the precise structures of the O-glycans but can be used to identify different overall patterns of O-glycosylation (Allen,1999). Mucin-type O-glycans expressed on certain proteins in various cells/tissues are highly heterogeneous. Therefore, the aim was to identify the α-GalNAc moiety of O-glycans at the reducing end, because such an approach would cover most kinds of mucin-type O-glycosylation. Various lectins specific for GalNAc or α- GalNAc containing disaccharides (T-antigen) were considered and finally, jacalin, HAA and PNA were chosen. Jacalin is known as a specific tool to capture O-glycoproteins, and its sugar-binding preference is well established for T- as well as Tn-antigens (α- GalNAc) (Jeyaprakash et al., 2003: Wu et al., 2003). HAA is highly specific for terminal GalNAc; how ever it binds substituted GalNAc also (Smith et al., 2006). PNA on the other hand binds only to desialylated T structures on glycoproteins.

The lectin binding assays (both ELLA and the dot blots) showed that Lp(a), LDL apo(a) and apo B possessed N- and O-linked sugars. As reported elsewhere, N-linked sugars are found on both the apo(a) and apo B in Lp(a), whereas O-linked sugars are found only on apo(a) accounting for approximately 83% of the total oligosaccharide structures (Garner et al.,2001; Harazono et al.,2005). Notwithstanding this knowledge, little is known about the O-glycosylation profiles in apo B or LDL. Strong binding of the T-antigen specific lectins (jacalin, PNA and HAA) to microtiter well-coated and dot-

blotted lipoproteins indicated abundance of T-antigen in Lp(a) and, to a much lesser extent, in LDL as well (Fig.13 and 14). O-glycosylation in apo B or LDL, as far as we know, had not been reported earlier.

4.2.3.1. Specificity of alkaline β- elimination: determination by dot blotting

Alkaline β-elimination is viewed as the most reliable and universal method in existence for the release of O-linked oligosaccharides; however, under the alkaline β-elimination conditions, significant peptide degradation occurs which may lead to the loss of N-linked sugars also. Lp(a) and LDL dot blots after alkaline β-elimination in the presence of borohydride were checked for the specificity of β-elimination (i.e. removal of O-linked sugars and not N-linked sugars) with HRP-conjugated jacalin (T-antigen specific lectin) and con A(N-glycosylation- specific lectin). Jacalin did not recognize

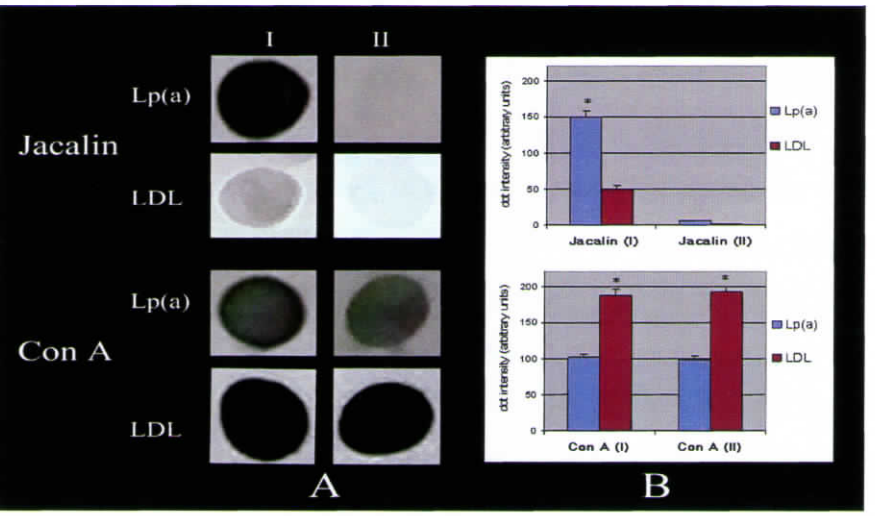


Figure 15. Specificity of alkaline β- elimination: determination by dot blotting. **A**: Native (panel I) and β-eliminated (panel II) lipoproteins were dot-blotted on PVDF membranes (1 μg protein/dot) and probed with HRP-conjugated lectins, jacalin and con A (70 μg lectin/ml), followed by detection of the bound lectin conjugate with 4-chloro-1-naphthol. **B**: Densitometric analysis of the dot blot experiments carried out using Image J 1.37 v software (NIH IMAGE). Each intensity value represents the mean + S.D of four intensity measurements of a defined area adjusted for background intensity. The lectin reactivity with Lp(a) and LDL was compared by unpaired t-test. *$P < 0.05$.

β-eliminated Lp(a) and LDL dot blots, which demonstrates that the O-linked sugars were removed by β-elimination (Fig.15 A & B); on the other hand, con A reactivity with the β-

eliminated dot blots were intact which indicates that there was no significant removal of N-linked sugars from Lp(a) and LDL by the β-elimination reaction (Fig.15 A & B).

4.3. Elucidation of the galectin-1 recognition of Lp(a) and LDL

Mechanisms of the accumulation of [Lp(a)] in atherosclerotic vessels is lacking in literature. This PhD study analyzed the role of carbohydrate structures of Lp(a) in atherogenesis. The role of Lp(a) glycans, if any, in the selective accumulation of Lp(a) mediated by a potential binding with human arterial galectin-1 was analyzed for the first time- a novel concept in the mechanisms of cholesterol accumulation hitherto unnoticed in atherosclerosis research.

Since Lp(a) is rich in T-antigens compared to LDL, some sequestering mechanisms or molecules in the arterial milieu that favorably binds the T-antigens in Lp(a), likely may be responsible for the selective accumulation of Lp(a) in atherosclerotic plaques. Galectin-1, a T-antigen binding lectin, is abundant in arterial tissues. The role of galectin-1 in the pathogenesis of malignant diseases including cancer is well documented. However, galectin-1 is least heard-of in atherosclerosis research with practically no reports except for two reports that highlighted the role of galectin-1 in SMC proliferation (Moiseeva et al., 1999, 2000).

This study sought to investigate the complementarities of the carbohydrate structures on Lp(a) and LDL with galectin-1 and whether endogenous galectin-1 binds Lp(a) *in situ*. Galectin-1 was isolated from the human placenta (human placental lectin, HPL) and also from bovine heart (bovine heart lectin, BHL). The differential response of galectin-1 with Lp(a) and LDL, and the T-antigen specificity of the binding was analyzed by enzyme-linked lectin assay and dot blotting. Localization of endogenous galectin-1 within histological sections of human internal mammary artery and human umbilical vein endothelial cells were analyzed by immuno histo/cytochemistry for *in situ* Lp(a) binding.

4.3.1 Isolation of galectin-1

Human galectin-1 is a dimeric carbohydrate binding protein having subunit mol wt around 14 KDa. Galectin-1 was purified from human placenta (HPL) by affinity chromatography using lactose-Sepharose. Lactose was used in the extraction buffer; according to deWaard et al.(1976), extraction of the lectin from the tissue into buffer is enhanced by the presence of specific sugar lactose. To protect galectin-1 against oxidative inactivation it was treated with a cystein modifying agent, iodoacetamide, during elution from the affinity resin. Alkylation with iodoacetamide yields carboxamidomethyl-galectin, which is fully active and stable to atmospheric oxygen (Whitney et al., 1986). Alkylation of lectin not only enabled the affinity chromatography experiment to be performed in the absence of reducing agents but also protected the lectin against inactivation by oxidation of the labile, free sulphydryl groups, during chromatography, dialysis and storage. Presence of the hapten sugar also helps in protecting the lectin from denaturation and precipitation, especially at higher temperatures.

The elute obtained from the affinity matrix not only contained the 14 KDa lectin but also a group of endogenous proteins as evidenced by the SDS PAGE (12%) of galectin-1 isolates (Fig.16A). Five bands were visible, the 14 KDa band is pure galectin-1, and the other four bands are lectin-glycoconjugate complexes co-eluted during the isolation of the lectin. The presence of 1M NaCl or 1% Triton X-100 in the column washing buffer did not prevent the co-purification of the proteins along with the lectins suggesting that ionic or hydrophobic interaction was not the reason for the binding of co-purified glycoproteins. Addition of 50mM lactose to the crude galectin-1 extract (after 70% ammonium sulphate precipitation) prevented the binding of both galectin-1 as well as endogenous glycoproteins to the matrix indicating the sugar dependent nature of association of galectin-1 and co-purified glycoproteins. Separation of galectin-1 from co-purified protein could not be achieved by gel filtration at atmospheric pressure.

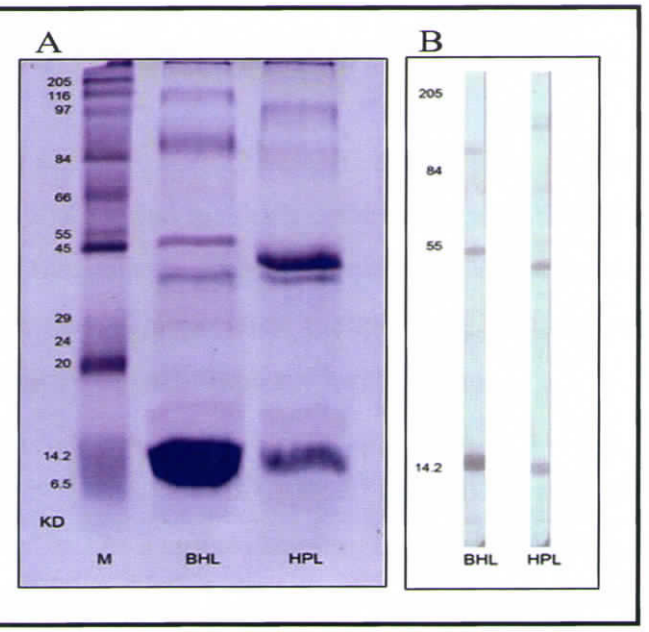


Figure 16. Characterization of human placental galectin-1 (HPL). **A**. 12% SDS PAGE of HPL along with galectin-1 isolated from bovine heart (BHL) and molecular weight markers(M). The 14 KDa band is pure galectin-1, and the other four bands are lectin-glycoconjugate complexes co-eluted during the isolation of the lectin. KD. Kilo Daltons. **B**. Western blot of HPL and BHL immunodetected with anti-galectin-1antibody and second antibody peroxidase conjugate confirms that the other proteins in the galectin-1 preparation were actually galectin-1-glycoconjugate complexes.

Galectin-1 was also isolated from bovine heart (BHL), and was used to upplement our studies with the human galectin-1. Both human and bovine galectin -1 ontain 134 amino acids but are different at 17 residues resulting in 87% amino acid lentity, and several of these differences are not conservative (Abbott et al., 1989, irabayashi et al., 1989). Endogenous glycoproteins had been shown to co-purify with HL (Appukuttan, 2002); it was suggested that the divalent galectin-1 binds to ultivalent endogenous glycoproteins and the resulting complexes contain enough sugar inding sites to facilitate erythrocyte receptor aggregation during hemagglutination ssays as well as its attachment to lactose sepharose during its isolation. The bovine heart alectin-1 binding to endogenous glycoproteins co purified with it during nromatography solely depended on the terminal α-linked galactose moiety on the latter here as these groups are absent on endogenous glycoproteins co-purified with human lacental galectin-1. If galectin-1 has an extracellular function then the liability of its inding activity in the presence of oxygen can put stringent restriction on the latitude of s activity. In tissues and cell extracts there is some evidence that galectin-1 is not free ut associated with its biological partners to form high mass complexes and that the ontaneous inactivation of native galectin-1 in the absence of reducing agents can be revented if the lectin is bound to glyco-conjugates (Cho and Cummings,1995). This

concentration); 60 to 75% reduction in lectin reactivity was observed after inhibition with lactose (Fig. 18).

Dot blot assays were carried out to support the enzyme linked lectin analysis data. Lp(a) and LDL sugar chains were cleaved in sequential exoglycosidase digestions and also by alkaline β-elimination reaction. The human galectin-1 response to sugar structures in Lp(a) and LDL were then analyzed (Fig.19.A). Individual responses or the blot intensity were quantified with image analysis software (NIH IMAGE) (Fig.19 B). The results substantiated the enzyme linked lectin analysis data. Galectin-1 response with Lp(a) was significantly higher than that with LDL, particularly after desialylation of the lipoproteins.

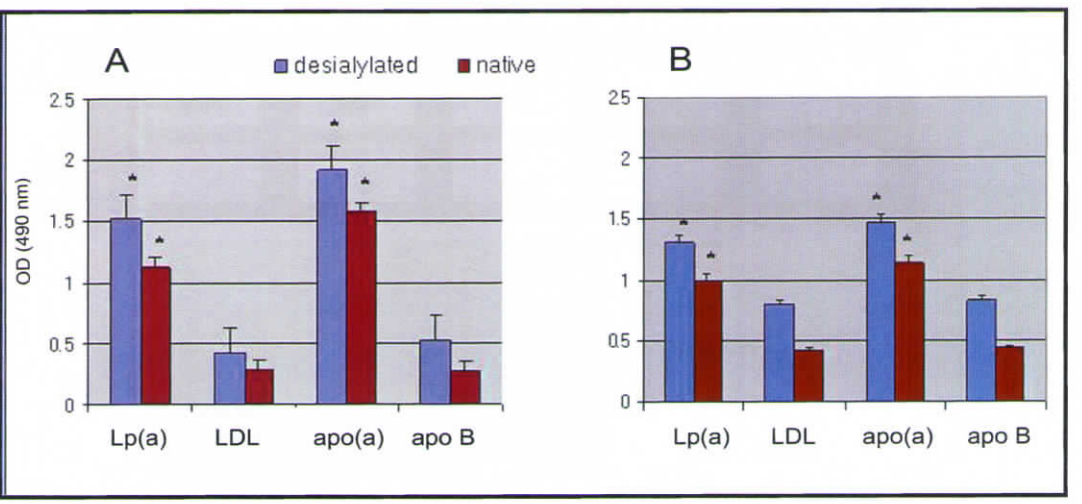


Figure 17. Enzyme linked lectin analysis of Lp(a), LDL, apo(a) and apo B with .A. galectin-1 from human placenta (HPL) , B. galectin-1 from bovine heart (BHL). Microtiter wells coated with purified Lp(a), LDL, apo(a) or apo B (1μg protein/well) were probed with the HRP-conjugated lectin (7.5 μg lectin /ml) followed by detection of the bound lectin conjugate with OPD. Desialylation of the coated proteins was also done in separate experiments by incubating with neuraminidase (from Clostridium perfringens) prior to incubation with the lectin-HRP solution. The values are expressed as optical density (OD) units at 490 nm. Mean + S.D of triplicate trials is shown. Lectin reactivity with Lp(a) vis-à-vis LDL and also apo(a) vis-à-vis apo B was compared by unpaired t-test , *$P < 0.05$.

There is considerable uncertainty surrounding the carbohydrate binding requirements of the galectins, particularly in regard to galectin-1.Dimeric bovine galectin-1 was reported to bind weakly to N-acetyllactosamine (Gal β1-4GlcNAc) and a

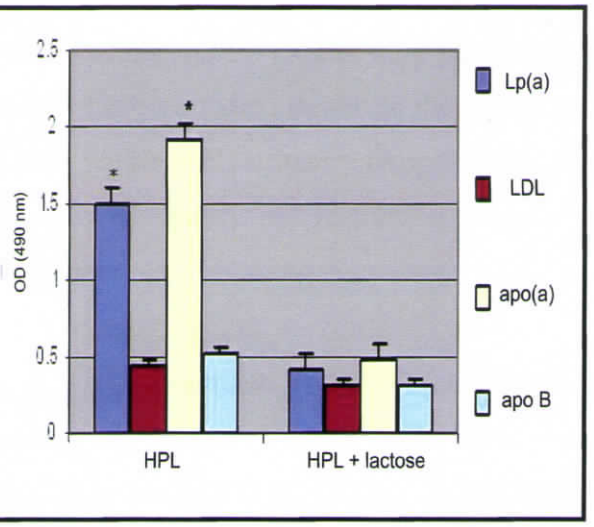
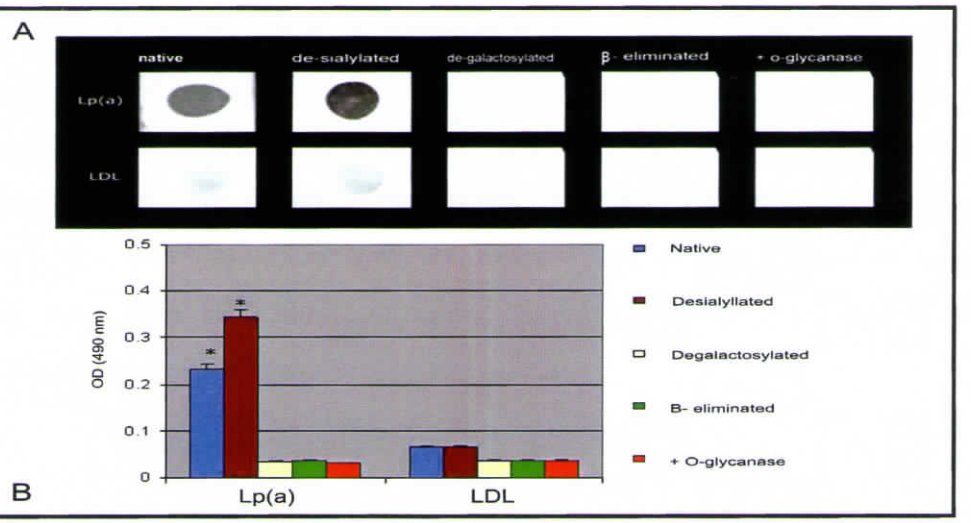


Figure.18. Sugar specificity analysis of galectin-1 reactivity with the lipoproteins. Microtiter wells coated with purified Lp(a), LDL, apo(a) or apo B (1µg protein/well) were probed with HRP-conjugated HPL (7.5 µg lectin /ml) followed by detection of the bound lectin-conjugate with OPD. In separate experiments, the galectin-1-HRP solution was incubated with lactose (50 mM final concentration in blocking buffer) prior to incubation with the coated proteins. The values are expressed as optical density (OD) units at 490 nm. Mean + S.D of triplicate trials is shown. Lectin reactivity with Lp(a) vis-à-vis LDL and also apo(a) vis-à-vis apo B was compared by unpaired t-test , *P < 0.05.



igure 19. Dot blot analysis of galectin-1 (HPL) reactivity with Lp(a) & LDL. A: Native and ariously treated Lp(a) and LDL were dot-blotted on PVDF membranes (1 µg protein/dot) and robed with HRP-conjugated lectins (70 µg lectin/ml) followed by detection of the bound lectin onjugate with 4-chloro-1-naphthol; Top panel: galectin-1 response with Lp(a), Bottom panel: alectin-1 response with LDL. Each panel shows native, desialylated, degalactosylated, β-iminated and O-glycosidase treated lipoprotein dots sequentially from the left treated with the ctin- HRP conjugate. B: Densitometric analysis of the dot blot experiments carried out using nage J 1.37 v software (NIH IMAGE). Each intensity value represents the mean + S.D of four tensity measurements of a defined area adjusted for background intensity. The lectin reactivity ith Lp(a) was compared to that with LDL by unpaired t-test. *P < 0.05.

ariety of other related structures (Abbott et al., 1988; Appukuttan, 2002; Solomon et al., 991) but preferential binding was observed toward poly-N-acetyllactosamine sequences

provides a mechanism for the stability of the lectin in the extracellular environment. Present results suggest lectin carbohydrate recognition a primary reason for association of co purified glycoproteins with HPL within intact tissue or after homogenization.

The western blot after SDS page showed 3 bands when probed with galectin-1 antibodies and the other bands were not visible (Fig.16 B). This confirms that the proteins in the galectin-1 preparation were actually galectin-1-glycoconjugate complexes. Co-purified glycoproteins therefore are chosen endogenous glycoproteins for HPL and contain sugar ligands apt for the lectin.

The yield of galectin (both BHL and HPL) from 35g tissue varied from 700 to 900µg.The average concentration of galectin-1 in different tissues may vary; for example, in rat lung and intestine, it was about 30 to 70µg/g and constitute up to 1% of total protein in cells that synthesize it (Allen et al., 1987). The activity of galectin-1 preparation was checked using trypsinized human RBC and the minimum agglutinating concentration was found to be between 2.5 to 5 µg/ml. Human erythrocytes contain cryptic T-antigens and terminal alpha linked gal groups which becomes exposed on trpysin mediated proteolysis. These may be the ligands involved in hemagglutination displayed by the lectin

4.3.2. Enzyme linked lectin analysis and dot- blot assays

Lp(a), LDL,apo(a) and apo B coated to microtiter wells were analyzed by enzyme linked lectin analysis (ELLA) using HRP conjugated galectin-1 (both HPL and BHL). The response of both BHL and HPL with Lp(a) was significantly higher than that with LDL. Similarly, the response of BHL and HPL with apo(a) was significantly higher than that with apo B (Fig 17 A&B). In addition, the galectin-1 response with apo(a) was found to be more than that with the native Lp(a).

The sugar specificity of galectin-1 interaction with the lipoproteins was verified by inhibition assays with lactose, a well known inhibitor of galectin-1(50mM final

(PL; [-3Gal β1-4GlcNAc βl-] $_n$) containing multiple, linear N-acetyllactosamine (LN) units (Merkle and Cummings, 1988; Zhou and Cummings, 1993) suggesting that LN motifs within the PL chains were possibly recognized by bovine galectin-1. Other recent results have also shed doubt on the ability of bovine galectin-1 to recognize internal LN motifs within a PL structure (Appukuttan, 2002).There is conflicting evidences regarding the lectins ability to recognize N-acetyllactosamine and poly-N-acetyllactosamine sequences.

The T-antigen as ligand for galectin-1 is a comparatively recent concept that is gaining acceptance (Glinsky et al., 2001; Sangeetha and Appukuttan, 2005; Hernandez et al., 2006; Jeschke et al., 2006). In our study, galectin-1 reactivity with Lp(a) and LDL was found to be exclusively T-antigen specific. Galectin -1 failed to recognize both Lp(a) and LDL in either situation, (1) after removal of the terminal sialic acid and penultimate β-linked gal by enzymatic desialylation followed by degalactosylation which exposes the α-linked GalNAc moiety or the Tn antigen, or (2) after complete removal of O-linked sugars by alkaline β-elimination or O-glycosidase (Fig.19 A). This clearly demonstrates that galectin-1 specifically bound to the T-antigens in Lp(a) and LDL. The binding was irrespective of sialylation; however, desialylation of Lp(a) enhanced galectin-1 binding by more than 30% and comparable increase was not seen with desialylated LDL (Fig.19.B).

The results from this study was reported in a paper titled "Galectin-1, an endogenous lectin produced by arterial cells, binds lipoprotein(a) [Lp(a)] *in situ* : Relevance to atherogenesis" (Chellan et al., in press). The higher *in vitro* reactivity of galectin-1 with Lp(a) compared to that with LDL may be relevant to atherogenesis if we are able to extent the results to the *in vivo* scenario as well. Nevertheless, the observation is novel and strong enough to support the galectin-1 mediated selective accumulation of Lp(a) in the arterial plaques *vis-à-vis* LDL Additionally, desialylation of the lipoproteins increased galectin-1 binding which may be of added significance since the desialylated lipoprotein are reportedly more atherogenic(Lin et al.,1989; Mel'nichenko et al.,2005). The galectin-1 response with apo(a) which was found to be more than that with the native

Lp(a) is also noteworthy in this context, since it is the structural components apo(a) and apo B that is found in abundance in the arterial plaques rather than the native Lp(a) itself.

4.3.3. Glyco histochemical analysis of tissue galectin-1 binding Lp(a) *in situ*

Lp(a) reportedly binds extra cellular matrix components such as proteoglycans, fibronectin and other structural elements, wherein, the proteoglycans are the major ligands for the lipoproteins (Williams et al.,1992).These apo B-containing lipoproteins bind weakly to proteoglycans in physiological ionic-strength environments (Khalil et al., 2004) suggesting that other matrix components may be involved in the efficient arterial retention of lipoproteins in atherogenesis. Galectin-1 is abundant in tissues of mesodermal origin including muscular tissues (Colnot et al., 1996) and may likely be a candidate protein involved in Lp(a) retention. Lectin binding is a purely carbohydrate-dependent phenomenon that commonly occurs in the *in vivo* physiological milieu. Therefore, we investigated whether endogenous galectin-1 in human internal mammary artery histological sections binds Lp(a) *in situ* sugar specifically. Trimmed internal mammary artery (IMA) segments were obtained from patients undergoing coronary artery bypass surgery at our institution. IMA segments were used for the study because of its easy availability and well preserved structural integrity after processing. Specimens were fixed in 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin. Five -µm transverse histological sections were cut. The sections were de-paraffinised and rehydrated using standard procedure, including pre-incubation with 0.3% hydrogen peroxide in PBS to inhibit endogenous peroxidase activity.

Lysine coated slides were not used since Lp(a) is known to bind to lysine and in place, egg albumin coated slides were used. Although high concentrations of Lp(a) [> 70 mg/dl] were found to bind weakly to albumin, albumin coated slides were ideal for the purpose. The tissue processing media or conditions did not inhibit tissue galectin-1 activity. However, processing temperatures and the period of exposure were very critical.

At no stages were the temperature allowed to go above 60° C and the exposure periods were strictly maintained, lest it will result in inactivation of tissue galectin-1.

4.3.3.1. Localization of galectin-1

Immunohistochemical analysis for the localization of galectin-1 in IMA sections with anti-galectin-1 antiserum showed extensive antiserum staining in the medial region of the artery and also in some regions around the intima which indicated the abundance of tissue galectin-1 within the arterial tissues (Fig.20A). The staining was particularly towards the external elastic lamina, whereas the external connective tissue layer was not stained. The medial region is made up of the smooth muscle tissue; considerable staining of the medial region may be in accordance with the abundance of galectin-1 in smooth muscle cells (Franklin et al., 1980). Endothelial cells are also known to express galectin-1 (Baum et al ,1995). However, in our study, endothelial galectin-1 expression was not as conspicuously evident as the medial smooth muscle galectin-1. It may be because the endothelium is a single-cell layer in comparison to the more voluminous underlying smooth muscle layer. The galectin anti-serum was able to recognize the galectin-1 epitopes on the tissue even without processing for antigen retrieval and the intensity of anti- galectin staining was more or less same in both cases, i.e. with and without antigen retrieval (data not shown).

4.3.3.2. Localization of tissue galectin-1 binding Lp(a).

Extensive Lp(a) binding was observed when the tissues were immunoanalysed for Lp(a) binding using a polyclonal antibody to human Lp(a) (Dako,Denmark). Serial sections were incubated with pure Lp(a) and immunostained with anti-Lp(a).The staining was optimum when Lp(a) was incubated at a concentration of 50 mg/dl. The staining pattern of anti- human Lp(a) suggests that the tissue binding of Lp(a) overlaps with the localization of endogenous galectin-1(Fig.20A, B and D). The tissue sections showed an almost normal intima and media (Fig.20A, B, C, D,E & F). To determine whether galectin-1 actually binds Lp(a) *in situ,* sections were pre-incubated with 50 mM lactose (a known inhibitor of galectin-1) prior to the incubation with Lp(a), while in separate

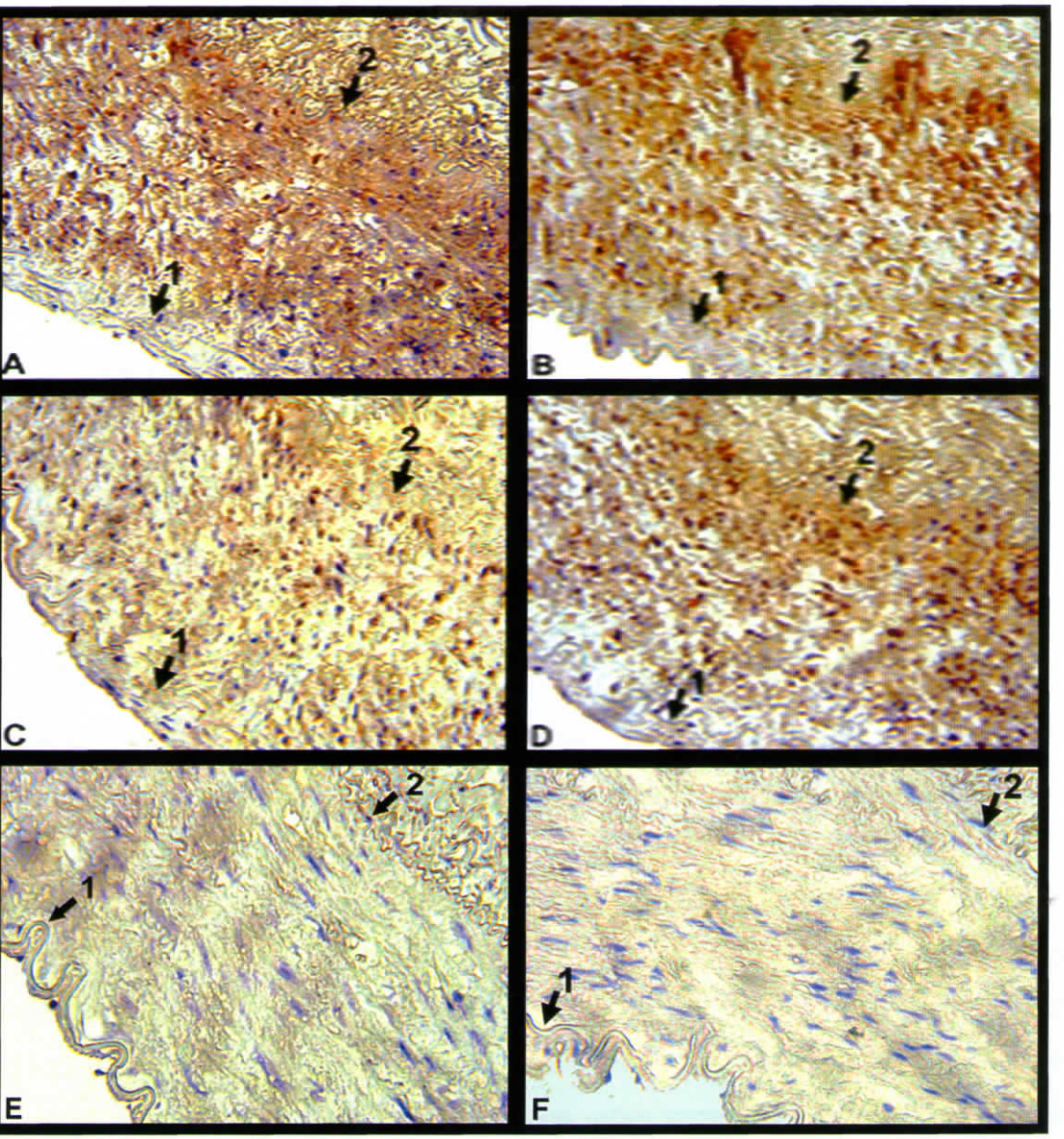


Figure 20. Glyco histochemical analysis of galectin-1 binding to Lp(a). Paraffin- embedded serial sections of human internal mammary artery were immunostained with antiserum to galectin-1 along with HRP-conjugated second antibody (**A**). Sections were incubated with 50 mg/dl Lp(a) (**B**) and also pre-incubated with 50 mM lactose (**C**) or 50 mM maltose (**D**) prior to incubation with Lp(a), followed by immunostaining with anti-Lp(a) and HRP-conjugated second antibody. **E**. negative control without primary antibody(anti –galectin-1 antiserum) in studies for the localization of galectin-1. **F**. negative control without primary antibody [anti-human Lp(a)] in Lp(a)-binding studies. Nuclei were counterstained with Mayer's hematoxylin. The arrows 1and 2 indicate the internal and the external elastic lamina respectively.

experiments, 50 mM maltose was used in place of lactose. Lactose is the specific sugar of galectin-1; maltose is nonspecific to galectin-1 and was used as the control sugar.

Proceeding sugar incubation, the sections were drained of sugar solutions (not washed) and incubated directly with Lp(a) [refer methods for details]. Lp(a) appears to bind poorly to the lactose incubated tissue (Fig.20.C) in comparison to that with the maltose incubated tissue (Fig.20.D). There was no non-specific binding by the second antibody–HRP conjugate as is evident from the negative control where the primary antibody, anti-galectin-1antiserum, was omitted in the galectin-1-localization experiments (Fig.20 E). Similarly, the negative control for the Lp(a)-binding studies without the primary antibody [anti-human Lp(a)] showed the absence of any nonspecific binding by the second antibody-HRP conjugate(Fig.20 F). These results clearly demonstrate that tissue galectin-1 binds Lp(a) *in situ.*

In atherosclerotic plaques, Lp(a) appears to be retained more avidly than LDL (Beisiegel,1991; Dangas et al., 1998; Krcuzer et al., 1994; Nielsen, 1999) suggesting an efficient trapping mechanism in the arterial intima for Lp(a). *In vitro*, Lp(a) has been shown to bind to arterial proteoglycans (Bihari Varga et al.,1988; Dahlen et al.,1978). The proteoglycan contribution to sub-endothelial retention of Lp(a) is, however, a much debated issue. Lp(a) arguably bind to proteoglycans structures via the proteoglycan binding sequences in its apo B (Bihari Varga et al.,1988). However; the proteoglycan binding site in apo B is masked in Lp(a) as suggested by Khalil et al.(2004), and apo(a) does not have GAG-binding consensus sequences such as those present in apo B or apo E (Khalil et al.,2004; McLean et al.,1987). LDL and other apo B containing lipoproteins were shown to bind weakly to heparin and other proteoglycans in physiologic ionic strength environments (Camejo et al., 1998).Weather proteoglycans can contribute to Lp(a) retention *in vivo* is not clear and needs further study.

In this perspective, much of the retention of Lp(a) within arteries appears to be due to specific inherent properties in its apo(a) component differing from the LDL retention which is thought to involve apo B. Differential glycosylation in apo(a) and apo B might have a say in the process. As mentioned earlier, significantly high levels of T-antigen in apo(a) or Lp(a) compared to low levels in apo B or LDL was reported by the present studies (Chellan et al., in press); galectin-1 was found to have a high T-antigen

specific reactivity with Lp(a) in comparison to a poor response with LDL. The glyco histochemical analysis of tissue galectin-1 binding Lp(a) *in situ,* formed another part of the reported paper. In this study, the smooth muscle tissue showed significant expression of galectin-1 (Fig.20.A), which is indicative of the relative abundance of galectin-1 in the arterial tissues. The extracellular matrix in and around the intima may be rich in galectin-1 since galectin-1 is also secreted to the extracellular matrix by the cells (Baum et al., 1995). Consequently, galectin-1, which is ubiquitous and abundant in the arterial histological organization, may possibly contribute to the accumulation or retention of Lp(a) within these tissues. In addition, more importantly, lectin binding is exclusively sugar specific, independent of charge or other physiological conditions.

4.3.4. Immunofluorescence analysis of the *in situ* binding of Lp(a) by human umbilical vein endothelial cell (HUVEC) membrane- expressed galectin-1

Galectin-1 is expressed by most tissue types; it is expressed abundantly by the smooth muscle cells and the endothelium of the arterial wall (Moiseeva et al., 1999, 2000; Baum et al., 1995). The endothelial layer forms the first barrier between arterial cells and the circulating lipoproteins. Localization of galectin-1 in the endothelium and its sugar specific *in situ* binding of Lp(a) was analyzed by immunofluorescence so as to support the glycohistochemical analysis data and investigate the possibility of a galectin-1 mediated Lp(a)binding. Additionally, colocalization of Lp(a) and the membrane-expressed galectin-1 in human umbilical vein endothelial cells was analyzed by double-staining immunocytochemistry and confocal microscopy. The results substantiated the study hypothesis that galectin-1 binds human Lp(a) and possibly may be involved in atherogenesis.

4.3.4.1. Localisation of galectin-1.

Endothelial cells were isolated from human umbilical vein (HUVECs) as described by Gimbrone et al. (1974) with slight modifications. Isolation and culture of human umbilical vein endothelial cells are done routinely at the Thrombosis research unit in the BMT wing of our institution. The HUVECs used in the studies were courtesy of Dr. Lissy K Krishnan (Head, Thrombosis research unit). The immunofluorescence analysis studies were performed at the Thrombosis research unit.

Cells from the third passage were used in the study. The membrane bound galectin-1 was immunodetected with anti-galectin-1 antibody (Santa Cruz ,USA) and second antibody-TRITC conjugate. In separate experiments, cells were activated with minimally oxidized LDL (MM- LDL) prior to immunodetection with anti- galectin-1 antibody and second antibody-TRITC conjugate. MM-LDL was prepared either by cold storage or iron oxidation (refer to methods). Lipid peroxidation or the extent of LDL oxidation was measured by thiobarbiturate-reactive substances (TBARS) assay as described by Agil et al. (1995) and contained 2 to 3.7 nmol of TBARS as malondialdehyde equivalents per mg protein. Fully oxidized LDL (Ox-LDL) is cytotoxic to cells; TBARS is in the range 7.5 to 32.7 nmol/mg protein normally for Ox-LDL. Cytotoxicity of MM-LDL was determined by MTT assay and the cells were not sensitive to MM-LDL. The cytoviability was 100% after 4h at the test concentration of MM-LDL (100µg/ml) which indicates cell proliferation rather than cell death.

In the immunofluorescence analysis for the localization of membrane expressed galectin-1 in normal HUVECs, the expression of galectin was too low as evidenced by the comparatively faint anti-galectin staining (Fig.21.A). However, galectin-1 expression was considerably elevated after cell activation with MM-LDL (Fig 21.B) as reported elsewhere (Baum et al., 1995). The staining pattern was very dispersive with almost the entire cell surface intensively stained indicating the abundance of membrane bound galectin-1.

4.3.4.2. Localization of bound Lp(a) in HUVECs after incubation with Lp(a).

HUVECs were incubated with Lp(a) and the bound Lp(a) was immunodetected with anti human Lp(a) (Dako, Denmark) and second antibody- FITC conjugate. The staining was optimum when Lp(a) was incubated at a concentration of 50 mg/dl. Both native and MM-LDL activated cells were used in separate assays. Strong anti-human Lp(a) staining was observed in cells activated with MM- LDL prior to incubation with Lp(a) (Fig.21.D), while native cells showed comparatively weaker anti-human Lp(a) staining (Fig.21.C).These results demonstrate that Lp(a) binding by the cells substantially increases following cell activation.

Immunofluorescence analysis of the localization of bound Lp(a) in HUVECs was done with an aim to determine the role of the endothelial surface expressed galectin-1, if any, in Lp(a) binding. The cells were fixed only after incubation with Lp(a), lest it may lead to the inactivation of the endothelial galectin-1. We have already seen that there is substantial increase in galectin-1 expression by the cells following cell activation (Fig.21.A&B). Endothelial galectin-1 may, therefore, likely be involved in the increased Lp(a) binding following cell activation (Fig.21.C & D). To determine whether galectin-1 actually binds Lp(a) in situ, MM-LDL activated cells were pre-incubated with 50 mM lactose (a known inhibitor of galectin-1) prior to the incubation with Lp(a). Proceeding sugar incubation, the sugar solutions were drained of (not washed) and incubated directly with Lp(a) [refer methods for details]. Lp(a) appears to bind poorly to the lactose incubated cells (Fig.21.E) indicating a galectin-1 mediated Lp(a) binding by the cells.

Colocalization of endothelial surface expressed galectin-1 and the bound Lp(a) following incubation of the activated cells with Lp(a) was analyzed by double-staining immunocytochemistry and confocal microscopy (refer methods). The results of the double-staining analysis for the galectin-1 expression by the cells, the corresponding Lp(a) binding pattern and the antigen colocalization are shown in figures 21 F, G and H respectively. The cells within a particular field are shown in the colocalization analysis. The TRITC stain (red) shows the galectin-1 expression; green staining of the FITC

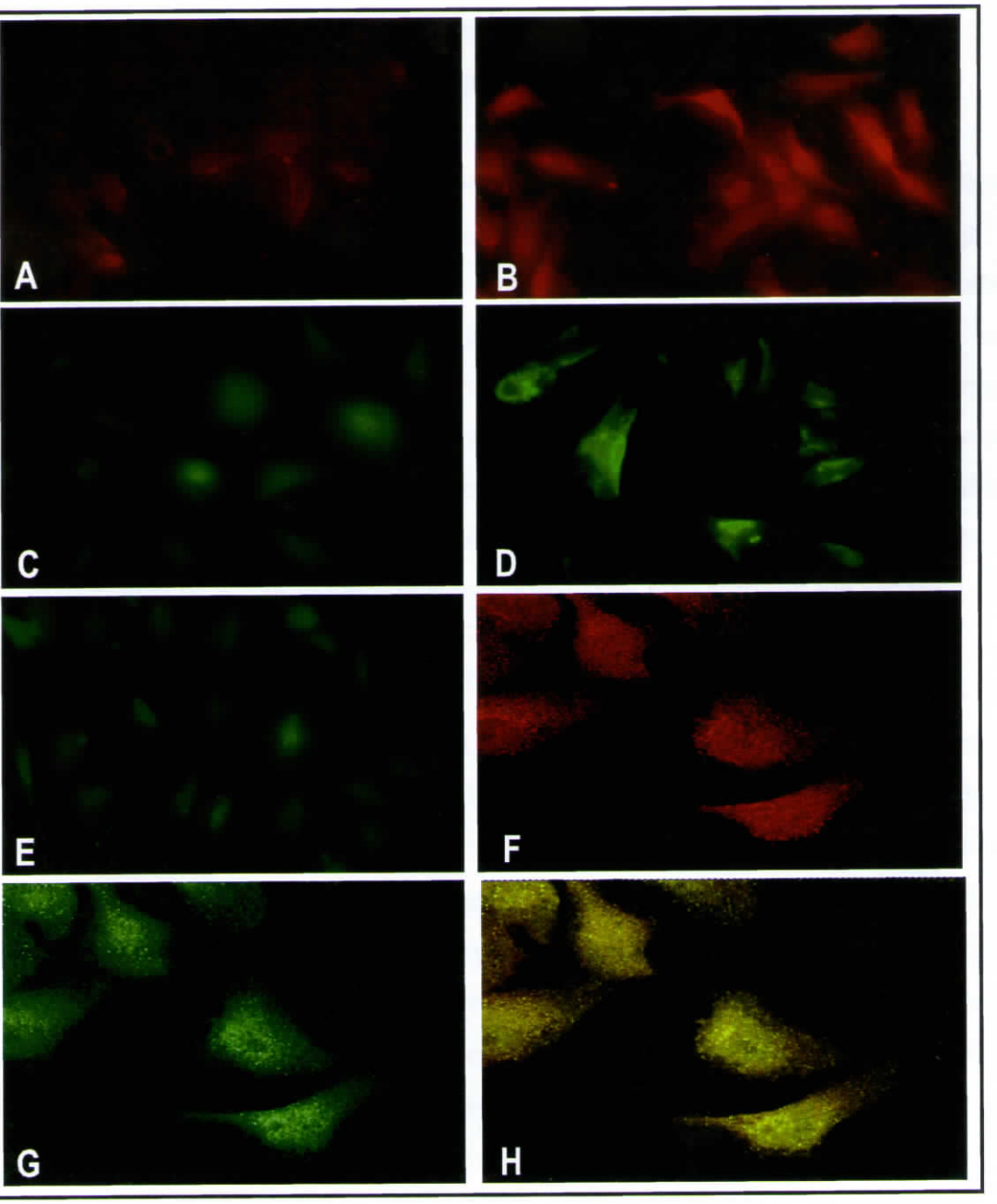


Figure 21. Immunofluorescence analysis of the in situ binding of Lp(a) by human umbilical vein endothelial cell(HUVEC) membrane-expressed galectin-1. 1st Panel: normal cells (**A**) and cells activated with minimally oxidized LDL (**B**) were probed with anti-galectin antibody followed by second antibody TRITC-conjugate for cell surface galectin expression. 2nd Panel: normal cells (C) and cells activated with minimally oxidized LDL (**D**) were incubated with Lp(a) [500 µg /ml] and immunodetected by anti-Lp(a) and secondary antibody FITC-conjugate. 3rd Panel: activated cells (**E**) incubated with 50 mM lactose (galectin-inhibitor) prior to incubating with Lp(a) and its immunodetection. In confocal microscopic analysis, activated cells were incubated with Lp(a) and double- stained with a mixture of anti-galectin-1 and anti-Lp(a) followed by the corresponding TRITC and FITC- conjugated second antibody: **F**. galectin-1 expression by the cells, **G**. Lp(a) binding by the cells, **H**. colocalization of membrane expressed galectin-1 and Lp(a) binding by the cells

conjugate is specific for Lp(a),while the yellow stain or areas represent the colocalization of galectin-1 and Lp(a). Substantial colocalization of galectin-1 and Lp(a) (Fig.20.H) is indicative of a possible galectin-1 mediated Lp(a) binding by these cells.

Endothelial cells form the gateway barrier for circulating lipoproteins seeking the internal arterial milieu. Lp(a) presumably traverses the endothelium through some receptor independent mechanisms (Scanu and Fless,1990). Galectin-1 in the endothelium may possibly bind Lp(a); extensive galectin-1 expression in the endothelial surface possibly may lead to a concentration of Lp(a) and favor its transendothelial transport. Endothelial cell activation is a primary event in atherogenesis mediated by proinflammatory agents. Galectin-1 expression is shown to be up regulated after cell activation by inflammatory agents (Baum et al., 1995). Increased galectin-1 expression, therefore, may be a consequence in atherogenesis that may augment the Lp(a)-binding by these cells.

The biological role of galectin-1 in the cardiovascular system is largely unknown. It has been shown to be involved in vascular smooth muscle cell proliferation, which is a key step in the development of atherosclerosis (Moiseeva et al., 1999, 2000). In the present studies the differential response of galectin-1 with Lp(a) and LDL, and the T-antigen specificity of the binding was analyzed by enzyme-linked lectin assay and dot blotting. Furthermore, localization of endogenous galectin-1 within histological sections of human internal mammary artery and human umbilical vein endothelial cells were analyzed by immunohisto/cytochemistry for *in situ* Lp(a)binding. These studies demonstrate compelling evidences for a T-antigen specific *in vitro* or *in situ* Lp(a) binding by human galectin-1. Based on these observations, it is reasonable to assume that specific carbohydrate determinants (T-antigens) on Lp(a) and LDL may initiate a pathogenic association with cell surface or extracellular matrix galectin-1 in early atherogenesis. Additional investigation will be required to analyze in greater detail, the role of galectin-1 in Lp(a) accumulation and to delineate the biological significance of this interaction in atherogenesis.

4.4. Galectin-1 in the extracellular matrix may crosslink glycoproteins with Lp(a): a possible mechanism for Lp(a) retention in the subendothelial intima

The extracellular matrix (ECM) is composed of a complex array of glycoproteins and glycosaminoglycans. The major adhesive macromolecules include fibronectin, vitronectin, laminin, and multiple types of collagen. Laminin is highly abundant in the basal laminae that underlie the vascular endothelium, suggesting that the ability of Lp(a) to bind this macromolecule could serve as a means of retaining Lp(a) within developing atheromas and enhancing its atherogenic effects. However, Lp(a) was found to bind very weakly to laminin and not at all to vitronectin or collagen (van der Hoek et al.,1994). On the other hand, Lp(a) has been shown to bind tightly to fibronectin and Lp(a) binding to fibronectin was lysine independent which was contradictory to the characteristic lysine binding property of apo(a) (Salonen et al.,1989)

Galectin-1 is present both extracellularly and intracellularly. Galactin-1 is secreted by the cells into the matrix where it may be associated with its natural ligands. Laminin and fibronectin are two ECM proteins proposed as the main receptors for galectin-1 (van den Brule et al.,2003; Zhou and Cummings, 1993) In human placenta extracts, two major galectin-1 receptors were obtained by affinity chromatography and identified as fibronectin and laminin. Additionally, galectin-1, fibronectin and laminin were found to colocalize in the extracellular matrix of placental tissue. In cell attachment assays, they further demonstrated that tissue fibronectin and laminin function as endogenous receptors for galectin-1 (Ozeki etal., 1995). Other ECM proteins such as thrombospondin and vitronectin and, to a lesser extent, osteopontin can also bind to galectin-1 (Moiseeva et al., 2000). Galectin-1 binds to several ECM proteins in a dose-dependent manner in the following order: laminin>cellular fibronectin > thrombospondin> plasma fibronectin > vitronectin > osteopontin. This binding is reduced by half in the presence of lactose for all proteins except vitronectin. Interactions between vitronectin and galectin-1 seem to depend on vitronectin conformation: vitronectin exists

either as a folded inactive monomer or an unfolded multimer able to interact with ECM components. Vitronectin shows significant binding to galectin-1 in the presence of lactose, probably because lactose induces unfolding of vitronectin (Moiseeva et al., 2000).

The present study had already reported *in vitro* interaction of human galectin-1 and Lp(a) (Chellan et al., in press), which is one of the few reports of an apo(a)-substrate interaction that is not lysine-dependent. In this study, we sought to investigate the possibility of a galectin-1 mediated crosslinking of human Lp(a) with ECM proteins, laminin and fibronectin, which may likely be a mechanism for the subendothelial retention of Lp(a) in atherogenesis. Galectin-1 mediated cross-linking of Lp(a) with fibronectin and laminin was determined by an ELISA based assay and also by immunoprecipitation with anti-fibronectin antibody followed by western blotting with anti- Lp(a).

4.4.1. Isolation of fibronectin by affinity chromatography

Laminin from human placenta (Sigma, USA) and human fibronectin (from both plasma and placenta) were used in the study. Fibronectin was isolated by affinity

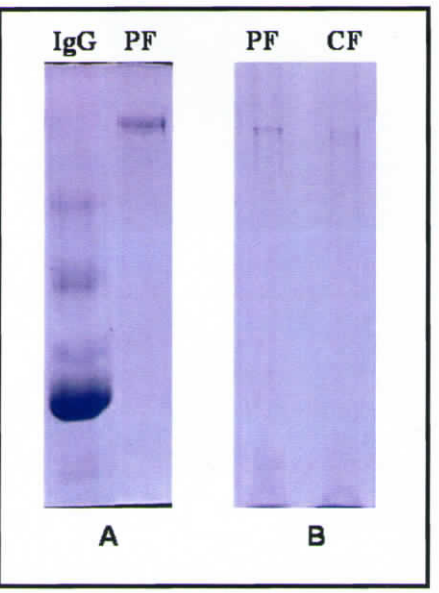


Figure. 22. Purity check of fibronectin isolates by SDS-PAGE(5%). **A**. isolated plasma fibronectin alongside human IgG. **B**. isolated placental fibronectin alongside plasma fibronectin.; **PF**. Plasma fibronectin; **CF**.cellular (placental) fibronectin.

hromatography on gelatin-Sepharose and purity checked by SDS-PAGE. The isolated lasma fibronectin was confirmed pure with no trace of human IgG (Fig.22 A). IgG is he most common contaminant found in fibronectin isolates. The characteristically broad iffuse band of placental fibronectin migrated similarly with the characteristic doublet and of plasma fibronectin (Fig.22 B). There were no other proteins in the fibronectin reparations as evidenced by the SDS-PAGE analysis.

.4.2. Lp(a)binding by human placental fibronectin and laminin: letermination by an ELISA based assay

Cellular fibronectin is the insoluble type of fibronectin found associated with issues in their extracellular matrix. This study was initiated to investigate the response of uman cellular (placental) fibronectin and placental laminin with human Lp(a) while arlier studies had used synthetic fibronectin and laminin with recombinant apo(a) (van er Hoek et al.,1994). Lp(a) binding by human placental fibronectin and laminin was

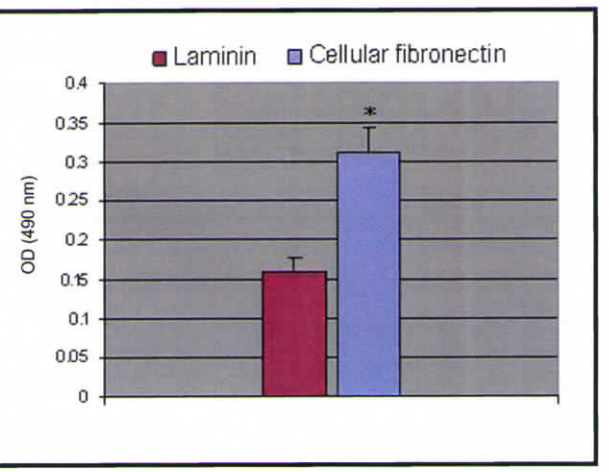


Figure 23. Lp(a)-binding by human cellular (placental) fibronectin and laminin: determination by an ELISA based assay. Coated laminin or fibronectin was incubated with Lp(a) [250 µg/ml] and immunodetected by anti-Lp(a) and HRP-conjugated second antibody (details in methods). Mean + S.D of triplicate trials is shown. The Lp(a)-binding by cellular fibronectin and laminin was compared by unpaired t-test *P < 0.05.

ompared by an ELISA based assay slightly modified from that described by van der Hoek et al.(1994). Lp(a) binding by human placental fibronectin was approximately wice than that by the placental laminin(Fig.23). Lp(a) binding by laminin was nsignificant when compared to that by cellular fibronectin.

4.4.3. Enzyme linked lectin analysis of the galectin-1 recognition of fibronectin and laminin

Galectin-1 recognition of human cellular fibronectin and laminin was analyzed by enzyme linked lectin assays essentially as described before for the galectin-1 interaction with Lp(a). Human galectin-1(HPL) reactivity was slightly higher with laminin. The galectin-1 response with both laminin and cellular fibronectin was more than twice that with plasma fibronectin (Fig.24) which was in agreement with previous reports (Moiseeva et al., 2000).

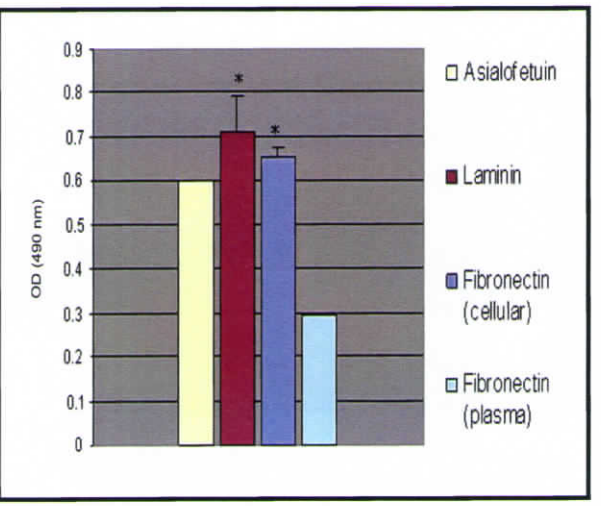


Figure 24. Galectin-1 reactivity with human laminin and fibronectin. Microtiter wells coated with human laminin, fibronectin and asialofetuin (1µg protein/well) were probed with the HRP-conjugated galectin-1 (HPL; 7.5 µg lectin /ml) followed by detection of the bound lectin conjugate with OPD. The values are expressed as optical density (OD) units at 490 nm. Mean + S.D of triplicate trials is shown. Lectin reactivity with laminin and cellular (placental) fibronectin was compared to that with plasma fibronectin by unpaired t-test, $*P < 0.05$. Asialofetuin, a strong ligand for galectin-1, was used for comparing individual galectin-1 responses.

Plasma fibronectin contains about 5% carbohydrate in the form of partially desialylated N-linked biantennary structures, with a small amount of larger oligosaccharide present (Fisher and laine,1979).Cellular fibronectin on the other hand has a similar structure, but contain about 9% carbohydrate with increased galactose and glucosamine (Pande et al., 1981) which explains its higher affinity for galectin-1.

4.4.4. Galectin-1 mediated cross-linking of Lp(a) with fibronectin and laminin: determination by ELISA based assay

Galectin-1 mediated cross linking of Lp(a) with human cellular (placental) fibronectin and laminin was investigated by an ELISA based assay (refer methods for details). Both laminin and fibronectin bound increasing concentrations of Lp(a) in the presence of galectin-1; however, Lp(a) binding by fibronectin was substantially more than laminin both in the presence and absence of galectin-1 at every tested concentration of Lp(a) (Fig.25)

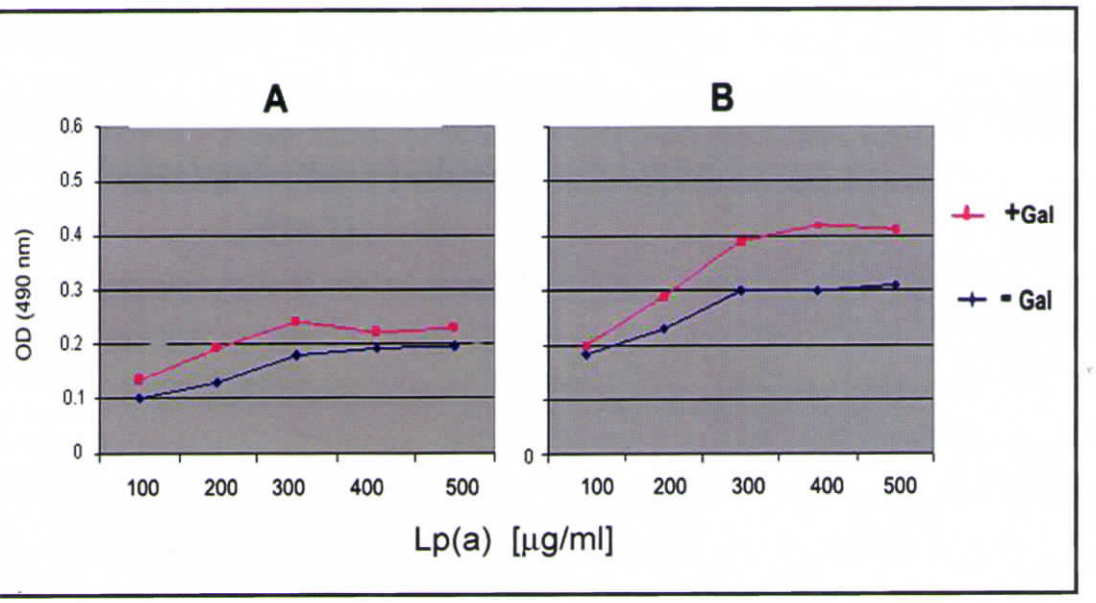


Figure 25.Galectin-1 mediated cross-linking of Lp(a) with fibronectin and laminin: determination by ELISA based assay. Coated human laminin (**A**) or placental fibronectin (**B**) was incubated with a mixture of increasing concentrations of Lp(a) [1 to 500 µg/ml] and human galectin-1 (HPL; at a constant concentration of 600 µg/ml) and probed with anti-Lp(a) and HRP-conjugated second antibody. (Mean + S.D of triplicate trials is shown)

A concentration dependent increase in Lp(a) binding was found only in the case of fibronectin (Fig.25 B),corresponding increase in Lp(a) binding by laminin was not very significant(Fig.25 A). Lp(a) binding by laminin in the presence of galectin-1 seems to have saturated at higher concentrations of Lp(a) [about 300 to 500µg/ml].Laminin compared to fibronectin binds weekly to Lp(a); however, laminin shows more affinity

towards galectin-1 than fibronectin. At saturation, the dimeric galectin-1 may bind more with the laminin glycans than cross linking Lp(a) with laminin since galectin-1 reactivity with human laminin is more than that with Lp(a). On the other hand, fibronectin shows strong binding with Lp(a) even in the absence of galectin-1 (Fig.25B). These observations demonstrate, at least in the case of fibronectin, that galectin-1 can crosslink ECM glycoproteins. Elevated levels of fibronectin (both from plasma and cellular sources), fibrinogen, and fibrin are found colocalized with Lp(a) in early atherosclerotic lesions and in atherosclerotic plaques (Stenman et al., 1980;Walton et al.,1974); binding to these substrates could provide a means of anchoring Lp(a) within developing atheromas, thereby potentiating the atherogenic effects of this lipoprotein.

4.4.5. Galectin-1 mediated cross-linking of Lp(a) with fibronectin: determination by immunoprecipitation with anti-fibronectin antibody followed by western blotting with anti-Lp(a):

Galectin-1 crosslinking Lp(a) with cellular fibronectin was further investigated by an immunoprecipitation based assay. Lp(a) and placental fibronectin were incubated in solution with or without human galectin-1 (HPL) and the complexes that formed between Lp(a) and fibronectin were immunoprecipitated with an antibody directed against fibronectin. The complexes were then subjected to electrophoresis and analyzed for the incorporation of Lp(a) by Western blotting with an antibody directed against Lp(a) [Dako, Denmark].

Lp(a) was incubated at concentrations ranging from 100 to 400 μg/ml [based on the protein content of Lp(a)] with human placental fibronectin and galectin-1(HPL) for 4 hours. The results showed that Lp(a) became cross-linked to fibronectin in a concentration-dependent manner (Fig.26 A). Meaningful amount of Lp(a) was found to bind to fibronectin even in the absence of galectin-1 which were in agreement with the above ELISA data; however, at all experimental concentrations, Lp(a) binding to fibronectin in the presence of galectin-1 was significant compared to that in the absence of galectin-1. Also, with increasing amounts of Lp(a) added to the reaction, there

appeared to be complexes formed of varying molecular weight. This was evident as a "tail" that existed above each band that had been detected by Western blotting (Fig.26 A).

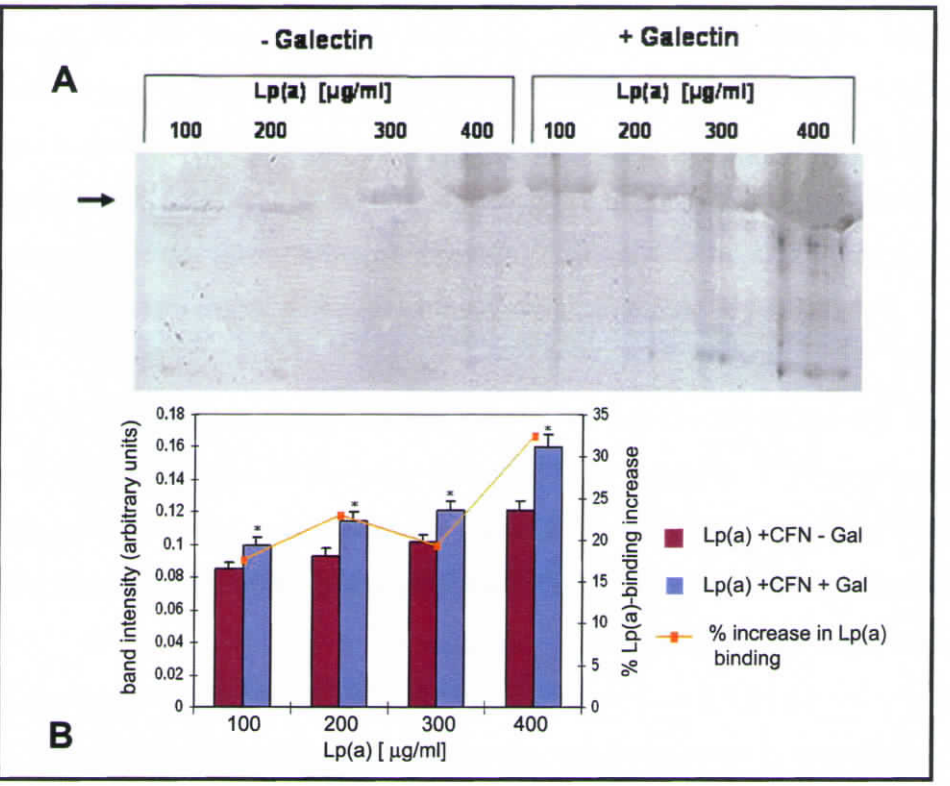


Figure 26. Galectin-1 mediated cross-linking of Lp(a) with fibronectin. **A** complexes formed between Lp(a) and fibronectin in the presence of galectin-1 were retrieved by immunoprecipitation with an anti-fibronectin antibody and then evaluated for incorporation of Lp(a) by western blotting using anti-Lp(a). Fibronectin (100 µg/ml) was incubated with increasing amounts of Lp(a) [100 to 400µg/ml] in the presence or absence of galectin-1. In the presence of galectin-1, Lp(a) became crosslinked to fibronectin in a concentration dependent manner which was significant when compared to that without galectin-1. Arrow indicate Lp(a) immunoreactivity in complexes immunoprecipitated with anti-fibronectin antibody. **B.** Densitometric analysis of Lp(a) cross-linking with fibronectin. Each intensity value represents the mean + S.D of four intensity measurements of a defined area adjusted for background intensity. Lp(a) binding to fibronectin in the presence of galectin-1 was compared to that without galectin-1 by unpaired t-test. *P < 0.05.

Densitometric analysis of the band intensities were carried out using Image J 1.37 software (NIH IMAGE) [Fig.26B]. At the highest tested concentration of Lp(a) [400 µg/ml], there was approximately 32% increase in Lp(a) binding in the presence of galectin-1 over that in the absence of galectin-1.These results indicate that galectin-1 mediates crosslinking between Lp(a) and fibronectin.

Mechanisms for Lp(a) retention in the vascular extracellular matrix is lacking in literature. The present study have already provided substantial evidences for a galectin-1 mediated Lp(a)retention: surface expressed or membrane bound galectin-1 in IMA tissue sections as well as HUVECs were shown to bind Lp(a) *in situ.* Galectin-1 secreted into the ECM by vascular cells also may likely play a role in Lp(a) retention and atherogenesis. In tissues and cell extracts there is some evidence that galectin-1 is not free but associated with its biological partners to form high mass complexes and that the spontaneous inactivation of native galectin-1 in the absence of reducing agents can be prevented if the lectin is bound to glyco-conjugates (Cho and Cummings,1995). Cellular fibronectin is a major galectin-1 ligand and is abundant in the ECM.

Atherogenesis is a complex process in which endothelial cell and smooth muscle cell activation appears to play a central role. Galectin-1 expression is shown to be upregulated upon cell activation (Baum et al.,1995) and also elevated levels of fibronectin are found colocalized with Lp(a) in early atherogenesis (Stenman et al., 1980;Walton et al.,1974). Considerable increase in galectin-1 expression is well documented in cancer and related diseases. It remains to be seen if galectin-1 expression is elevated in atherogenesis. Nevertheless, it is reasonable to assume that galectin-1 may possibly crosslink Lp(a) with cellular fibronectin as also other major glycoproteins of the ECM such as laminin and vitronectin, and contribute to Lp(a) retention in atherogenesis.

Chapter 5

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

5.1. SUMMARY AND CONCLUSIONS

Atherosclerotic cardiovascular diseases which include the CAD are one of the major causes of mortality in the world. It is fast becoming a major killer in India and is predicted to assume epidemic proportions by the year 2020. Atherosclerosis is a complex, multi-factorial disease involving a number of different cell types and mechanisms. Exactly how atherosclerosis begins or what causes it isn't known. The work of many investigators strongly supports the subendothelial retention of atherogenic lipoproteins as the central pathogenic process in atherogenesis originally proposed as the response-to-retention hypothesis of early atherogenesis by Williams and Tabas in 1995.

Lipoprotein(a) or Lp(a) is a type of plasma lipoprotein highly associated with atherosclerotic diseases, stroke and myocardial infarction. Lp(a) is a modified LDL molecule; Lp(a) closely resembles LDL in its content of cholesterol, phospholipid and apo B (apolipoprotein B-100). What makes Lp(a) unique is the disulfide linkage of a specific glycoprotein apolipoprotein(a) or apo (a), varying in mass between 300 and 800 KDa to a single copy of apo B (approximately 500 KDa).

Lp(a) has also been implicated in cholesterol deposition. Lp(a) has been shown to be readily bound and internalized by macrophages, after oxidation or prior stimulation of the cells. Lp(a) is ten-times more atherogenic than LDL and has been detected in the vessel wall, where it appears to be retained more avidly than LDL. The mechanism of accumulation of these apo B- containing lipoproteins [Lp(a) and LDL] within the arterial wall is not very clear. Positively charged domains in apo B of LDL was shown to bind with the negatively charged matrix components, including proteoglycans, collagen and fibronectin. However, considerable gaps still remain in our understanding of how Lp(a) accumulate in atherosclerotic vessels. The proteoglycan binding site in apo B is masked in Lp(a) as suggested by Khalil et al.(2004) and apo(a) does not have stretches of positively charged residues that would predict interaction with the proteoglycans. Additionally, it is not known as to what are the molecular mechanisms responsible for the binding avidity of Lp(a)[compared to LDL] for the arterial wall. All these suggests the involvement of some unexplored matrix components in the arterial accumulation of Lp(a), some molecules that may preferentially bind Lp(a) over LDL

The diverse structural variations possible in oligosaccharide chains (glycans) attached to cell surface and extracellular proteins are known to mediate numerous biological roles and are implicated in events such as fertilization, development and differentiation, cancer metastasis, host pathogen interactions and apoptosis. Apart from carbohydrate specific antibodies and enzymes, lectins are a major group of mammalian sugar-binding proteins. Among galectins (galactose-binding mammalian lectins), galectin-1 is the most abundant in tissues and is present on endothelial and smooth muscle cell surfaces of the human artery. The role of galectin-1 in the pathogenesis of malignant diseases including cancer is well documented. However, galectin-1 is least heard-of in atherosclerosis research with practically no reports except for two reports that highlighted the role of galectin-1 in SMC proliferation (Moiseeva et al., 1999, 2000). The present study was an attempt to elucidate the role of galectin-1, if any, in lipoprotein retention. Glycosylation profiles in Lp(a) and LDL were analyzed using specific lectins and the complementarities of these glycans with the human galectin-1 was analyzed in specific *in vitro* and *in situ* assays.

Human Lp(a) was isolated by sequential ultracentrifugation from serum. Highly purified Lp(a) was required to study its specific glycosylation properties *vis-à-vis* LDL. A new simple method was developed to purify Lp(a) after its isolation from serum (Chellan et al.,2006).The method was based on the fact that Lp(a), LDL and HDL exhibits pre-beta, beta and alpha migration respectively in native polyacrylamide gel electrophoresis (PAGE). Lp(a) was selectively electroeluted after native PAGE of serum Lp(a) isolates using L-proline at 0.1 M concentration to prevent the aggregation of Lp(a) with LDL. The system described in text is simple and can be reproduced in any laboratory with basic instrumentation, while other methods for purification of Lp(a) such as gel filtration or affinity chromatography employs sophisticated detection and fractionating systems to accurately fractionate the closely eluting similar sized Lp(a) and LDL. Pure LDL can also be simultaneously prepared by this method since LDL preparations in the density range 1.05-1.063 g/ml consistently contains Lp(a) as contaminant. Apo(a) and apo B, the glycoprotein components in Lp(a) were then isolated from Lp(a); both these glycoproteins were also used in the studies since it is these glycoproteins that are found in atherosclerotic plaques rather than the intact Lp(a) molecule.

Other than that reported for sialic acids (Tertov et al., 1993), the role of carbohydrate structures on Lp(a) and LDL in the development of atherosclerotic plaques is lacking in literature. An understanding of the types carbohydrate structures on Lp(a) *vis-à-vis* LDL is necessary to elucidate the role of galectin, if any, in lipoprotein retention. The types of sugars present on Lp(a), LDL, and their glycoprotein components, apo(a) and apo B, were analyzed by enzyme linked lectin assay (ELLA). Microtiter well coated Lp(a), LDL apo(a) and apo B were analyzed using HRP-conjugated lectins. N-linked sugar specific lectins, con A and WGA; O-linked sugar specific PNA, jacalin and HAA; and RCA-1 reactive to both O and N-linked sugars were used in the study. The findings of the study were that both Lp(a) and LDL [also apo(a) and apo B] possessed N-linked sugars. Lp(a) [also apo(a)] was rich in O-linked sugars, especially the T-antigens, as evidenced by the high reactivity with the typically T-antigen specific lectins PNA, jacalin and HAA. The O-specific lectins showed a highly significant response with Lp(a)

compared to a much poor response with LDL. A significant finding of the assays was that LDL [also apo B] appeared to possess O-linked sugars which are hitherto unreported in literature. To support the ELLA data and to further confirm the T-antigen profiles in Lp(a) and LDL, dot blot assays were carried out. Lp(a) and LDL sugar chains were cleaved in sequential exoglycosidase digestions and also by alkaline β-elimination reaction. The O-linked sugars were then analyzed for T-antigen structures using HRP-conjugated PNA, jacalin and HAA. These experiments confirmed that Lp(a) compared to LDL was rich in T-antigens.

Studies were then carried out to analyze the complementarities of the T-antigen structures on Lp(a) and LDL with galectin-1 and to investigate whether endogenous galectin-1 binds Lp(a) *in situ*. Galectin-1 was isolated from the human placenta. The differential response of human galectin-1 with Lp(a) and LDL, and the T-antigen specificity of the binding was analyzed by enzyme-linked lectin assay and dot blotting. Galectin-1 reactivity with Lp(a) and LDL was found to be exclusively T-antigen specific and galectin-1 showed a significantly increased sugar- specific response towards Lp(a) compared to LDL. More importantly, galectin-1 showed increased reactivity with desialylated Lp(a) and LDL. The higher *in vitro* reactivity of galectin-1 with Lp(a) compared to that with LDL may be relevant to atherogenesis if we are able to extent the results to the *in vivo* scenario as well. Nevertheless, the observation is novel and strong enough to support a galectin-1 mediated selective accumulation of Lp(a) *vis-à-vis* LDL in the arterial plaques. Additionally, desialylation of the lipoproteins increased galectin-1 binding which may be of added significance since the desialylated lipoprotein are reportedly more atherogenic. Another observation which is noteworthy in this context is that, galectin-1 response with apo(a) was more than that with the native Lp(a). It is to be read together with the fact that the structural components apo(a) and apo B is found in abundance in the arterial plaques rather than the native Lp(a) itself.

Lp(a) reportedly binds extra cellular matrix components such as proteoglycans, fibronectin and other structural elements. These apo B-containing lipoproteins bind weakly to proteoglycans in physiological ionic-strength environments suggesting that

other matrix components may be involved in the efficient arterial retention of lipoproteins in atherogenesis. Galectin-1 is expressed by most tissue types; it is expressed abundantly by the smooth muscle cells and the endothelium of the arterial wall. Lectin binding is purely carbohydrate dependent, therefore, the present study investigated whether endogenous galectin-1 in human internal mammary artery (IMA) histological sections binds Lp(a) *in situ* sugar specifically. The smooth muscle tissue in IMA histological sections showed significant expression of galectin-1 which is indicative of the relative abundance of galectin-1 in the arterial tissues. Extensive Lp(a) binding was observed when serial sections of the tissue were incubated with pure Lp(a) and immunoanalysed for Lp(a) binding. More importantly, much of the Lp(a) failed to bind to the tissues when the sections were pretreated with lactose, a galectin-1 inhibitor, clearly demonstrating the involvement of tissue galectin-1 in the Lp(a) binding. The extracellular matrix in and around the intima may be rich in galectin-1 since galectin-1 is also secreted to the extracellular matrix by the cells. Consequently, galectin-1, which is ubiquitous and abundant in the arterial histological organization, may possibly contribute to the accumulation or retention of Lp(a) within these tissues. Lectin binding is exclusively sugar specific, independent of charge or other physiological conditions.

The endothelial layer forms the first barrier between arterial cells and the circulating lipoproteins. Localization of membrane bound galectin-1 in human umbilical vein endothelial cells (HUVEC) and its sugar specific *in situ* binding of Lp(a) was analyzed by immunoflourescence to investigate the possibility of a galectin-1 mediated Lp(a) binding by HUVECs in support for the glycohistochemical analysis data. The expression of galectin was low in normal cells; however, galectin-1 expression was considerably elevated after cell activation with minimally oxidized LDL (MM-LDL).The staining pattern was very dispersive with almost the entire cell surface intensively stained indicating the abundance of membrane bound galectin-1. Substantial Lp(a) binding was observed when the tissue was incubated with pure Lp(a) and immunoanalysed for Lp(a) binding which again was low after lactose pretreatment of the tissue as in the case of the IMA sections. Further more, colocalization of membrane bound galectin-1 and the membrane bound Lp(a) following incubation of the activated cells with Lp(a) was

analyzed by double-staining immunocytochemistry and confocal microscopy. The results demonstrated significant colocalization of cell surface galectin-1 and the incubated Lp(a). These results demonstrate that HUVEC surface galectin-1 binds Lp(a) *in situ*. Significant Lp(a) binding was seen only in cells activated with MM-LDL. This is to be read with the significant surface expression of galectin-1 on cell activation and the fact that activated endothelium induces cellular inflammatory responses leading to atherogenesis

Endothelial cells form the gateway barrier for circulating lipoproteins seeking the internal arterial milieu. Lp(a) presumably traverses the endothelium through some receptor independent mechanisms. Galectin-1 in the endothelium may possibly bind Lp(a); extensive galectin-1 expression in the endothelium possibly may lead to a concentration of Lp(a) and favor its transendothelial transport. Endothelial cell activation is a primary event in atherogenesis mediated by proinflammatory agents. Galectin-1 expression is shown to be up regulated after cell activation by inflammatory agents. Increased galectin-1 expression, therefore, may be a consequence in atherogenesis that may augment the Lp(a) binding by these cells.

The biological role of galectin-1 in the cardiovascular system is largely unknown. However, it has been shown to be involved in vascular smooth muscle cell proliferation, which is a key step in the development of atherosclerosis. The present studies demonstrate compelling evidences for a T-antigen specific *in vitro* or *in situ* Lp(a)-binding by human galectin-1. Based on these observations, it is reasonable to assume that specific carbohydrate determinants (T-antigens) on Lp(a) and LDL may initiate a pathogenic association with cell surface or extracellular matrix galectin-1 in early atherogenesis.

The extracellular matrix (ECM) is composed of a complex array of glycoproteins and glycosaminoglycans. The major adhesive macromolecules include fibronectin, vitronectin, laminin, and multiple types of collagen. Lp(a) reportedly binds weakly to laminin and not at all to vitronectin or collagen. On the other hand, Lp(a) has been shown to bind tightly to fibronectin and Lp(a) binding to fibronectin was lysine independent

which was contradictory to the characteristic lysine binding property of apo(a). Galectin-1 is present both extracellularly and intracellularly. Galactin-1 is secreted by the cells into the matrix where it may be associated with its natural ligands. Laminin and fibronectin are two ECM proteins proposed as the main receptors for galectin-1. The present study, already had reported the *in vitro* interaction of human galectin-1 and Lp(a) (Chellan et al., in press), which is one of the few reports of an apo(a)-substrate interaction that is not lysine-dependent. The possibility of a galectin-1 mediated crosslinking of human Lp(a) with ECM proteins, laminin and fibronectin, which may likely be a mechanism for the subendothelial retention of Lp(a) in atherogenesis was investigated. Galectin-1 mediated cross-linking of Lp(a) with cellular fibronectin and laminin was determined by an ELISA based assay. Essentially, Lp(a) binding by laminin and fibronectin in the presence or absence of human galectin-1 was compared. Both laminin and fibronectin bound more of Lp(a) in the presence of galectin-1; however, Lp(a) binding by fibronectin was substantially more than that by laminin. A concentration dependent increase in Lp(a) binding was found only in the case of fibronectin and corresponding increase in Lp(a) binding by laminin was not very significant.

Galectin-1 crosslinking Lp(a) with cellular fibronectin was further investigated by an immunoprecipitation based assay. Lp(a) and placental fibronectin were incubated in solution with or without human galectin-1 and the complexes that formed between Lp(a) and fibronectin were immunoprecipitated with an antibody directed against fibronectin. The complexes were then subjected to electrophoresis and analyzed for the incorporation of Lp(a) by Western blotting with an antibody directed against Lp(a). The results showed that Lp(a) became cross-linked to fibronectin in the presence of galectin-1 in a concentration-dependent manner which were in agreement with the above ELISA data.

Cellular fibronectin is a major galectin-1 ligand and is abundant in the ECM. In tissues and cell extracts there is some evidence that galectin-1 is not free but associated with its biological partners to form high mass complexes and that the spontaneous inactivation of native galectin-1 in the absence of reducing agents can be prevented if the lectin is bound to glyco-conjugates. Galectin-1 expression is shown to be upregulated

upon cell activation and also elevated levels of fibronectin are found colocalized with Lp(a) in early atherogenesis. Atherogenesis is a complex process in which endothelial cell and smooth muscle cell activation appears to play a central role. Considerable increase in galectin-1 expression is well documented in cancer and related diseases. However, it remains to be seen if galectin-1 expression is elevated in atherogenesis. Nevertheless, it is reasonable to assume that galectin-1 may possibly crosslink Lp(a) with cellular fibronectin as also other major glycoproteins of the ECM such as laminin and vitronectin, and contribute to Lp(a) retention in atherogenesis.

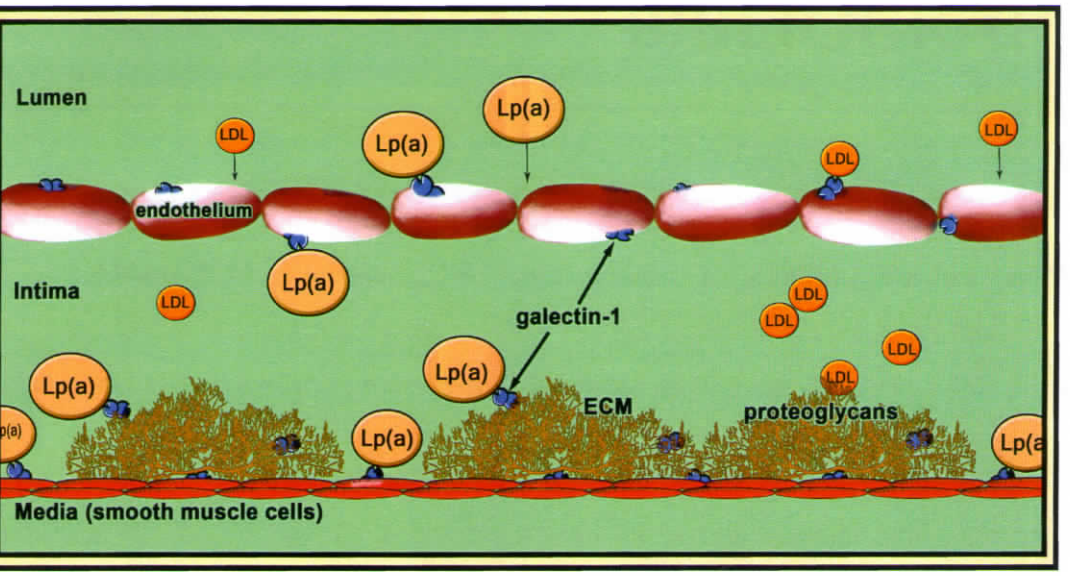
Figure 27 shows the schematic summary of the proposed hypothesis of this PhD study which envisages a potential role for arterial tissue galectin-1 (endothelial, smooth muscle cell, and the extracellular matrix galectin-1) in binding and retention of lipoprotein(a) in atherogenesis

5.2. FUTURE DIRECTIONS

Mechanisms for Lp(a) retention in the vascular extracellular matrix is lacking in literature. The present studies demonstrated compelling evidences for a T-antigen specific *in vitro* or *in situ* Lp(a) binding by human galectin-1. The higher *in vitro* reactivity of galectin-1 with Lp(a) compared to that with LDL may be relevant to atherogenesis if we are able to extent the results to the *in vivo* scenario as well. Additional investigation will be required to analyze in greater detail, the role of galectin-1 in Lp(a) accumulation and to delineate the biological significance of this interaction in atherogenesis.

The strength of a possible physiological association of Lp(a) and LDL with galectin-1 has to be assessed. Quantitative analysis or the determination of equilibrium dissociation constant of human galectin-1 binding to Lp(a) and LDL may provide some insights. Further, localization of galectin-1 in atherosclerotic plaques and demonstration of its sugar specific *in situ* binding to Lp(a) vis-à-vis LDL will be testimony to the

therogenic role of galectin-1. Additionally, the function of galectin-1 and other galectins n other cell types namely, the SMC and macrophages, is unknown with regard to a role n atherogenesis. Lp(a) internalization by macrophages is another unknown phenomenon. The role of specific receptors and or galectin-1, if any, in the phenomenon may also be ddressed in future studies.



igure 27. Schematic summary of the PhD hypothesis envisaging a potential role for arterial ssue galectin-1 in the selective lipoprotein(a) retention during atherogenesis: The endothelial alectin-1 may favorably bind Lp(a) more than it does for LDL and may help in the ansendothelial transport of galectin into the intima of the artery. Inside the intima, ECM alectin-1 may bind Lp(a) or ECM galectin-1 may crosslink Lp(a) with ECM glycoproteins such s fibronectin and laminin. Underlying the intima is the smooth muscle cell layer (SMC). SMC alectin-1 also may favorably bind Lp(a) over LDL. Thus galectin-1, which is ubiquitous and oundant in the arterial histological organization, may possibly contribute to the accumulation or etention of Lp(a) within these tissues during atherogenesis.

Chapter 6

BIBLIOGRAPHY

Abbott,W.M., Hounsell,E.F., and Feizi, T. (1988). Further studies of oligosaccharide recognition by the soluble 13 kDa lectin of bovine heart muscle. Ability to accommodate the blood-group-H and -B-related sequences, *Biochem J* 252, 283-287.

Abbott, W. M., Mellor, A., Edwards, Y., and Feizi, T. (1989). Soluble bovine galactose-binding lectin. cDNA cloning reveals the complete amino acid sequence and an antigenic relationship with the major encephalitogenic domain of myelin basic protein, *Biochem J* 259, 283-290.

Abe, A., and Noma, A. (1992). Studies on apolipoprotein(a) phenotypes. Part 1. Phenotype frequencies in a healthy Japanese population, *Atherosclerosis* 96, 1-8.

Agil, A., Fuller, C. J., and Jialal, I. (1995). Susceptibility of plasma to ferrous iron/hydrogen peroxide-mediated oxidation: demonstration of a possible Fenton reaction, *Clin Chem* 41, 220-225.

Ahmad, N., Gabius, H. J., Sabesan, S., Oscarson, S., and Brewer, C. F. (2004). Thermodynamic binding studies of bivalent oligosaccharides to galectin-1, galectin-3, and the carbohydrate recognition domain of galectin-3, *Glycobiology* 14, 817-825.

Akazawa, C., Nakamura, Y., Sango, K., Horie, H., and Kohsaka, S. (2004).

Distribution of the galectin-1 mRNA in the rat nervous system: its transient upregulation in rat facial motor neurons after facial nerve axotomy, *Neuroscience* 125, 171-178.

Albers, J. J., and Hazzard, W. R. (1974) Immunochemical quantification of human plasma Lp(a) lipoprotein, *Lipids* 9, 15-26

Albers, J. J., Kennedy, H., and Marcovina, S. M. (1996). Evidence that Lp[a] contains one molecule of apo[a] and one molecule of apoB: evaluation of amino acid analysis data, *J Lipid Res* 37, 192-196.

Albers, J. J., Marcovina, S. M., and Lodge, M. S. (1990). The unique lipoprotein(a): properties and immunochemical measurement, *Clin Chem* 36, 2019-2026.

Albers, J. J., Taggart, H. M., Applebaum, D., Haffner, S., Chesnut, C. H., and Hazzard, W. R. (1984). Reduction of lecithin-cholesterol acyltransferase, apolipoprotein D and the Lp(a) lipoprotein with the anabolic steroid Stanozolol, *Biochim Biophys Acta* 795, 293-296

Allen, A.C. (1999). Methodological approaches to the analysis of IgA1 O-glycosylation in IgA nephropathy, *J Nephrol* 12, 76-84.

Allen, H. J., Cywinski, M., Palmberg, R., and DiCioccio, R. A. (1987). Comparative analysis of galactoside-binding lectins isolated from mammalian spleens, *Arch Biochem Biophys* 256, 523-533.

Andersen, H., Jensen, O. N., Moiseeva, E. P., and Eriksen, E. F. (2003). A proteome study of secreted prostatic factors affecting osteoblastic activity: galectin-1 is involved in differentiation of human bone marrow stromal cells, *J Bone Miner Res* 18, 195-203.

Anitschokow, N. (1933). Experimental arteriosclerosis in animals, in *Arteriosclerosis: A Survey of the Problem.* (Cowdry, E. V., Ed.), pp 271-322., New York: Macmillan

Appukuttan, P. S. (2002). Terminal alpha-linked galactose rather than N-acetyl lactosamine is ligand for bovine heart galectin-1 in N-linked oligosaccharides of glycoproteins, *J Mol Recognit 15*, 180-187.

Appukuttan, P. S., Surolia, A., and Bachhawat, B. K. (1977). Isolation of two galactose binding proteins from Ricinus communis by affinity chromatography, *Indian J Biochem Biophys* 14 382-384.

Armstrong, V. W., Cremer, P., Eberle, E., Manke, A., Schulze, F., Wieland, H., Kreuzer, H., and Seidel, D. (1986). The association between serum Lp(a) concentrations and angiographically assessed coronary atherosclerosis. Dependence on serum LDL levels, *Atherosclerosis* 62, 249-257.

Armstrong, V. W., Harrach, B., Robenek, H., Helmhold, M., Walli, A. K., and Seidel, D. (1990). Heterogeneity of human lipoprotein Lp[a]: cytochemical and biochemical studies on the interaction of two Lp[a] species with the LDL receptor, *Journal of lipid research* 31, 429-441.

Armstrong, V. W., Walli, A. K., and Seidel, D. (1985). Isolation, characterization, and uptake in human fibroblasts of an apo(a)-free lipoprotein obtained on reduction of lipoprotein(a), *J Lipid Res* 26, 1314-1323.

Ascherio, A., Cho, E., Walsh, K., Sacks, F. M., Willett, W. C., and Faruqui, A. (1996). Premature coronary deaths in Asians, *BMJ* 312, 508.

Autio, I., Jaakkola, O., Solakivi, T., and Nikkari, T. (1990). Oxidised low density lipoprotein is chemotactic for arterial smooth muscle cells in culture. *FEBS Lett* 277, 247-249.

Baenziger, J. U.(1985). The role of glycosylation in protein recognition. Warner-Lambert Parke-Davis Award lecture, *The Am J Path* 121, 382-391.

Baron, J. (1827). *The Life of Edward Jenner, M.D.* , London: Henry Colburn.

Barondes, S. H., Cooper, D. N., Gitt, M. A., and Leffler, H. (1994). Galectins. Structure and function of a large family of animal lectins, *J Biol Chem* 269, 20807-20810.

Baum, L. G., Seilhamer, J. J., Pang, M., Levine, W. B., Beynon, D., and Berliner, J. A. (1995). Synthesis of an endogeneous lectin, galectin-1, by human endothelial cells is up-regulated by endothelial cell activation, *Glycoconj J* 12, 63-68.

Bedford, D. E. (1968). Harvey's third circulation. De circulo sanguinis in corde, *Br Med J* 4, 273-277.

Beisiegel, U. (1991). Lipoprotein (a) in the arterial wall, *Curr Opin Lipidol* 2, 317-323.

Beisiegel, U., Niendorf, A., Wolf, K., Reblin, T., and Rath, M. (1990). Lipoprotein(a) in the arterial wall, *Eur Heart J* 11 Suppl E, 174-183.

Berg, K. (1963). A New Serum Type System in Man--the Lp System, *Acta pathologica et microbiologica Scandinavica* 59, 369-382.

Berg, K., Dahlen, G., and Frick, M. H. (1974). Lp(a) lipoprotein and pre-beta1-lipoprotein in patients with coronary heart disease, *Clin Genet* 6, 230-235.

Bhatnagar,D., Anand, I., and Durington, P.N.(1995). Coronary risk factors in people from Indian subcontinent in West London and their siblings in India, *Lancet* 345, 405-9.

Bhavanandan,V.P.(1991).Cancer-associated mucins and mucin-type glyco proteins, *Glycobiology* 1, 493-503.

BihariVarga, M., and Vegh, M. (1967). Quantitative studies on the complexes formed between aortic mucopolysaccharides and serum lipoproteins, *Biochim Biophys Acta* 144, 202-210.

BihariVarga, M., Gruber, E., Zechner, R., and Kostner, G. M. (1988). Interaction of lipoprotein Lp(a) and low density lipoprotein with glycosaminoglycans from human aorta., *Arteriosclerosis* 8, 851-857

Bimmermann, A., Boerschmann, C., SchwartzkopfT, W., von Baeyer, H., and Schleicher, J. (1991). Effective therapeutic measures for reducing lipoprotein(a) in patients with dyslipidemia. Lipoprotein(a) reduction with sustained-release bezafibrate, *Curr Ther Res* 49, 635-643.

Bing, R. J. (1992). Atherosclerosis, in *Cardiology: The Evolution of the Science and the Art* (Bing, R. J., Ed.), pp 127-143, Harwood Academic Publishers.

Blaser, C., Kaufmann, M., Muller, C., Zimmermann, C., Wells, V., Mallucci, L., and Pircher, H. (1998). Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells, *Eur J Immunol* 28, 2311-2319.

Boerwinkle, E., Leffert, C. C., Lin, J., Lackner, C., Chiesa, G., and Hobbs, H. H. (1992). Apolipoprotein(a) gene accounts for greater than 90% of the variation in plasma lipoprotein(a)concentrations, *J Clin Invest* 90, 52-60.

Boren, J., Gustafsson, M., Skalen, K., Flood, C., and Innerarity, T. L. (2000). Role of extracellular retention of low density lipoproteins in atherosclerosis, *Curr Opin Lipidol* 11, 451-456.

Boren, J., Lee, I., Taylor, S., and Innerarity, T. L. (1998). Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100, *J Clin Invest* 101, 1084-1093.

Bornstein, P. (1992). Thrombospondins: structure and regulation of expression, *Faseb J* 6, 3290-3299.

Borth, W., Chang, V., Bishop, P., and Harpel, P. C. (1991). Lipoprotein(a) is a substrate for factor XIIIa and tissue transglutaminase, *J Biol Chem* 226, 18149-18153

Bostom, A. G., Gagnon, D. R., Cupples, L. A., Wilson, P. W., Jenner, J. L., Ordovas, J. M., Schaefer, E. J., and Castelli, W. P.(1994). A prospective investigation of elevated lipoprotein (a) detected by electrophoresis and cardiovascular disease in women. The Framingham Heart Study, *Circulation* 90, 1688-1695.

Bottalico, L. A., Keesler, G. A., Fless, G. M., and Tabas, I. (1993). Cholesterol loading of macrophages leads to marked enhancement of native lipoprotein(a) and apoprotein(a) internalization and degradation, *J Biol Chem* 268, 8569-8573.

Brown, M. S., and Goldstein, J. L. (1986). A receptor-mediated pathway for cholesterol homeostasis, *Science* 232, 34 - 47.

Brown, S. A., Morrisett, J., Patsch, J. R., Reeves, R., Gotto, A. M., and Patsch, W. (1991). Influence of short term dietary cholesterol and fat on human plasma Lp(a) and LDL levels, *J Lipid Res* 32, 1281-1289.

Camby, I., Belot, N., Lefranc, F., Sadeghi, N., de Launoit, Y., Kaltner, H., Musette, S., Darro, F., Danguy, A., Salmon, I., Gabius, H. J., and Kiss, R. (2002). Galectin-1 modulates human glioblastoma cell migration into the brain through modifications to the actin cytoskeleton and levels of expression of small GTPases, *Journal of neuropathology and experimental neurology* 61, 585-596.

Camby, I., Le Mercier, M., Lefranc, F., and Kiss, R. (2006). Galectin-1: a small protein with major functions, *Glycobiology* 16, 137R-157R.

Camejo, G., HurtCamejo, E., and Bondjers, G. (1990). Effect of proteoglycans on lipoprotein-cell interactions: possible contribution to atherogenesis, *Curr Opin Lipidol* 1, 431-436.

Camejo, G., HurtCamejo, E., Olsson, U., and Bondjers, G. (1993a). Proteoglycans and lipoproteins in atherosclerosis, *Curr Opin Lipidol* 4, 385-391.

Camejo, G., Rosengren, B., HurtCamejo, E., and Bondjers, G. (1993 b). Binding of low density lipoproteins by proteoglycans synthesized by proliferating and quiescent

human arterial smooth muscle cells. , *J Biol Chem 268*, 14131-14137

Camejo, G., Hurt-Camejo, E., Wiklund, O., and Bondjers, G. (1998). Association of apo B lipoproteins with arterial proteoglycans: pathological significance and molecular basis, *Atherosclerosis* 139, 205-222.

Camejo, G., Olsson, U., Hurt-Camejo, E., Baharamian, N., and Bondjers, G. (2002). The extracellular matrix on atherogenesis and diabetes-associated vascular disease, *Atheroscler Suppl* 3, 3-9.

Capron, L. (1989). Pathogenesis of atherosclerosis: an update on the three main theories, *Ann Cardiol Angeiol (Paris)* 38, 631-634.

Cardoso, L. E., and Mourao, P. A. (1994). Glycosaminoglycan fractions from human arteries presenting diverse susceptibilities to atherosclerosis have different binding affinities to plasma LDL, *Arterioscler Thromb* 14, 115-124.

Carew, T. E., Pittman, R. C., Marchand, E. R., and Steinberg, D. (1984). Measurement in vivo of irreversible degradation of low density lipoprotein in the rabbit aorta: predominance of intimal degradation., *Arteriosclerosis* 4, 214-224.

Carlson, L. A., Hamsten, A., and Asplund, A. (1989). Pronounced lowering of serum levels of lipoprotein Lp(a) in hyperlipidaemic subjects treated with nicotinic acid., *J Int Med Res* 226, 271-276.

Carsons, S., Lavietes, B. B., Slomiany, A., Diamond, H. S., and Berkowitz, E. (1987). Carbohydrate heterogeneity of fibronectins. Synovial fluid fibronectin resembles the form secreted by cultured synoviocytes but differs from the plasma form, *J Clin Invest* 80, 1342-1349.

Chait, A., and Wight, T. N. (2000). Interaction of native and modified low-density lipoproteins with extracellular matrix, *Curr Opin Lipidol* 11, 457-463.

Chacko,B.K., and Appukuttan,P.S.(2001). Peanut (Arachis hypogaea) lectin

recognizes alpha-linked galactose, but not N-acetyl lactosamine in N-linked oligosaccharide terminals, *Int J Biol Macromol* 28, 365-71

Chandler, W. L., and Loo, S. (1990). Lipoprotein(a) does not show circadian variations, *Thromb Haemostasis* 63, 151.

Chang, M. Y., Lees, A. M., and Lees, R. S. (1993). Low-density lipoprotein modification and arterial wall accumulation in a rabbit model of atherosclerosis, *Biochemistry* 32, 8518-8524.

Chellan, B., Appukuttan, P. S., and Jayakumari, N. (2006). Electroelution of lipoprotein(a) [Lp(a)] from native polyacrylamide gels: a new, simple method to purify Lp(a), *J Biochem Biophys meth* 68, 43-53.

Chellan, B., Narayani, J., and Appukuttan, P. S. (2007). Galectin-1, an endogenous lectin produced by arterial cells, binds lipoprotein(a) [Lp(a)] in situ : Relevance to atherogenesis., *Exp Mol Path* . In press.

Cheng, L., Tachibana, K., Iwasaki, H., Kameyama, A., Zhang, Y., Kubota, T., Hiruma, T., Tachibana, K., Kudo, T., Guo, J. M., and Narimatsu, H. (2004). Characterization of a novel human UDP-GalNAc transferase, pp-GalNAc-T15, *FEBS letters* 566, 17-24.

Childs, R. A., and Feizi, T. (1979). beta-Galactoside-binding muscle lectins of man and monkey show antigenic cross-reactions with those of bovine origin, *Biochem J* 183, 755-758.

Chisolm, G. M. (1991). Antioxidants and atherosclerosis: a current assessment *Clin Cardiol* 14, I25-I30.

Chisolm, G. M. (1993). Oxidized lipoproteins and leukocyte-endothelial interactions: growing evidence for multiple mechanisms, *Lab Invest* 68, 369-371.

Cho, M., and Cummings, R. D. (1995). Galectin-1, a beta-galactoside-binding

lectin in Chinese hamster ovary cells. I. Physical and chemical characterization, *J Biol Chem* 270, 5198-5206.

Chung, C. D., Patel, V. P., Moran, M., Lewis, L. A., and Miceli, M. C. (2000). Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction, *J Immunol* 165, 3722-3729.

Clausse, N., van den Brule, F., Waltregny, D., Garnier, F., and Castronovo, V. (1999). Galectin-1 expression in prostate tumor-associated capillary endothelial cells is increased by prostate carcinoma cells and modulates heterotypic cell-cell adhesion, *Angiogenesis* 3, 317-325.

Cobbaert, C., and Kesteroot, H. (1992). Serum lipoprotein(a) levels in racially different populations., *Am J Epidemiol* 136, 441-449.

Collins, E., and Carew, T. E. (1997). Residence time of low density lipoprotein in the normal and atherosclerotic rabbit aorta, *Circ Res* 80, 208-218.

Colnot, C., Ripoche, M. A., Scaerou, F., Foulis, D., and Poirier, F. (1996). Galectins in mouse embryogenesis, *Biochem Soc Trans* 24, 141-146.

Colognato, H., and Yurchenco, P. D. (2000). Form and function: the laminin family of heterotrimers, *Dev Dyn* 218, 213-234.

Cook, G. M. (1986). Cell surface carbohydrates: molecules in search of a function?, *J Cell Sci* 4, 45-70.

Cooper, D. N., and Barondes, S. H. (1990). Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism, *J Cell Biol* 110, 1681-1691.

Cooper, D. N., and Barondes, S. H. (1999). God must love galectins; he made so many of them, *Glycobiology* 9, 979-984.

Cooper, D. N., Boulianne, R. P., Charlton, S., Farrell, E. M., Sucher, A., and Lu, B. C. (1997). Fungal galectins, sequence and specificity of two isolectins from Coprinus cinereus, *J Biol Chem* 272, 1514-1521.

Corsetti, J. P., Sterry, J. A., Sparks, J. D., Sparks, C. E., and Wientraub, M. (1991). Effect of weight loss on serum lipoprotein(a) concentrations in an obese population., *Clin Chem* 37, 1191-1195

Craig, W. Y., Neveux, L. M., Palomaki, G. E., Cleveland, M. M., and Haddow, J. E. (1998). Lipoprotein(a) as a risk factor for ischemic heart disease: metaanalysis of prospective studies, *Clin Chem* 44, 2301-2306.

Crook, D., Sidhu, M., Seed, M., O'Donnell, M., and Stevenson, J. C. (1992). Lipoprotein Lp(a) levels are reduced by danazol, an anabolic steroid, *Atherosclerosis* 92, 41-47

Cushing, G. L., Gaubatz, J. W., Guyton, J. R., Weilbaecher, D., and Morrisett, J. D. (1989). Quantitation and localization of apolipoproteins (a) and B in coronary artery bypass vein grafts resected at re-operation, *Arteriosclerosis* 9, 593-603

Cushing, S. D., Berliner, J. A., Schwartz, C. J., and Fogelman, A. M. (1990). Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endotihelial cells and smooth muscle cells, *Proc Natl Acad Sci USA* 87, 5134-5138.

Dahlen, G. H., Guyton, J. R., Attar, M., Farmer, J. A., Kautz, J. A., and Gotto, A. M. (1986). Association of levels of lipoprotein Lp(a) plasmid lipids, and other lipoproteins with coronary artery disease documented by angiography, *Circulation* 74, 758-765

Dahlen, G., Ericson, C., and Berg, K. (1978). In vitro studies of the interaction of isolated Lp (a) lipoprotein and other serum lipoproteins with glycosaminoglycans, *Clin Genet* 14, 36-42.

Dallongeville, J., Selinger, E., Davigon, J., and Lussier, S. (1992). Fish-oil supplementation reduces Lp(a) concentrations in type III dysbetalipoproteinemia, *Clin Chem* 38, 1510-1511.

Dangas, G., Mehran, R., Harpel, P. C., and Fallon, J. T. (1998). Lipoprotein(a) and inflammation in human coronary atheroma: association with the severity of clinical presentation, *J Am Coll Cardiol* 32, 2035-2042.

Daugherty, A., Dunn, J. L., Rateri, D. L., and Heinecke, J. W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions., *J Clin Invest* 94, 437-444.

de Waard, A., Hickman, S., and Kornfeld, S. (1976). Isolation and properties of beta-galactoside binding lectins of calf heart and lung, *J Biol Chem* 251, 7581-7587.

Dean, P. D. G., Johnson, W. S., and Middle, F. A. (1985). *Affinity Chromatography: A Practical Approach,* , IRL Press, Oxford England.

Denhardt, D. T., and Guo, X. (1993). Osteopontin: a protein with diverse functions, *Faseb J* 7, 1475-1482.

DePaola, N., Gimbrone, M. A., Davies, P. F., and Dewey, C. F. (1992). Vascular endothelium responds to fluid shear stress gradients, *Arterioscler Thromb* 12, 1254-1257.

DePergola, G., iorgino, F., Cospite, M. R., Giagulli, V. A., Cignarelli, M., Fern, G., and Giorgino, R. (1993). Relation between sex hormones and serum lipoprotein and lipoprotein(a) concentrations in premenopausal obese women., *Arterioscler Thromb* 13, 675-679

deRijke, Y. B., Jurgens, G., Hessels, E. M., and vanBerkel, T. J. (1992). In vivo fate and scavenger receptor recognition of oxidized lipoprotein(a) isoforms in rats, *J Lipid Res* 33, 1315-1325.

DiCorleto, P. E., and Soyombo, A. A. (1993). The role of the endothelium in

atherogenesis, *Curr Opin Lipidol* 4, 364-372.

Dionysosiou-Asteriou, A., and Katimertzi, M. (1993). Endogenous testosterone and serum apolipoprotein(a) levels, *Atherosclerosis* 100, 123-126

Durrington, P. N., Ishola, M., Hunt, L., Arrol, S., and Bhatnager, D. (1988). Apolipoproteins (a), A1 and B and parental history in men with early onset of ischaemic heart disease, *Lancet* 1, 1070-1073

Edelberg, J. M., and Pizzo, S. V. (1991). Lipoprotein(a) inhibits plasminogen activation in a template-dependent manner, *Blood Coagulation Fibrinol.* 2, 759-764

Edelberg, J. M., and Pizzo, S. V. (1992). Why is lipoprotein(a) relevant to thrombosis? , *Am.J.Clin.Nutr.* 56, 791S-792S.

Edelberg, J. M., Gonzalez-Gronow, M., and Pizzo, S. V. (1989). Lipoprotein(a) inhibits strep-tokinase-mediated activation of human plasminogen., *Biochemistry* 28, 2370-2374.

Eden, S., Wiklund, O., Oscarsson, J., Rosen, T., and Bengtsson, B. A. (1993). Growth hormone treatment of growth hormone deficient adults results in a marked increase in lipoprotein(a) and HDL-cholesterol concentration., *Atheroscler Thromb* 13, 296-301

Egnaczyk, G. F., Pomonis, J. D., Schmidt, J. A., Rogers, S. D., Peters, C., Ghilardi, J. R., Mantyh, P. W., and Maggio, J. E. (2003). Proteomic analysis of the reactive phenotype of astrocytes following endothelin-1 exposure, *Proteomics* 3, 689-698.

Ehnholm, C., Jauhiainen, M., and Metso, J. (1990). Interaction of lipoprotein(a) with fibronectin and its potential role in atherogenesis., *Eur Heart J* 11, 190-195.

Eisenberg, S., Sehayek, E., Olivecrona, T., and Vlodavsky, I. (1992). Lipoprotein lipase enhances binding of lipoproteins to heparan sulfate on cell surfaces and

extracellular matrix., *J Clin Invest* 90, 2013-2021.

Emancipator, K. (1992). Low bias in assayed values of lipoprotein antigens-lipoprotein(a) and apo-lipoproteins A-l and B- in midday postprandial blood specimens compared with morning fasting specimens, *Clin Chem* 38, 431-433.

Enas, E. A. (1998). High rates of CAD in Asian Indians in the United States despite intensive modification of life style. What next?, *Current Science* 74.

Enas, E. A., and Mehta, J. (1995). Malignant coronary artery disease in young Asian Indians: thoughts on pathogenesis, prevention, and therapy. Coronary Artery Disease in Asian Indians (CADI) Study, *Clin Cardiol* 18, 131-135.

Enas, E. A., Dhawan, J., and Petkar, S. (1996). Coronary artery disease in Asian Indians: Lessons learnt and the role of lipoprotein(a)., *Indian Heart J* 49, 25-34.

Enas, E. A., Yusuf, S., and Sharma, S. (1998). Coronary artery disease in South Asians: second meeting of the International Working Group, March 16, 1997, Anaheim, California., *Indian Heart J.* 50, 105-113.

Erichsen, J. E. (1844). *Observations on Aneurism Selected from the Works of the Principal Writers on that Disease from the Earliest Periods to the Close of the Last Century*, London: Sydenham Society.

Erickson, H. P. (1993). Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions, *Curr Opin Cell Biol* 5, 869-876.

Etingin, O. R., Hajjar, D. P., Hajjar, K. A., and Nachman, R. L. (1991). Lipoprotein(a) regulates piasminogen activator inhibitor-1 expression in endothelial cells: a potential mechanism in thrombogenesis., *J Biol Chem* 266, 2459-2465.

Evanko, S. P., Raines, E. W., Ross, R., and Wight, T. N. (1998). Proteoglycan contribution in lesions of atherosclerosis depends on lesion severity, mural characteristics, and the proximity of platelet-derived growth and transforming growth

factor-β., *Am J Pathol* 152, 533-546.

Famier, M., Bonnefous, F., Debbas.N, and Irvine, A. (1994). Comparative efficacy and safety of micronized fenofibrate and simvastatin in patients with primary type IIa or IIb hyperlipidemia, *Arch Intern Med 154*, 441-449

Farish, E., Rolton, H. A., Barnes.J.F, and Hart, D. M. (1991). Lipoprotein(a) concentrations in postmenopausal women taking norethisterone, *BMJ* 303, 694-695.

Fieseler, H. G., Armstrong, V. W., Wieland, E., Thiery, J., Schiitz, E., Walli, A. K., and Seidel, D. (1991). Serum Lp(a) concentrations are unaffected by treatment with HMG-CoA reductase inhibitor, Pravastatin: Results of a 2-year investigation *Clin Chim Acta* 204, 291-300

Fisher, S. J., and Laine, R. A. (1979). Carbohydrate structure of the major glycopeptide from human cold-insoluble globulin, *J Supramol Struct* 11, 391-399.

Fless, G. M., and Snyder, M. L. (1996). Quantitation of lipoprotein (a) after lysine-sepharose chromatography and density gradient centrifugation, *Meth Enzymol* 263, 238-251.

Fless,G.M., ZumMallen, M.E, and Scanu. A.M.(1986).Physicochemical properties of apolipoprotein(a) and lipoprotein(a-) derived from the dissociation of human plasma lipoprotein(a), *J Biol Chem* 261, 8712- 718,

Fless, G. M., Pfaffinger, D., and Scanu, A. M. (1989). Relative affinity of human Lp(a), ApoB-100-Apo(a) and Apo(a) for various lipoproteins., *Circulation* 80, 180.

Floren, C. H., Albers, J. J., and Bierman, E. (1981). Uptake of Lp(a) by cultured fibroblasts., *Biochem Biophys Res Commun* 102, 636-639.

Franklin, G. I., Walsh, F. S., and Thompson, E. J. (1980). Endogenous lectins of human muscle, *FEBS Lett* 118, 200-204.

Frings, C. S., Foster, L. B., and Cohen, P. S. (1971). Electrophoretic separation of serum lipoproteins in polyacrylamide gel, *Clin Chem* 17, 111-114.

Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M., and Timpl, R. (1988). Structure and distribution of N-linked oligosaccharide chains on various domains of mouse tumour laminin, *Biochem J* 252, 453-461.

Futterman, L.G., and Lemberg, L(1998). Fifty percent of patients with coronary artery disease do not have any of the conventional risk factors, *Am J Crit Care* 7,240-244.

Fye, W. B. (1986). The delayed diagnosis of acute myocardial infarction: It took half a century, *Circulation* 72, 262-271.

Galvan, M., Tsuboi, S., Fukuda, M., and Baum, L. G. (2000). Expression of a specific glycosyltransferase enzyme regulates T cell death mediated by galectin-1, *J Biol Chem* 275, 16730-16737.

Garner, B., Merry, A. H., Royle, L., Harvey, D. J., Rudd, P. M., and Thillet, J. (2001). Structural elucidation of the N- and O-glycans of human apolipoprotein(a): role of o-glycans in conferring protease resistance, *J Biol Chem* 276, 22200-22208.

Gaubatz, J. W., Hoogeveen, R. C., Hoffman, A. S., Ghazzaly, K. G., Pownall, H. J., Guevara, J., Jr., Koschinsky, M. L., and Morrisett, J. D. (2001). Isolation, quantitation, and characterization of a stable complex formed by Lp[a] binding to triglyceride-rich lipoproteins, *J Lipid Res* 42, 2058-2068.

Gavish, D., Azrolan, N., and Breslow, J. L. (1989). Plasma Lp(a) concentration is inversely correlated with the ratio of kringle IV/ kringle V encoding domains in the apo(a) gene, *J Clin Invest* 84, 2021-2027.

Gaw, A., Boerwinkle, E., Cohen, J. C., and Hobbs, H. H. (1994). Comparative analysis of the apo(a) gene, apo(a) glycoprotein, and plasma concentrations of Lp(a) in three ethnic groups, *J Clin Invest* 93, 2526-2534.

Gayton, J., and Klemp, K. (1996) Development of the lipid-rich core in human atherosclerosis, *Atheroscler Thromb Vasc Biol* 16, 4-11.

Genest, J., Jenner, J. L., McNamara, J. R., Ordovas, J. M., and Silberman, S. R. (1992). Prevalence of lipoprotein(a) excess in coronary artery disease, *Am J Cardiol* 67, 1039-1045

Gerdes, L. U., Schmidt, E. B., Klausen, I. C., Kristensen, S. D., Ernst, E., Faergeman, O., and Dyerberg, J. (1992). Plasma concentration levels of apolipoprotein A-I. apolipoprotein B and lipoprotein(a) in Greenland Inuit (Eskimos), *J Intern Med* 231, 623-625.

Gero, S., Gergely, J., Szerely, L., and Virag, S. (1960). Role of mucoid substances of the aorta in the deposition of lipids., *Nature* 187, 152-153.

Giachelli, C. M., Schwartz, S. M., and Liaw, L. (1995). Molecular and cellular biology of osteopontin: potential role in cardiovascular disease, *Trends Cardiovasc Med* 5, 88-95.

Gillenwater, A., Xu, X. C., el-Naggar, A. K., Clayman, G. L., and Lotan, R. (1996). Expression of galectins in head and neck squamous cell carcinoma, *Head & neck* 18, 422-432.

Gimbrone, M. A., Jr., Cotran, R. S., and Folkman, J. (1974). Human vascular endothelial cells in culture. Growth and DNA synthesis, *J Cell Biol* 60, 673-684.

Gitt, M. A., Colnot, C., Poirier, F., Nani, K. J., Barondes, S. H., and Leffler, H. (1998). Galectin-4 and galectin-6 are two closely related lectins expressed in mouse gastrointestinal tract, *J Biol Chem 273*, 2954-2960.

Gitt, M. A., Wiser, M. F., Leffler, H., Herrmann, J., Xia, Y. R., Massa, S. M., Cooper, D. N., Lusis, A. J., and Barondes, S. H. (1995). Sequence and mapping of galectin-5, a beta-galactoside-binding lectin, found in rat erythrocytes, *J Biol Chem* 270, 5032-5038.

Glinsky, V. V., Glinsky, G. V., Rittenhouse-Olson, K., Huflejt, M. E., Glinskii, O. V., Deutscher, S. L., and Quinn, T. P. (2001). The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium,*Cancer research* 61, 4851-4857.

Gottschalk, A., and Thomas, M. A. (1961). Studies on mucoproteins. V. The significance of N-acetylneuraminic acid for the viscosity of ovine submaxillary gland mucoprotein, *Biochim Biophys Acta* 46, 91-98.

Grainger, D. J., Kirschenlohr, H. L., and Lawn, R. M. (1993). Proliferation of human smooth muscle cells promoted by lipoprotein(a), *Science* 260, 1655-1658.

Gregory, W. L., Game, F. L., Fairer, M., Idle, J. R., Laker, M. F., and James, O. F. (1994). Reduced serum lipoprotein(a) levels in patients with primary biliary cirrhosis, *Atherosclerosis* 105, 43-50.

Gries, A., Malle, E., Wurm, H., and Kostner, G. M. (1990). Influence of dietary fish oils on plasma Lp(a) levels., *Thromb Res* 58, 667-668

Gries, A., Nimpf, J., Nimpf, M., Wurm, H., and Kostner, G. M. (1987). Free and apo B-associated Lpa-specific protein in human serum, *Clin Chim Acta* 164, 93-100.

Grinstead, G. F. (1990). Lipoprotein(a): Review and update, *Fats Life* 4, 1-8.

Guevara, J., Knapp, R. D., Honda, S., Northup, S. R., and Morrisett, J. D. (1992). A structural assessment of the apo(a) protein of human lipoprotein(a), *Proteins* 12, 188-199.

Gupta, G., and Lowe, C. R. (2004). An artificial receptor for glycoproteins, *J Mol Recognit* 17, 218-235.

Gupta, R., and Gupta, V. P. (1996). Meta-analysis of coronary heart disease prevalence in India, *Indian Heart J* 48, 241-245.

Gurakar, A., Hoeg, J. M., Kostner, G., Papadopoulos, N. M., and Brewer, H. B. (1985) Levels of lipoprotein Lp(a) decline with neomycin and niacin treatment, *Atherosclerosis* 57, 293-301.

Guyton, J. R., Dahlen, G. H., Poutsch, W., Kantz, J. A., and Gotto, M. A. (1985). Relationship of plasma lipoprotein(a) levels to race and to apolipoprotein B, *Arteriosclerosis* 5, 265-272.

Haberland, M. E., Fless, G. M., Scanu, A. M., and Fogelman, A. M. (1992). Malondialdehyde modification of lipoprotein(a) produces avid uptake by human monocyte-macrophages, *J Biol Chem* 267, 4143-4151.

Haberland, M. E., Fless, G., Scanu, A. M., and Fogelman, A. M. (1989). Modification of Lp(a) by malondialdehyde leads to avid uptake by human monocyte-macrophages *Circulation* 80, II 63.

Hadari, Y. R., Paz, K., Dekel, R., Mestrovic, T., Accili, D., and Zick, Y. (1995). Galectin-8. A new rat lectin, related to galectin-4., *J Biol Chem* 270, 3447-3453.

Haffner, S. M., Gruber, K. K., Morales, P. A., Hazuda, H. P., Valdez, R. A., Mitchell, B. D., and Stem, M. P. (1992). Lipoprotein(a) concentrations in Mexican Americans and non-Hispanic whites: The San Antonio Heart Study., *Am J Epidemiol* 136, 1060-1068.

Hajjar, K. A., Gavish, D., Breslow, J. L., and Nachman, R. L. (1989). Lipoprotein(a) modulation of endothelial cell surface fibrinolysis and its potential role in atherosclerosis., *Nature* 339, 303-305.

Halliwell, B. (1988). Albumin- an important extracellular antioxidant?, *Biochem Pharmacol* 37, 569-571.

Hannun, Y. A. (1994). The sphingomyelin cycle and the second messenger function of ceramide ., *J Biol Chem* 269, 3125-3128.

Harazono, A., Kawasaki, N., Kawanishi, T., and Hayakawa, T. (2005). Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS, *Glycobiology* 15, 447-462.

Harpel, P. C., Chang, V. T., and Borth.W. (1992). Homocysteine and other sulfhydryl compounds enhance the binding of lipoprotein(a) to fibrin: A potential biochemical link between thrombosis, atherogenesis, and sulfhydryl compound metabolism., *Proc Natl Acad Sci USA* 89, 10193-10197.

Harpel, P. C., Gordon, B. R., and Parker, T. S. (1989). Plasmin catalyzes binding of lipoprotein(a) to immobilized fibrinogen and fibrin., *Proc Natl Acad Sci USA* 86, 3847-3851.

Harris, C. R. S. (1973). *The Heart and Vascular System in Ancient Greek Medicine from Alcmaeon to Galen.*, Oxford.

Harvey, S., Zhang, Y., Landry, F., Miller, C., and Smith, J. W. (2001). Insights into a plasma membrane signature., *Physiological genomics* 5, 129-136.

Havekes, L., Vermeer, B. J., Brugman, T., and Emeis, J. (1981). Binding of Lp(a) to the low density lipoprotein receptor of human fibroblasts., *FEBS Lett* 132, 169-173

Heam, J. A., Demaio, S. J., Roubin, G. S., Hammerstrom.M, and Sgoutas, D. (1990). Predictive value of lipoprotein(a) and other serum lipoproteins in angiographic diagnosis of coronary artery disease., *Am J Cardiol* 66, 1176-1180

Heberden, W. (1772). Some account of a disorder of the breast, *Med Trans Coll Physicians(Lond)* 2, 59-67.

Hedin, U., Bottger, B. A., Forsberg, E., Johansson, S., and Thyberg, J. (1988). Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells., *J Cell Biol* 107, 307-319.

Heinrich, J., Sandkamp, M., Kokott, R., Schulte, H., and Assmann, G. (1991).

Relationship of lipoprotein(a) to variables of coagulation and fibrinolysis in a healthy population., *Clin Chem* 37, 1950-1954.

Helmhold, M., Bigge, J., Muche, R., Mainoo, J., Thiery, J., Seidel, D., and Armstrong, V. (1992). Contribution of the apo(a) phenotype to plasma Lp(a) concentrations shows considerable ethnic variation., *J Lipid Res* 32, 1919-1928.

Henriksson, P., Angelin, B., and Berglund, L. (1992). Hormonal regulation of serum Lp(a) levels., *J Clin Invest* 89, 1166-1171

Hernandez, J. D., Nguyen, J. T., He, J., Wang, W., Ardman, B., Green, J. M., Fukuda, M., and Baum, L. G. (2006). Galectin-1 binds different CD43 glycoforms to cluster CD43 and regulate T cell death., *J Immunol* 177, 5328-5336.

Heyderman, E., Strudley, K., and Richardson, T. C. (1989). *Handbook of experimental immunology*, Vol. 129, Oxford: Blackwell

Hirabayashi, J., and Kasai, K. (1993) The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution, *Glycobiology* 3, 297-304.

Hirabayashi, J., Ayaki, H., Soma, G., and Kasai, K. (1989). Cloning and nucleotide sequence of a full-length cDNA for human 14 kDa beta-galactoside-binding lectin., *Biochim Biophys Acta* 1008, 85-91.

Hirabayashi, J., Ubukata, T., and Kasai, K. (1996). Purification and molecular characterization of a novel 16-kDa galectin from the nematode Caenorhabditis elegans., *J Biol Chem* 271, 2497-2505.

Hoff, H. F., and Wagner, W. D. (1986). Plasma low density lipoprotein accumulation in aortas of hypercholesterolemic swine correlates with modifications in aortic glycosaminoglycan composition., *Atherosclerosis* 61, 231-236.

Horie, H., and Kadoya, T. (2000). Identification of oxidized galectin-1 as an

initial repair regulatory factor after axotomy in peripheral nerves., *Neuroscience research* 38, 131-137.

Horie, H., Inagaki, Y., Sohma, Y., Nozawa, R., Okawa, K., Hasegawa, M., Muramatsu, N., Kawano, H., Horie, M., Koyama, H., Sakai, I., Takeshita, K., Kowada, Y., Takano, M., and Kadoya, T. (1999). Galectin-1 regulates initial axonal growth in peripheral nerves after axotomy., *J Neurosci* 19, 9964-9974.

Hughes, J. T. (1989). Raised serum lipoprotein during treatment with lovastatin., *Lancet* 1, 911

Hunt, L. A., Davidson, S. K., and Golemboski, D. B. (1983). Unusual heterogeneity in the glycosylation of the G protein of the hazelhurst strain of vesicular stomatitis virus., *Arch Biochem Biophys* 226, 347-356.

Ikeo, K., Takahashi, K., and Gojobori, T. (1991). Evolutionary origin of numerous kringles in human and simian apolipoprotein(a)., *FEBS Lett* 287, 146-148.

Ishibashi, S., Goldstein, J. L., Brown, M. S., and Bums, D. K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice., *J Clin Invest* 93, 1885-1893.

Ismail, N. A., Alavi, M. Z., and Moore, S. (1994). Lipoprotein-proteoglycan complexes from injured rabbit aortas accelerate lipoprotein uptake by arterial smooth muscle cells., *Atherosclerosis* 105, 79-87.

Iverius, P. H. (1972). The Interaction between Human Plasma Lipoproteins and Connective Tissue Glycosaminoglycans., *J Biol Chem 247*, 2607-2613.

Jackson, C. L., Raines, E. W., Ross, R., and Reidy, M. A. (1993). Role of endogenous platelet-derived growth factor in arterial smooth muscle cell migration after balloon catheter injury., *Arterioscler Thromb* 13, 1218-1226.

Janus, E. D., Postiglione, A., and Singh, R. B. (1996). The modernization of Asia:

Implications for coronary heart disease., *Circulation* 94, 2671-2673.

Jauhiainen, M., Koskinen, P., Ehnholm, C., Frick, M. H., Manttari, M., Manninen, V., and Huttunen, J. K. (1991 a). Lipoprotein (a) and coronary heart disease risk: a nested case-control study of the Helsinki Heart Study participants., *Atherosclerosis* 89, 59-67.

Jauhiainen, M., Metso, J., Koskinen, P., and Ehnholm,C.(1991b). Characterization of the enzyme activity of human plasma lipoprotein(a) using synthetic peptide substrates., *Biochem J* 274, 491-496.

Jeschke, U., Karsten, U., Wiest, I., Schulze, S., Kuhn, C., Friese, K., and Walzel, H. (2006). Binding of galectin-1 (gal-1) to the Thomsen-Friedenreich (TF) antigen on trophoblast cells and inhibition of proliferation of trophoblast tumor cells in vitro by gal-1 or an anti-TF antibody., *Histochemistry and cell biology* 126, 437-444.

Jeyaprakash, A. A., Katiyar, S., Swaminathan, C. P., Sekar, K., Surolia, A., and Vijayan, M. (2003). Structural basis of the carbohydrate specificities of jacalin: an X-ray and modeling study., *J Mol Biol* 332, 217-228.

Johnson, R. R., McGregor, J. L., Taylor, P. R., and Poston, R. N. (1994). Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques: coexpression with intercellular adhesion molecule-1., *Am J Pathol* 144, 952-961.

Jovicid, A., Ivanisevic, V., and Ivanovic, I. (1993). Lipoprotein(a) in patients with carotid atherosclerosis and ischemic cerebrovascular disorders.,*Atherosclerosis* 98, 59-65.

Jurgens, G., Ashy, A., and Zenker, G. (1989). Raised serum lipoprotein during treatment with lovastatin., *Lancet* 1(8643), 911-912.

Kalant, N., and McCormick, S. (1992). Inhibition by serum components of oxidation and collagen-binding of low density lipoprotein., *Biochim Biophys Acta* 1128, 211-219.

Karadi, I., Kostner, G. M., Cries, A., Romics, L., and Malle, E. (1988). Lipoprotein(a) and plasminogen are immunochemically related., *Biochim Biophys Acta* 960, 91-97.

Keele, K. D. (1952) *Leonardo da Vinci on the Movement of the Heart and Blood.* London: Harvey and Blythe Ltd.

Khalil, M. F., Wagner, W. D., and Goldberg, I. J. (2004). Molecular interactions leading to lipoprotein retention and the initiation of atherosclerosis., *Arterioscler Throm Vasc Biol* 24, 2211-2218.

Kim, J. Q., Song, J. H., Lee, M. M., Park, Y. B., Chung, H. K., Tchai, B. S., and Kim, S. K. (1992). Evaluation of Lp(a) as a risk factor of coronary artery disease in the Korean population., *Ann Clin Biochem* 29, 226-228.

Kopitz, J., von Reitzenstein, C., Andre, S., Kaltner, H., Uhl, J., Ehemann, V., Cantz, M., and Gabius, H. J. (2001). Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3., *J Biol Chem* 276, 35917-35923.

Kopitz, J., von Reitzenstein, C., Burchert, M., Cantz, M., and Gabius, H. J. (1998). Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture., *J Biol Chem* 273, 11205-11211.

Kostner, G. M., and BihariVarga, M. (1990). Is the atherogenicity of Lp(a) caused by its reactivity with proteoglycans?, *Eur Heart J* ll, 184-189.

Kostner, G. M., Gavish, D., Leopold, B., Bolzano, K., Weintraub, M. S., and Breslow, J. L. (1989). HMG-CoA-reductase inhibitors lower LDL cholesterol without reducing Lp(a) levels., *Circulation* 80, 1313-1319.

Kostner, G. M., Ibovnik, A., Holzer, H., and Grillhofer, H. (1999). Preparation of a stable fresh frozen primary lipoprotein[a] (Lp[a]) standard., *J Lipid Res* 40, 2255-2263.

Kostner, M. (1993). Interaction of Lp(a) and apo(a) with liver cells., *Arterioscler Thromb* 13, 1101-1109

Kosugi, K., Morel, D. W., DiCorleto, P. E., and Chisolm, G. M. (1987). Toxicity of oxidized low-density lipoprotein to cultured fibroblasts is selective for S phase of the cell cycle., *J Cell Physiol* 130, 311-320.

Kraft, H. G., Menzel, H. J., Hoppichler, F., Vogel, W., and Utermann, G. (1991). Changes of genetic apolipoprotein phenotypes caused by liver transplantation., *J Clin Invest* 83, 137-142.

Krcuzer, J., Lloyd, M. B., Bok, D., Fless, G. M., and Lusis, A. J. (1994). Lp(a) displays increased accumulation compared to LDL in the murine arterial wall., *Chem Phys Lipids* 67/68, 175-190.

Krempler, F., Kostner, G. M., Bolzano, K., and Sandhofer, F. (1980). Turnover of lipoprotein(a) in man., *J Clin Invest* 65, 1483-1490.

Krempler, F., Kostner, G. M., Haslauer, F., and Bolzano, K. (1983). Studies on the role of specific cell surface receptors in the removal of lipoprotein(a) in man., *J Clin Invest* 71, 1431-1441.

Kumar,G.S., Appukuttan,P.S., and Basu,D.(1982).Purification and characterization of an alpha-galactose binding lectin from jack fruit (Artocarpus integrifolia)., *J Biosci* 4, 257-261.

La Belle, M., and Krauss, R.M.(1990). Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns, *J Lipid Res* 31, 1577–1588.

Labeur,C., DeBacquer,D., DeBacker,G., Vincke,J., Muyldermans, L., Vandekerckhove, Y., Varderstichele, E., and Rosseneu, M. (1992). Plasma lipoprotein(a) values and the severity of coronary heart disease in a large population of patients undergoing coronary angiography., *Clin Chem* 38, 2261-2266.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4., *Nature* 227, 680-685.

Lahm, H., Andre, S., Hoeflich, A., Kaltner, H., Siebert, H. C., Sordat, B., von der Lieth, C. W., Wolf, E., and Gabius, H. J. (2004). Tumor galectinology: insights into the complex network of a family of endogenous lectins., *Glycoconj J* 20, 227-238.

Lawn,R.M.(1992). Lipoprotein (a) in heart disease, *Sci Am* 266, 54-60.

Leerink, C. B., Gimpel, J. A., Kortlandt, W., and Bouma, B. N. (1991). Kinetic analysis of lipoprotein(a) inhibition of plasminogen activation by tissue plasminogen activator in vitro., *Fibrinolysis* 5, 233-238.

Leffler, H., and Barondes, S. H. (1986). Specificity of binding of three soluble rat lung lectins to substituted and unsubstituted mammalian beta-galactosides., *J Biol Chem* 261, 10119-10126.

Leonidas, D. D., Elbert, B. L., Zhou, Z., Leffler, H., Ackerman, S. J., and Acharya, K. R. (1995). Crystal structure of human Charcot-Leyden crystal protein, an eosinophil lysophospholipase, identifies it as a new member of the carbohydrate-binding family of galectins., *Structure* 3, 1379-1393.

Leppanen, A., Stowell, S., Blixt, O., and Cummings, R. D. (2005). Dimeric galectin-1 binds with high affinity to alpha2,3-sialylated and non-sialylated terminal N-acetyllactosamine units on surface-bound extended glycans., *J Biol Chem* 280, 5549-5562.

Levi, G., and Teichberg, V. I. (1981). Isolation and physicochemical characterization of electrolectin, a beta-D-galactoside binding lectin from the electric organ of Electrophorus electricus, *J Biol Chem* 256, 5735-5740.

Levi, G., and Teichberg, V. I. (1982). Antigenic cross-reactivity between electrolectins., *FEBS Lett* 148, 145-148.

Li, H., Cybulsky, M. I., Gimbrone, M. A., and Libby, P. (1993). An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium., *Arterioscler Thromb* 13, 197-204.

Lin, S. J., Hong, C. Y., Chang, M. S., Chiang, B. N., and Chien, S. (1992). Long-term nicotine exposure increases aortic endothelial cell death and enhances transendothelial macromolecular transport in rats., *Arterioscler Thromb* 12, 1305-1312.

Lin, S. J., Jan, K. M., Weinbaum, S., and Chien, S. (1989). Transendothelial transport of low density lipoprotein in association with cell mitosis in rat aorta., *Arteriosclerosis* 9, 230-236.

Lindahl, G., Gersdorf, E., Menzel, H. J., Duba, D., Cleve, H., Humphries, S., and Utermann, G. (1989). The gene for the Lp(a)-specific glycoprotein is closely linked to the gene for plasminogen on chromosome 6., *Hum Genet* 81, 149-152.

Linden, T., Bondjers, G., Bergstrand, R., and Wiklund, O. (1989). Affinity of LDL to a human arterial proteoglycan among male survivors of jnyocardial infarction., *Eur J Clin Invest* 19, 38-44.

Lis, H., and Sharon, N. (1993). Protein glycosylation. Structural and functional aspects., *Eur J Biochem* 218, 1-27.

Liu, F. T. (2000). Galectins: a new family of regulators of inflammation., *Clin Immunol* 97, 79-88.

Liu, F. T., Hsu, D. K., Zuberi, R. I., Kuwabara, I., Chi, E. Y., and Henderson, W. R., Jr. (1995). Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages., *Am J Path* 147, 1016-1028.

Liu, J., Harpel, P. C., and Gurewich.V. (1994). Fibrin-bound lipoprotein(a) promotes plasminogen binding but inhibits fibrin degradation by plasmin., *Biochemistry* 33, 2554-2560.

Lobsanov, Y. D., Gitt, M. A., Leffler, H., Barondes, S. H., and Rini, J. M. (1993). X-ray crystal structure of the human dimeric S-Lac lectin, L-14-II, in complex with lactose at 2.9-A resolution., *J Biol Chem* 268, 27034-27038.

Lopez-Lucendo, M. F., Solis, D., Andre, S., Hirabayashi, J., Kasai, K., Kaltner, H., Gabius, H. J., and Romero, A. (2004). Growth-regulatory human galectin-1: crystallographic characterisation of the structural changes induced by single-site mutations and their impact on the thermodynamics of ligand binding., *J Mol Biol* 343, 957-970.

Loris, R. (2002). Principles of structures of animal and plant lectins., *Biochim Biophys Acta* 1572, 198-208.

Loscalzo, J. (1990). Lipoprotein(a): A unique risk factor for atherothrombotic disease., *Arteriosclerosis* 9, 672-679.

Loscalzo, J., Weinfeld, M., Fless, G. M., and Scanu, A. M. (1990). Lipoprotein(a), fibrin binding, and plasminogen activation., *Arteriosclerosis* 10, 240-245.

Lowe, J.B. (2001).Glycosylation, immunity, and autoimmunity., *Cell* 104, 809-812.

Lowry,O. H.,Rosebrough, N. J., Farr, A. L.,and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent., *J Biol Chem* 193, 265-275.

Lutomski, D., Joubert-Caron, R., Lefebure, C., Salama, J., Belin, C., Bladier, D., and Caron, M. (1997). Anti-galectin-1 autoantibodies in serum of patients with neurological diseases., *Clin Chim Acta*, 131-138.

Mackie, E. J., Scott-Burden, T., Hahn, A. W., Kern, F., Bernhardt, J., Regenass, S., Weller, A., and Buhler, F. R. (1992). Expression of tenascin by vascular smooth muscle cells. Alterations in hypertensive rats and stimulation by angiotensin II., *Am J Path* 141, 377-388.

Madri, J. A., and Basson, M. D. (1992). Extracellular matrix-cell interactions: dynamic modulators of cell, tissue and organism structure and function., *Lab Invest* 66, 519-521.

Magnaldo, T., Bernerd, F., and Darmon, M. (1995). Galectin-7, a human 14-kDa S-lectin, specifically expressed in keratinocytes and sensitive to retinoic acid., *Dev Biol* 168, 259-271.

Majack, R. A., Goodman, L. V., and Dixit, V. M. (1988). Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation., *J Cell Biol* 106, 415-422.

Malle, E., Steinmetz, A., Kostner, G. M., and Sattler, W. (1994). Identification of glycoprotein IIb as the lipoprotein(a)-binding protein on platelets., *Arterioscler Thromb* 14, 345-352.

Marcovina,S.M., Zhang, Z. H., Gaur, V. P., and Albers, J. J. (1993). Identification of 34 apolipoprotein(a) isoforms: Differential expression of apolipoprotein(a) allelles between American blacks and whites., *Biochem Biophys Res Commun* 191, 1192-1196.

Marshall, R. D. (1972). Glycoproteins, *Ann Rev Biochem* 41, 673-702.

Maserei, J. R., Rouse, I. L., Lynch, W. J., Robertson, K., Vandongen, R., and Beilin, L. J. (1984). Effects of a lacto-ovo vegetarian diet on serum concentrations of cholesterol, triglyceride, HDL-C, HDL2-C, HDL3-C, apoprotein B and Lp(a)., *Am J Clin Nutr* 40, 468-479

Matsumoto, R., Matsumoto, H., Seki, M., Hata, M., Asano, Y., Kanegasaki, S., Stevens, R. L., and Hirashima, M. (1998). Human ecalectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes., *J Biol Chem* 273, 16976-16984.

McLean, J. W., Tomlinson, J. E., Kuang, W. J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M., and Lawn, R. M. (1987). cDNA sequence of human

apolipoprotein(a) is homologous to plasminogen., *Nature* 300, 132-137.

McMurray, H. F., Parthasarathy, S., and Steinberg, D. (1993). Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes., *J Clin Invest* 92, 1004-1008.

Mel'nichenko, A. A., Tertov, V. V., Ivanova, O. A., Aksenov, D. V., Sobenin, I. A., Popov, E. V., Kaplun, V. V., Suprun, I. V., Panasenko, O. M., and Orekhov, A. N. (2005). Desialylation decreases the resistance of apo B-containing lipoproteins to aggregation and increases their atherogenic potential., *Bull Exp Biol Med* 140, 51-54.

Mensink, R. P., Zock, P. L., Katan, M. B., and Homstra, G. (1992). Effect of dietary cis and trans-fatty acids on serum lipoprotein(a) levels in humans., *J Lipid Res* 33, 1493-1501

Merkle, R. K., and Cummings, R. D. (1987). Relationship of the terminal sequences to the length of poly-N-acetyllactosamine chains in asparagine-linked oligosaccharides from the mouse lymphoma cell line BW5147. Immobilized tomato lectin interacts with high affinity with glycopeptides containing long poly-N-acetyllactosamine chains., *J Biol Chem* 262, 8179-8189.

Merrilees, M. J., Campbell, J. H., Spanidis, E., and Campbell, G. R. (1990). Glycosaminoglycan synthesis by smooth muscle cells of differing phenotype and their response to endothelial cell conditioned medium., *Atherosclerosis* 81, 245-254.

Miles, L. A., Fless, G. M., Levin, E. G., Scanu, A. M., and Plow, E. F. (1989). A potential basis for the thrombotic risks associated with lipoprotein(a), *Nature* 339, 301-305.

Mills, G. L., and Taylaur, C. E. (1971). The distribution and composition of serum lipoproteins in eighteen animals., *Comp Biochem Physiol [B]* 40, 489-501.

Minick, C. R., Stemerman, M. G., and Insull, W. (1977). Effect of regenerated endothelium on lipid accumulation in the arterial wall., *Proc Natl Acad Sct USA* 74, 1724

-1728.

Moiseeva, E. P., Javed, Q., Spring, E. L., and de Bono, D. P. (2000). Galectin 1 is involved in vascular smooth muscle cell proliferation., *Cardiovasc Res* 45, 493-502.

Moiseeva, E. P., Spring, E. L., Baron, J. H., and de Bono, D. P. (1999). Galectin 1 modulates attachment, spreading and migration of cultured vascular smooth muscle cells via interactions with cellular receptors and components of extracellular matrix., *J Vasc Res* 36, 47-58.

Moiseeva, E. P., Williams, B., Goodall, A. H., and Samani, N. J. (2003). Galectin-1 interacts with beta-1 subunit of integrin., *Biochem Biophys Res Commun* 310, 1010-1016.

Mosmann,T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays., *J Immunol Meth* 65, 55-63.

Murai, A., Miyahara, T., Fujimoto, N., Matsuda, M., and Kameyama, M. (1986). Lp(a) lipoprotein as a risk factor for coronary heart disease and cerebral infarction, *Atherosclerosis* 59, 199-204.

Nagel, T., Resnick, N., Atkinson, W. J., Dewey, C. F., and Gimbrone, M. A. (1994). Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells., *J Clin Invest* 94, 885-891.

Naruszewicz, M., Selinger, E., Dufour, R., and Davignon, J. (1992). Probucol protects lipoprotein(a) against oxidative modification., *Metab Clin Exp* 41, 1225-1228

NCEP. (1994). National Cholesterol Education Program. Second Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel II)., *Circulation* 89, 1333-1445.

Newby, A. C. (2000). An overview of the vascular response to injury: a tribute to the late Russell Ross., *Toxicol Lett* 112-113, 519-529.

Nickel, W. (2005). Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells, *Traffic (Copenhagen, Denmark)* 6, 607-614.

Nielsen, L. B. (1999). Atherogenecity of lipoprotein(a) and oxidized low density lipoprotein: insight from in vivo studies of arterial wall influx, degradation and efflux., *Atherosclerosis* 143, 229-243.

Nievelstein, P. F. E. M., Fogelman, A. M., Mottino, G., and Frank, J. S. (1991). Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein: a deep-etch and immunolocalization study of ultrarapidly frozen tissue., *Arterioscler Thromb* 11, 1795-1805.

Nogues, X., Seni, M., Pedro-Botet, J., Rubies-Prat, J., and Vidal-Baraquez, F. (1991). Serum lipoprotein(a) levels in men with peripheral vascular disease., *Angiology* 8, 659-664

Nordestgaard, B. G., and Nielsen, L. B. (1994). Atherosclerosis and arterial influx of lipoproteins., *Curr Opin Lipidol* 5, 252-257.

Norrgard, O., Angquist, K. A., and Dahlen, G. H. (1991). Lp(a) lipoprotein in patients with arterial insufficiency of the lower extremities., *Eur J Vasc Surg* 5, 277-282

Oda, Y., Herrmann, J., Gitt, M. A., Turck, C. W., Burlingame, A. L., Barondes, S. H., and Leffler, H. (1993). Soluble lactose-binding lectin from rat intestine with two different carbohydrate-binding domains in the same peptide chain., *J Biol Chem* 268, 5929-5939.

Offner, H., Celnik, B., Bringman, T. S., Casentini-Borocz, D., Nedwin, G. E., and Vandenbark, A. A. (1990). Recombinant human beta-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis., *J Neuroimmunol* 28, 177-184.

Ogden, A. T., Nunes, I., Ko, K., Wu, S., Hines, C. S., Wang, A. F., Hegde, R. S., and Lang, R. A. (1998). GRIFIN, a novel lens-specific protein related to the galectin

family., *J Biol Chem* 273, 28889-28896.

Ohyama, Y., Hirabayashi, J., Oda, Y., Ohno, S., Kawasaki, H., Suzuki, K., and Kasai, K. (1986). Nucleotide sequence of chick 14K beta-galactoside-binding lectin m RNA., *Biochem Biophys Res Commun* 134, 51-56.

OlinLewis, K., Krauss, R. M., Wight, T. N., and Chait, A. (2002). ApoC-III content of apoB-containing lipoproteins is associated with binding to the vascular proteoglycan biglycan., *J Lipid Res* 43, 1969-1977.

Osler, W. (1889). Rupture of the heart., *Med News* 54, 129-130.

Outenreath, R. L., and Jones, A. L. (1992). Influence of an endogenous lectin substrate on cultured dorsal root ganglion cells., *J Neurocytol* 21, 788-795.

Ozaki, K., Inoue, K., Sato, H., Iida, A., Ohnishi, Y., Sekine, A., Sato, H., Odashiro, K., Nobuyoshi, M., Hori, M., Nakamura, Y., and Tanaka, T. (2004). Functional variation in LGALS2 confers risk of myocardial infarction and regulates lymphotoxin-alpha secretion in vitro., *Nature* 429, 72-75.

Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., Sato, H., Hori, M., Nakamura, Y., and Tanaka, T. (2002). Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction., *Nature genetics* 32, 650-654.

Ozeki, Y., Matsui, T., Yamamoto, Y., Funahashi, M., Hamako, J., and Titani, K. (1995). Tissue fibronectin is an endogenous ligand for galectin-1, *Glycobiology* 5, 255-261.

Pande, H., Corkill, J., Sailor, R., and Shively, J. E. (1981). Comparative structural studies of human plasma and amniotic fluid fibronectins., *Biochem Biophys Res Commun* 101, 265-272.

Papadopoulos, N. M. (1979). Abrnomal lipoprotein patterns in human serum as

determined by agarose gel electrophoresis., *Clin Chem* 25, 1885-1887.

Parra, H. J., Luyeye, J., Bouramoue, C., Demarquilly, C., and Fruchart, J. C. (1987). Black white differences in serum Lp(a) lipoprotein levels., *Clin Chim Acta* 167, 27-31.

Pentikainen, M. O., Oksjoki, R., Oorni, K., and Kovanen, P. T. (2002). Lipoprotein lipase in the arterial wall: linking LDL to the arterial extracellular matrix and much more., *Arterioscler Thromb Vasc Biol* 22, 211-217.

Pentikainen, M. O., Oorni, K., Lassila, R., and Kovanen, P. T. (1997) The proteoglycan decorin links low density lipoproteins with collagen type I.., *J Biol Chem* 272, 7633-7638.

Pepin, J. M., O'Neil, J. A., and Hoff, H. F. (1991). Quantification of apo[a] and apo B in human atherosclerotic lesions., *J Lipid Res* 32, 317-327.

Perillo, N. L., Marcus, M. E., and Baum, L. G. (1998). Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death., *J Mol Med* 76, 402-412.

Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995). Apoptosis of T cells mediated by galectin-1., *Nature* 378, 736-739.

Pfeifer, K., Haasemann, M., Gamulin, V., Bretting, H., Fahrenholz, F., and Muller, W. E. (1993). S-type lectins occur also in invertebrates: high conservation of the carbohydrate recognition domain in the lectin genes from the marine sponge Geodia cydonium, *Glycobiology* 3, 179-184.

Pierce-Cretel, A., Pamblanco, M., Strecker, G., Montreuil, J., and Spik, G. (1981). Heterogeneity of the glycans O-glycosidically linked to the hinge region of secretory immunoglobulins from human milk, *Eur J Biochem* 114, 169-178.

Pillarisetti, S., Paka, L., Berglund, L., and Goldberg, I. J. (1997). Subendothelial retention of lipoprotein (a). Evidence that reduced heparan sulfate promotes lipoprotein

binding to subendothelial matrix., *J Clin Invest* 100, 867-874.

Podrez, E. A., Schmitt, D., Hoff, H. F., and Hazen, S. L. (1999). Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro., *J Clin Invest* 103, 1547-1560.

Quinn, M. T., Parthasarathy, S., Fong, L. G., and Steinberg, D. (1987). Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis., *Proc Natl Acad Sci USA* 84, 2995-2998.

Rabinovich, G. A., Ariel, A., Hershkoviz, R., Hirabayashi, J., Kasai, K. I., and Lider, O. (1999). Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1., *Immunology* 97, 100-106.

Rath, M., Niendorf, A., Reblin, T., Dietel, M., Krebber, H. J., and Beisiegel, U. (1989). Detection and quantification of lipoprotein(a) in the arterial wall of 107 coronary bypass patients., *Arteriosclerosis* 9, 579-592.

Rennick, R. E., Campbell, J. H., and Campbell, G. R. (1988). Vascular smooth muscle phenotype and growth behaviour can be influenced by macrophages in vitro., *Atherosclerosis* 71, 35-43.

Rhoads, G. G., Dahlen, G. H., Berg, K., Morton, N. E., and Dannenberg, A. L. (1986). Lp(a) lipoprotem as a risk factor for myocardial infarction., *JAMA* 256, 2540-2544

Ridker, P. M., Hennekens, C. H., and Stampfer, M. J. (1993). A prospective study of lipoprotein(a) and the risk of myocardial infarction., *JAMA* 270, 2195-2199.

Risau, W., and Lemmon, V. (1988). Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis., *Dev Biol* 125, 441-450.

Ritter, M. M., Siihler, K., Richter, W., and Schwandt, P. (1990). Short and long-term effects of LDL-apheresis on lipoprotein(a) serum levels., *Clin Chim Acta* 195, 9-16.

Romanic, A. M., Arleth, A. J., Willette, R. N., and Ohlstein, E. H. (1998). Factor XIIIa cross-links lipoprotein(a) with fibrinogen and is present in human atherosclerotic lesions, *Circ Res* 83, 264-269.

Rosenfeld, M. E., Butler, S., Curtiss, L. K., and Witztum, J. L. (1993). Abundant expression of apoprotein E by macrophages in human and rabbit atherosclerotic lesions., *Arterioscler Thromb* 13, 1382-1389.

Rosengren, J., Wilhemsen, L., Erikssen, E., Risberg, B., and Wedel, H. (1990). Lipoprotein(a) and coronary heart disease: A prospective case control study in a general population sample of middle aged men., *BMJ* 301, 1248-1251.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s., *Nature* 362, 801-809.

Ross, R., Glomset, J., and Harker,L.(1977). Response to injury and athero genesis., *Am J Pathol* 86, 675-684.

Rubinstein, N., Alvarez, M., Zwirner, N. W., Toscano, M. A., Ilarregui, J. M., Bravo, A., Mordoh, J., Fainboim, L., Podhajcer, O. L., and Rabinovich, G. A. (2004). Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege., *Cancer cell* 5, 241-251.

Rubinstein, N., Ilarregui, J. M., Toscano, M. A., and Rabinovich, G. A. (2004). The role of galectins in the initiation, amplification and resolution of the inflammatory response., *Tissue antigens* 64, 1-12.

Salonen, E. M., Jauhiainen, M., Zardi, L., Vaheri, A., and Ehnholm, C. (1989). Lipoprotein(a) binds to fibronectin and has serine proteinase activity capable of cleaving it., *EMBO J* 8, 4035-4040.

Sandholzer, C., Boerwinkle, E., Saha, N., Tong, M. C., and Utermann, G. (1992). Apolipoprotein(a) phenotypes, Lp(a) concentration and plasma lipid levels in relation to coronary heart disease in a Chinese population: Evidence for the role of the apo(a) gene in coronary heart disease., *J Clin Invest* 89, 1040-1046

Sandholzer, C., Hallman, D. M., Saha, N., Sigurdson, G., Lackner, C., Csaszar, A., Boerwinkle, E., and Utermann.G. (1991). Effects of the apolipoprotein(a) size polymorphism on the lipoprotein(a) concentration in 7 ethnic groups., *Hum Genet* 86, 607-614

Sandison, A. T. (1967). Degenerative vascular disease, in *Diseases in Antiquity: A Survey of the Diseases, Injuries and Surgery of Early Populations* (Brothwell, D., Sandison AT, Ed.), pp 474-488, Springfield, IL.

Sangeetha, S. R., and Appukuttan, P. S. (2005). IgA1 is the premier serum glycoprotein recognized by human galectin-1 since T antigen (Galbeta1-->3GalNAc-) is far superior to non-repeating N-acetyl lactosamine as ligand., *Int J Biol Macromol* 35, 269-276.

Sano, R., Fujino, A., Shimazu, H., Kobayashi, M., Yahata, Y., Ogyuu, A., Suzuki, K., Saito, T., and Inokuchi, H. (1990). Serum Lp(a) lipoprotein concentrations of Japanese patients with coronary heart disease., *Tohoku J. Exp. Med.* 162, 261-267.

Sasaki, T., Hirabayashi, J., Manya, H., Kasai, K., and Endo, T. (2004). Galectin-1 induces astrocyte differentiation, which leads to production of brain-derived neurotrophic factor., *Glycobiology* 14, 357-363.

Saxena, U., Ferguson, E., and Bisgaier, C. L. (1993). Apolipoprotein E modulates low density lipoprotein retention by lipoprotein lipase anchored to the subendothelial matrix., *J Biol Chem* 268, 14812-14819.

Scanu, A. M. (1988). Lipoprotein(a). A potential bridge between the fields of atherosclerosis and thrombosis., *Arch Pathol Lab Med* 112, 1045-1047.

Scanu, A. M., and Fless, G. M. (1990). Lipoprotein (a). Heterogeneity and biological relevance., *J Clin Invest* 85, 1709-1715.

Schachter, H., and Brockhausen, I. (1992). in *Glycoconjugates: Composition, Structure and Function* (Allen, H. H., and Kisailus, E. C., Eds.), p 63, Marcel Dekker, New York.

Schissel, S. L., Williams, K. J., and Tabas, I. (1994). Sphingomyelinase (SMase)-induced LDL aggregation: mechanism and relevance., *Circulation* 90, I-403.

Schurnacher, M., Kessler, A., Meier, A., Wiegert, A., and Wood, W. G (1994) Lipoprotein(a) concentrations in cord and capillary blood from newborns and in serum from in-patient children, adolescents and adults., *Eur J Clin Chem Clin Biochem* 32, 341-347.

Schwarzbauer, J. E (1991). Alternative splicing of fibronectin: three variants, three functions., *Bioessays* 13, 527-533.

Schwenke,D(1995). Selective increase in cholesterol at atherosclerosis-susceptible aortic sites after short-term cholesterol feeding., *Arterioscler Thromb Vasc Biol* 15, 1928-1937.

Schwenke, D. C., and Carew, T. E. (1989,b). Initiation of atherosclerotic lesions in cholesterol-fed rabbits, II: selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries., *Arteriosclerosis* 9, 908-918.

Schwenke, D. C., and Carew, T. E. (1989,a). Initiation of atherosclerotic lesions in cholesterol-fed rabbits, I: focal increases in arterial LDL concentration precede development of fatty streak lesions., *Arteriosclerosis* 9, 895-907.

Schwenke, D. C., and StGlair, R. W. (1993). Influx, efflux, and accumulation of LDL in normal arterial areas and atherosclerotic lesions of white Carneau pigeons with naturally occurring and cholesterol-aggravated aortic atherosclerosis., *Arterioscler Thromb* 13, 1368-1381.

Sharon, N., and Lis, H. (1981). Glycoproteins: Research booming on long-ignored, ubiquitous compounds., *Chem Eng News* 59, 21-44.

Small, D. M (1988). Progression and regression of atherosclerotic lesions: insights from lipid physical biochemistry., *Arteriosclerosis* 8, 103-129.

Smith, A. C., de Wolff, J. F., Molyneux, K., Feehally, J., and Barratt, J. (2006). O-glycosylation of serum IgD in IgA nephropathy., *J Am Soc Nephrol* 17, 1192-1199.

Smith, E. B., and Cochran, S. (1990). Factors influencing the accumulation in fibrous plaques of lipid derived from low density lipoprotein, II: preferential immobilization of lipoprotein (a) (Lp(a))., *Atherosclerosis* 84, 173-181

Smith, E. B., and Crosbie, L. (1991). Does lipoprotein(a) (Lp(a)) compete with plasminogen in human atherosclerotic lesions and thrombi., *Atherosclerosis* 89, 127-136

Smith, E. B., and Staples, E. M. (1980). Distribution of plasma proteins across the human aortic wall: Barrier functions and endothelium and internal elastic lamina., *Atherosclerosis* 37, 579-590.

Smith, T. H. F. (1960). A chronology of atherosclerosis., *Am J Pharmacy* 132, 390-405.

Solomon, J. C., Stoll, M. S., Penfold, P., Abbott, W. M., Childs, R. A., Hanfland, P., and Feizi, T. (1991). Studies of the binding specificity of the soluble 14,000-dalton bovine heart muscle lectin using immobilised glycolipids and neoglycolipids., *Carbohydrate Research* 213, 293-307.

Sorrentino, M. J., Velhauer, C., Eisenbart, J. D., Fless, G., Scanu, A. M., and Feldman, T. (1992) Plasma lipoprotein(a) protein concentration and coronary artery disease in black patients compared with white patients., *Am J Med* 93, 658-662.

Sparrow, C. P., and Olszewski, J. (1993). Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions., *J*

Lipid Res 34, 1219-1228.

Springer, G. F., and Desai, P. R. (1982). Extent of desialation of blood group MM, NN, and MN antigens required for reactivity with human anti-T antibody and *Arachis hypogaea* lectin, *J Biol Chem* 257, 2744-2746.

Stary, H. C., Chandler, A. B., Guyton, J. R., Rosenfeld, M. E., Wagner, W. D., and Wissler, R. W. (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association., *Arterioscler Thromb* 14, 840-856.

Steinberg, D. (1999). At last, direct evidence that lipoxygenases play a role in atherogenesis., *J Clin Invest* 103, 1487-1488.

Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity., *New Eng J Med* 320, 915-924.

Stenman, S., von Smitten, K., and Vaheri, A. (1980). Fibronectin and atherosclerosis., *Acta Medica Scandinavica* 642, 165-170.

Stowell, S. R., Dias-Baruffi, M., Penttila, L., Renkonen, O., Nyame, A. K., and Cummings, R. D. (2004). Human galectin-1 recognition of poly-N-acetyllactosamine and chimeric polysaccharides., *Glycobiology* 14, 157-167.

Symons, A., Cooper, D. N., and Barclay, A. N. (2000). Characterization of the interaction between galectin-1 and lymphocyte glycoproteins CD45 and Thy-1., *Glycobiology* 10, 559-563.

Tabas, I., Xu, S. W., Swenson, T. L., and Williams, K. J. (1993). Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogerric lipoproteins with smooth muscle cells and extracellular matrix: a possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation., *J Biol Chem* 268, 20419-20432.

Tachibana, K., Nakamura, S., Wang, H., Iwasaki, H., Tachibana, K., Maebara, K., Cheng, L., Hirabayashi, J., and Narimatsu, H. (2006). Elucidation of binding specificity of Jacalin toward O-glycosylated peptides: quantitative analysis by frontal affinity chromatography, *Glycobiology* 16, 46-53.

Tavazzi,L(1999).Clinical epidemiology of acute myocardial infarction, *Am Heart J,*138(2 pt 2):S48-S54.

Taylor, K. E., Glagov, S., and Zarins, C. K. (1989). Preservation and structural adaptation of endothelium over experimental foam cell lesions: quantitative ultrastructural study., *Arteriosclerosis* 9, 881-894.

Teichberg, V. I., Silman, I., Beitsch, D. D., and Resheff, G. (1975). A beta-D-galactoside binding protein from electric organ tissue of Electrophorus electricus., *Proc Nat Acad Sci USA* 72, 1383-1387.

Tertov, V. V., Orekhov, A. N., Sobenin, I. A., Morrisett, J. D., Gotto, A. M., Jr., and Guevara, J. G., Jr. (1993). Carbohydrate composition of protein and lipid components in sialic acid-rich and -poor low density lipoproteins from subjects with and without coronary artery disease., *J Lipid Res* 34, 365-375.

Thubrikar, M. J., Keller, A. C., Holloway, P. W., and Nolan, S. P. (1992). Distribution of low density lipoprotein in the branch and non-branch regions of the aorta., *Atherosclerosis* 97, 1-9.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc Nat Acad Sci USA* 76, 4350-4354.

Tyrrell, J., Cooke, T., Reilly, M., Colgan, M., Moore, D., Shanik, D. G., and Berlin, C. (1992). Lipoprotein [Lp(a)] and peripheral vascular disease., *J Intern Med* 232, 349-352.

Utermann, G., and Weber, W. (1983). Protein composition of Lp(a) lipoprotein

from human plasma., *FEBS letters* 154, 357-361.

Utermann, G., Duba, C., and Menzel, H. J. (1988 b) Genetics of the quantitative Lp(a) lipoprotein trait. II. Inheritance of Lp(a) glycoprotein phenotypes., *Hum Genet* 78, 47-50.

Utermann, G., Kraft, H. G., Menzel, H., Hopferwieser, T., and Seitz, C. (1988 a). Genetics of the quantitative Lp(a) lipoprotein trait. 1. Relation of Lp(a)-glycoprotein phenotypes to Lp(a) lipoprotein concentration in plasma., *Hum Genet* 78, 41-46.

van den Brule, F., Califice, S., Garnier, F., Fernandez, P. L., Berchuck, A., and Castronovo, V. (2003). Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin., *Lab Invest* 83, 377-386.

van der Hoek, Y. Y., Sangrar, W., Cote, G. P., and Koschinsky, M. L. (1994). Binding of recombinant apolipoprotein(a) to extracellular matrix proteins., *Arterioscler Thromb Vasc Biol* 14, 1792-1798.

van der Leij, J., van den Berg, A., Blokzijl, T., Harms, G., van Goor, H., Zwiers, P., van Weeghel, R., Poppema, S., and Visser, L. (2004). Dimeric galectin-1 induces IL-10 production in T-lymphocytes: an important tool in the regulation of the immune response., *Journal of Pathology* 204, 511-518.

vanBerkel, T. J., deRijke, Y. B., and Kruijt, J. K. (1991). Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats: recognition by various scavenger receptors on Kupffer and endothelial liver cells., *J Biol Chem* 266, 2282-2289.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct., *Glycobiology* 3, 97-130.

Vas, V., Fajka-Boja, R., Ion, G., Dudics, V., Monostori, E., and Uher, F. (2005). Biphasic effect of recombinant galectin-1 on the growth and death of early hematopoietic

cells., *Stem Cells* 23, 279-287.

Vasile, E., Simionescu, M., and Simionescu, N. (1983). Visualization of the binding, endocytosis, and transcytosis of low density lipoprotein in the arterial endothelium in situ., *J Cell Biol* 96, 1677-1689.

Vessby, B., Kostner, G., Lithell, H., and Thomis, J. (1982). Diverging effects of cholestyramine on apolipoprotein B and lipoprotein Lp(a)., *Atherosclerosis* 44, 61-71.

Virchow, R. (1854). Ortliche Storungen des Kreislaufes, in *Handbuch der Speciellen Pathologie und Therapie* (Virchow, R., Vogel, J, Stiebel, S F S, Ed.), pp 95-270, Erlangen: Ferdinand Enke.

von Wolff, M., Wang, X., Gabius, H. J., and Strowitzki, T. (2005). Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation., *Mol Hum Reprod* 11, 189-194.

Wada, J., and Kanwar, Y. S. (1997). Identification and characterization of galectin-9, a novel beta-galactoside-binding mammalian lectin., *J Biol Chem* 272, 6078-6086.

Wada, M., Ono, S., Kadoya, T., Kawanami, T., Kurita, K., and Kato, T. (2003). Decreased galectin-1 immunoreactivity of the skin in amyotrophic lateral sclerosis., *J Neurol Sci* 208, 67-70.

Wagner, W. D., Salisbury, G. J., and Rowe, H. A. (1986). A proposed structure of chondroitin 6-sultate proteoglycan of human normal and adjacent atherosclerotic plaque., *Arteriosclerosis* 6, 407-417.

Walton, K. W., Hitchens, J., Magnani, H. N., and Khan, M. (1974). A study of methods of identification and estimation of Lp(a) lipoprotein and of its significance in health, hyperlipidaemia and atherosclerosis., *Atherosclerosis* 20, 323-346.

Wang, L., Friess, H., Zhu, Z., Frigeri, L., Zimmermann, A., Korc, M., Berberat, P.

O., and Buchler, M. W. (2000). Galectin-1 and galectin-3 in chronic pancreatitis., *Lab Invest* 80, 1233-1241.

Wang, P.,Mariman, E., Keijer, J., Bouwman, F., Noben, J. P., Robben, J., and Renes, J (2004).Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines., *Cell Mol Life Sci* 61, 2405-2417.

Watanabe, N., Yamada, S., Ishikawa, Y., and Yokoyama, M. (1993). Reduction of plasma lipoprotein(a) by allelestrenol., *Atherosclerosis* 102, 229-230

Watkins, W. M., and Morgan, W. T. (1952). Neutralization of the anti-H agglutinin in eel serum by simple sugars., *Nature* 169, 825-826.

Wells, V., Davies, D., and Mallucci, L. (1999). Cell cycle arrest and induction of apoptosis by beta galactoside binding protein (beta GBP) in human mammary cancer cells. A potential new approach to cancer control., *Eur J Cancer* 35, 978-983.

Whitney, P. L., Powell, J. T., and Sanford, G. L. (1986). Oxidation and chemical modification of lung beta-galactoside-specific lectin., *Biochem J* 238, 683-689.

WHO (Feb,2007). Facts about cardiovascular diseases, in *Fact sheet N°317*.

Wight, T. N., Curwen, K. D., Litrenta, M. M., and Alonso, D. R (1983). Effect of endothelium on glycosaminoglycan accumulation in injured rabbit aorta., *Am J Pathol* 113, 156-164.

Wight, T. N., Raugi, G. J., Mumby, S. M., and Bornstein, P. (1985). Light microscopic immunolocation of thrombospondin in human tissues., *J Histochem Cytochem* 33, 295-302.

Williams, K. J., and Tabas, I. (1995). The response-to-retention hypothesis of early atherogenesis., *Atheroscler Thromb Vasc Biol* 15, 551-561.

Williams, K. J., and Tabas, I.(1998). The response-to-retention hypothesis of

atherogenesis reinforced, *Curr Opin Lipidol* 9, 471-474.

Williams, K. J., Fless, G. M., Snyder, M. L., and Swenson, T. L. (1992). Mechanisms by which lipoprotein lipase alters cellular metabolism of lipoprotein(a), low density lipoprotein, and nascent lipoproteins: roles for low density lipoprotein receptors and heparan sulfate proteoglycans., *J Biol Chem* 267, 13284-13292.

Wissler, R. W. (1991). Morphological characteristics of the developing atherosclerotic plaque: animal studies and studies of lesions from young people., *Atheroscler Rev* 23, 91-103.

Witztum, J. L., and Steinberg, D. (1991). Role of oxidized low density lipoprotein in atherogenesis., *J Clin Invest* 88, 1785-1792.

Wollina, U., Schreiber, G., Gornig, M., Feldrappe, S., Burchert, M., and Gabius, H. J. (1999). Sertoli cell expression of galectin-1 and -3 and accessible binding sites in normal human testis and Sertoli cell only-syndrome., *Histology and histopathology* 14, 779-784.

Wu, A. M., Wu, J. H., Lin, L. H., Lin, S. H., and Liu, J. H. (2003). Binding profile of Artocarpus integrifolia agglutinin (Jacalin)., *Life sciences* 72, 2285-2302.

Xia, J., May, L. F., and Koschinsky, M. L. (2000). Characterization of the basis of lipoprotein [a] lysine-binding heterogeneity., *J Lipid Res* 41, 1578-1584.

Yamada, K. M. (1991). Fibronectin and other cell interactive glycoproteins, in *Cell Biology of Extracellular Matrix* (Hay, E., Ed.) 2 ed., pp 111-146, Plenum Press, New York.

Yashiro, A., O'Neil, J., and Hoff, H. F. (1993). Insoluble complex formation of lipoprotein(a) with low density lipoprotein in the presence of calcium ions., *J Biol Chem* 268, 4709-4715.

Ye,O., Rahman,M.N., Koschinsky,M.L .and Jia,Z (2001). High resolution crystal

structure of apolipoprotein(a) kringle IV type7: insight into ligand binding., *Protein Sci* 10, 1124–1129.

Ye, S. Q., Trieu, V. N., Stiers, D. L., and McConathy, W. J. (1988). Interactions of low density lipoprotein and other apolipoprotein B-containing lipoproteins with lipoprotein(a)., *J Biol Chem* 263, 6337-6343.

YlaHerttuala, S., Sumuvuori,H., Karkola,K., and Nikkari, T. (1986). Glycosamino glycans in normal and atherosclerotic human coronary arteries., *Lab Invest* 54, 402-407.

Zambon, A., Torres, A., Hayden, M. R., and Brunzell, J. D. (1993). Prevention of raised low density lipoprotein cholesterol in a patient with familial hypercholesterolaemia and lipoprotein lipase deficiency., *Lancet* 341, 1119-1121.

Zand, T., Majno, G., Nunnari, J. J., Hoffman, A. H., MacWilliams, B., and Joris, I (1991). Lipid deposition and intimal stress and strain: a study in rats with aortic stenosis., *Am J Pathol* 139, 101-101 113.

Zawadski, Z., Terce, F., Seman, L. J., Theolis, R. T., Breckenridge, W. C., Milne, R. W., and Marcel, Y. L. (1988). The linkage with apolipoprotein(a) in lipoprotein(a) modifies the immunological and functional properties of apolipoprotein B., *Biochemistry* 27, 8474-8481

Zenker, G., Koltringer, P., Bone, G., Niederkorn.K, Pfeiffer, K., and Jurgens, G. (1986). Lipoprotein(a) as a strong indicator for cerebrovascular disease, *Stroke* 17, 942-945.

Zhou, Q., and Cummings, R. D. (1993). L-14 lectin recognition of laminin and its promotion of in vitro cell adhesion., *Arch Biochem Biophys* 300, 6-17.

Zhu,Q., and Bhavanandan, V.P(1995). Analysis of serine/threonine-linked oligosaccharides derived by alkaline-borohydride treatment of mucin glycoproteins electroblotted onto membranes: comparison of the saccharide profiles of the 390 kDa and 350 kDa forms of epitectin., *Glycoconj J* 12, 639-644.

Zilversmit, D. B. (1979). Atherogenesis: a postprandial phenomenon., *Circulation* 60, 473-485.

Zuniga, E., Gruppi, A., Hirabayashi, J., Kasai, K. I., and Rabinovich, G. A. (2001). Regulated expression and effect of galectin-1 on Trypanosoma cruzi-infected macrophages: modulation of microbicidal activity and survival, *Infection and immunity* 69, 6804-6812.

LIST OF PUBLICATIONS

1. **Chellan, B.**, Narayani, J., and Appukuttan, P. S. (2007). Galectin-1, an endogenous lectin produced by arterial cells, binds lipoprotein(a) [Lp(a)] *in situ* : Relevance to atherogenesis., *Exp Mol Path* . In press.

2. **Chellan, B.**, Appukuttan, P. S., and Jayakumari, N. (2006). Electroelution of lipoprotein(a) [Lp(a)] from native polyacrylamide gels: a new, simple method to purify Lp(a), *J Biochem Biophys meth* 68, 43-53.