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# Characterization of lipoprotein-proteoglycan complexes in balloon catheter deendothelialized aorta of rabbits and the uptake of these complexes by smooth muscle cells and macrophages

by
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# Lipoprotein-proteoglycan complexes in injured aortas of normocholesterolemic rabbits

*To*

*My Parents*

ABSTRACT

The injury-induced alterations in sulfated proteoglycans (PG) were studied, in neointima, developed in response to a selective deendothelialization of the aorta, of normocholesterolemic rabbits. Light microscopic radioautography and size exclusion chromatography revealed differences in PG between neointima not covered by regenerated endothelium, and reendothelialized neointima or normal aorta. These differences included radioautographic reaction, concentration, size distribution and composition. Further studies were conducted to examine the putative role of the altered PG in lipoprotein (LP) sequestration and lipid accumulation during atherogenesis. LP-PG complexes were isolated by anti apo-B affinity column, from intima-medial tissues from normal and injured aortas. These complexes contained low density and very low density LP, chondroitin sulfate PG and hyaluronic acid. It appeared that endothelial injury enhanced the formation of LP-PG complexes, particularly in areas covered by regenerated endothelium. The uptake and degradation of LP-PG complexes, derived from normal (LP-NPG) or injured aortas (LP-IPG), by arterial smooth muscle cells (SMC) and blood monocyte-derived macrophages (BMDM) were also examined. LP-PG complexes stimulated LP binding, internalization and degradation by SMC and BMDM. Both cell types showed a higher affinity for LP-IPG than LP-NPG. The uptake of LP-PG complexes was mediated mainly by the LDL receptor pathway and phagocytosis. The scavenger receptor played a minor part in the uptake of LP-PG complexes. Data from this study provide evidence that endothelial injury could trigger alterations in neointimal PG, which in turn, facilitate LP accumulation both extracellularly and intracellularly during atherogenesis.

## RÉSUMÉ

Nous avons étudié les altérations des protéoglycans (PG) sulfatés, développées en réponse à une désendothélialisation sélective de l'aorte de lapins normocholestérolémiques, lors de lésions induites dans la néointima. L'autoradiographie microscopique et la chromatographie par exclusion de taille ont démontré des différences dans les PG de la néointima, entre la néointima normale et celle non recouverte par un endothélium régénéré, en ce qui a trait à leur réaction autoradiographique, leur concentration, leur distribution et leur composition. Des études ultérieures furent menées pour examiner le rôle putatif des PG altérés dans la séquestration des lipoprotéines (LP) et l'accumulation des lipides durant l'athérogénèse. Les complexes LP-PG furent isolés à l'aide d'une colonne à affinité anti-apo-B à partir des tissus intima-média d'aortes normales et lésées. Ces complexes contenaient des LP de densité faible et très faible, des PG de sulfate de chondroitine et de l'acide hyaluronique. Il nous est apparu que les lésions endothéliales augmentaient la formation de complexes LP-PG, particulièrement dans les zones recouvertes d'endothélium régénéré. L'incorporation et la dégradation des complexes LP-PG, dérivés d'aortes normales (LP-NPG) ou lésées(LP-LPG), par les cellules musculaires lisses artérielles (CML) et les macrophages dérivés des monocytes sanguins (MDMS) furent aussi étudiées. Les complexes LP-PG stimulèrent la liaison des LP, l'internalisation de même que la dégradation par les CML et les MDMS. Les deux types de cellules ont démontré une plus grande affinité pour les LP-LPG que pour les LP-NPG. L'incorporation de complexes LP-PG est régulée principalement par la voie des récepteurs LDL et par la phagocytose. Le récepteur de LDL oxydés ne jouait qu'un rôle mineur dans l'incorporation des complexes LP-PG. Les données recueillies par cette étude apportent l'évidence que les lésions endothéliales pourraient induire des altérations dans les PG néointimaux qui, à leur tour, facilitent l'accumulation des LP tant extracellulaires qu'intracellulaires pendant l'athérogénèse.

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Abbreviations

BMDM: blood monocyte-derived macrophage

CS: chondroitin sulfate

DS: dermatan sulafate

ECM: extracellular matrix

HA: hyaluronic acid

HS: heparan sulfate

HDL: high density lipoproteins

IPG: proteoglycan from injured aortas

LDL: low density lipoproteins

LM: light microscopy

LP: lipoprotein(s)

NPG: proteoglycan from normal aorta

PG: proteoglycan(s)

PIA: polyinosinic acid

SMC: smooth muscle cell(s)

UPA: urokinase plasminogen activator

VLDL: very low density lipoproteins

# PROLOGUE

This investigation is part of the interest developed by Dr. Moore and others on the importance of endothelial injury in the development of atherosclerosis. My study focuses on the injury-induced modifications in the aortic neointimal proteoglycans (PG), and the contribution of PG to lipid accumulation during atherogenesis. The experimental model of atherosclerosis used in this study was an injury model of atherosclerosis, in which aortas of normocholesterolemic rabbits, were deendothelialized using a balloon catheter.

There is a great body of evidence suggesting that PG synthesis and composition is modulated during atherogenesis. Moreover, the accumulation of extracellular matrix PG early in the course of atherosclerosis is proposed to trap lipoproteins by virtue of their ability to form complexes with apo-B containing lipoproteins. This interaction between PG and apo-B lipoproteins has been implicated as a mechanism for lipoprotein sequestration in the arterial intima and subsequent cellular processes. This hypothesis is supported by the following: 1) there is evidence for colocalization of apo-B containing lipoproteins and chondroitin sulfate rich PG in atherosclerotic lesions, 2) LP-PG complexes have been extracted from human atherosclerotic lesions as well as from hypercholesterolemic animal models, 3) LP-PG complexes have been shown to induce lipid accumulation and foam cell formation, when incubated with monocytes/macrophages.

## RATIONALE

This thesis is presented as a manuscript style, each chapter has its own abstract, introduction, methods, results, discussion and references. A general introduction covering the literature on related studies is presented, followed by four chapters:

CHAPTER 1: Injury-induced alterations in newly synthesized sulfated proteoglycans from aortic neointima covered by regenerated endothelium

CHAPTER 2: Characterization of lipoprotein-proteoglycan complexes isolated by affinity chromatography from aortic neointima of normocholesterolemic rabbits

CHAPTER 3: Lipoprotein-proteoglycan complexes from injured aortas accelerate lipoprotein uptake by arterial smooth muscle cells

CHAPTER 4: Interaction of apo-B containing lipoproteins with proteoglycans from injured aortas stimulates its uptake by monocyte-derived macrophages

Finally, an overall conclusion and the original contributions made by these studies, in the field of atherosclerosis research, is presented.

# CHAPTER 1: INTRODUCTION

## 1.1 ATHEROSCLEROSIS

### 1.1.1 *Definition and Background*

The word atherosclerosis derives from two Greek terms: *athere*, porridge, and *sklerosis*, a hardening. The atherosclerotic lesion's porridge-like part, mainly contained lipid; sclerosis refers to thickening and loss of elasticity of arterial walls (Moore, 1990). It is a disease of large and medium sized muscular and elastic arteries, and is recognized to be the prime disorder leading to death and morbidity in economically developed societies. Research in the field of atherosclerosis has for many years attracted an intense and significant interest which has been sustained not only for the complexity of the problems involved but also because of the far reaching importance in human beings.

Atherosclerosis is not a modern disease, but was prevalent in ancient times and has proved a common finding in the Egyptian mummies (Ruffer, 1911). Interestingly enough, the first observation of this condition attended the opening of the body of Menephtah, one of the most widely known of all the ancient royal Egyptians, whose aorta showed advanced sclerosis and calcium phosphate deposits as examined by Shattock in 1908 (Long, 1967). Rudolf Virchow in the nineteenth century, was the first to study atherosclerosis with histological detail not attempted before. During all this time, continued speculation went on about the origin of the disease as well as to the various lesions of atherosclerosis.

### 1.1.2 *Lesions of Atherosclerosis*

The lesions of atherosclerosis have a characteristic anatomic distribution and are located principally in the innermost layer of the artery wall, the intima, although secondary changes are occasionally found in the media too. Although any artery may be affected, the aorta and the coronary and cerebral systems are the prime targets, making myocardial and cerebral infarction the two major consequences of this disease. Atherosclerosis is a slowly progressive disease, that begins in childhood, but does not become manifest until middle age or later, when the arterial lesions precipitate clinical manifestations. Intimal changes in arteries appear in infancy and childhood, that manifest **intimal thickening**, with excess cellularity, matrix formation and little or no lipid accumulation (DeSa, 1979). Some have a patchy nodular appearance and others show diffuse intimal thickening. The first changes in the arteries that are visible to the naked eye are the **fatty streaks or spots**. Fatty streaks are seen in adolescence and in young adults; usually composed of lipids, fibrinogen, glycosaminoglycans and some collagen (Velican and Velican,1980). The basic lesion of atherosclerosis, **the fibrous plaque or atheroma**, consists of a raised focal plaque within the intima, having a lipid core and a covering fibrous cap. These atheromas are sparsely distributed at first, but as the disease advances, they become numerous and sometimes cover the entire intimal surface of severely affected arteries. As the plaques increase in size, they progressively encroach on the lumen of the artery as well as on the subjacent media. Consequently, atherosclerosis compromises arterial blood flow and weakens affected arteries. The essential features of atheroma are: lipid accumulation, connective tissue elaboration,

smooth muscle cell proliferation and foam cell formation. Fully developed atheromatous plaques may undergo a series of changes that result in so-called **complicated lesions**. In advanced disease, atheroma may undergo calcification, ulceration, superimposed thrombosis, haemorrhage and/or aneurysmal dilatation (Moore, 1983).

### 1.1.3 *Theories of Atherosclerosis*

The nature of the lesions of atherosclerosis have been described for over a century, yet our understanding of the complexity and the pathogenesis of this disease is still incomplete. Many theories of the pathogenesis of atherosclerosis have been evolved, and still the etiology is not fully comprehended. The impact of cellular and molecular biology on research in atherosclerosis has provided an insight into the fine structure of the lesions of atherosclerosis as well as the cellular mechanisms that may contribute to this complex process. The role of the different cell types involved in the atheromatous lesion has yet not been clarified. The factors that trigger endothelial dysfunction, smooth muscle cell proliferation or macrophage activation, have yet to be determined.

#### 1.1.3.1 *The imbibition hypothesis*

The first historical hypothesis, the imbibition hypothesis, was proposed by Virchow in 1856. Virchow explained the cellular proliferation in the intima as a form of low-grade inflammatory reaction to increased filtration of plasma proteins and lipids from the blood. He considered the early deposits, were due to the primary imbibition from the passing blood, which loosened the connective tissue ground substance and

prepared the way for degenerative changes. An almost neoplastic proliferation of the connective tissue of the intima, together with an increase in the ground substance occured, before degenerative changes set in. The latter consisted of fatty metamorphosis of the connective tissue cells associated with softening of the intermediate ground substance. This led to localized thickenings, which were prone to erosion from continued softening and the passing blood (Long, 1967).

#### 1.1.3.2 *The lipid infiltration hypothesis*

Over the years the concept of the imbibition hypothesis has undergone modification to become the "lipid," "insudation," or "infiltration" hypothesis. Anitschkow (1913) postulated an altered permeability of the vessel wall for lipids, based on his observation that lipid-rich intimal vascular lesions could be produced in experimental animals by a high cholesterol diet. For decades, atherosclerosis research in animal models was concentrated on cholesterol feeding. The question how lipids move into and out of the cells in the lesions was revitalized with the concept of receptor-regulated uptake in the sixties and seventies (Brown and Goldstein, 1979). Further support came from the rapidly accumulating, advanced knowledge of the lipoprotein subclasses and their role in the distribution of lipids not only in vascular tissues, but also in other tissues, such as the liver. With evolving knowledge of the molecular biology of the apoproteins, as well as of receptor proteins, the important linkage to the apparent genetic aspects of atherosclerosis is being made (Brewer et al., 1988).

#### 1.1.3.3 *The encrustation hypothesis*

This hypothesis is ascribed to Rokitansky, who postulated that plaque initiation and progression is the consequence of repeated cycles of thrombosis and remodeling (Duguid,1948). However, autopsy studies of vessels of children and experiments with cholesterol fed animals have shown that thrombosis is not the initial event in atherogenesis; in fact, thrombosis appear to be a feature of advanced disease. Hence, this hypothesis is more applicable to the problem of plaque progression rather than formation of plaques. Furthermore, it does not explain how lipid and smooth muscle cells accumulate in the lesion.

#### 1.1.3.4 *The monoclonal hypothesis*

This hypothesis, postulated by Benditt and Benditt (1973) interprets the monotypic nature of smooth muscle cells in the plaque as likely to arise as a clone from a single progenitor smooth muscle cell. There may be genetic or acquired aberrations of growth control of medial cells, leading to their proliferation. This hypothesis is based on the observation that individual plaques of human females heterozygous for the x-linked marker glucose-6-phosphate dehydrogenase (G-6-PD) frequently exhibit one, but not both, of the G-6-PD isotypes. At a certain moment in time, single cells might be stimulated to enter the growth cycle and undergo several rounds of division leading to the formation of a monoclonal lesion. Although the universal monoclonal nature of the plaques has been questioned (Thomas and Kim, 1983), there is evidence in animals that certain oncogenic viruses and carcinogens cause plaques in the aorta (Fabricant, 1985).

### 1.1.3.5 *The response to injury hypothesis*

Recently, the imbibition hypothesis and the lipid infiltration hypothesis have been incorporated into the response to injury hypothesis. Since then, it has been modified and extended by many investigators; Moore (1973; 1975; 1976; 1979), Duguid (1975), Mustard and Packman (1975) who played an important role in its development. The hypothesis deals with the proliferative and cellular aspects of atheromatous plaques, explaining the abnormal growth of the vascular tissue as a variation of wound healing. The response to injury hypothesis, formulated by Ross and Glomset in 1976 and modified in 1986, states that the lesions of atherosclerosis are initiated as a response to some form of injury to the arterial endothelium. Endothelial injury may be induced by mechanical denudation, hemodynamic force, immune complex deposition, irradiation and chemical or toxic agents. Focal sites of injury lead to increased permeability to plasma constituents including lipids and permit blood platelets and monocytes to adhere to endothelial and subendothelial connective tissue. Many studies have shown that disruption of the endothelial barrier induces a complicated tissue response that involves platelets, leucocytes, smooth muscle cells (SMC) as well as endothelial cells themselves (Groves et al., 1979; Hansson et al., 1981). Platelets contain mitogens and chemoattractants for SMC (Ross et al.,1974; Moore et al., 1976; Paul and Piaseck, 1984) and these are released into the injured vessel wall (Goldberg et al., 1980; Richardson et al., 1984). The second component of the hypothesis is SMC proliferation in the intima. The proliferating SMC originate from cells migrating from the tunica media and possibly from preexisting myointimal cells (Moore, 1985). Medial SMC are heterogeneous in their

proliferating and chemotactic potential (Haudenschild and Grunwald, 1985). Only 50% of those medial cells which migrate to form the neointima subsequently proliferate ( Clowes and Schwartz, 1985). A number of SMC mitogens derived from cells or serum have been implicated in such proliferation. Among these are: platelet derived growth factor (PDGF), monocyte derived growth factor (MDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and transforming growth factor α and ß (TGF). The third component of the hypothesis is the inflammatory cell infiltration. Leucocytes form a small part, less than 10% of the total cell population, but a significant fraction of cells within the vascular neointima (Verheyen et al.,1988; Ferns et al., 1991). Monocytes adhere to endothelium early in the course of experimental hypercholesterolemia, emigrate into the intima, and differentiate into macrophages. Macrophages participate in many phases of the injury response, from directing restorative cellular proliferation to restructuring of the extracellular matrix (ECM). The process of matrix degradation by macrophages begins as the cell homes along a trail of chemoattractant toward a site of injury. During chemotaxis the macrophage becomes polarized and concentrates the UPA-UPA receptor complex to the leading edge of the cell. This activator and downstream proteinase activity are thus focused on the cellular margin responsible for breaching the matrix barrier. *Invadolysin*, the metalloproteinase most strongly implicated in the invasive behavior of other cell types, is also the major secreted metalloproteinase of macrophages (Alexander and Werb, 1989).

In the lesion, macrophages contribute also to lipid accumulation and foam cell formation (Verheyen et al., 1988; Rosenfeld et al, 1991).

### 1.1.4 *Experimental Atherosclerosis*

#### 1.1.4.1 *Diet induced atherosclerosis*

Ignatovski (1908) was the first to use rabbits in feeding experiments to induce atherosclerosis, by giving a diet composed of milk, meat and eggs. The rabbits developed intimal lesions, but the investigator concluded that the high protein diet was responsible for lesion production. Four years later, Anitschkow (1912) identified that cholesterol was the atherogenic component of the diet. Anitschkow initiated an extended series of studies of the effects of cholesterol feeding in rabbits, which became the classic modality and dominated subsequent research. Anitschkow's observations indicated the importance of two elements in atherosclerosis; local enhanced permeability of the inner vascular lining and excessive intake of cholesterol. Many investigators repeated these studies, and gave description of the lesions found. Furthermore, it was learned that atheromatous lesions could be produced in other animals than rabbits, such as Guinea pigs, cats, dogs and chickens. Lyman Duff (1935), observed that swelling of subendothelial ground substance precedes the earliest deposition of lipoid material. Duff concluded that arterial injury is the initial event, which is followed by the deposition of lipid. The morphological changes of diet induced atherosclerosis are of the fatty streak type and included endothelial alterations, ground substance elaboration, lipid-filled foam cells and extracellular lipid.

#### 1.1.4.2 *Injury-induced atherosclerosis*

There were a number of early observations consistent with endothelial injury and atherogenesis in the absence of hyperlipidemia including the relationship of spontaneous atherosclerosis to possible arterial injury by Duff (1936) and later by Haust and More (1965). Alterations described as fibroelastic or atherosclerotic plaques in the aorta of normolipemic rabbits were considered as part of the repair process in response to the creation of a small defect in the vessel wall. Bjorkerud (1969) described the response to a superficial mechanical injury to the rabbit aortic intima using a microsurgical instrument. The first observations that arterial injury in normolipemic rabbits would result in lipid-containing lesions which had features of human atherosclerosis were reported by Moore in 1973. The model used in these experiments was one of continuous intimal injury caused by an indwelling aortic catheter.

Injury can be caused by balloon catheter deendothelialization, air-dry injury to the endothelium, immunologic and endotoxin-induced injury.

Balloon catheter deendothelialization, is one of the several forms of arterial injury that have been used to investigate specific aspects of atherogenesis among different species. This technique was first introduced experimentally by Baumgartner in 1963. A partially inflated Fogarty embolectomy catheter is repeatedly ( usually 2-3 times) drawn through a vessel bed to ensure that the endothelium is completely removed. Healing is characterized by intimal and medial remodelling, endothelial cell regrowth, medial and intimal cell proliferation and elaboration of ECM. Mechanical removal of the endothelium from an artery results in an almost immediate response by circulating

platelets (Groves et al., 1979) and leucocytes (Hansson et al., 1981). The luminal surface of the de-endothelialized vessel is rapidly covered by a layer of platelets and occasional clusters of adherent leucocytes. The loss of the cell number and DNA content of the vascular wall that occurs in balloon injury is followed by a period of intense cellular activity. Regeneration of endothelium takes place adjacent to branch vessel orifices (Moore, 1983; 1985). Some areas of extensively de-endothelialized vessel do not become recovered with endothelium but instead develop a non thrombogenic "pseudoendothelium" composed of phenotypically altered SMC (Reidy, 1985). This is due to the migration of SMC from tunica media and their proliferation within the intima (Moore, 1985; Clowes and Schwartz, 1985). The number of cells continues to increase, in the neointima, for up to two years (Moore et al., 1982). Increased ECM production contributes to the development of the thickened intima. At three months after injury, almost half of the endothelium that has been denuded is covered by regenerated endothelium. The areas of the luminal surface which are not reendothelialized, are freely permeable to circulating plasma proteins (Richardson and Moore, 1980). The neointima continues to increase in thickness and to accumulate lipid for up to two years (Moore et al., 1982).

## 1.2 PROTEOGLYCANS

### 1.2.1 *Structure and Assembly*

Proteoglycans (PG) are complex macromolecules that each contain a core protein with one or more covalently bound glycosaminoglycan (GAG) chains (Wight et al., 1991). PG are a subclass of glycoproteins with unique features of carbohydrate structure, containing GAG, that make them distinctive from glycoproteins. The amount of carbohydrate may comprise up to 95% of the weight of a PG molecule. GAG are linear polymers of repeating disaccharide units that contain one hexosamine and either a carboxylate or a sulfate ester, or usually both. These simple definitions encompass an exceptionally large range of structures involving different core proteins, different classes of GAG and different numbers and length of individual GAG chains.

GAG exist in seven main forms: heparan sulfate, heparin, chondroitin 4 sulfate, chondroitin 6 sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid. The first six contain sulfate substituents and form PG, whereas hyaluronic acid lacks sulfate and occurs as a free glycosaminoglycan. All GAG except keratan sulfate are present in normal aortas of various species (Salisbury and Wagner,1981). The types of GAG are the same in the three layers of the aorta (intima, media, adventitia) but their concentration varies considerably (Murata et al., 1975). PG have different types of core proteins, reflecting the varied locations and functions of these molecules, (Ruoslahti, 1989). Some PG have membrane-embedded core proteins, for example heparan sulfate PG (Saunders et al.,1989). Others have the core attached to a cell or to an extracellular

macromolecule, where it assumes an anchoring function, which may be required for locating GAG chain at specific sites. Information regarding the structure of many core proteins is still lacking. However, DNA of some PG core protein has been sequenced (Bourdon et al., 1985).

Each GAG chain is attached to the core protein through a glycosidic bond between the hydroxyl group of a serine and a xylose residue at the reducing end of the chain (Roden, 1980). Two galactose residues and one glucuronic acid residue complete the specialized linkage region upon which the repeating disaccharide units are subsequently assembled.

Assembly of a PG molecule requires the orchestration of a large number of specialized biosynthetic pathways. There are probably many factors required to ensure that the different substituents are added to the core in the proper sequence and that the completed macromolecule proceeds through the intracellular compartments in an orderly fashion. The synthesis of the core protein begins in the ribosomes of the endoplasmic reticulum. While the protein is still elongated, initial stages in the assembly of the N-linked oligosaccharides begin through a highly coordinated series of enzymatic steps. Once released from the ribosomes into the endoplasmic reticulum cisternae, the core protein begins a journey to the Golgi region of the cell where GAG chains and O-linked oligosaccharides are assembled onto the hydroxyls of appropriate serine, and serine and threonine residues respectively (Thonar et al., 1983). Once the GAG chains have been initiated on a core protein, the PG is completed very rapidly. The completed molecules are collected into secretory vacuoles and over a time period, are secreted from the cell

by exocytosis (Kimura et al., 1980). Some PG molecules remain associated with the cell surface, either the apical or the lateral surface or both. PG monomers are not aggregated when they leave the cell (Kimura et al., 1980). Thus assembly of these large aggregates occurs some time after secretion. The aggregation process is critical for holding the PG molecule in the tissue, and the unbound monomers can diffuse out of the matrix.

### 1.2.2 *Functions related to the arterial wall*

Analyses of arterial PG reveal that endothelial cells synthesize multiple species of heparan sulfate, while SMC synthesize little heparan sulfate but significant quantities of chondroitin and dermatan sulfate. Each family of PG synthesized by each cell type, differs with regard to charge density, hydrodynamic size, GAG type and size, oligosaccharide content and ability to form high molecular weight aggregates (Wight et al., 1986). Many studies have shown that PG contribute significantly to several physical and biological properties of the arterial wall in health and disease. Such regulatory functions include proliferation, migration, adhesion, viscoelasticity, permeability, lipid metabolism, haemostasis and thrombosis (Camejo, 1982; Berenson et al., 1985; Wight, 1989). Heparin and heparan sulfate PG have been shown to inhibit proliferation of several cell types including vascular SMC (Castellot et al., 1985). It is suggested that the antiproliferative effect of heparin and heparan sulfate PG is a result of the inhibition of the protein kinase C pathway and this activity increases with the chain length and the degree of sulfation of the saccharide molecule (Wright et al,1989). In atherosclerotic lesions where endothelium is functionally altered, SMC are able to proliferate. However,

in regression lesions of atherosclerosis, there is increased synthesis and secretion of heparan sulfate PG (Berenson et al., 1985).

PG play an important role in mediating the uptake of lipoproteins by arterial cells. A specific recognition binding sequence in the apo B containing lipoprotein has been identified to interact selectively with GAG chains (Camejo et al.,1988; Cardin and Weintraub, 1989). Moreover, the protein binding site in a GAG chain can be a specific sugar sequence (Bray et al., 1989). This interaction between PG and lipoproteins (LP) is considered to be one of the major mechanisms by which lipid accumulates both intracellularly and extracellularly during the course of atherosclerosis. The protein moiety of LP which binds to GAG is modulated by molecular sieving, steric exclusion and electrostatic interaction between the anionic moieties of sulfated GAG and the protein moiety of LP (Iverius, 1973). Chondroitin sulfate PG is potent and selective in lipoprotein binding (Vijayagopal et al., 1981). Goldstein and collaborators (1976) have demonstrated that sulfated GAG are able to release low density lipoproteins (LDL) from their cell surface receptors, which in turn modulate LDL internalization and accumulation within arterial cells. Recently, it has been proposed that PG modify LP to be recognized and internalized avidly by arterial cells. Such modification includes oxidation (Camejo et al., 1991). Heparin and heparan sulfate are believed to affect the release of lipoprotein lipase at the luminal surface of endothelium. Lipoprotein lipase is a key enzyme involved in the hydrolysis of plasma chylomicrons and very low density lipoproteins-triglycerides.

Heparin, heparan sulfate and dermatan sulfate possess anticoagulant activity as they inhibit thrombin-induced platelet aggregation (De Agostini et al., 1990). Heparan

sulfate PG also participate in the maintenance of an anticoagulant surface by accelerating certain hemostatic enzyme-protease inhibitor interactions.

Recently, the role of PG as modulators of growth factors and cytokines has been explored. PG can control the access of regulatory molecules such as growth factors to cell surface receptors (Rouslahti and Yamaguchi, 1991). Transforming growth factor ß receptor type III has been identified to be heparan sulfate PG molecule (Segarini and Seyedin, 1988). Heparan sulfate chains of PG bind growth factors such as fibroblast growth factor (FGF) and this binding appears to protect the growth factor from degradation and to provide a reservoir for overlying cells (Burgess and Maciag, 1989; Sakasela and Rifkin, 1990). Growth factors and cytokines that are known to bind to PG include: interferon gamma, granulocyte-macrophage colony stimulating factor, interleukin-3, pleiotrophin, a neurite promoting factor, platelet factor IV and transforming growth factor ß. Therefore, PG provide a means of concentrating growth factor activities and directing them into a geometry appropriate for the architecture of the tissue to allow them to regulate intercellular and cell to matrix interactions ( Lortat-Jacob et al., 1991).

### 1.2.4 *Implication in atherosclerosis*

PG, previously known as mucosubstances, were recognized to be involved in the pathogenesis of atherosclerosis as early as the time of Virchow, 1856. Since the sixties, progress has been made to investigate arterial carbohydrates as they exist *in situ*, i.e. PG. Proteoglycans are one of the principal components of the vascular extracellular matrix known to accumulate within the intima of blood vessels during the early stages of

atherosclerosis, and this accumulation is thought to predispose the vessel wall to further complications of this disease. In human and experimental models of atherosclerosis, there have been many reports demonstrating the increased biosynthesis and accumulation of PG within the arterial wall ( Kumar et al., 1967; Smith, 1974; Murata et al., 1975; Alavi and Moore 1985; 1987 and Berenson et al., 1988). The high concentration of sulfated PG in the intima observed in atherosclerotic lesions is proposed to trap circulating LP that gained entry into the vessel wall (Iverius, 1973; Camejo, 1982; Alavi et al., 1983; Alavi and Moore 1984; Day et al., 1985). Alteration in PG composition has also been reported in atherosclerotic lesions. Enhanced chondroitin sulfate PG synthesis and accumulation has been shown in developing and advanced lesions of atherosclerosis. Chondroitin sulfate PG synthesized by arterial SMC is capable of interacting with hyaluronic acid and link proteins to form link-stabilized aggregates and in this respect resemble chondroitin sulfate PG present in the cartilage (Chang et al., 1983). Biochemical studies revealed that this PG contains a shorter protein core and few but longer GAG chains. Another feature is the relatively high content of O-linked oligosaccharides per GAG chain present in chondroitin sulfate PG (Wagner et al., 1986).

When the atherogenic stimuli are removed, although lipid material is depleted, connective tissue components, including PG persist in an altered state for a long period. The GAG composition depends on the nature of the lesion and the degree of regression (Berenson et al., 1985). Berenson and collaborators (1981) compared the effect of various anti-atherogenic regimens on GAG concentration in diet-induced atherosclerosis. The total GAG concentration among the different groups did not differ significantly but

correlated positively with the severity of the lesions. Heparan sulfate and hyaluronic acid increased with regression, suggesting regeneration of endothelium, whereas, chondroitin sulfate and dermatan sulfate followed an opposite trend, indicating a reduced proliferative activity of SMC. Hollander and coworkers (1984) reported that in regressive lesions of atherosclerosis, dermatan sulfate concentration of the arteries increased significantly. All these studies reveal that matrix PG are intimately involved in the repair process and remodelling of the aorta.

The biological importance and the manifold physiological functions of GAG, made them useful as therapeutic agents in the treatment of various diseases. The anti-atherogenic effect of chondroitin 4 sulfate was first described by Morrison (1969) and it is still used in the treatment in Japan. The mode of action of chondroitin 4 sulfate is not yet clarified, but it might interfere with the complex formation between intimal GAG and serum LDL and thus reverse lipid deposition (Bihari-Varga et al., 1981). Heparan sulfate and dermatan sulfate containing products have a lipid clearing and serum cholesterol and LDL reducing effect (Seethanathan et al., 1975). A modified heparin containing heparan sulfate and dermatan sulfate lowered the involvement of the intima by atherosclerotic lesions by 20% in rabbits (Radhakrishnamurthy, 1978), supposedly via release of lipoprotein lipase at the luminal surface.

## 1.3 LIPOPROTEINS

### 1.3.1 *Nomenclature*

Fat absorbed from the diet and lipids synthesized by the liver and adipose tissues, is transported in plasma as lipoproteins (LP). LP are spherical macromolecular complexes originally described in horse serum by Machebouef in 1929 (Chapman,1980). They can be divided into many subclasses on the basis of their physicochemical properties. Plasma LP can be divided into five types, depending on their sedimentation properties: 1) **chylomicrons**, which have the lightest density, are composed predominantly of triglycerides and are found in plasma after meal; 2) **very low density lipoproteins** (VLDL),which mainly transport triglycerides that have been synthesized in the liver; **intermediate density lipoproteins** (IDL), which are remnants generated from the action of lipoprotein lipase on VLDL; **low density lipoproteins** (LDL), and **high density lipoproteins** (HDL) function primarily in the transport of endogenous cholesterol to body cells. About 70% of the total plasma cholesterol level, in humans, is contained in LDL. A relatively newly discovered variant of LDL, called lipoprotein (a) [Lp (a)], possesses all the characteristics of LDL, but has its B apoprotein linked to a distinct lipoprotein (a) by disulfide bridges. Although its function is unknown, its plasma levels are correlated with increased incidence of coronary artery disease (Scanu, 1988).

### 1.3.2 *Composition of lipoproteins*

Lipoproteins are marvelously constructed with a thin outer shell surrounding a central core of hydrophobic fat. The shell contains proteins, phospholipids, and free cholesterol lined up in a parallel array, one molecule thick. The free cholesterol and phospholipids have detergent-like properties that make the particle soluble in the blood. They are oriented with their water-soluble (polar) ends facing outward and the fat-soluble (nonpolar) ends facing the lipoprotein core. The apoproteins, the protein moiety of LP, are embedded in the shell with a similar polar arrangement. Some apoproteins are integral and cannot be removed, except when the particle is destroyed, whereas others are transferable to other lipoproteins. One or more apoproteins are present in each LP particle. According to the ABC nomenclature, the major apoproteins of HDL are designated A, while LDL is designated B which is also found in VLDL and chylomicrons. However, apo-B of chylomicrons is smaller (B-48) than the apo-B of LDL or VLDL (B-100). In humans and rabbits, apo B-48 is synthesized in the intestine and apo B-100 in the liver. Apo-C are smaller polypeptides freely transferable between several different LP. Apo-D, also designated apo A-III, is found mainly in HDL. Apo-E is arginine rich and is a constituent of VLDL and HDL. Apoproteins are important in regulating the transport of lipids and the complex interactions between the different lipoprotein classes. They serve as enzyme cofactors, for example for lipoprotein lipase and lecithin:cholesterol acyl transferase (LCAT). They can act as ligands for internalization with lipoprotein receptors in tissues as apo B-100, apo-E for LDL receptor. They can also act as lipid transfer proteins (Brewer et al., 1988).

The centre of the lipoprotein sphere contains two prominent forms of fat in the blood stream: triglycerides and cholesterol esters. They are completely insoluble in the plasma. Cholesterol ester is constructed from free (non esterified) cholesterol linked to a fatty acid molecule via an ester bond. Once non-esterified cholesterol enters arterial plaque, most of it becomes esterified.

### 1.3.3 *Lipoproteins and Atherosclerosis*

A large and consistent body of evidence indicates that the total plasma blood cholesterol level is a strong and independent predictor of atherosclerosis (Stamler et al., 1986). Total plasma cholesterol represents the sum of cholesterol distributed in LDL, IDL, VLDL and HDL. The VLDL and IDL are precursors of LDL, which in turn, is positively associated with atherosclerosis while HDL has an inverse relationship (Castelli et al., 1977). Several lines of evidence support this notion; LDL are the causative factors in familial hypercholesterolemia (FHC). Lowering LDL level in hypercholesterolemia significantly reduced the risk of death from coronary heart disease (CHD). Apo-B-100 interacts with sulfated PG by means of regions rich in basic amino acids to form PG-LP complexes which are implicated as a means of LP accumulation, extracellularly and intracellularly, during atherogenesis.

LDL particles transport approximately three fourths of the total blood cholesterol, delivering cholesterol to tissues throughout the body. If the LDL concentration in the blood rises, some of its cholesterol can be deposited in arterial walls. The concentration of LDL in the blood is determined by its rate of production and removal, both of which

are dramatically affected by diet. Dietary saturated fat and cholesterol increase the liver's production of VLDL, some of which are transformed into LDL particles within the blood stream. Saturated fat and cholesterol also suppress the withdrawal of LDL by the liver, which raises LDL levels even further.

LDL are a heterogeneous collection of particles with distinct physical and chemical characteristics. Certain LDL subpopulations are more atherogenic than others. Newton and collaborators (1975), described an increase in the content of saturated cholesteryl esters in LDL of familial hypercholesterolemic (FHC) subjects. Patsch et al.(1982) have shown that LDL of patients from FHC were larger in size and contained more cholesteryl esters and less triglycerides than LDL of normal individuals. Sniderman and collaborators (1980), found that LDL with high apo-B to cholesterol ratio were more prevalent in humans with coronary heart disease. The higher apo-B to cholesterol ratio may be due to the presence of a relatively cholesteryl ester-poor, triglyceride-rich, LDL occurring concomitantly with elevated plasma triglyceride concentration. In hypertriglyceridemic individuals, the plasma LDL have been found to be smaller in size and to have a higher average hydrated density. It is not certain that small LDL particles, in CHD individuals are atherogenic (Rudel et al.,1986). The factors associated with the formation of small LDL, including overproduction of VLDL apo-B and slow LDL catabolism, may be responsible for the predisposition of some hypertriglyceridemic individuals to premature atherosclerosis. A contrasting situation occurs in non human primates; many cholesterol fed monkeys have large LDL-cholesterol size, which is positively correlated with the severity of coronary artery atherosclerosis (Rudel et

al.,1986). Other modifications of LDL including ß-VLDL and arginine rich VLDL may have a role in atherogenesis (Mahley and Innerarity, 1983).

Several mechanisms are suggested by which LDL might initiate or promote atherogensis other than via delivering lipids to the cells of the arterial wall including; injury to the endothelium, growth stimulation of SMC, platelet aggregation.

Recently, it has been indicated that "modified" forms of LDL are especially menacing. The most important instance occurs when LDL combines with oxygen to form a chemically reactive oxidized LDL (Steinberg et al.,1989). Oxidized LDL is potentially more atherogenic than native LDL in several ways: 1) It is chemotactic for circulating monocytes 2) It is an inhibitor of motility of resident macrophages. 3) It is cytotoxic for cells in culture. 4) It can stimulate the release of a chemotactic factor from endothelial cells in culture. 5) It can stimulate the release of colony stimulating factor and a monocyte chemotactic factor for endothelial cells in culture (Steinberg, 1991).

### 1.3.4 *Metabolism of lipoproteins in the arterial wall*

The endothelium normally regulates the entrance of LP into the artery wall. Huttner and collaborators (1973) have demonstrated that arterial endothelial cells, in contrast to capillary endothelium, are linked together by highly interdigitated cell junctions that preclude penetration of molecules as small as 40,000 daltons. Stein and Stein (1973) have provided evidence that endothelial cells can allow transport of vesicles of 75 nm in diameter. These and other studies support the concept that passage of macromolecules is restricted to certain focal areas such as arterial branch points or sites

of endothelial injury or desquamation. Lipid accumulation in the intima of atherosclerotic lesions occurs intracellularly and extracellularly.

Most lipids of the atherosclerotic lesion originate from apo-B containing lipoproteins (Walton et al., 1974 and Hoff, 1975). Plasma LP that have leaked through altered endothelium and penetrated into the tissue of the artery wall, is taken up by macrophage and SMC and cholesterol is stored in the cytoplasm in the form of cholesteryl esters. The mechanisms by which SMC and macrophages can internalize LP include: 1) receptor mediated endocytosis via the apo-B/E receptor for the native LDL, 2) non-regulated scavenger receptor for certain chemically modified LDL, 3) phagocytosis (Brown and Goldstein, 1979; Pitas, 1990 and Ross, 1981). The LDL that enters the intima suffers multiple modification in its lipid and protein moiety. Some of these are caused by the actual hydrolytic and proteolytic enzymes and further changes take place by the activities of the oxygen-free radicls generated by cells in the intima. This oxidatively modified LDL is taken up avidly by SMC and macrophages and thus can generate foam cells (Steinberg et al., 1989). LP can suffer other modification such as LDL aggregation, LP-PG complex formation and immunologic complexes ( Berenson et al., 1972; Griffith et al., 1988; Khoo et al., 1992).

The membranous lipid deposits found within the extracellular matrix appear to coincide with regions characteristically occupied by PG. These lipid deposits may be formed by two mechanisms; the trapping of LP by PG or as a result of cell degeneration and necrosis resulting in the release of intracellular lipid moieties into the extracellular environment (Iverius, 1973).

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## CHAPTER 2

# INJURY-INDUCED ALTERATIONS IN NEWLY SYNTHESIZED SULFATED PROTEOGLYCANS FROM NEOINTIMA COVERED BY REGENERATED ENDOTHELIUM

## 2.1 ABSTRACT

Proteoglycan (PG) synthesis is a highly regulated dynamic process that is known to be altered during atherogenesis. Endothelial injury, which may be the primary event in atherosclerosis, has been reported to stimulate PG synthesis and accumulation in the arterial extracellular matrix. The objective of this investigation was to study injury-induced alterations in PG synthesis and accumulation in the neointima, developed in response to a selective balloon catheter-deendothelialization of aorta of normocholesterolemic rabbits. One group of rabbit aortas was incubated with $^{35}S$-$Na_2SO_4$ for 8 hours, to study *in vitro* the *de novo* synthesis of sulfated PG. Another group of rabbit aortas was used to study PG accumulated in aortic neointima versus PG present in intima-media of normal aortas. Newly synthesized sulfated PG was characterized by light microscopic radioautography and size exclusion chromatography. Purified Intimal-medial PG extracts from unlabeled aortas, were analyzed for protein and GAG content and GAG distribution pattern. Results from this study revealed that neointima of injured aortas synthesized sulfated PG at a significantly higher concentration than intima of normal aortas. Silver grains exhibited a gradient concentration pattern, in injured aortas covered with regenerated endothelium only. Size exclusion chromatography revealed that neointima synthesized larger polymer size PG of the high molecular weight population, and at higher proportion, compared with its counterpart PG from normal aortas. PG accumulated in neointima of injured aortas showed a significantly altered GAG distribution pattern. These data confirm that neointima developed in response to injury, synthesizes and accumulates altered PG compared with intima of normal aorta.

## 2.2 INTRODUCTION

Proteoglycans (PG) have been shown to influence many cellular processes in the vessel wall including proliferation, adhesion, lipid metabolism and haemostasis ( Camejo, 1982; Berenson et ., 1984; Wight, 1989). PG synthesis and composition appeared to be modulated early in the course of atherosclerosis ( Berenson et al., 1984; Wight et al., 1986), diet-induced atherosclerosis (Radhakrishnamurthy et al., 1988) and injury-induced atherosclerosis ( Wight and Ross, 1975; Wight et al., 1985; Alavi and Moore 1985, Alavi et al., 1992; Galis et al, 1992). The increased biosynthesis of PG early in the course of atherosclerosis is proposed to facilitate lesion development and progression (Moore, 1983).

The endothelial cells synthesize multiple species of heparan sulfate (HS), while smooth muscle cells synthesize little heparan sulfate but significant quantities of chondroitin sulfate (CS) and dermatan sulfate (DS). Each family of PG synthesized by each cell type, differs with regard to charge density, hydrodynamic size, GAG type and size, oligosaccharide content and the ability to form high molecular weight aggregates (Wight et al., 1986). Thus, the alteration of the versatile PG molecules are important determinants of cell regulation and the composition and architecture of the extracellular matrix (ECM). For these reasons, both *in vivo* and *in vitro* studies are needed for metabolic assessment and biochemical characterization of the PG molecules in the arterial wall.

We used freshly dissected aortas maintained in culture medium, which has the advantage, over cell culture studies, in providing conditions somewhat similar to the *in*

*situ* situation. We studied: 1) *in vitro* the *de novo* synthesis of intimal-medial sulfated PG in normal and injured aortas; 2) biochemical characteristics of PG extracted from intima-media of normal and injured aortas.

Size exclusion chromatography permitted the direct assessment of altered synthesis of PG by injured aortas. Glycosaminoglycan content and distribution pattern and Light-Microscopic (LM) radioautography enabled us to examine the distribution of intimal-medial PG from injured aortas of normocholesterolemic rabbits. Results from this study provide a possible explanation for enhanced PG accumulation in early atherosclerotic lesions.

## 2.3 MATERIALS AND METHODS

### *2.3.1 Animals*

Male and female New Zealand white rabbits (2-3 kg) were maintained on regular rabbit chow for 2 weeks before surgery. Deendothelialization of the aorta was achieved using a 4F Fogarty balloon catheter ( Model 12-010, Edward lab. Inc., Santa Anna, Calif.). The catheter was introduced into the femoral artery up to the aortic arch and the balloon was inflated with 0.5 ml saline. This process was repeated twice, then the catheter was withdrawn. In sham operated rabbits (control), the femoral artery was exposed but no catheter was introduced. The incision was closed, and the rabbits were allowed to recover. All experiments were performed 12 weeks following surgery, by that time, reendothelialization was well established involving at least half of the area of the aorta.

### *2.3.2 Organ culture*

Rabbits were sacrificed by an overdose of pentobarbital (60 mg/Kg BW) and the entire aorta was quickly removed and placed in chilled Hanks' balanced salt solution. Extraneous tissue was dissected from aortas, which were then slit longitudinally and incubated with Dulbeco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and supplemented with sodium pyruvate, non essential amino acids, sodium carbonate and antibiotics (Gibco, Canada Inc.). One hundred $\mu$Ci of $^{35}$S-sodium sulfate /ml medium was added to measure the production of newly synthesized sulfated PG. Cultures were maintained in a humidified gas mixture ( 95% air, 5% $CO_2$), at 37 °C for 8 hours. After incubation, aortas were washed 3 times with PBS before further processing.

*2.3.2.1 Light microscopy-radioautography*

Small pieces from each aorta were fixed with 2.5% glutaraldehyde in 0.1M PB for 2 h, washed with 0.2M PB overnight, dehydrated in ascending grades of ethanol and embedded in Epon. Semi-thin sections (1 $\mu$m thick) were cut and stained with iron hematoxylin. Stained slides were coated with Ilford photographic emulsion, exposed for 4 weeks and developed for fine silver grains, (Kopriwa and Leblond, 1962).

*2.3.2.2 Quantitation of newly synthesized sulfated PG*

Fifteen micrographs were taken at the same magnification (x400), from each aorta per tissue type (intima of normal aortas, neointima covered by regenerated endothelium, REA, and neointima with denuded endothelium, DEA). The numbers of silver grains, over the cellular and extracellular compartments, were counted in the intima from control and injured aortas according to the method described by Kanwar et al. (1983). The relative area of each compartment was computed, using a PAD software package. The grain density was then calculated by dividing the number of grains over a given compartment by the total area. Finally, the mean grain density and SD about the mean were calculated.

*2.3.2.3 Size Exclusion chromatography of newly synthesized sulfated PG*

Intima-media from the labeled aortas were separated from adventitia, homogenized in 10 volumes (wt/vol) of 4M guanidine hydrochloride (Gu-HCl) containing 20mM Tris, 10mM EDTA and protease inhibitors (100mM $\epsilon$-aminocaproic acid 1mM phenylmethylsulfonyl fluoride and 1mM benzamidine), pH 7.0 [Buffer A] for 48 h at 4°C with gentle shaking. The homogenate was centrifuged at 105,000 x g for 60 min.

at 4°C. The amount of radioactivity was measured in the supernatant before and after dialysis against distilled water to determine the amount of free radioactivity. Dialyzed extracts were lyophilized, dissolved in 1 ml of 4M Gu-HCL (Buffer A) and then individually applied to a Sepharose CL-4B column (50 x 1.6 cm). The column was previously equilibrated with buffer A and fractions were eluted at a flow rate of 0.2 ml/min. Five hundred microlitre fractions were collected and an aliquot from each fraction was used for counting incorporated radioactivity. A chromatogram of eluted fractions was plotted against $^{35}S$-incorporation. Fractions of corresponding peaks were pooled, dialyzed against distilled water and lyophilized. GAG electrophoresis was done for individual peaks after proteolytic digestion, GAG precipitation and specific enzymatic digestion.

*2.3.3.1 Extraction and purification of intimal-medial PG from unlabeled aortas*

To study the biochemical characteristics of intimal-medial PG of normal and injured aortas, PG was extracted from freshly excised aortas. Intima-media was peeled off from the adventitia, cut into small pieces and extracted in 10 volumes of 4M Gu-HCl (Buffer A) for 48 h at 4°C with gentle shaking. The pellet was delipidated with chloroform :methanol (2:1, vol:vol). The resulting delipidated residue was dried under $N_2$ and weighed. The dried defatted tissue (DDT) was used as a reference unit for protein and GAG measurement. The extract was centrifuged, and the supernatant was dialyzed for 36 h at 4°C against 7M urea, 0.15M NaCl containing protease inhibitors, pH 7.0 [Buffer B]. The dialyzed extract was loaded on a DEAE-Sephacel column (10 x 1 cm), which had been equilibrated with buffer B. Fractions were eluted with the urea

buffer, followed by a linear NaCl gradient from 0.15M to 1.0M in the same buffer (buffer B), at a flow rate of 0.25 ml/min. Fractions adsorbed on a cellulose acetate membrane and stained with 0.2% Alcian Blue were tested for the presence of PG. The proteoglycan containing fractions were pooled, dialyzed against distilled water and lyophilized. The lyophilized PG were dissolved in a buffer containing 500 $\mu$l 0.5M NaCl, 10mM Tris-HCl, pH7.0 and passed through a Superose 6 HR column (30 x 1 cm) connected to an FPLC system. Fractions were eluted with the same buffer in which the sample was dissolved, at a flow rate 0.2ml/min. Fractions positive for PG were pooled, dialyzed, lyophilized and considered as purified PG for further characterization.

*2.3.3.2 Characterization of intimal-medial aortic PG extracts*

The amount of protein and GAG were determined in the purified extracted PG, according to the methods of Bradford (1976) and Bartold and Page (1985) respectively. One aliquot of the concentrated PG was subjected to proteolytic digestion with papain followed by precipitation with absolute ethanol and saturated solution of sodium acetate in a ratio of 1:4:1. The precipitate was suspended in distilled water and analyzed for GAG composition by electrophoresis on cellulose acetate membrane (Breen et al., 1970), using 0.3M cadmium acetate buffer, pH 8.1, at 4mA/strip for 60 min. One aliquot was treated with Chondroitinase AC enzyme before electrophoresis. For identification, mobility of tissue GAG was compared to the mobility of standard GAG in the same system. The membrane was stained with 0.2% Alcian blue in 0.03M $MgCl_2$, 0.1% glacial acetic acid and 10% ethanol. GAG distribution was assessed by densitometric evaluation on stained electrophorograms, using a Hoefer GS 300 scanning densitometer

equipped with GS-360, densitometry software (Alavi et al., 1992).

*2.3.4 Statistical analyses*

One way analysis of variance and Tukey's Honest Significant Difference (HSD) comparing mean difference between control, regenerated endothelial areas (REA) and denuded endothelial areas (DEA) were performed using SYSTAT software package (5.01) of Systat Incorporation, Evanston, IL.

## 2.4 RESULTS

### *2.4.1 Characterization of Newly Synthesized Sulfated PG*

#### *2.4.1.1 Morphology*

The intima of normal rabbit aorta consisted of a continuous monolayer of endothelium resting on a basement lamina, and underlying stroma with few cells and scant extracellular matrix (ECM). After labeling aortas with $^{35}S$-$Na_2SO_4$, one could determine the localization of the newly synthesized, sulfated PG and their site of accumulation within the aortic intima. Radioautography of semi-thin sections revealed that in aortas incubated with $^{35}S$-$Na_2SO_4$ for 8 hours, the isotope was incorporated into the cytoplasm of endothelial cells and SMC. Silver grains were accumulated at the cell surface as well as in the extracellular space (Fig. 1a).

Twelve weeks following deendothelialization of the aorta, almost half of the intima was covered by regenerated endothelium (REA) while the second half remained denuded (DEA). The neointima of REA was thickened and the subendothelial space showed more cells and ECM components. In DEA, the neointima showed fewer cells but a fair amount of ECM. REA from injured aortas exhibited enhanced radioautographic reaction over normal aortas. Silver grains were associated with endothelial and smooth muscle cells both intracellularly and at the cell surface (Fig. 1b). The ECM demonstrated an increase in the concentration of silver grains. Newly synthesized sulfated PG accumulation appeared in a gradient concentration pattern, with the denser concentration toward the luminal front, decreasing gradually toward the media

(Fig. 1c). This gradient concentration pattern was only noticed in neointima from REA, whereas it was not observed in either normal aortas or DEA from injured aortas. DEA showed less radioautographic reaction than that observed in REA, Fig. 1d.

Radioautographic silver grains were counted over the cellular and extracellular compartments and the relative areas of each compartment were calculated and the results are represented in the bar graph (Fig. 2). There was a highly significant difference between injured and normal aortas; REA were also significantly different from DEA. ($p<0.001$ control vs REA; $<0.001$ control vs DEA; $<0.01$ REA vs DEA).

Figure 1: Radioautographs of transverse sections from rabbit aortas, incubated with $^{35}$S-sodium sulfate for 8 h, coated with Ilford emulsion, exposed for 4 weeks and developed for fine silver grains. a) Control rabbit aorta. b) Injured rabbit aorta from areas with regenerated endothelium c) Injured rabbit aortas from REA illustrating the gradient concentration pattern of the label, with denser concentration toward the luminal front. d) Injured rabbit aorta from denuded endothelial areas. L,lumen. x1000.

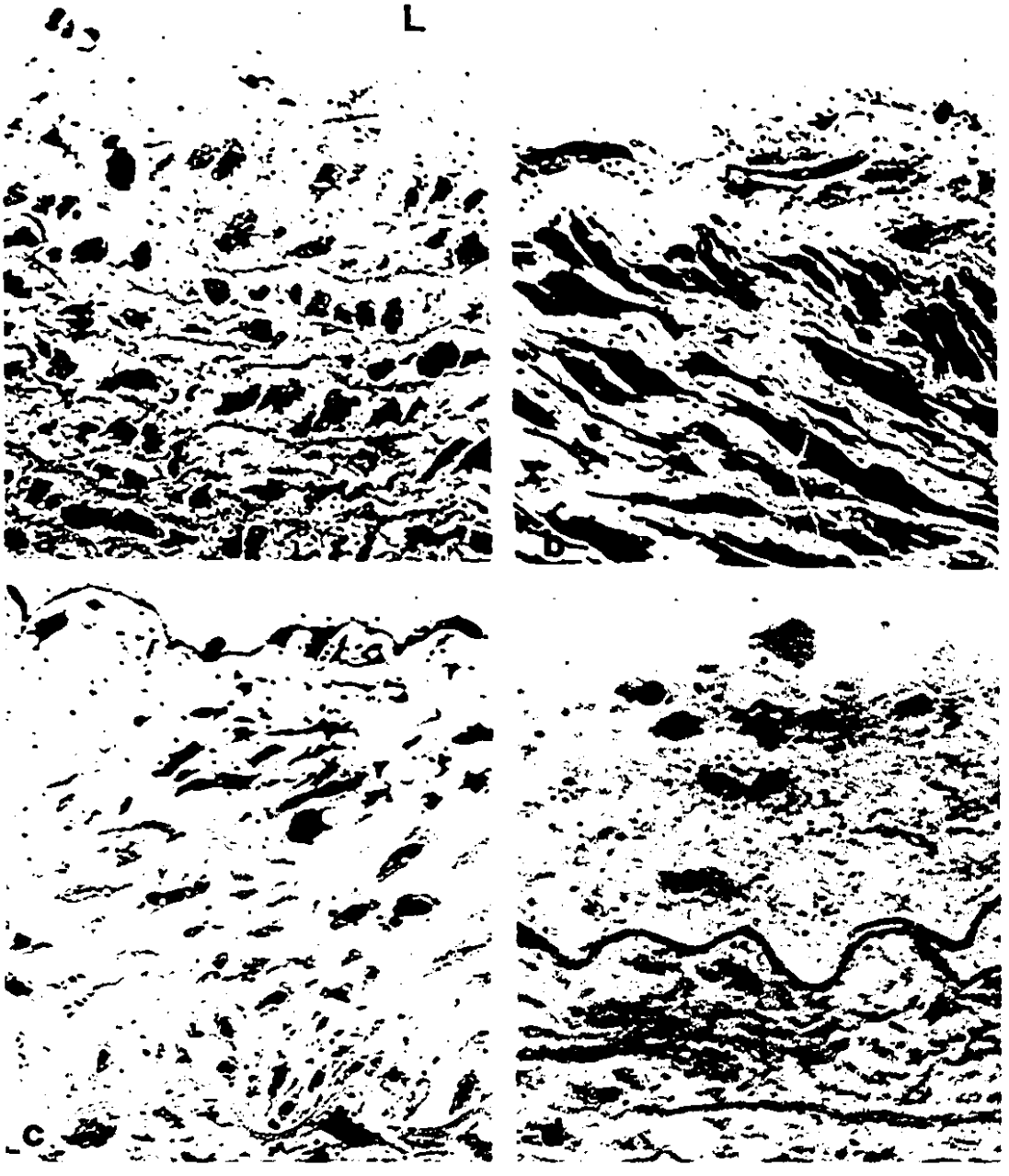
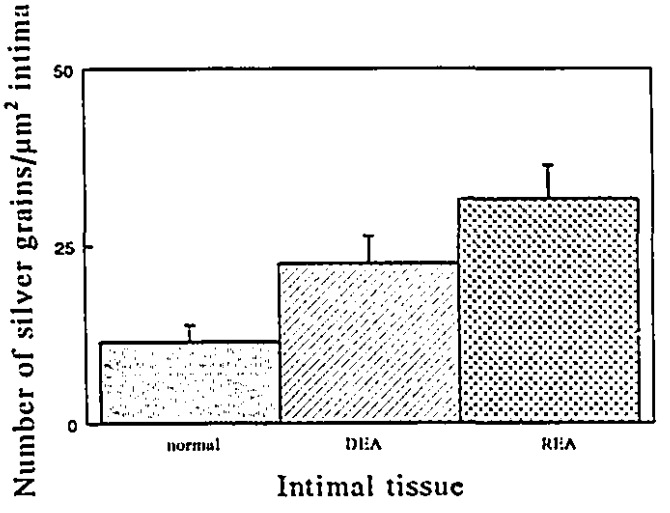

L
C
D

Figure 2: Bar graph illustrates the newly synthesized sulfated PG within aortic intimal tissues. Values represents the average number of silver grains per $\mu m^2$ of aortic intima. ( mean $\pm$ SEM; n=45)


Number of silver grains/μm² intima
50
25
0
normal
DEA
REA
Intimal tissue

*2.4.1.2 Size exclusion chromatography*

Newly synthesized sulfated PG, extracted from intima-media of either normal or injured aortas (REA), eluted into two peaks of radioactivity (Fig. 3a,b). The amount of radioactivity present in the extract after dialysis was greater than 75%, indicating that more than 75% of $^{35}S$-$Na_2SO_4$ was bound to the aortic intimal-medial tissues. The void volume ($V_o$) of an equilibrated Sepharose CL 4B column (50 x 1.6 cm) was determined by applying 1mg/ml Blue Dextran (Pharmacia, Canada) into the column. The total volume ($V_t$) was determined by applying 0.5mg/ml Tryptophan (MW 204 D) into the column. The partition coefficient value ($k_{av}$) was determined for each PG population from different aortas.

$$K_{av} = \frac{(V_e - V_o)}{(V_t - V_o)}$$

The high molecular weight PG population, from injured aortas, eluted four fractions earlier than the high molecular weight population from normal aortas, as determined by the partition coefficient value ($K_{av}$). The partition coefficient value ($K_{av}$) for normal aortas was 0.06, whereas, the high molecular weight population of PG from injured aortas was 0.03, indicating that intima-media from injured aortas synthesized PG of larger polymer size than intima-media of normal aortas. The lower molecular weight population of PG eluted at a $K_{av}$ value of 0.4 for both normal and injured aortas.

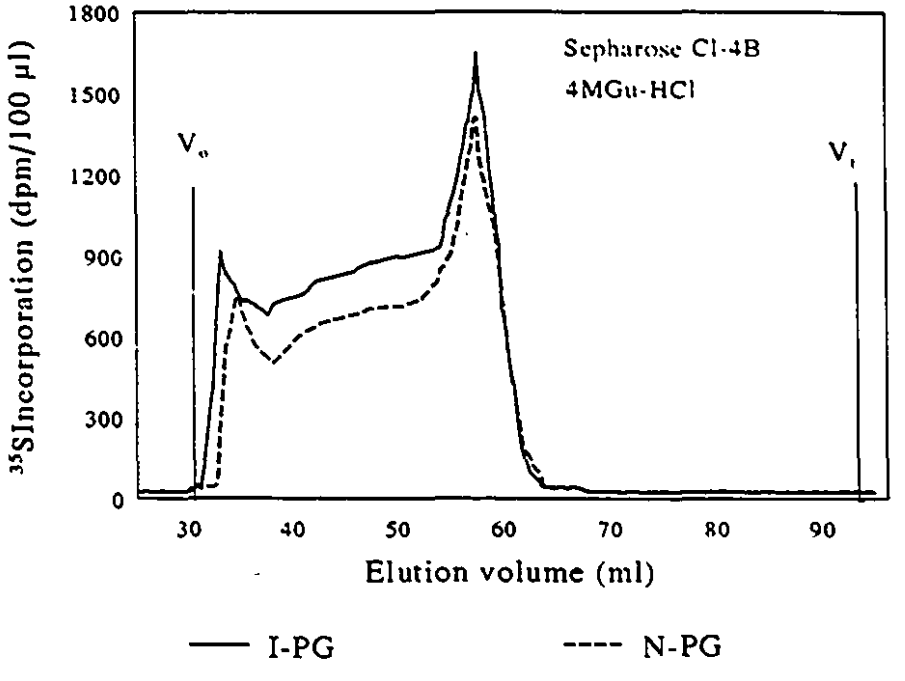
The area under each peak was computed, using PAD software package, to determine the

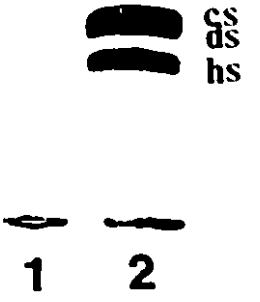
proportion of high and low molecular weight populations of PG possessed by each tissue. Newly synthesized sulfated PG extracted from intima-media of normal aortas contained 23% of the higher molecular weight population and 77% of the lower molecular weight population of PG. In injured aortas, this distribution of higher and lower molecular weight populations of PG was found to be 30% and 70% respectively, indicating that injured aortas synthesized a higher proportion of sulfated PG of the high molecular weight population than normal aortas.

Electrophoresis of the high and low molecular weight populations of PG before and after enzymatic digestion with Chondroitinase AC revealed that the high molecular weight population of PG was mainly composed of chondroitin sulfate, whereas, the lower molecular weight population was mainly composed of dermatan sulfate. Heparan sulfate was present in both populations.

Figure 3: Size exclusion chromatography of newly synthesized sulfated PG from intima-media of normal and injured aortas. PG extract from labeled aortas was applied to a Sepharose CL 4B column (50 x 1 cm), eluted with 4M Gu-HCl buffer at a flow rate of 0.2 ml/min., and 500 $\mu$l fractions were collected. PG eluted as two main populations of different molecular sizes. The high molecular weight population of PG eluted earlier in the case of injured aortas. The lower molecular weight population of PG eluted at an identical peak in both normal and injured aortas. ($V_o$ = 31 ml, $V_t$ = 94 ml)

X-ray exposure of electrophorograms of the high molecular weight population (1) and the lower molecular population (2) is also presented.


1800
1500
1200
900
600
300
0
$^{35}$SIncorporation (dpm/100 µl)
Sepharose Cl-4B
4MGu-HCl
$V_o$
$V_t$
30
40
50
60
70
80
90
Elution volume (ml)
I-PG
N-PG


ss
ds
hs
1
2

*2.4.2 Biochemical characterization of intimal-medial PG from unlabeled aortas*

The elution profile of intimal-medial PG extracts from unlabeled aortas was consistent, and PG were eluted at about 0.5 M NaCl on DEAE Sephacel column, whereas the large majority of other macromolecules in extracts were eluted earlier, (Fig.4). PG fractions were pooled and purified on gel filtration chromatography. The use of a Superose 6 HR column resolved PG into a high molecular weight fraction (Fig. 5). The peak was bimodal for both PG isolated from normal and injured aortas. The amount of GAG and protein was determined in those fractions which were considered purified PG fractions. GAG was $0.93\pm0.2$, $1.9\pm0.4$ and $1.1\pm0.2$ mg/g DDT for normal, REA and DEA respectively (mean $\pm$SD, n=4). When the amount of GAG was determined in the residue, it was revealed that 30-40% of the total intimal-medial GAG was extracted with 4M Gu-HCl buffer. The amount of protein constituted about 25-35% of the PG molecules from each tissue type.

GAG composition was analyzed by electrophoresis on a cellulose acetate membrane which resolved GAG into four main classes: Chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and hyaluronic acid (HA), (Fig.6). Pretreatment with enzyme chondroitinase AC and densitometric evaluation revealed that CS comprised about 55% of total GAG extracted from normal aortas whereas, it comprised about 64% of total GAG extracted from injured aortas. HA was significantly reduced in neointima of injured aortas compared to that from normal aortas. There was no significant difference between DS and HS proportion in either injured or normal aortas, (Fig. 7).

Figure 4: Elution profile of PG, extracted from neointima of REA, obtained during ion exchange chromatography. The extract was loaded on a DEAE Sephacel column connected to an FPLC system. PG were eluted with 7M urea followed by a linear NaCl gradient from 0.15M to 1.0M in the same buffer and at a flow rate of 0.25 ml/min. Fractions containing PG eluted at 0.5M NaCl.

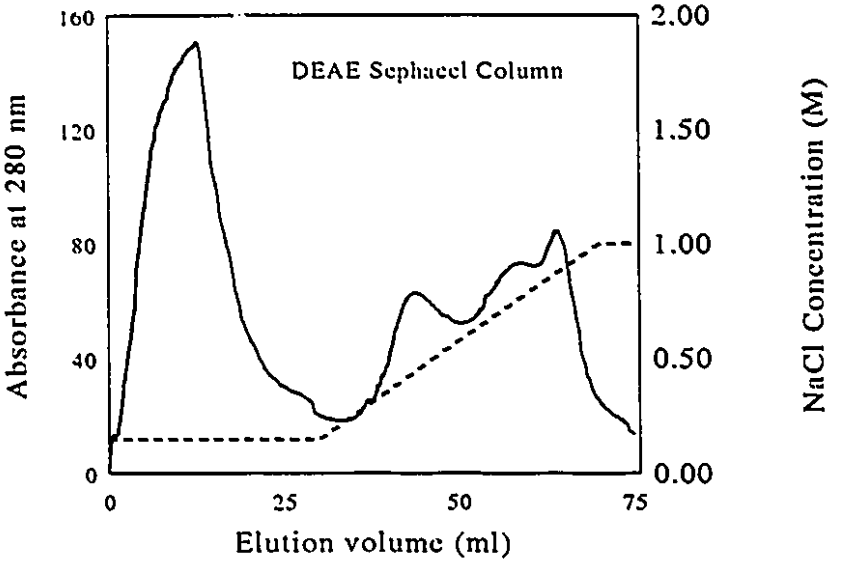

DEAE Sephacel Column
Absorbance at 280 nm
NaCl Concentration (M)
Elution volume (ml)
160
120
80
40
0
2.00
1.50
1.00
0.50
0.00
0
25
50
75

Figure 5: Elution profile of PG fractions obtained after ion exchange chromatography and subjected to gel filtration chromatography using Superose 6 column. Fractions were eluted with 0.5M NaCl, at a flow rate of 0.2 ml/min., and one ml fractions were collected. Fractions forming the first peak after the void volume were positive for PG. ( $V_o$ =6.5 ml, $V_t$ =25 ml).

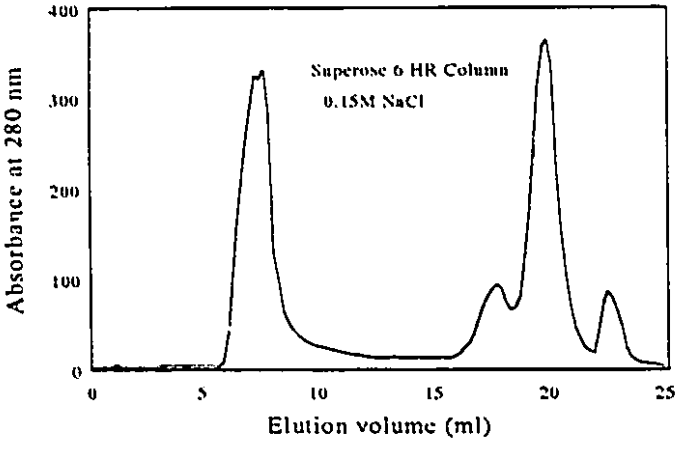

Superose 6 HR Column
0.15M NaCl
Absorbance at 280 nm
400
300
200
100
0
0
5
10
15
20
25
Elution volume (ml)

Figure 6: Electrophorograms, on cellulose acetate membrane, showing GAG composition of aortic intimal-medial extracts after staining with 0.2% Alcian blue. Lane 1 represents GAG standards: CS, DS, HS and HA. Lanes 2, 3 and 4 represents GAG composition in REA, DEA and normal aortic extracts.

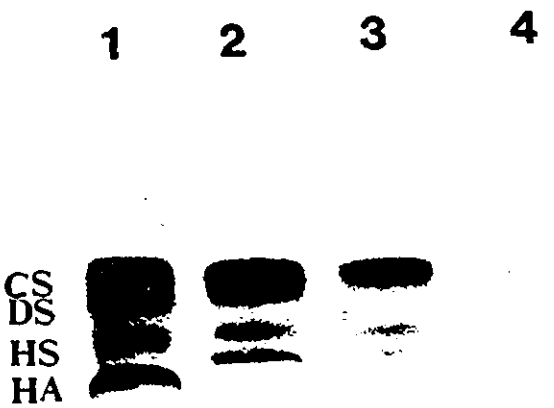
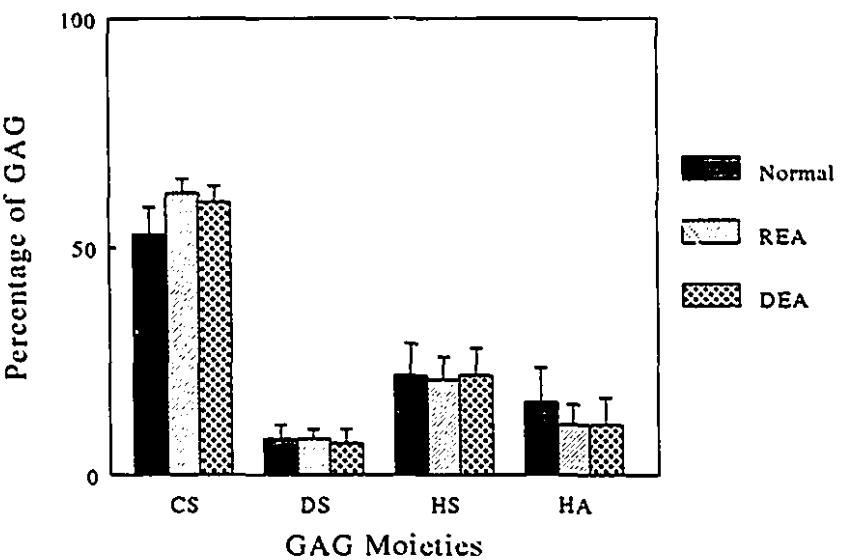

1
2
3
4
CS
DS
HS
HA

Figure 7: Bar graph represents GAG distribution pattern after densitometer evaluation. Bars represent the percentage of GAG moiety in aortic intimal-medial extracts. (values are mean $\pm$ SD, n=4)


100
50
0
Percentage of GAG
CS
DS
HS
HA
GAG Moieties
Normal
REA
DEA

## 2.5 DISCUSSION

The data included in the present investigation indicate that the *de novo synthesis* of PG determined in neointima covered by regenerated endothelium versus intima of normal aorta was greatly increased (2-3 fold). This finding is similar to previous finding in our laboratory (Alavi and Moore, 1985; Alavi et al., 1992; Galis et al., 1992), but extends further. In this study, we emphasized the overall assessment of sulfated PG molecules with regard to the site of synthesis and accumulation, molecular size characteristics, proportion of the different PG population in the aortic intima-media, and GAG distribution.

The technique of organ culture to study $^{35}$S-PG was previously employed by Yanagishita and Hascall (1983), Radhakrishnamurthy et al. (1988) and Galis et al. (1992). Since the incubation time was 8 hours, this enabled us to localize the newly synthesized sulfated PG as well as areas of accumulation of the PG produced.

Since extensive washing was performed, throughout all the steps included in processing the tissues till they were coated with the radioautographic reaction, the possibility of passive diffusion of the radioisotope could be eliminated. Furthermore, greater than 75% of radioactivity was found to be bound in aortic intimal-medial extracts, and only 40% of sulfated PG was extracted by 4M Gu-HCl, as determined in this study, this means that silver grains overlying intimal tissues would represent actual binding to the newly synthesized sulfated PG and minimal, if any, grains would account for unbound or free radioactivity due to passive diffusion.

Radioautographs demonstrated that $^{35}$S-sulfate was incorporated into endothelial and SMC, both at the cell surface and within the cells, and in the ECM. The cell associated PG are HS and CS (Ruoslahti, 1989). CS has been described to be the main PG produced by SMC, HS by endothelial cells while DS are produced by both SMC and fibroblasts and derive mainly from the medial tissue (Wight, 1989). The association of silver grains over the cell surface may suggest a critical role played by sulfated PG in the regulation of cell growth and differentiation. The extensive accumulation of newly synthesized sulfated PG in the ECM of injured aortas, may be attributed to the phenotypic modulation of SMC (Merrilees et al., 1990; Li et al., 1993), which in turn contribute most of the PG in the ECM (Young, 1973). The high concentration of sulfated PG towards the luminal front, observed in injured aortas with regenerated endothelium, substantiates the role played by PG in trapping lipoproteins during atherogenesis, and the accumulation of lipids in areas covered by regenerated endothelium (Hajjar et al., 1981; Alavi et al., 1984; Galis et al., 1992). The accumulation of PG, at the luminal front in neointima from REA, would also suggest that PG through their adhesion properties may facilitate the interaction of invading cells, such as platelets and monocytes, to the vessel wall and hence contribute to lesion development and progression.

The biosynthesis, by neointima of injured aortas, of PG with the high molecular weight population at a higher proportion and of larger polymer size as determined by size exclusion chromatography, is reminiscent of PG extracted from atherosclerotic lesions. The high molecular weight population of PG, which is composed mainly of CS, revealed larger polymer size PG in the case of injured aortas than its counterpart in normal aortas,

suggesting longer CS chains might be present in neointima of injured aortas. Wagner and collaborators (1986) described the properties of PG extracted from human atherosclerotic lesions, and proposed that these lesions might contain longer CS side chains. Radhakrishnamurthy and collaborators (1988) found that PG synthesized by aortic explants, derived from hypercholesterolemic rabbits, differ from PG of normal aorta in their longer CS chains. Galis et al. (1992), using the same injury-model, suggested through their immunocytochemical, ultrastructural observations, that CS chains might be longer in neointima of injured aortas than that of normal aortas.

The significance of the larger polymer size CS chains in neointima, may be their capacity for capturing soluble molecules such as growth factors in mediating cell to cell or cell to matrix signalling during atherosclerosis (Ruoslahti, 1989). Another important aspect of the longer CS chains could be as a potent means for the interaction with lipoproteins within the intima and hence they could augment lipoprotein sequestration in the aortic wall during atherogenesis.

Biochemical analyses of GAG composition of extracted PG from unlabeled aortas confirmed previous findings that aortic intimal-medial extracts were composed mainly of CS. In addition to DS, HS and HA, we again found a significantly higher concentration of GAG in injured aortas along with an increase in the proportion of CS. These data substantiate the previous finding with labeled PG, where injured aortas exhibited a higher proportion of the high molecular weight PG, which was composed mainly of CS.

Chondroitin sulfate exhibits potent atherogenic properties and is found to accumulate more extensively in early atherosclerotic lesions, (Berenson et al., 1984; Hoff

and Wagner, 1986; Wight et al., 1991). It is selective in lipoprotein binding, forming complexes with apo-B containing lipoproteins, (Wagner et al., 1986; Alavi et al., 1989). This interaction between CS and lipoprotein is considered one of the major mechanisms by which lipid accumulates both intracellularly and extracellularly during atherogenesis.

In summary, the newly synthesized sulfated PG were found to be characteristically different in concentration, distribution and composition, in neointima of injured aortas compared to intima of normal aortas. Chondroitin sulfate is the major class of PG accumulated within the neointima and exhibits longer chains than its counterpart in normal aortas. Results from this study may provide a possible explanation for enhanced PG accumulation in early atherosclerotic lesions and its potential role in lesion development and progression.

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## CHAPTER 3

# CHARACTERIZATION OF LIPOPROTEIN-PROTEOGLYCAN COMPLEXES ISOLATED BY AFFINITY CHROMATOGRAPHY FROM AORTIC NEOINTIMA OF NORMOCHOLESTEROLEMIC RABBITS

## 3.1 ABSTRACT

Lipoprotein-Proteoglycan (LP-PG) complexes have been extracted from atherosclerotic lesions. Earlier we have shown that the neointima, developed in response to a selective deendothelialization of the aorta of normolipemic rabbits, has many features in common with developing atherosclerotic lesions. Consequently, we asked whether LP-PG complexes are present in the neointima. Intima-media from normal and injured rabbit aortas were extracted in 0.16M NaCl for 24h at 4°C with gentle shaking. The extract was passed through an anti-apo-B affinity column using an FPLC system. Adsorbed material, dissociated with 4M Gu-HCl buffer was checked for LP and GAG content. Fractions found positive for both were further characterized by molecular-sieve gel chromatography, electrophoresis, immunodiffusion and electron microscopy. Results showed the presence of LP-PG complexes in both normal and injured aortas. LP-PG complexes consisted of apo-B associated with chondroitin sulfate and hyaluronic acid. These results confirmed that LP-PG complexes are present in normal and injured aortas of normocholesterolemic rabbits. Furthermore, our data revealed that injury to the aorta increased the amount of LP-PG complexes within the reendothelialized neointima of normolipemic rabbits.

## 3.2 INTRODUCTION

Atherosclerosis is characterized by lipid accumulation in the fibromuscular thickened intima. The mechanisms of lipoprotein (LP) interaction with the arterial wall that lead to the accumulation of LDL-cholesterol have not been fully elucidated. Proteoglycans (PG) of the vascular extracellular matrix could be responsible for LP accumulation by virtue of their ability to form complexes with lipoproteins (Camejo, 1982; Vijayagopal et al., 1985; Salisbury et al., 1985; Alavi et al., 1989). It has been proposed that PG through its interaction with lipoproteins can modify lipoproteins to be recognized and internalized avidly by arterial cells (Wight, 1989; Camejo et al., 1991). LP-PG complexes have been isolated from early human atherosclerotic lesions (Srinivasan et al., 1972; Camejo et al., 1980; Vijayagopal et al., 1992) as well as from hypercholesterolemic animal models of atherosclerosis (Mawhinney et al., 1978). To date no information is available on whether these complexes are also present in the neointima, developed in response to injury using a balloon catheter, of normocholesterolemic rabbits. The importance of these complexes resides in the mechanism by which they mediate LP sequestration in the arterial intima and subsequent cellular processes, setting the stage for the development of atherosclerosis.

In the present study, we investigated the presence of LP-PG complexes in neointima of injured aortas of normocholesterolemic rabbits. We utilized mild extraction procedures to avoid dissociation of the components forming the complexes. The extract was then applied to an anti-apo-B affinity column to specifically isolate apo-B bound PG.

Data from these results indicate that deendothelialization of the aorta enhances the accumulation of LP-PG complexes within the arterial wall, even in the absence of hyperlipidemia.

## 3.3 MATERIALS AND METHODS

### *3.3.1 Animals*

Male and female New Zealand white rabbits (2-3 kg) were maintained on regular rabbit chow for 2 weeks before surgery. De-endothelialization of the aorta was achieved using a 4F Fogarty balloon catheter (Model 12-010, Edward Lab. Inc., Santa Anna, Calif.) as previously described (§ 2.3.1). Rabbits were maintained on regular diet and water ad libitum for 12 weeks, at which time experiments were performed.

### *3.3.2 Antibody purification and preparation of affinity column chromatography*

To isolate anti rabbit apo-B immunoglobulins, purified rabbit apo-B lipoproteins were injected into a goat for immunization. The antisera was precipitated three times with a saturated solution of ammonium sulfate (pH 7.8) and IgG fraction was further purified by DEAE Sephacel chromatography equilibrated with 10mM potassium phosphate buffer. Fractions were eluted with the same buffer, followed by a linear NaCl gradient from 0 to 0.5M in the same buffer at a flow rate of 0.1 ml/min. Eluted fractions, were checked against rabbit apo-B containing lipoproteins and HDL by radial immunodiffusion. The purity of IgG fraction was ascertained by SDS-PAGE electrophoresis. The anti-apo B LP immunoglobulins (26 mg protein) were coupled to 3 g of cyanogen bromide activated Sepharose 4B (Pharmacia, Canada) in 0.1M $NaCO_3$, o.5M NaCl pH 8.3. The mixture was allowed to rotate end-over-end for 2h at room temperature. Excess ligand was washed with coupling buffer, and remaining active groups were blocked with 0.1M Tris-HCL pH 8 for 2h at room temperature. An 8 ml

column (10 x 1 cm) was prepared and was washed for three cycles with alternating pH. The column was then equilibrated with 0.15M NaCl, 10mM Tris-HCl pH 7.4 and checked for efficient coupling by passing $^{125}$I-LDL (110,000 cpm) into the column which was allowed to dissociate by 4M Gu-HCl buffer pH 7.2 according to Camejo et al. (1985). Eluted fractions were tested for radioactivity.

### *3.3.3 Extraction of LP-PG complexes*

*In vivo* LP-PG complexes were extracted from intima-media of control and injured aortas by a modification of the procedure of Vijayagopal et al. (1992). Briefly, intimal-medial tissues from control, areas of regenerated endothelium (REA) and areas denuded of endothelium (DEA) were separated from the adventitia, and carefully minced to minimize any trauma to the intimal surface. The aortic tissues were separately extracted with 7 vol/g wet tissue of 0.15M NaCl, 10mM Tris-HCl (pH 7.4) containing protease inhibitors (10mM $\epsilon$-aminocaproic acid, 10mM benzimidine, 10mM phenylmethylsulfonyl fluoride), 10mM EDTA and 0.1% sodium azide [Buffer A] at 4°C for 24 hours with gentle shaking. The extracts were centrifuged at 800 g for 30 min at 4°C. The opalescent middle layer was separated from the upper and lower layers. Each layer, upper, middle and lower, was applied to the anti-apo B affinity column, that has been equilibrated with buffer A. The column was washed with buffer A at a flow rate 0.2 ml/min until no absorbing material was detected in the eluent at 280 nm. To elute the adsorbed material, the column was washed with 4M guanidinum-hydrochloride (pH 7.4). Sequential 1 ml fractions were collected and tested for PG by staining with alcian blue, and for apo-B by serial immunodiffusion against anti apo-B. Fractions positive for

both PG and LP were pooled, dialyzed against buffer A, concentrated and used for characterization.

### *3.3.4 Gel Filtration Chromatography*

The amount of GAG and LP determined in LP-PG complexes isolated by the affinity column was too little for further analysis. In order to have a better yield, another well established technique, described by Srinivasan and collaborators (1972, 1992), for the extraction of LP-PG complexes was employed. Briefly, the opalescent middle layer, formed after centrifugation of LP-PG complex extracts, was applied to a gel filtration column (Superose 6 HR, 30x1cm) to purify LP-PG complexes. The column was equilibrated with buffer A and fractions were eluted at a flow rate of 0.2 ml/min. One ml fractions were collected and tested for PG and apo-B. Fractions positive for both PG and apo-B were pooled, dialyzed and used for further characterization.

In order to determine whether LP-PG fractions isolated by the gel filtration column were in a complex form, one aliquot of rabbit aortic PG extract was applied to the same column. PG elution was shifted to the right from LP-PG elution pattern, indicating that LP-PG complexes isolated by the gel filtration column was in a complex form.

#### *3.3.4.1 Characterization of LP-PG complexes*

##### *3.3.4.1.1 Proteoglycan characterization*

All characterization techniques were done from the complexes isolated by gel filtration column. Glycosaminoglycan (GAG) characterization was carried out by electrophoresis on a cellulose acetate membrane, after papain digestion and ethanol

precipitation of GAG as described by Alavi et al. (1992). Densitometer evaluation comparing GAG distribution, obtained from the electrophorograms before and after enzymatic digestion, with Chondroitinase AC enzyme, was assessed using a Hoefer GS 300 scanning densitometer equipped with GS-360 densitometry software (Alavi et al., 1989).

*3.3.4.1.2 Lipoprotein characterization*

To characterize the LP component of LP-PG complexes, one aliquot of the LP-PG complex was adjusted to a solvent density of 1.063 g/ml by KBr and centrifuged for 22 h at 45000 rpm. Lipoproteins were recovered from the top of the centrifuge tube, dialyzed against buffer A and characterized by radial immunodiffusion against anti apo-B. Another aliquot of the LP recovered by density centrifugation was spread on a formvar-carbon coated grid, stained with phosphotungestic acid then examined on Philips 300 Electron Microscope.

## 3.4 RESULTS

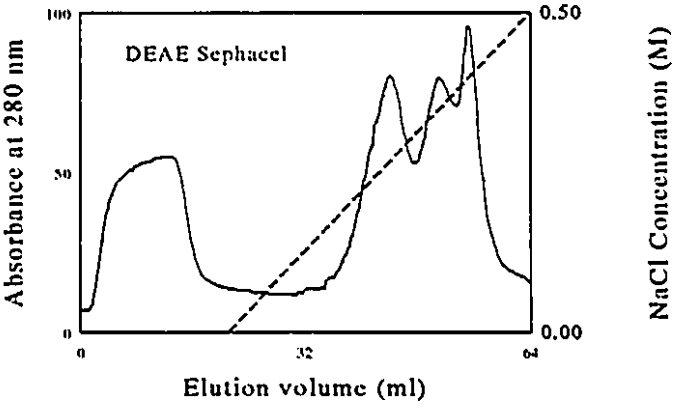
### *3.4.1 Antibody purification and affinity chromatography*

The elution profile of anti-apo-B IgG, in a DEAE Sephacel column, is presented in Fig. (1). Only the first and second peak after the gradient showed immunoreactivity with rabbit apoprotein B. No immunoreactivity was observed to rabbit HDL, indicating the specificity of anti apo-B, with these two peaks. SDS-PAGE , under reducing conditions, of all peaks resolved after ion exchange chromatography, demonstrated that the first and second peak after the gradient were purified IgG fractions. Purified IgG fractions resolved into two bands of molecular weight around 50 KD and 23 KD corresponding to the heavy and light chains respectively (Fig. 2).

The specificity of the affinity column for apo-B was ascertained after passing rabbit $^{125}$I-LDL and PG. Rabbit PG did not show any appreciable absorption while greater than 60% of the $^{125}$I-LDL was found to be adsorbed.

Figure 1: Elution profile of anti apo-B antisera, following a passage through an 8 ml DEAE Sephacel column, after the third precipitation with ammonium sulfate. Apo-B IgG eluted with 10mM ammonium phosphate buffer followed by a linear NaCl gradient from 0 to 0.5M in the same buffer at a flow rate of 0.1 ml/min. Fractions containing apo-B IgG, as detected by immunodiffusion, were included within the first and second peak after the gradient.

Figure 2: Electrophorograms developed after subjecting all peaks, resolved by antisera purification by ion exchange chromatography, to 12 % SDS-PAGE under reducing conditions. Lane 1 represents molecular weight markers (numbers represent MW in KD). Lane 2 represents fractions eluted before gradient. Lanes 3, 4 and 5 represent first, second and third peak after gradient respectively.


DEAE Sephacel
Absorbance at 280 nm
100
50
0
0
32
64
Elution volume (ml)
0.50
0.00
NaCl Concentration (M)

*3.4.2 Determination of LP-PG complexes from normocholesterolemic rabbit aortas*

Lipoprotein-Proteoglycan (LP-PG) complexes were extracted from aortic intimal-medial tissues of three different sources; normal, REA and DEA. Low centrifugation of the extract from each tissue, resolved into three layers: an upper and a lower transparent layer and a middle opalescent layer. The three layers were carefully separated and passed individually through the anti apo-B affinity column. The upper and lower layers from each extract had almost the same elution profile, with no absorbance detected with buffer B. Only the opalescent layer from each extract showed affinity for the anti apo-B column. Figure 3 represents the elution profile of LP-PG complexes that had been passed through the affinity column. Eluted fractions from the adsorbed material showed a single band with anti-apo-B in radial immunodiffusion wells, Fig. 4. All of the immunoreactive apo-B were associated only with the adherent fractions. To check for the presence of PG, in the eluted fractions, GAG were determined in both adherent and non adherent fractions. The non adherent fractions accounted for most of the protein and GAG (75-80%) in the middle layer. The amount of protein and GAG determined in adherent fractions isolated by the affinity column are represented in Table I:

| Tissue Type | Protein ($\mu$g/g DDT) | GAG ($\mu$g/g DDT) |
|---|---|---|
| Normal | 172 | 62 |
| REA | 213 | 95 |
| DEA | 170 | 60 |


180
116
84
58
48
36
26
5
4
3
2
1

Figure 3: Elution profile of soluble extracts of aortic intimal-medial tissues applied to anti apo-B affinity column. An 8 ml column (10 x 1 cm) was equilibrated with 0.15M NaCl, 10mM Tris, pH 7.4 at a flow rate of 0.2 ml/min. Adsorbed material containing LP-PG complexes were eluted with 4M Gu-HCl buffer.

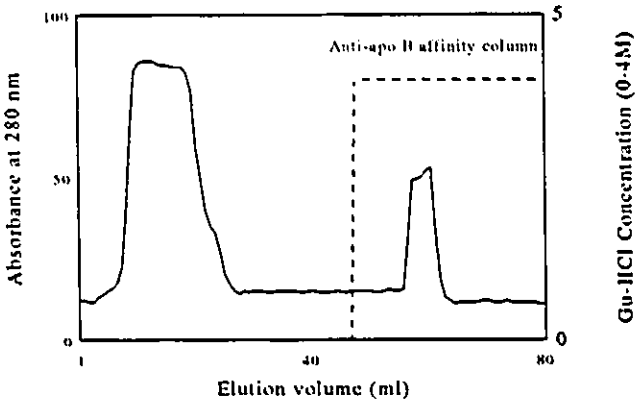
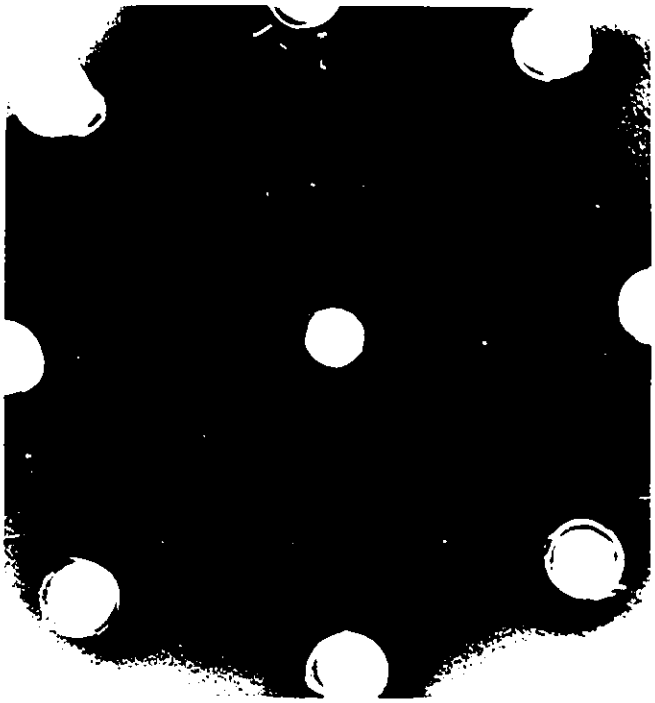

Anti-apo B affinity column
Absorbance at 280 nm
100
50
0
1
40
80
5
0
Gu-HCl Concentration (0-4M)
Elution volume (ml)

Figure 4: Immunoreactivity of eluted fractions from adsorbed material on the apo-B affinity column. A single arc is clearly visible in radial immunodiffusion of LP-PG complexes, isolated by the affinity column, with anti apo-B.



### *3.4.3 Proteoglycan composition of LP-PG complexes*

Figure (5) shows a representative elution profile of LP-PG complexes on Superose 6 HR column. Fractions positive for both PG and LP, as checked by Alcian blue staining and by immunodiffusion against anti apo-B, were considered as LP-PG complexes. Those fractions, positive for LP-PG, eluted as a single peak at the void volume of the column. When PG extract was applied to the same column, it was resolved into a high molecular weight fraction shifted to the right towards the inclusive volume of the column. This suggested that LP associated with PG in those fractions should be in a complex form.

The identification of the types of GAG present in LP-PG complexes was determined by GAG electrophoresis on cellulose acetate membrane. LP-PG complexes were composed of chondroitin sulfate and hyaluronic acid (Fig. 6). Treatment by Chondroitinase AC revealed that no dermatan sulfate was associated in LP-PG complexes. Densitometric evaluation comparing GAG distribution pattern, obtained from electrophorograms, revealed that chondroitin sulfate comprised the most of GAG components of the complexes and it represented 60% of GAG in complexes extracted from normal aortas and 62% of the GAG composition from injured aortas (REA).

Figure 5: Elution profile of LP-PG complexes purified by gel filtration chromatography. LP-PG complexes eluted as a single peak at the void volume of Superose 6 HR column (30 x 1 cm). The column was equilibrated with 0.15M NaCl, 10mM Tris, pH 7.4 and one ml fractions were collected at a flow rate of 0.2 ml/min.

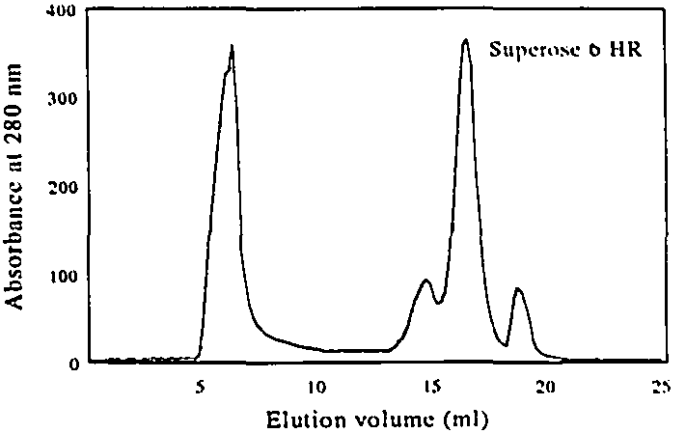
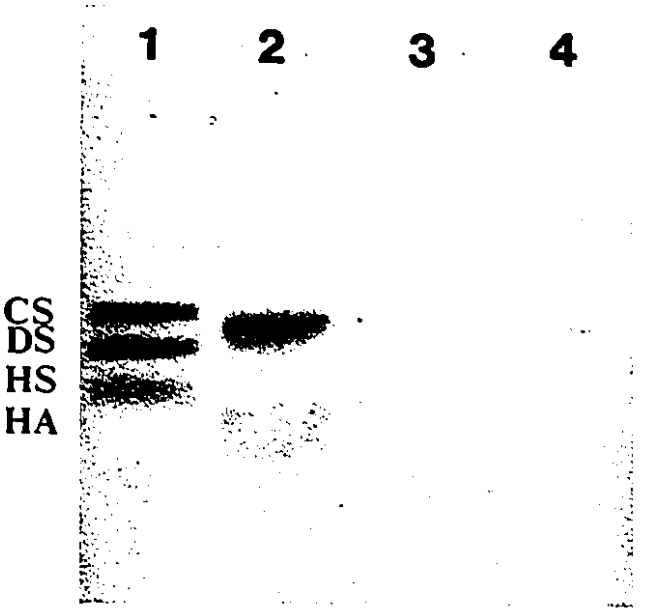

Superose 6 HR
Absorbance at 280 nm
400
300
200
100
0
5
10
15
20
25
Elution volume (ml)

Figure 6: Electrophorogram illustrating GAG composition of LP-PG complexes. Lane 1 represents GAG standards. GAG composition of LP-PG complexes isolated from injured aortas (REA) are represented in lane 2, injured aortas (DEA) in lane 3 and normal aortas in lane 4.

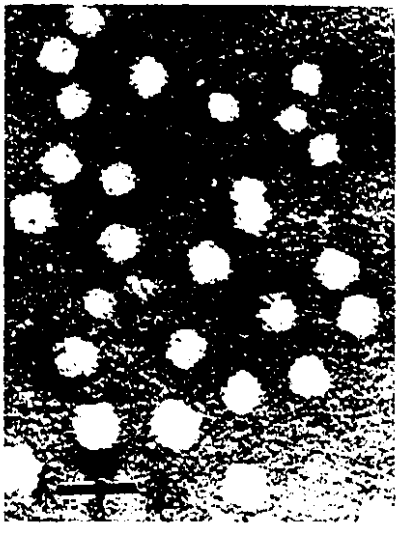

1
2
3
4
CS
DS
HS
HA

### *3.4.4 Lipoprotein composition of LP-PG complexes*

Lipoprotein (LP) of LP-PG complexes is apo-B containing LP as revealed from the affinity chromatography and immunodiffusion against anti-apoB antibody. The ratio of protein to GAG was much higher than that present in aortic proteoglycans, reflecting the association of lipoproteins with the complexes.

Electron microscopic examination of negatively stained aliquots of lipoproteins recovered from density centrifugation ($< 1.063$ g/ml) revealed lipoprotein particles of 20 and 100 nm in diameter. The majority of the particles had an average diameter size of 80-100 nm (Fig. 7) identical to those of VLDL particles as widely reported by Chapman (1980).

Figure 7: Electron micrograph of negatively stained aliquot of lipoproteins recovered from density centrifugation (< 1.063 g/ml) of LP-PG complexes. Lipoprotein particles having a diameter size ranging from 20 to 100 nm. The majority of the particles are of 100 nm in diameter. Bar =200 nm



## 3.5 DISCUSSION

Our data demonstrate for the first time, that normal and injured aortas of normocholesterolemic rabbits contain intact LP-PG complexes. The interaction between LP and PG is mainly ionic in nature ( Iverius, 1973). Moreover, multiple binding regions in the protein moiety (apo-B) of LDL that interact with specific recognition binding sequences in the GAG chains have been identified (Hiroshe et al., 1987; Camejo et al., 1988; Cardin and Weintraub, 1989). LP-PG complexes have been implicated as a mechanism for LP sequestration in the arterial intima and subsequent cellular processes (Camejo, 1982).

Anti-apo-B affinity column to isolate LP-PG complexes from atherosclerotic lesions was recently used by Camejo et al.(1985) and Vijayagopal et al.(1992) and appeared to be a very useful tool for isolation. Purified LP-PG complexes isolated by affinity column indicated that PG that eluted with the adherent apo-B should have been tightly associated with LP, since PG was found in the adherent fraction and confirmed that PG was complexed with LP. The amount of GAG and protein determined in LP-PG complexes isolated by affinity chromatography, indicated that injured aortas from REA possess more complexes than normal aortas or DEA. These findings are consistent with the observations that lipoprotein accumulates in the arterial wall of injured aortas preferentially in areas covered by regenerated endothelium (Clowes et al., 1978; Hajjar et al., 1981; Moore et al., 1982; Alavi and Moore 1984).

By gel filtration chromatography, the crude extract from aortic intimal-medial

tissues was resolved into high molecular weight fractions containing LP and PG. When rabbit aortic PG extract was applied to the same column, its elution profile was shifted to the right suggesting that the fractions positive for apo-B and PG, should be in a complex form.

Data from this study revealed that glycosaminoglycan composition of rabbit LP-PG complexes, as determined by electrophoresis was composed of chondroitin sulfate and hyaluronic acid. The ratio of chondroitin sulfate to hyaluronic acid was higher in injured aorta than in control rabbit aorta. It should be emphasized that this GAG distribution is specific to the LP-PG complexes isolated by a mild extraction procedure, whereas, the aortic intimal-medial extracts comprising 4 types of GAG, is distinctly different. LP-PG complexes isolated from human fatty streaks and fibrous plaque also contained chondroitin sulfate and hyaluronic acid (Srinivasan et al., 1972) and very little dermatan sulfate (Vijayagopal et al., 1992); whereas the GAG composition of hypercholesterolemic rabbit LP-PG complexes were found to consist of heparan sulfate, chondroitin sulfate and hyaluronic acid (Mawhinney et al., 1978). Other investigators (Camejo et al., 1985) have not been able to detect hyaluronic acid in LP-PG complexes isolated from human atherosclerotic lesions. This could be due to different extraction procedures or different lesion composition. Chondroitin sulfate is the predominant glycosaminoglycan observed in high quantity during the course of atherosclerosis (Wagner et al., 1986; Alavi et al., 1987; Berenson et al., 1988). Vijayagopal and collaborators (1981) have demonstrated that chondroitin sulfate PG is potent and selective in binding to lipoprotein.

Heparan sulfate was not determined in LP-PG complexes, isolated in this study.

This may be due to the absence of heparan sulfate in LP-PG complexes of injured rabbit aortas, as in the case of the human atherosclerotic plaque. Alternatively, a mild extraction procedure was unable to extract heparan sulfate, which is firmly bound to the elastic tissue (Vijayagopal et al., 1983). HS binding ability to LP is relatively poorer than chondroitin sulfate PG (Vijayagopal et al., 1983).

As in human lesions, which contained both LDL and VLDL (Srinivasan et al., 1972; Vijayagopal et al., 1992), LDL and VLDL were determined in the LP-PG complexes from rabbit aortas. VLDL is the main lipoprotein in rabbit plasma (Chapman, 1980). That could be the reason we found more VLDL than LDL in isolated complexes. VLDL particles have a density greater than 0.94 g/ml but less than 1.006 g/ml and have an average diameter of 100 nm. Lipoproteins of the LP-PG complexes were characterized in this study by immunodiffusion against anti apo-B and by electron microscopy of negatively stained aliquot purified by differential, density flotation.

In the present investigation, LP-PG complexes in neointima formed in response to deendothelialization of aortas of normocholesterolemic rabbits were studied for the first time. This animal model of injury-induced atherosclerosis is widely used in the rabbit as well as in other experimental animals and has been observed to reproduce lesions of a composition, similar to that of human atherosclerotic plaques. Consequently, this study provides a better understanding of the role of arterial injury and lipid accumulation during atherogenesis.

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## CHAPTER 4

# LIPOPROTEIN-PROTEOGLYCAN COMPLEXES FROM INJURED AORTAS ACCELERATE LIPOPROTEIN UPTAKE BY ARTERIAL SMOOTH MUSCLE CELLS

## 4.1 ABSTRACT

Lipoprotein-Proteoglycan (LP-PG) complexes are taken up more avidly by smooth muscle cells (SMC) and macrophages than native lipoproteins (LP). The enhanced uptake may contribute to lipid accumulation and foam cell formation during atherogenesis. Endothelial injury is known to alter proteoglycan (PG) synthesis and distribution in neointima, developed in response to injury. The present study examines the uptake and degradation of LP-PG complexes, derived from PG of injured aortas by arterial SMC. Rabbit apo-B lipoprotein (LP), including VLDL, IDL and LDL was isolated by ultracentrifugation and coupled with PG extracted from normal aortas (NPG) or with PG from injured aortas (IPG). Rabbit aortic SMC were cultured from intima-media explants, incubated with $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG for 20 h at 37°C. LP binding, internalization and degradation was markedly increased ($p<0.001$) for LP-NPG and LP-IPG over native LP. Competition experiments indicated that more than 50% of the LP-PG complexes are taken up by the apo-B/E receptor pathway. Phagocytosis was the second important route of uptake of these complexes, whereas the scavenger receptor played a minor part in the uptake and degradation of LP-PG complexes. Data from this study indicate that LP-PG complexes accelerate LP uptake and degradation by SMC more than native LP. Therefore, these complexes may contribute to lipid accumulation by SMC, thus generating foam cells. Furthermore, LP-PG complexes prepared from PG of injured aortas are more effective in lipid accumulation than LP-PG complexes from PG of normal aortas.

## 4.2 INTRODUCTION

It is well established that the lipid-laden foam cells within atherosclerotic plaques are smooth muscle cells (SMC) and blood monocyte-derived macrophages (BMDM) (Klurfeld, 1985; Rosenfeld et al., 1987; Katsuda et al., 1992). The uptake of modified lipoproteins (LP) by macrophages is thought to contribute to lipid accumulation, foam cell formation and the development of atherosclerotic lesions (Steinberg et al., 1989; Stiko-Rahm et al., 1992)). Scavenger receptors, which bind certain chemically modified forms of LP are expressed by endothelial cells, macrophages and SMC (Goldstein et al., 1979; Nagelkerke et al., 1983; Pitas et al., 1985; Pitas, 1990). Other modifications such as LDL aggregation, LP aggregates formed in response to monoclonal antibodies and the formation of lipoprotein-proteoglycan (LP-PG) complexes were also found to cause accelerated uptake of LP by macrophages, although the mechanisms of uptake were different (Salisbury et al., 1985; Hurt et al., 1990, Khoo et al., 1992). Aggregates of LP complexed with PG have been shown to induce cholesteryl ester accumulation in BMDM in vitro and in subendothelial cells from human aortic intima (Bondjers et al., 1990; Hurt-Camejo et al., 1992; Vijayagopal et al., 1992). We have previously shown that LP-PG complexes are present in normal and deendothelialized aortas of normocholesterolemic rabbits (Ismail et al., 1991). PG synthesized by intima-media of injured aortas differ significantly from PG of normal aortas in both physical and chemical properties (Wight et al., 1983; Alavi and Moore 1985; 1987).

The mechanism by which SMC become foam cells in vivo has not been

established. The current investigation was undertaken to examine the hypothesis that LP-PG complexes could stimulate the uptake of apo-B LP by SMC and thus contribute to foam cell formation. Since endothelial injury has been shown to induce alterations in PG synthesis and distribution, we studied the effect of LP-PG complexes on SMC, utilizing PG derived from normal and injured aortas.

## 4.3 MATERIALS AND METHODS

### *4.3.1 Smooth muscle cell culture conditions*

SMC were cultured from intima-media explants of normal rabbit aortas in a tissue culture incubator (95% $O_2$, 5% $CO_2$) at 37°C. Cells were allowed to grow for three passages in Dulbecco's Modified Eagle's Medium ( Gibco Canada Inc) supplemented with 10% fetal bovine serum, sodium bicarbonate, L-glutamine, non essential amino acids, sodium pyruvate and penicillin-streptomycin (medium A). SMC were identified by morphology and immunologically with antibody specific for α-smooth muscle actin. After the third passage, confluent cells were removed from their dishes by trypsinization in the presence of EDTA (0.5% Trypsin, 0.2% EDTA) and were counted. Cells were reseeded into 35 mm dishes at a density of $10^5$ cells/dish. Incubation studies were performed after two days at which time SMC were confluent and quiescent.

### *4.3.2 Rabbit arterial proteoglycans*

Intima-media PG from normal and injured aortas were prepared as described in detail previously (§ 2.3.3). The PG preparations from normal aortas (NPG) and from injured aortas (IPG) were lyophilized and stored at -20°C until used.

### *4.3.3 Lipoproteins and lipoprotein-deficient serum*

Apo-B lipoproteins (VLDL, IDL, LDL) were prepared from plasma (1mg/ml EDTA) of healthy, fasted rabbits by ultracentrifugation for 22 h after raising the density to 1.063g/ml by KBr. After centrifugation, the isolated LP were dialyzed against 0.16M NaCl, 10mM Tris-HCl pH 7.2 containing 10mM EDTA (Buffer A) and analyzed by cellulose acetate electrophoresis. Labeling of the LP with $^{125}$I-iodide was performed by

the Iodogen method according to the manufacturer's instructions (Pierce, Rockford, IL). The specific activity was 120 cpm/ng of protein and 93% of the radioactivity could be precipitated with 15% TCA (w/v). Iodinated LP were dialyzed against interaction buffer (5mM Tris-HCl, 2mM $MgCl_2$, 5mM$CaCl_2$, 5mM KCl pH 7.2).

Lipoprotein deficient serum (d > 1.22g/ml) was prepared by ultracentrifugation of the infranatant obtained by apo-B LP isolation. Lipoprotein deficient serum was dialyzed against Buffer A, checked by immunodiffusion for absence of apo-B, filtered through a 0.22 $\mu$m filter and stored at -20°C for use.

### *4.3.4 In vitro LP-PG complexes*

LP-PG complexes were prepared from two different sources of PG (NPG or IPG) and $^{125}$I-LP in interaction buffer. The PG was added to the LP at a ratio of 10-20 $\mu$g GAG/mg cholesterol of apo-B LP and the mixture was incubated for 1h at 4°C. The aggregated LP-PG complexes were collected by centrifugation at 10,000 g for 15 min at 4°C. The pellet was washed once with the interaction buffer, redissolved in culture medium containing LP free serum and filtered through a 0.45 $\mu$m filter. The radioactivity was measured in an LKB gamma counter. Identical aliquots of radioiodinated LP were added into the incubation medium so that every dish received an equal amount of apo-B LP.

### *4.3.5 Lipoprotein binding, internalization and degradation*

SMC were incubated for 24 h with culture medium containing LP deficient serum (medium B), then washed 3 times with PBS. The cells were then incubated with a fresh medium B containing $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG, at a cholesterol concentration

of 100μg/ml. For competition experiments, a 40 fold excess of unlabeled LP was added to the labeled LP-PG complexes. To examine the possible routes of uptake of LP-PG complexes by SMC, polyinosinic acid (50 μg/ml), an inhibitor of the scavenger receptors, was added to SMC together with PG-LP complexes. Cytochalasin D (10 μg/ml), an inhibitor of phagocytosis, was added to another group of SMC that had been incubated with LP-PG complexes. After incubation for 20 h, the cells were washed twice with PBS. Medium and washes were pooled and the extent of degradation of $^{125}$I-LP or $^{125}$I-LP-PG was determined according to the method of Stiko-Rahm et al (1992). Briefly, the degradation process was arrested by cooling the medium to 4°C , protein was precipitated by trichloroacetic acid (TCA) at a final concentration of 10% and removed by centrifugation for 10 min at 2000g. Free iodide was precipitated by adding $AgNO_3$ (final concentration of 2.5%) to the supernatant. After centrifugation at 2000g for 10 min, the TCA soluble $^{125}$I in the supernatant was measured in a gamma counter.

Cells were then treated with 5mg/ml trypsin in 1ml Hank's balanced salt solution for 10 min at 37°C. The trypsin solution was transferred to a tube and the trypsinized cells were washed twice with PBS. The trypsin solution and the two washes were pooled and radioactivity corresponding to the cell surface bound LP was measured. The cell layer was dissolved in 1ml of 0.2N NaOH in order to determine the amount of LP internalized by the cells. Parallel dishes without cells were always included as controls for non specific adsorption of the label. Every experiment was performed on 4 dishes per group. The experiments were repeated three times.

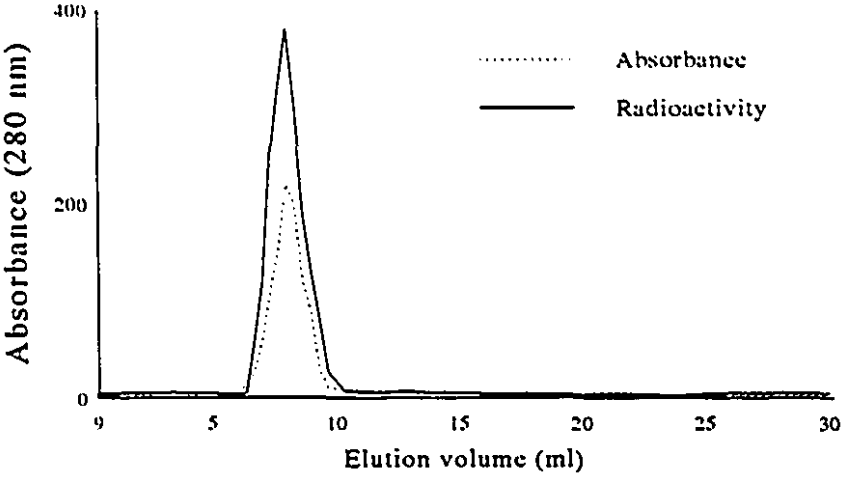
## 4.4 RESULTS

### *4.4.1 LP-PG complexes*

Although LP-PG complexes were prepared under identical experimental conditions with the same ratio of GAG to LP cholesterol, 60% of the added LP was identified in LP-IPG whereas only 45% of the added LP was formed in LP-NPG complexes. Therefore, IPG has more affinity for apo-B LP than NPG. These findings are in agreement with previous data from our laboratory (Alavi et al., 1989) demonstrating that injury-induced changes in PG influence their interaction with LP.

To check whether the medium used to dissolve the complexes might dissociate the complex into its individual components, one aliquot from the labeled complex dissolved in medium B was dialyzed, and passed through a gel filtration column (Superose 6 HR). Fig. 1 shows the elution profile of LP-NPG on gel filtration chromatography. It can be seen that LP-NPG eluted as a single peak and this peak comprised greater than 95% of the radioactivity, indicating that LP-PG complexes had not dissociated.

Figure 1: Gel filtration chromatogram of $^{125}$I-LP-NPG complexes after being dissolved in culture medium. Aliquots of 500 $\mu$l of $^{125}$I-LP-NPG (120,000 cpm) were passed through a Superose 6 HR column (30 x 1 cm) equilibrated with 5mM Tris, 0.15M NaCl, 4mM $CaCl_2$, 2mM $MgCl_2$ pH 7.2. The column was eluted at a flow rate of 0.25 ml/min. One mililitre fractions were collected and radioactivity was measured in 100 $\mu$l aliquots.

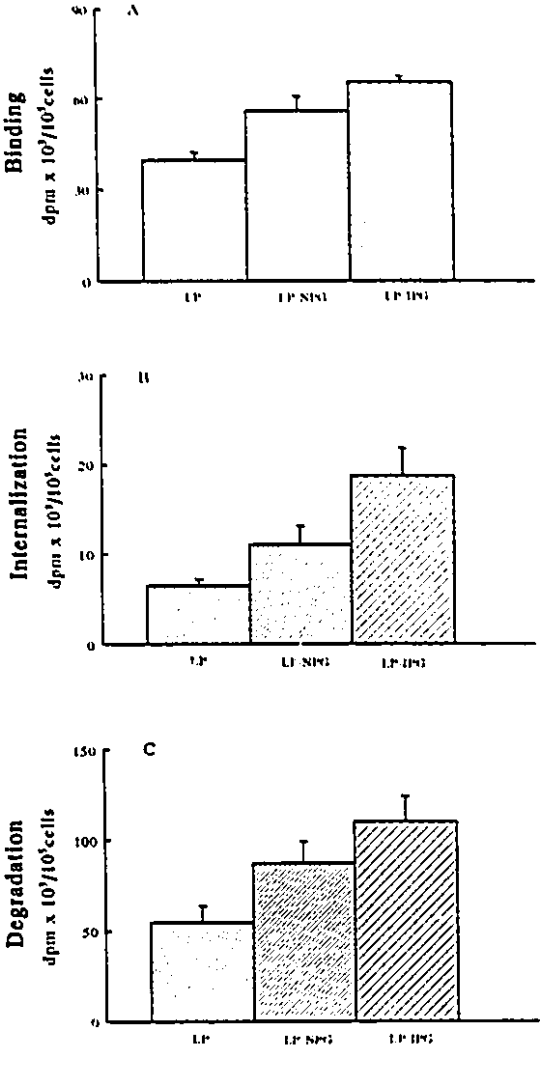

400
200
0
Absorbance (280 nm)
Absorbance
Radioactivity
9
5
10
15
20
25
30
Elution volume (ml)

*4.4.2 Lipoprotein binding, internalization and degradation*

Labeling of lipoprotein with $^{125}I$ enabled us to determine the difference in the uptake of PG-LP and native LP by SMC. The interaction of arterial PG with apo-B LP stimulated binding, internalization and degradation of LP ( Fig. 2). Comparatively, the LP-PG complexes induced 2-3 fold higher LP internalization by SMC than the native LP for LP-NPG ($p<0.02$) and LP-IPG ($p<0.001$) respectively. Moreover, SMC accumulated significantly more LP from LP-IPG than from LP-NPG in the form of internalized LP ($p<0.002$, $n=5$). Similarly, cell surface bound LP was significantly higher in the case of LP-PG complexes than native LP ($p<0.001$, $n=5$). SMC incubated with complexes prepared from IPG showed more bound LP than complexes prepared from NPG ($p<0.001$).

The degradation of internalized LP and LP-PG by SMC was studied and the results are presented in Fig. 2c. Our data showed a high affinity degradation of LP and LP-PG at a single LP cholesterol concentration for 20 h. $^{125}I$-LP-PG was degraded to a higher extent than $^{125}I$-LP. The degradation of complexes was enhanced 1.5 and 2 fold for LP-NPG and LP-IPG respectively, over the native LP.

Figure 2: Bar graphs of cell surface bound, internalized and degraded $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG by rabbit arterial SMC. Arterial SMC were incubated with $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG at 37°C. After 20 h incubation, the amount of labeled LP: A; binding to the cell surface, B; internalization and C; degradation, determined as described in Methods. values represent the mean $\pm$SD, (n=5).


A
Binding
dpm x 10³/10⁴cells
B
Internalization
dpm x 10³/10⁴cells
C
Degradation
dpm x 10³/10⁴cells
100
50
0

### *4.4.3 Mechanism of uptake of LP-PG complexes*

a) Effect of unlabelled native LP: Once the uptake and internalization experiments had shown that LP-PG complexes appeared to be taken up and internalized by SMC, competition experiments were performed to determine whether LP-PG uptake was mediated through the apoB/E (LDL) receptor. Unlabeled native LP inhibited the binding and internalization of LP-PG complexes up to 60% in both LP-NPG and LP-IPG (Fig. 3). Moreover, there was a 50% decrease in the degradation in the presence of cold LP, indicating that at least 50% of the uptake of the LP-PG complexes was mediated by the LDL receptor pathway (Fig. 3c).

b) Effect of polyinosinic acid: We tested the ability of the polyinosinic acid, an inhibitor of the scavenger receptor on the uptake of LP from LP-PG complexes by arterial SMC. Polyinosinic acid inhibited both cell binding and internalization of LP-PG complexes. LP uptake was inhibited by 13% in the case of LP-NPG whereas, it was inhibited by 6% in LP-IPG. The maximum inhibition of LP internalization was 35% and 30% for LP-NPG and LP-IPG complexes respectively (Fig.4).

The degradation studies revealed that there was an 8.5% decrease in the presence of polyinosinic acid, indicating that the scavenger receptor played a minor role in the uptake and degradation of LP-PG complexes (Fig. 4c).

c) Effect of cytochalasin D: This drug inhibits phagocytosis by blocking cytoskeleton dependent internalization (Carter, 1967). Cytochalasin D was used to test whether phagocytosis played any role in the uptake and internalization of LP-PG complexes. During 20 h incubation, LP-PG uptake and internalization was suppressed up to 48%. The same level of suppression was achieved when the drug was administered to SMC incubated with either LP-NPG or LP-IPG (Fig.5).

Figure 3: Bar graphs showing the ability of unlabeled LP to inhibit SMC cell srface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG in a culture medium containing a 40 fold excess of unlabeled LP. After 20 h incubation LP: A; binding, B; internalization, C; degradation was determined as described in Methods. Values are mean $\pm$ SD (n=5).

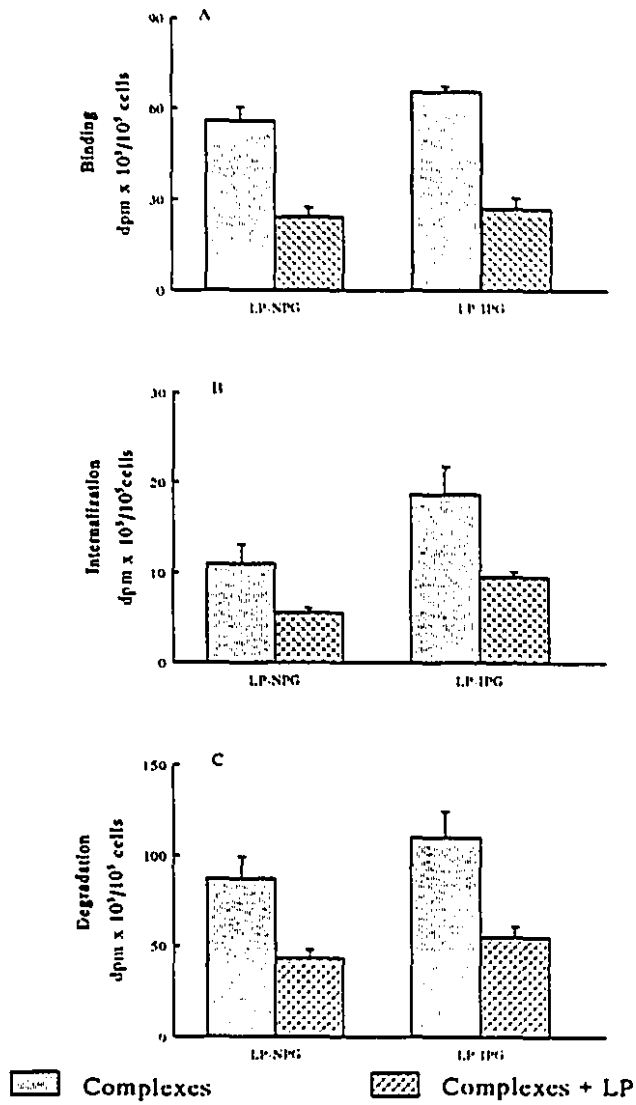

A
Binding
dpm x 10³/10⁵ cells
90
60
30
0
LP-NPG
LP-IPG
B
Internalization
dpm x 10³/10⁵cells
30
20
10
0
LP-NPG
LP-IPG
C
Degradation
dpm x 10³/10⁵ cells
150
100
50
0
LP-NPG
LP-IPG
Complexes
Complexes + LP

Figure 4: Bar graphs showing the ability of polyinosinic acid, a scavenger receptor inhibitor, to inhibit SMC cell surface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG complexes in a culture medium containing 50 $\mu$g/ml polyinosinic acid. After 20 h incubation, LP: A; binding, B; internalization and C; degradation was determined as described in Methods. Values are mean $\pm$ SD (n=5).

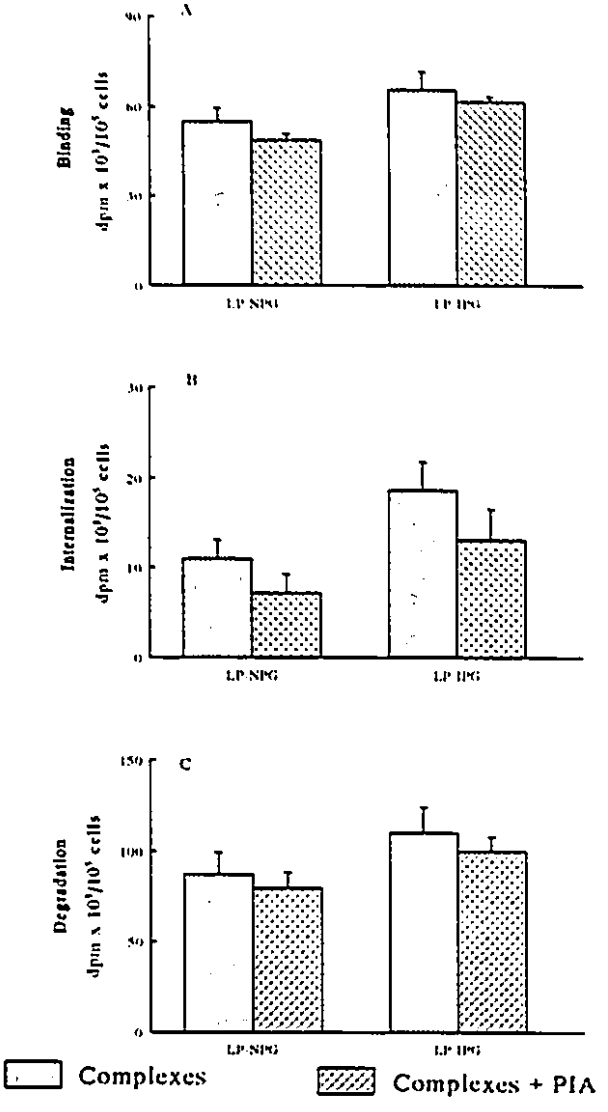
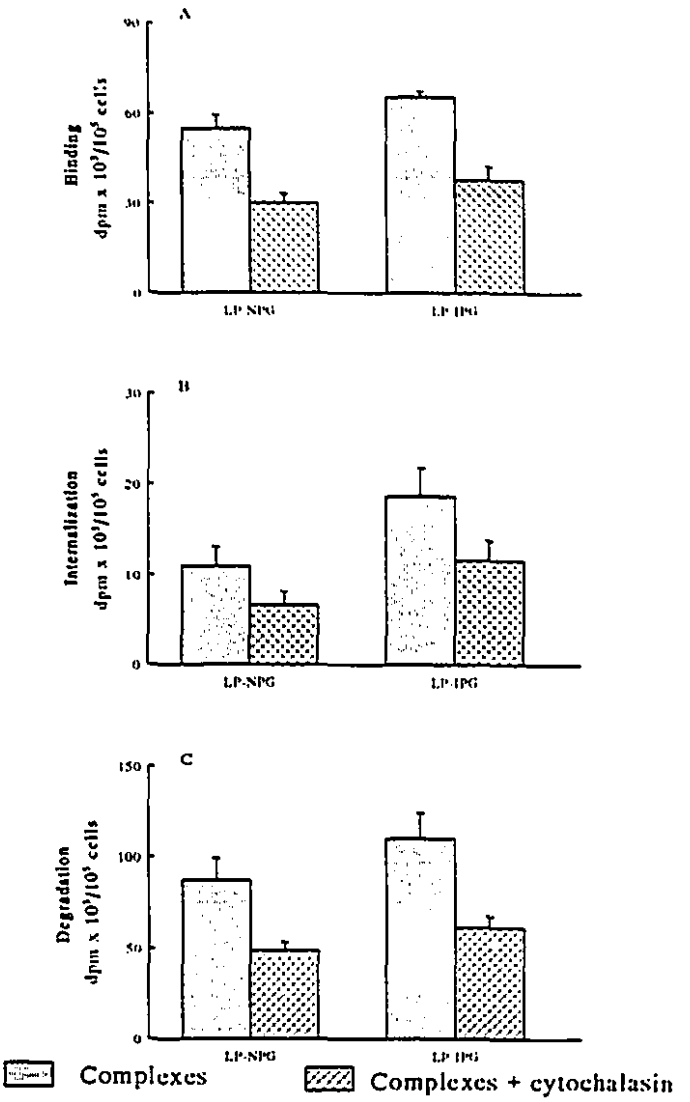

A
Binding
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
B
Internalization
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
C
Degradation
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
Complexes
Complexes + PIA

Figure 5: Bar graphs showing the ability of cytochalasin D, an inhibitor of phagocytosis, to inhibit SMC cell surface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG complexes in a culture medium containing 10 $\mu$g/ml cytochalasin D. After 20 h incubation, LP: A; binding B; internalization and C; degradation was determined as described in Methods. Values are mean $\pm$ SD (n=5).


A
Binding
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
B
Internalization
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
C
Degradation
dpm x 10³/10⁵ cells
LP-NPG
LP-IPG
Complexes
Complexes + cytochalasin

## 4.5 DISCUSSION

In the present study we analyzed the effect of LP-PG complexes on arterial SMC, utilizing PG derived from injured aortas and PG from normal uninjured aortas. LP-PG complexes have been implicated as a means for LP accumulation within macrophages and SMC (Salisbury et al., 1985; Hurt et al., 1990, Vijayagopal et al., 1992; Hurt-Camejo et al., 1992). The in vitro complexes prepared in this investigation revealed that apo-B LP had higher affinity for IPG than NPG. PG from injured aortas has been reported to differ significantly from PG of normal aortas (Wight et al., 1983; Alavi and Moore 1985; 1987) which may explain its pathogenic potential in atherogenesis. To date, the effects of LP-PG complexes, derived from injured aortas, on arterial SMC have not been studied.

In the experiments described here, LP-PG complexes were prepared in a low ionic strength buffer to achieve the reversible type of association. This type of association has been shown to be a more plausible model for stimulating cell-receptor interaction with LP-PG complexes, as the irreversible LP-PG complexes have been suggested to block the interaction between the LDL and the apo B,E receptor (Bihari-Varga et al., 1983; Mateu et al., 1984; Hurt et al., 1990). Results from this study revealed that these complexes remained associated after dissolving in culture medium.

Rabbit SMC were preincubated with LP deficient serum to enhance receptor expression for LP. The results obtained in this study for measuring the uptake of $^{125}$I-LP by arterial SMC in culture indicated that LP-PG complexes stimulated LP binding,

complexes on BMDM (Hurt-Camejo et al., 1990; Vijayagopal et al., 1992). Moreover, it appeared from our findings that LP-IPG complexes were more avidly taken up by SMC than LP-NPG complexes. This could be related to the qualitative difference in PG of injured aortas. Our laboratory and others have previously shown that the quantity and composition of GAG in injured aortas are significantly different from those of normal aortas (Wight et al., 1983; Alavi and Moore, 1985; 1987). Furthermore, sulfated GAG have shown to inhibit a variety of lysosomal enzymes isolated from leukocytes, spleen and liver (Avila, 1978; Hajjar et al., 1981).

The possible mechanisms by which vascular SMC can internalize LP include: 1) endocytosis through the apo-B/E receptor for native LDL, 2) non-regulated scavenger receptors for certain chemically modified LP and 3) non-receptor mediated endocytosis (Brown and Goldstein, 1979; Pitas, 1990; Ross, 1981).

Experiments designed to study the possible mechanism(s) of LP-PG complexes uptake by arterial SMC suggests that apo-B/E receptors play a significant part in the uptake of the complexes. This is supported by the observation that the binding and internalization of the complexes was suppressed up to 60% by excess unlabelled native LP. Next, phagocytosis also had an important role in the uptake and internalization of the PG-LP complexes. Cytochalasin D suppressed LP uptake and internalization by 45% when SMC were incubated with either LP-NPG or LP-IPG complexes. These findings are in accordance with Khoo and collaborators' observations (1992) who reported that phagocytosis was the main mechanism for uptake of LDL-aggregates by macrophages.

The partial inhibition of polyinosinic acid on the binding and internalization

of PG-LP complexes suggests that the scavenger receptors may play a minor part in the uptake of LP-PG complexes by SMC. Our findings agree with Hurt-Camejo et al. (1990) who reported that LP-PG complexes were mainly internalized by the apo B/E receptor pathway and little by the scavenger receptor in BMDM. The suppression of LP uptake by polyinosinic acid was inconsistent for LP-PG complexes. The maximum inhibition by polyinosinic acid was 13% and 6% for LP-NPG and LP-IPG respectively. This could be due to a higher scavenger receptor activity caused by IPG. However, there was no significant difference between the suppression of LP internalization or degradation for either complex.

This study is the first to examine the effect of LP-PG complexes, on arterial SMC in vitro utilizing PG extracted from injured aortas. It appeared that LP-PG complexes were taken up mainly by the apo B/E receptor and by phagocytosis. The scavenger receptor played a minor part in the uptake of LP-PG complexes by SMC. Arterial SMC exhibited a higher affinity for LP-IPG than LP-NPG, emphasizing the important role of injury in atherogenesis.

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CHAPTER 5

# INTERACTION OF APO-B CONTAINING LIPOPROTEINS WITH PROTEOGLYCANS FROM INJURED AORTAS STIMULATES ITS UPTAKE BY MONOCYTE-DERIVED MACROPHAGES

## 5.1 ABSTRACT

The biological behavior of lipoprotein-proteoglycan (LP-PG) complexes, from injured aortas of rabbits was studied in blood monocyte-derived macrophages (BMDM). LP-PG complexes were prepared *in vitro* by coupling rabbit apo-B lipoprotein (LP), with proteoglycan (PG) extracted from normal aortas (NPG) or with PG from injured aortas (IPG). Rabbit BMDM were cultured by the Ficol hypaque gradient technique, and incubated with $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG for 20 h at 37°C. LP binding, internalization and degradation was significantly increased for LP-NPG and LP-IPG over native LP. Moreover, BMDM exhibited higher affinity for LP-PG derived from PG of injured aortas than LP-PG of normal aortas. The uptake and degradation of LP by macrophages was inhibited by 50%, when cytochalasin, an inhibitor of phagocytosis was used indicating that the uptake of LP-PG complexes was mediated mainly by phagocytosis. Furthermore, the uptake of LP-PG complexes was reduced by competition for the apo-B/E receptor, and the results indicated that more than 40% of the LP-PG complexes are taken up by the apo-B/E receptor pathway. The scavenger receptor played a minor part in the uptake and degradation of LP-PG complexes. Data from this study indicate that the interaction of apo-B containing lipoproteins with PG from injured aortas stimulates LP uptake by BMDM. Therefore, LP-PG complexes may contribute to lipid accumulation by BMDM, and injury to the endothelium may accelerate the process.

## 5.2 INTRODUCTION

The macrophage exhibits diverse roles during the course of atherosclerosis, including thrombolysis, vascular immunologic processes, phagocytosis and remodelling of the extracellular matrix (Schwartz et al., 1985). The blood monocyte-derived macrophage (BMDM) appears to be the second most important cell type to generate foam cells, after smooth muscle cells, within atherosclerotic plaques (Klurfeld, 1985; Katsuda et al., 1992). There are several potential ways by which macrophages might accumulate cholesterol, including lipoprotein uptake via receptor-dependent mechanisms, phagocytosis or adsorptive endocytosis, and transport of free cholesterol along chemical gradients (Brown and Goldstein, 1983; Fielding, 1984).

The mechanism of transition of macrophages to foam cells *in vivo* is unknown. Recently, the uptake of modified lipoproteins (LP) by macrophages is thought to contribute to lipid accumulation, foam cell formation and the development of atherosclerotic lesions. Certain chemically modified forms of LP are taken up by macrophages through the scavenger receptor (Goldstein et al.,1979, Nagelkerke et al.,1983). Other modifications such as LDL aggregation, LP aggregates formed in response to monoclonal antibodies and the formation of lipoprotein-proteoglycan (LP-PG) complexes were also found to cause accelerated uptake of LP by macrophages, although the mechanisms of uptake were different (Hurt-Camejo et al., 1990; Vijayagopal et al., 1992; Khoo et al., 1992). Aggregates of LP complexed with PG have been shown to induce cholesteryl ester accumulation in BMDM in vitro and in subendothelial cells from

human aortic intima (Vijayagopal et al., 1992; Hurt-Camejo et al., 1992; Bondjers et al., 1990). We have previously shown that LP-PG complexes are present in normal and deendothelialized aortas of normocholesterolemic rabbits (Ismail et al., 1991). PG synthesized by intima-media of injured aortas differ significantly from PG of normal aortas in both physical and chemical properties (Wight et al., 1981; Alavi and Moore, 1985; 1987).

The current investigation was undertaken to examine the hypothesis that LP-PG complexes could stimulate the uptake of apo-B by blood-derived macrophages and thus contribute to foam cell formation. Since endothelial injury has been shown to induce alterations in PG synthesis and distribution, we studied the effect of LP-PG complexes on LP uptake by blood-derived macrophages, utilizing PG derived from normal and injured aortas.

## 5.3 MATERIALS AND METHODS

### *5.3.1 Blood monocyte-derived macrophage culture conditions*

Monocytes were isolated from blood (containing 1mg/ml EDTA) of healthy rabbits by the Ficol-Hypaque gradient method (Boyum, 1976). Ficol-Hypaque was prepared by adding ten parts of Isopaque (sodium metrizoate, 32.8% solution, Sigma, St. Louis) to 24 parts 8% Ficol, mixed and the density was adjusted to 1.077 g/ml with either Isopaque or distilled water. Blood was diluted with an equal volume of sterile phosphate-buffered saline (PBS), and 8 ml aliquots were layered over 6 ml Ficol-Hypaque in 15 ml tubes. The tube was centrifuged at 500g for 30 min at 20°C. The mononuclear cell preparation, collected from the interface region, was washed three times in sterile PBS containing 1mM EDTA to remove platelets. The cell preparation was suspended in RPMI-1640 (Gibco, Canada Inc.) containing 20% fetal bovine serum and supplemented with sodium bicarbonate, L-glutamine, non essential amino acids, sodium pyruvate and penicillin-streptomycin (medium A). Aliquots were distributed in 35 mm dishes at a density of $10^7$ cells/dish and incubated in a humidified tissue culture incubator (95% $O_2$, 5%$CO_2$) at 37°C. After three hours, non adherent cells were removed from the culture dishes by three washes with serum-free medium. The cells were then incubated in fresh medium A for 7 days before the experiments were initiated. At that time, cells were identified by morphology and immunologically with antibody specific for rabbit macrophages (RAM 11, Dako Corporation, Calif.).

*5.3.2 Rabbit arterial proteoglycans*

Intima-media PG from normal and injured aortas were prepared as described in detail previously (§ 2.3.3.1). The PG preparations from normal aortas (NPG) and from injured aortas (IPG) were lyophilized and stored at -20°C until used.

*5.3.3 Lipoproteins and lipoprotein-deficient serum*

Apo-B lipoproteins (VLDL, IDL, LDL) and lipoprotein deficient serum ($d > 1.22$g/ml) were prepared from plasma (1mg/ml EDTA) of healthy, fasted rabbits as described previously (§ 4.3.3). Iodination of apo-B lipoproteins (LP) was performed by the Iodogen method according to the manufacturer's instructions (Pierce, Rockford, IL.).

*5.3.4 In vitro LP-PG complexes*

LP-PG complexes were prepared from two different sources of PG (NPG or IPG) and $^{125}$I-LP as described previously (§ 4.3.4).

*5.3.5 Lipoprotein binding, internalization and degradation*

Seven days after initial culturing of monocytes, cells were incubated for 24 h with culture medium containing LP deficient serum (medium B), then washed 3 times with PBS. Cells were incubated with a fresh medium B containing $^{125}$I-LP, $^{125}$I-LP-NPG or $^{125}$I-LP-IPG, at a cholesterol concentration of 100$\mu$g/ml for 20 h at 37°C. To examine the possible routes of uptake of LP-PG complexes by BMDM, 40-fold excess of unlabeled LP, polyinosinic acid (50 $\mu$g/ml), and cytochalasin D (10 $\mu$g/ml), were added individually to BMDM together with LP-PG complexes as described previously (§ 4.3.5).

Parallel dishes without cells were always included as controls. Experiments were performed on 4 dishes per group and were repeated three times.

## 5.4 RESULTS

### *5.4.1 LP-PG complexes*

Although LP-PG complexes were prepared under identical experimental conditions with the same ratio of GAG to LP cholesterol, 60% of the added LP was identified in LP-IPG whereas only 45% of the added LP was formed in LP-NPG complexes. Therefore, IPG has more affinity for apo-B LP than NPG. These findings are in agreement with previous data from our laboratory (Alavi et al., 1989) demonstrating that injury-induced changes in PG influence their interaction with LP.

To check whether the medium used to dissolve the complexes might dissociate the complex into its individual components, one aliquot from the labeled complex dissolved in medium B was dialyzed, and passed through a gel filtration column (Superose 6 HR). LP-NPG eluted as a single peak and this peak comprised greater than 95% of the radioactivity, indicating that LP-PG complexes had not dissociated.

### *5.4.2 Lipoprotein binding, internalization and degradation*

Labeling of lipoprotein with $^{125}I$ enabled us to determine the difference in the uptake of LP-PG and native LP by BMDM. The interaction of arterial PG with apo-B LP stimulated binding, internalization and degradation of LP by macrophages (Fig. 1). Comparatively, the LP-PG complexes induced 3 and 4.5 fold higher LP binding by macrophages than the native LP ( $p=0.001$, for LP-NPG and $p<0.001$ for LP-IPG, $n=4$). Moreover, macrophages exhibited significantly more LP binding from LP-IPG than from LP-NPG ($p=0.02$). Similarly, LP internalization was significantly higher in

the case of LP-PG complexes than native LP ($p<0.001$). Macrophages incubated with complexes prepared from IPG showed more internalized LP than complexes prepared from NPG, $p=0.001$.

The degradation of internalized LP and LP-PG complexes by macrophages was studied and the results are presented in Fig. 1c. Our data showed a high degradation of LP and LP-PG at a single LP cholesterol concentration for 20 h. $^{125}$I-LP-PG was degraded to a higher extent than $^{125}$I-LP($p<0.001$). The degradation of complexes was enhanced 3.5 and 5.5 fold for LP-NPG and LP-IPG respectively, over the native LP.

*5.4.3 Mechanism of uptake of LP-PG complexes*

a) Effect of unlabelled native LP: Once the uptake and internalization experiments had shown that LP-PG complexes appeared to be taken up and internalized by macrophages, competition experiments were performed to determine whether LP-PG uptake was mediated through the apoB/E (LDL) receptor. Unlabeled native LP inhibited the binding and internalization of LP-PG complexes up to 50% in both NPG-LP and IPG-LP (Fig. 2). Moreover, there was a 48% decrease in the degradation of the complexes in the presence of unlabeled LP, indicating that at least 48% of the uptake of the LP-PG complexes was mediated by the LDL receptor pathway (Fig. 2c).

b) Effect of polyinosinic acid: We tested the effect of the polyinosinic acid, an inhibitor of the scavenger receptor on the uptake of LP from LP-PG complexes by macrophages. Polyinosinic acid inhibited both cell binding and internalization of LP-PG complexes. LP uptake was inhibited by 32% in the case of LP-NPG

whereas, it was inhibited by 7% in LP-IPG. The maximum inhibition of LP internalization was 24% and 17% for LP-NPG and LP-IPG complexes respectively (Fig.3). The degradation studies revealed that there was 9% decrease in the presence of polyinosinic acid, indicating that the scavenger receptor played a minor role in the uptake and degradation of LP-PG complexes (Fig. 3c).

c) Effect of cytochalasin D: This drug inhibits phagocytosis by blocking cytoskeleton dependent internalization (Carter, 1967). Cytochalasin D was used to test whether phagocytosis played any role in the uptake and internalization of LP-PG complexes. During 20 h incubation, LP-PG binding, internalization and degradation was suppressed up to 50%. The same level of suppression was achieved when the drug was administered to macrophages incubated with either LP-NPG or LP-IPG (Fig.4).

Figure 1: Bar graphs of cell surface bound, internalized and degraded $^{125}$I-LP, $^{125}$LP-NPG or $^{125}$I-LP-IPG at 37 °C. After 20 h incubation, the amount of labeled LP: A; binding to the cell surface, B; internalization and C; degradation, determined as described in Methods. [ Values represent the mean $\pm$ SD (n=4)].

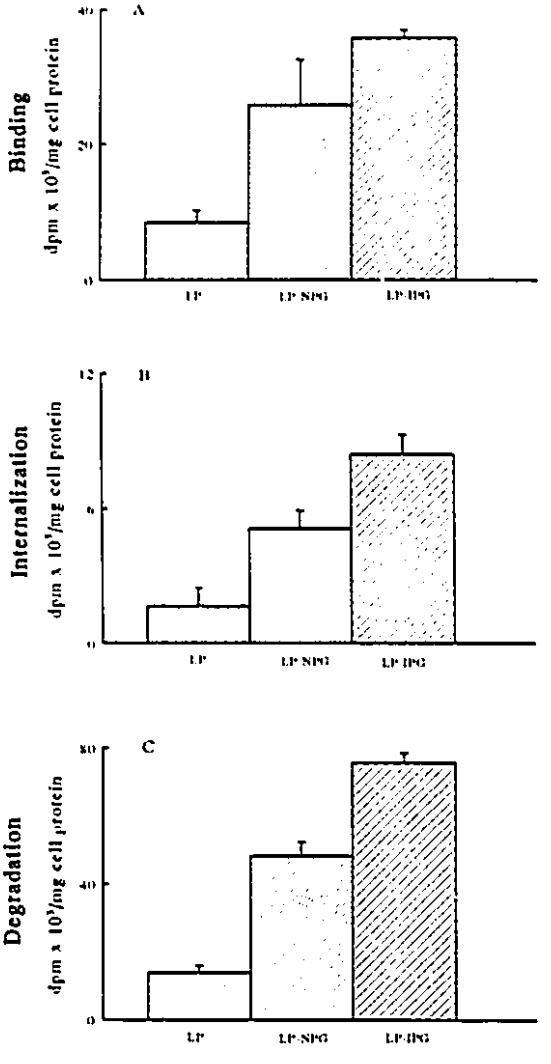

A
Binding
dpm x 10³/mg cell protein
LP
LP-NPG
LP-IPG
B
Internalization
dpm x 10³/mg cell protein
LP
LP-NPG
LP-IPG
C
Degradation
dpm x 10³/mg cell protein
LP
LP-NPG
LP-IPG

Figure 2: Bar graphs showing the ability of unlabeled LP to inhibit BMDM cell surface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG complexes in a culture medium containing a 40 fold excess of unlabeled LP. After 20 h incubation, LP: A; binding, B; internalization and C; degradation was measured as described in Methods. [ Values are mean $\pm$ SD (n=4)].

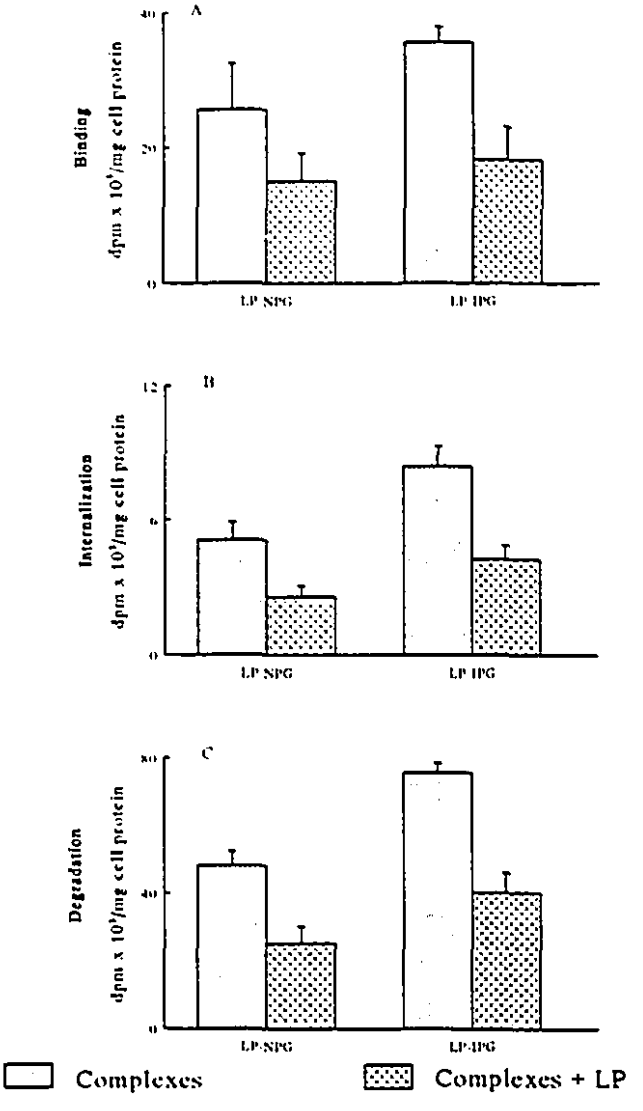

A
Binding
dpm x 10³/mg cell protein
40
20
0
LP SPG
LP IPG
B
Internalization
dpm x 10³/mg cell protein
12
6
0
LP SPG
LP IPG
C
Degradation
dpm x 10³/mg cell protein
80
40
0
LP-SPG
LP-IPG
Complexes
Complexes + LP

Figure 3: Bar graph showing the ability of polyinosinic acid, a scavenger receptor inhibitor, to inhibit monocyte/macrophage cell surface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG complexes in a culture medium containing 50 $\mu$g/ml polyinosinic acid. After 20 h incubation, LP: A; binding, B; internalization and C; degradation was determined as described in Methods. [ Values are mean $\pm$ SD (n=4)].

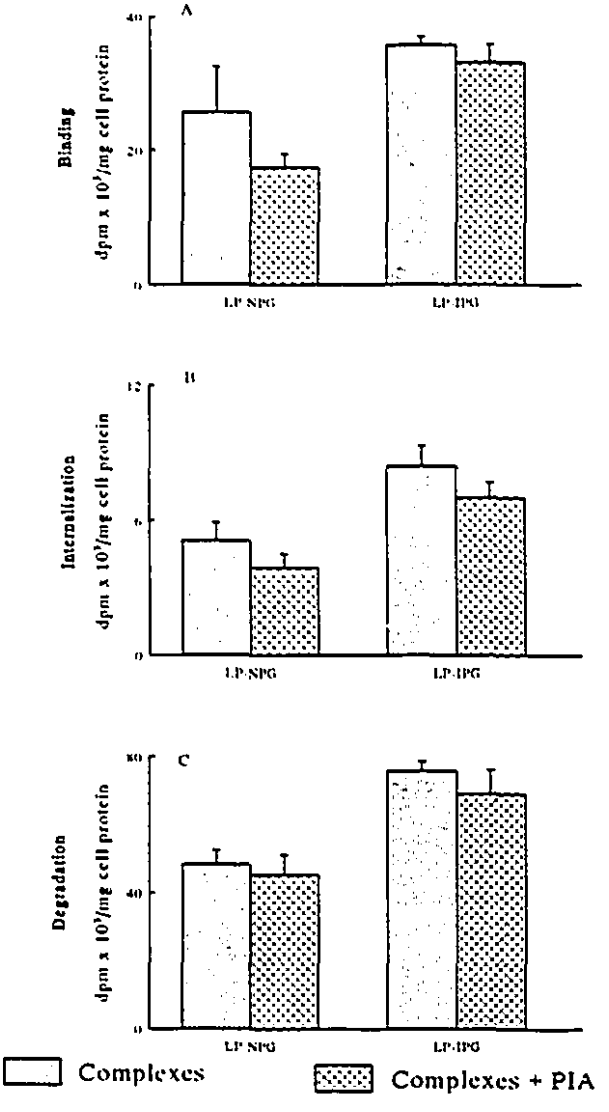
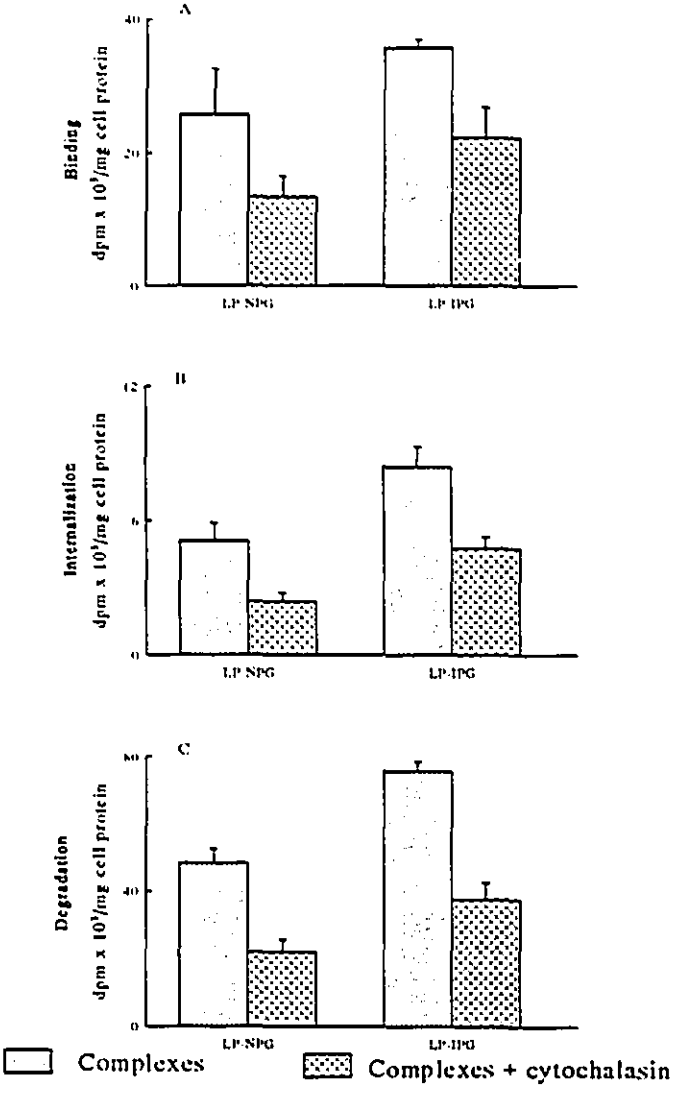

A
Binding
dpm x $10^3$/mg cell protein
LP-NPG
LP-IPG
B
Internalization
dpm x $10^3$/mg cell protein
LP-NPG
LP-IPG
C
Degradation
dpm x $10^3$/mg cell protein
LP-NPG
LP-IPG
Complexes
Complexes + PIA

Figure 4: Bar graphs showing the ability of cytochalasin D, an inhibitor of phagocytosis, to inhibit macrophage cell surface binding, internalization and degradation of $^{125}$I-LP-NPG or $^{125}$I-LP-IPG complexes in a culture medium containing 10 $\mu$g/ml cytochalasin. After 20 h incubation, LP: A; binding, B; internalization and C; degradation was determined as described in Methods. [Values are mean $\pm$ SD (n=4)].


A
Binding
dpm x 10³/mg cell protein
LP-NPG
LP-IPG
B
Internalization
dpm x 10³/mg cell protein
LP-NPG
LP-IPG
C
Degradation
dpm x 10³/mg cell protein
LP-NPG
LP-IPG
Complexes
Complexes + cytochalasin

## 5.5 DISCUSSION

Results presented in this study indicate that apo-B containing LP have higher affinity for IPG than NPG, confirming and extending earlier studies (Alavi et al., 1989). LP-PG complexes were prepared in a low ionic strength buffer and results demonstrate that these complexes prepared *in vitro* remained associated after dissolving in culture medium. It has been reported that LP-PG complexes promote cholesteryl ester accumulation in peritoneal macrophages and in monocyte-derived macrophages (Salisbury et al., 1985; Vijayagopal et al., 1992; Hurt et al., 1990; Hurt-Camejo et al.,1992). Furthermore, this is the first study, using LP-PG complexes, derived from injured aortas, and their influence on macrophage uptake.

Macrophages were preincubated with LP deficient serum to enhance receptor expression for LP. The results obtained in this study for measuring the uptake of $^{125}$I-LP by monocyte/macrophage in culture indicated that LP-PG complexes stimulated LP binding, internalization and degradation. These results are similar to the effect of LP-PG complexes observed in human BMDM (Hurt et al., 1990; Vijayagopal et al., 1992). Moreover, our findings indicate that LP-IPG complexes were more avidly taken up by macrophage than LP-NPG complexes. This could be related to the qualitative differences in PG of injured aortas. Reports from our laboratory and others have previously shown that the quantity and composition of GAG in injured aortas are significantly altered from those of normal aortas (Wight et al., 1983; Moore and Richardson, 1985; Alavi and Moore, 1985; 1987; Alavi et al., 1991). Furthermore, sulfated GAG have been shown

to inhibit a variety of lysosomal enzymes isolated from leukocytes, spleen and liver (Avila, 1978; Hajjar et al., 1981).

The possible mechanisms by which macrophages can internalize LP include: 1) endocytosis through the apo-B/E receptor for native LDL, 2) non-regulated scavenger receptors for certain chemically modified LP and 3) phagocytosis or non-receptor mediated endocytosis, 4) transport of free cholesterol along chemical gradients (Brown and Goldstein, 1979; Goldstein et al., 1979; Fielding, 1984).

Data presented in this investigation suggest that the uptake of LP-PG complexes by BMDM is mediated mainly by the apo-B/E receptor and phagocytosis. This is supported by the observation that the binding and internalization of the complexes was suppressed up to 48% by excess unlabelled native LP and 50% by cytochalsin D. These findings agree with the observations of Hurt and collaborators (1990) who reported that LP-PG complexes were mainly internalized by the apo B/E receptor pathway. The experiments of Khoo and collaborators (1992), is not fully supportive of these results but could not be ruled out as they observed that phagocytosis was the main mechanism for the uptake of LDL-aggregates, formed in response to monoclonal antibodies, by macrophages.

The scavenger receptor, for certain oxidized LDL, and the $F_c$ receptor, for LDL aggregates formed in response to monoclonal antibodies, were not downregulated, and the uptake by way of the receptor might continue unabated and lead to foam cell formation ( Goldstein et al., 1979; Khoo et al., 1992). However, the slight inhibition by polyinosinic acid on the binding and internalization of LP-PG complexes, noted in this

study, suggests that the scavenger receptor plays only a minor part in the uptake of LP-PG complexes by macrophages. These findings also agree with studies by Vijayagopal and collaborators (1987,1991) who observed that the scavenger receptor shared a minor part in the uptake and degradation of LP-PG complexes. These authors suggested that the binding sites for acetyl-LDL and LP-PG complexes were not identical and they proposed that the metabolism of LP-PG complexes in macrophages was dependant upon the G-protein coupled signal transduction mechanism.

Although at least 48% of the uptake of LP-PG complexes is mediated by the apo B/E receptor for the native LDL, which can normally be down regulated, another pathway which is not subject to down-regulation could be responsible for lipid accumulation and thus foam cell formation. Nevertheless, more LP-PG complexes were taken up than native LP which suggests an additional mechanism may be involved in the increased uptake of LP-PG complexes and subsequent lipid accumulation. LP-PG complexes may be responsible for the stimulation of phagocytosis and endocytosis, as revealed from our data. It has been reported that the uptake of insoluble LDL-immune complexes by human monocyte-derived macrophages, while leading to increased intracellular cholesteryl ester accumulation, paradoxically led to enhanced LDL receptor activity (Lopes-Virella et al., 1991).

In conclusion, the uptake of LP-PG complexes could significantly modify macrophage behavior in generating foam cells and thereby may influence the atherogenic process.

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# CHAPTER 6: OVERALL CONCLUSIONS

The importance of endothelial injury in the development of atherosclerosis is now a well recognized and a largely accepted mechanism. The concept is based on morphological, biochemical and molecular experimental studies and is supported by various examples of arterial wall injury in man. The balloon catheter deendothelialization of the aorta of normocholesterolemic rabbits, has been shown, by Moore and collaborators, to induce lesions of a composition similar to that of human atherosclerotic lesions. The neointima which forms, 12 weeks following balloon-catheter removal of the endothelium, resembles, in many features early atherosclerotic lesions. At six months following injury, the lesions closely resemble human fibrous plaques. All experiments reported in this thesis were carried out at 12 weeks following injury to study the role of proteoglycans in the early development of atherosclerosis. PG has been widely reported to be implicated in atherogenesis.

This type of injury can be induced in man by repeated or continuous injury, as in the case of placing an indwelling catheter in the aorta to monitor blood gases in neonates. Paradoxically, it is also a model that resembles balloon angioplasty, used for the treatment of coronary artery disease (CAD) patients. Moreover, age, hypertension, hypercholesterolemia, hyperglycaemia and hemodynamic factors were found to alter the properties of endothelium.

The characteristic composition and organization of the extracellular PG, in the vessel wall, leads them to coordinate manifold physiological functions. Any alteration

in these versatile molecules would likely influence the local properties of the vessel wall, such as permeability, elasticity, macromolecular movement in the interstitial space and cellular migration. It has been widely reported by our laboratory and others that PG are implicated in atherogenesis. Some physicochemical properties of PG have been proposed in such involvement, but the exact mechanism(s) by which PG is implicated in atherogenesis is still not comprehended.

In this study, the highest concentration of PG was detected in injured aortas within those areas covered by regenerated endothelium, as compared to neointima not covered by endothelium or uninjured aortas. Moreover, the neointima, covered by regenerated endothelium exhibited distinct characteristics in newly synthesized, sulfated PG, with regard to size, composition, distribution and affinity to apo-B, compared to that of normal aortas. These characteristics allow PG to facilitate lipoprotein (LP) sequestration in the intima, setting the stage for the development of atherosclerosis. Although collagen, elastin and fibrin can also interact with LP and may contribute to lipid accumulation in the intima, it appears that sulfated PG are the most potent candidates in this regard.

Furthermore, neointima covered by regenerated endothelium showed increased formation of LP-PG complexes compared to neointima not covered by endothelium or intima from normal aortas. Studies were extended to examine the biological behavior of LP-PG complexes, i.e. binding, internalization and degradation, in smooth muscle cells (SMC) and blood monocyte-derived macrophages (BMDM). Data from these

studies indicate that LP-PG complexes stimulate LP binding, internalization and degradation, by SMC and BMDM. The uptake of LP-PG complexes is mediated mainly by apo-B/E receptor endocytosis and by phagocytosis, in SMC and BMDM. The scavenger receptor, for certain oxidized forms of LDL, plays a minor part in the uptake of these complexes. Moreover, both cell types, SMC and BMDM, exhibit a higher affinity for LP-PG derived from PG of neointima, covered by regenerated endothelium, than LP-PG of normal aortas.

In summary, all these findings are consistent with the hypothesis that sulfated PG are produced in excess in areas of lipid accumulation, and thus may trap or retain apo-B containing LP. It is widely reported that LP are preferentially accumulated in neointima of injured aortas, in areas covered by regenerated endothelium. The failure of deendothelialized tissue to accumulate sulfated PG or LP-PG complexes in high quantities, compared to reendothelialized tissue, may be due to the "reverse" barrier function of the endothelium.

## ORIGINAL CONTRIBUTION

*The original contributions made by this thesis include the following*:

The characteristics of newly synthesized sulfated PG, presented in this thesis, include the overall assessment of sulfated PG molecules produced by neointima developed in response to injury, in a form not attempted before.

This is the first study to characterize LP-PG complexes in neointima developed in response to injury using a normocholesterolemic animal model.

The biological behavior of LP-PG complexes, i.e. uptake, internalization and degradation, was studied on arterial SMC and BMDM, utilizing for the first time LP-PG complexes derived from injured aortas. The mechanism of uptake of these complexes was also analyzed.