Changes in the human aortic glycosaminoglycans in atherosclerosis and diabetes.

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ABBREVIATIONS

AGE	Advanced Glycosylation Endproduct
AMP	Acid Mucopolysaccharide
Apo	Apolipoprotein
CS	Chondroitin Sulfate
CS-4	Chondroitin 4-Sulfate
CS-6	Chondroitin 6-Sulfate
DDT	Dry Defatted Tissue
DM	Diabetes Mellitus
DS	Dermatan Sulfate
FS	Fatty Streak
GAG	Glycosaminoglycan
Gal	Galactose
GalNAc	N-Acetyl Galactosamine
GlcNAc	N-Acetyl Glucosamine
GlcUA	Glucoronic Acid
HA	Hyaluronic Acid
HDL	High Density Lipoprotein
Hep.	Heparin
HS	Heparan Sulfate
IdUA	Iduronic Acid

KS	Keratan Sulfate
LDL	Low Density Lipoprotein
LP	Lipoprotein
PG	Proteoglycan
SMC	Smooth Muscle Cell
TCA	Trichloroacetic Acid
VLDL	Very Low Density Lipoprotein
Xyl	Xylose

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ABSTRACT

Arterial Glycosaminoglycans (GAGs) have gained importance in atherogenesis due to their ability to trap lipid inside the vessel wall. Atherosclerotic lesions have displayed an altered GAG content and distribution. Diabetes is a recognized risk factor for atherosclerosis, but no information is available on the arterial GAGs in human diabetics. To improve our understanding of the atherogenic process we examined GAGs in normal and atherosclerotic intima of nondiabetic and type-II diabetic humans

Intima was stripped from the autopsy samples of thoracic aortas, normal and plaque areas were separated. GAGs were isolated by delipidation, proteolytic digestion, and precipitation. They were assayed biochemically and their distribution evaluated by electrophoresis and densitometry.

Results indicate a significant decrease in total GAGs and a change in GAG distribution in plaques of nondiabetics. Similar changes of lesser magnitude were found in normal intima of diabetics, while changes in plaque areas were more pronounced. This indicates that changes in arterial GAGs precede the development of lesions in diabetes and may be important in atherogenesis.

RÉSUMÉ

Les glycosaminoglycans (GAGS) artériels ont gagnés de l'importance dans l'athérogénèse par leur habilité à s'associer aux lipides dans la tunique interne des vaisseaux sanguins. Les lésions d'athérosclérose montrent une distribution ainsi qu'une forme de GAGs altérés. Le diabète est un facteur de risque reconnu pour l'athérosclérose, par contre il n'existe aucune information sur les GAGs artériels de diabète humain. Pour approfondir notre compréhension sur le processus athérogénique, nous avons examiné les GAGs des parois internes de vaisseaux sanguins normales et atteintent d'athérosclérose, sur des sujets humains diabétiques de type II et non-diabétiques.

La paroi interne d'aortes thoraciques a été prélevée d'un échantillon d'autopsie; les régions normales et atteintent d'athérosclérose ont été séparées. Les GAGs ont été isolés par délipidation, digestion protéolitique et précipitation. Des tests biochimiques ont été fait et leur distribution évaluée par électrophorèse et densitométrie.

Les résultats montrent une diminution significative des GAGs totaux et un changement dans la distribution des GAGs des lésions d'athérosclérose de sujets non-diabétiques. Des changements similaires, mais de moindre amplitude, ont été retrouvés dans la paroi de vaisseaux normale chez des sujets diabétiques; par contre ces mêmes changements dans les lésions athérosclérotiques sont plus évidents. Ceci indique que ces changements dans les GAGs artériels sont des précurseurs au développement de lésions chez les diabétiques et peuvent entraîner des maladies athérogéniques potentielles.

1. INTRODUCTION

Glycosaminoglycans (GAGs) have been found to be actively involved in a number of biological processes. Their role in atherosclerosis is appreciated mainly due to their property of interacting with lipoproteins which could help in accumulation of lipid in the arterial wall. Moreover, they have also been found to be involved in cell adhesion, migration and proliferation which are important events in atherogenesis. For the last 3 decades many investigators have been studying GAGs in both human and experimental atherosclerosis, and they have detected both qualitative and quantitative changes. It has been suggested that these changes may be important in atherogenesis. Unfortunately, few of these findings agree with one another.

Prevalence of atherosclerosis and its clinical manifestations are markedly higher in diabetic individuals. Although scores of potentially atherogenic factors have been implicated to explain the phenomenon, it still remains poorly understood. It is logical to raise the question about the role of GAGs in this process. Very few investigators have tried to address this question. All of these studies have been performed in experimental diabetes and the changes reported in arterial GAGs have been diverse. With this background, the current study was initiated to achieve the following goals:

a. To detect changes in GAG concentration and distribution in

   i) human atherosclerotic plaques (in our hands)

   ii) intima of type-II diabetic humans.

b. To find a correlation between findings observed in i) and ii).

These findings will demonstrate changes in arterial GAGs in both atherosclerosis and diabetes and will help in understanding the role of GAGs in the macrovascular complications of diabetes in particular and atherogenesis in general.

Following is a brief overview of our current knowledge of GAGs, atherosclerosis, diabetes and the speculative triangular link between them. For the sake of simplicity and brevity in most situations only findings in humans will be discussed.

1.1 Glycosaminoglycans and Proteoglycans:

GAGs constitute a group of a special type of polysaccharides which exist in the form of a protein linked molecule called proteoglycan (PG). Therefore the terms GAG and PG are often used interchangeably. PGs are emerging as a group of one of the most important and versatile components in the extracellular matrix. Their importance is underscored by their involvement in a diverse array of phenomena in both health and disease (Wight, 1989). Initially they were only considered to be structural elements forming the ground substance and involved in mechanical support of body tissues. With the development of sophisticated skills in various domains of science, PGs have been found to be capable of performing more intricate roles in cellular and matrix biology and pathology.

PGs are almost ubiquitous in distribution within the body. They are not only important constituents of extracellular matrix but are also found both within and on the surface of various types of cells. They are also present in secretions and body fluids as well as in blood and urine. PGs are most abundant in cartilage (about 50 % of tissue weight), vitreous humour, and umbilical cord. They constitute only about 2 percent of the dry defatted tissue in the arterial intima-media where the major components are collagens (about 25 %) and elastin (about 20 %) (Smith, 1974).

1.1.1 History:

The history of PG (Kennedy, 1979; Heinegard et al., 1984) dates back to centuries ago when it was first extracted from human umbilical cord by Wharton in 1656 and was given the name of "Wharton's jelly". This substance was what is now described as hyaluronic

acid, but at that time its biochemical nature was not known. During the 19th century biochemical analyses of cartilage resulted in isolation and partial characterization of chondroitin sulfate. During the 20th century the structure of GAGs was elucidated largely by Levene and coworkers during the earlier part and Karl Meyer and collaborators during the mid-century. Other members of the family were recognized; hyaluronic acid in 1934 from vitreous humor, dermatan sulfate in 1941 from the skin, heparan sulfate in 1948, and keratan sulfate and chondroitin in 1953 from bovine cornea. In 1956 it was observed that original chondroitin sulfate could exist in three isomeric forms, A,B,C. Chondroitin sulfate B turned out to be the already isolated dermatan sulfate. Chondroitin sulfate A and B are now referred to as chondroitin sulfate-6 and chondroitin sulfate-4 respectively. They were then called acid mucopolysaccharides (AMPs).

At that time it was generally accepted that these AMPs were pure polysaccharide molecules, associated but not linked to proteins. In 1950s it was recognized that AMPs occur in nature coupled with protein, thus forming a GAG-protein complex. Later, largely as a result of the work of Muir and Roden and their colleagues a covalent link between AMP and protein was recognized. With this breakthrough attention was diverted to the protein portion and was fuelled by the recent advances in molecular biology. Today, sequencing of different PGs has been achieved and most of the elements regarding structure have been elucidated.

1.1.2 Structure and composition:

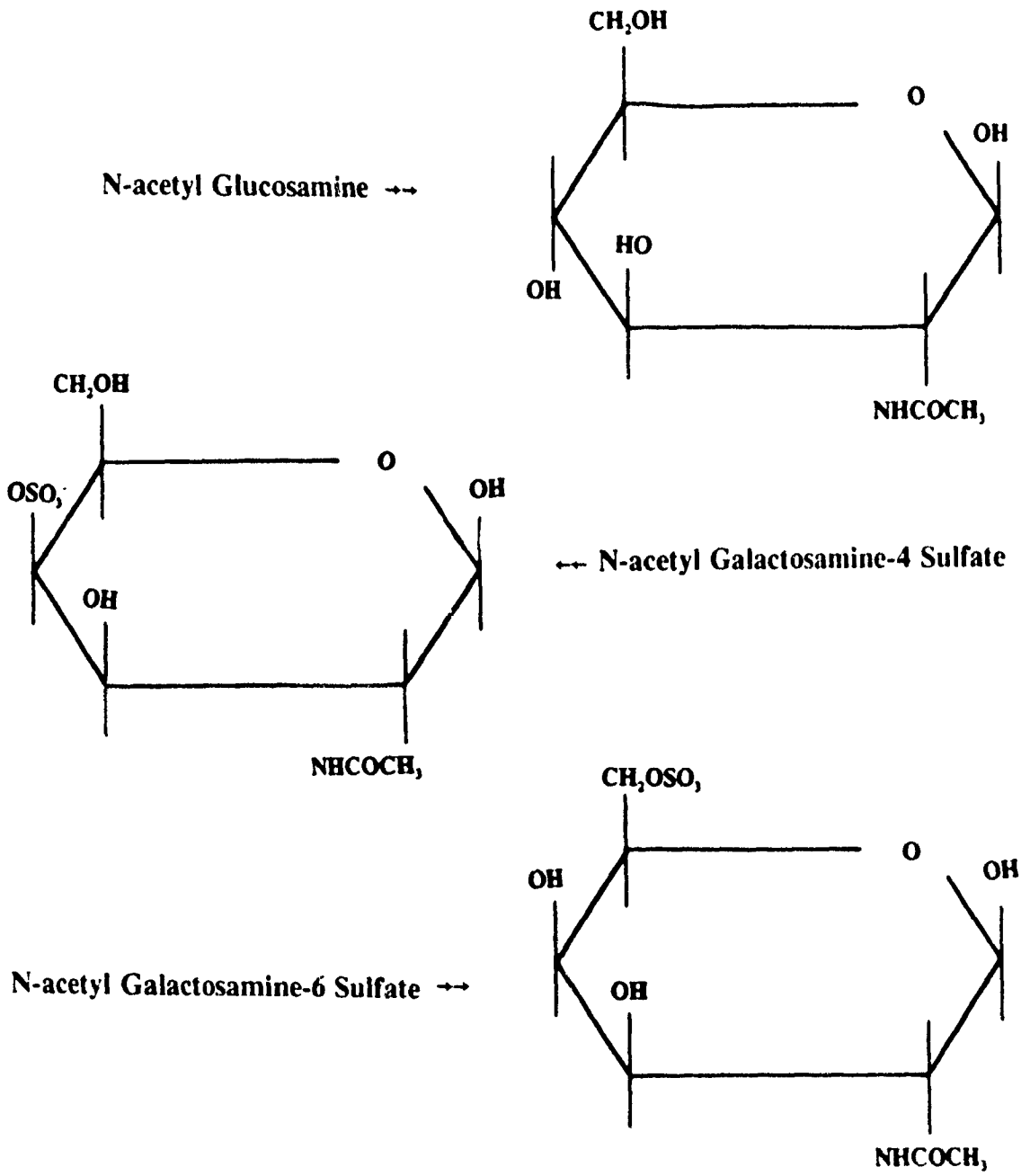
Each PG molecule is composed of GAG chains covalently linked to a protein called core protein. They are distinct from conventional glycoproteins in various aspects.

Glycoproteins generally have a greater proportion of protein than carbohydrate which is made up of branching oligosaccharide chains. PGs, on the other hand, consist of carbohydrate as the major constituent which is arranged in the form of linear chains of repeating disaccharide units forming GAGs. The number of GAG chains in a PG varies from 1 (as in thrombomodulin) to >100. A few N- and O- linked oligosaccharides are also present in PG. GAGs have a very high charge density which confers on them a highly extended structure in solution. This arrangement gives PG a structure resembling a "bottle brush". Different PG molecules in turn may form a big supramolecular complex by non-covalently interacting with hyaluronic acid and other components of extracellular matrix (Kennedy 1979; Roden 1980; Hassel et al., 1986; Kjellen et al., 1991).

1.1.2.1 Glycosaminoglycans:

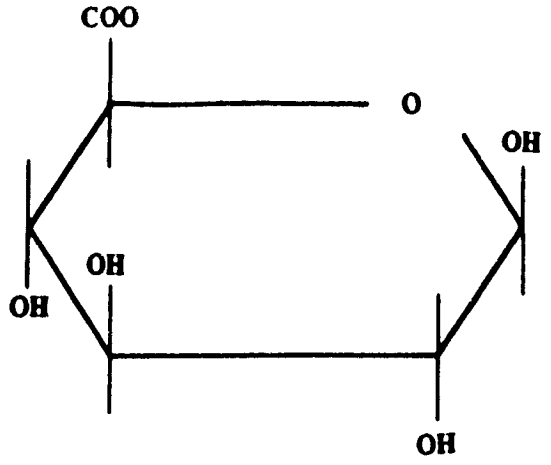
Each GAG molecule is composed of repeating disaccharide units which form a chain of varying length. One of the sugars in each unit must be a hexosamine while other can be a uronic acid or a hexose. GAGs are highly negatively charged due to the presence of sulfate and carboxyl groups. About 8 types of GAG are found in human and animal tissues which differ in their basic structure. These are chondroitin sulfate-6 (CS-6), chondroitin sulfate-4 (CS-4), dermatan sulfate (DS), heparan sulfate (HS), hyaluronic acid (HA), heparin (Hep.), chondroitin, and keratan sulfate (KS). Each GAG is characterized by its one (or more) specific type of disaccharide units. Disaccharides of different GAGs differ in the type of uronic acid (glucoronic acid or iduronic acid), type of hexosamine (glucosamine or galactosamine), and degree and position of sulfation (at position 4 or 6 or none at all). HA differs from other GAGs as it is not sulfated and is not covalently

linked to a protein. The structure and specificities of different GAGs are given in figures 1 & 2 and table 1. As evident from the table their chain size varies over a wide range. With the advent of complete sequencing, it is becoming increasingly evident that most GAGs exist as hybrids of two or more types of disaccharides. Sometimes it becomes difficult to decide which group a GAG should be assigned to. Such a controversy often exists between Hep. & HS and CS & DS and lies in the complex mechanism involved in GAG biosynthesis. Of special importance is DS which contains regions of CS type of disaccharides. Similarly Hep. and HS are often difficult to distinguish.

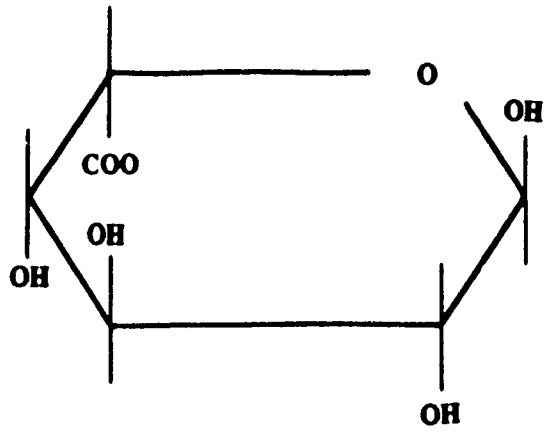

N-acetyl Glucosamine
CH2OH
O
OH
HO
OH
NHCOCH3
CH2OH
OSO3
O
OH
OH
NHCOCH3
N-acetyl Galactosamine-4 Sulfate
N-acetyl Galactosamine-6 Sulfate
CH2OSO3
O
OH
OH
OH
NHCOCH3

a

Figure 1 (a, b)



Glucoronic Acid



Iduronic Acid

b

Figure 1 (a, b)

Monosaccharide units of GAGs; hexosamines (a) & uronic acids (b).

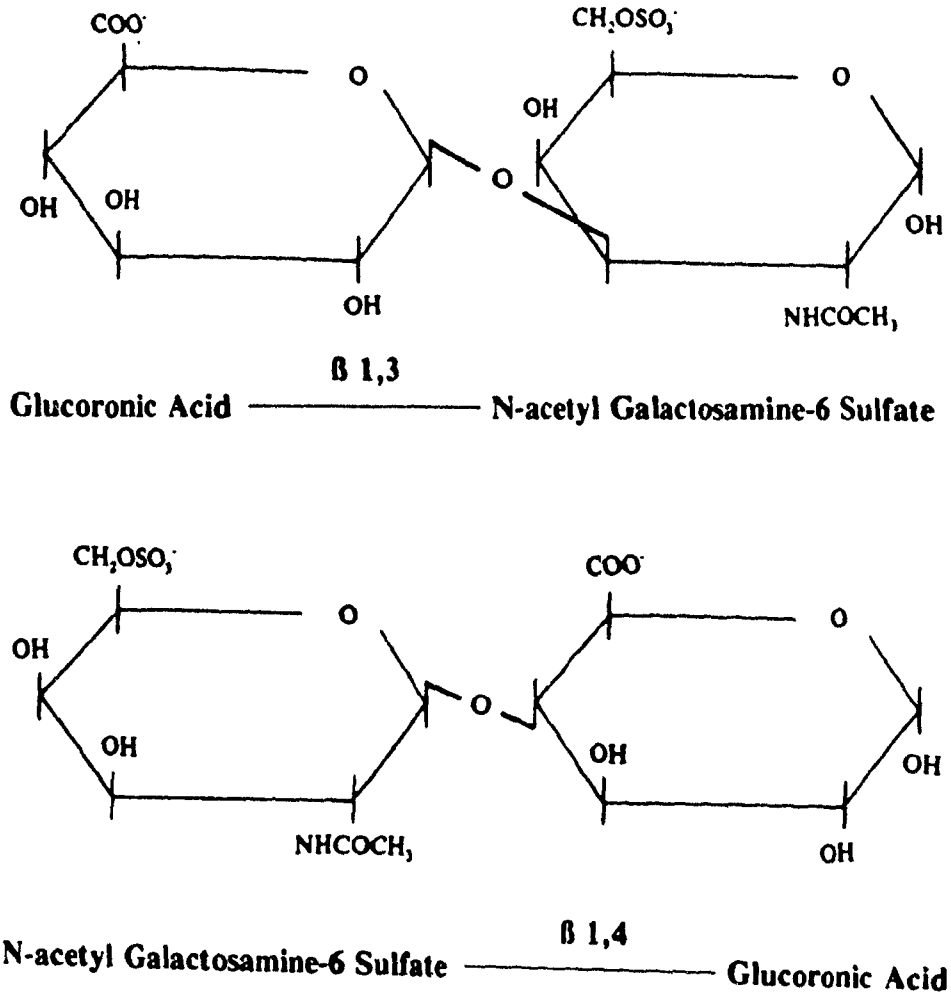


Figure 2

Disaccharide units of chondroitin 6-sulfate.

Table 1. *Characteristics of GAG Types.*

GAGs	Hexosamine	Other Sugar	Linkage*	Disaccharide Units	Distribution
C4-S	GalNAc 4-SO_3	GlcUA	ß 1-4 ß 1-3	20-60	Cartilage Intervertebral Disc Bone Arteries Cornea
C6-S	GalNAc 6-SO_3				
DS	GalNAc 4-SO_3	IdUA (2-SO_3^-)	ß 1-4 α 1-3	30-80	Fibrous Connective Tissue
HS	GlcNAc GlcN-SO_3^-	GlcUA IdUA (2-SO_3^-)	α 1-4 ß 1-4 or α 1 4	10-60	Cell Surface Basement Membrane
HA	GlcNAC	GlcUA	ß 1-4 ß 1-3	500-10,000	Umbilical Cord Vitreous Body Synovial Fluid
Hep	GlcN-SO_3^- GlcNAC3- and/or 6-SO_3	IdUA (2 SO_3^-) GlcUA	α 1-4 α 1-4 or ß 1-4	10-60	Mast Cells
KS	GlcNAc 6-SO_3^-	Gal (6 SO_3^-)	ß 1-4 ß 1-3	5-40	Cartilage Cornea

Table 1

* *The first linkage is of hexosamine to glucoronic acid and the second one is of glucoronic acid to hexosamine.*

Groups in parenthesis may be present in some of the units.

Hep. contains more N-sulfates and iduronic acids than HS.

DS chains also contain CS type of units of varying number.

GlcNAC = N-acetyl glucosamine; GalNAC = N-acetyl galactosamine

GlcUA = Glucoronic acid; IdUA = Iduronic acid; Gal = Galactose

1.1.2.2 Arterial GAGs:

The arterial wall in human contains only 5 types of GAG, viz., CS-6, CS-4, DS, HS, and HA, while Keratan sulfate, chondroitin and heparin are either completely absent or are in very minute amounts. Tables 2 and 3 display the results of many studies in humans aimed at measuring GAG in arterial wall. The total quantity of GAGs described in human arterial tissues varied between 1-2 % of dry defatted tissue weight. It is also evident that each study has described a different GAG distribution but the most abundant type is CS (CS-6 + CS-4 which are difficult to separate). DS and HS come next and HA is usually the least abundant. The cause of the difference in results lies in the techniques of GAG extraction and assay, thickness of arterial wall used, age of the subjects, part of the arterial system used as the source and, last but not least, different experimental hands.

Table 2 (a,b). *Total GAG Values in Human Arteries.*

2a. Aorta

References		GAG (mg/100 mg)	Denominator
Kaplan	1960	0.85-1.2	Dry Defatted Tissue (DDT)
Bottcher	1963	2.14	DDT
Smith	1965	2.62	Protein
Kumar	1967	~1	DDT
Nakamura	1968	1	DDT
Dalferes	1971	2	DDT
Stevens	1976	3.2	Decalcified DDT

2b. Coronaries

References		GAG (mg/100 mg)	Denominator
Tammi	1978	0.57	DDT
Murata	1982	1.65	DDT
Yla-Herttuala	1986	~1.33	DDT

Some of the values are taken from graphs and in a few cases uronic acid or hexosamine measurements have been converted to GAG weight by multiplying by 3.

Table 3 (a,b). *GAG Distribution in Human Arteries.*

3a. Aorta

References		HA (%)	HS (%)	DS + CS (%)
Buddecke	1962	15	10	60
Klynstra	1967	12.6	20.8	6.4 + 60.2
Nakamura	1968	33.3	16.8	48.6
Murata	1968	15	24	28 + 33
Dalferes	1971	8	40	52
Stevens	1976	4.1	15.9	14.7 + 65.3

3b. Coronaries

References		HA (%)	HS (%)	DS (%)	CS (%)
Tammi	1978	12	21	9	58
Murata	1982	6	36	14	44
Yla-Herttuala	1986	8	26	30	36

Some of the values are taken from graphs.

1.1.2.3 Link Region:

This consists of an oligosaccharide unit at the beginning of the GAG chains, which is linked to an amino acid of the core protein. In all GAGs except KS and HA this consists of Serine-O-Xylose-Galactose-Galactose. In KS this linkage resembles the oligosaccharide linkage of either O- or N- type. Phosphate esters have been found on some of the Xylose and Serine residues, the significance of which is unknown.

1.1.2.4 Proteoglycans:

PGs exhibit a great deal of heterogeneity and polydispersity. This property is due to the difference in the type, length and number of GAG chains attached to a PG molecule and difference in the structure of core protein. Due to these reasons PGs are difficult to classify. PGs have been arbitrarily named depending upon the major type of GAG found, e.g., CSPG, DSPG and HSPG but each group in itself contains a variable mixture of different GAGs of different chain lengths. Recently, attempts have been made to classify them according to their core proteins but the task is complicated (Heinegard et al., 1989; Oldberg et al., 1990; Kjellen et al., 1991).

Different types of PGs exist in mammalian tissues but only a few of them have been characterized. Some of the PGs which have been found in aorta and characterized (Salisbury et al., 1981; Radhakrishnamurthy et al., 1986; Wagner et al., 1986; Morgelin et al., 1989) are described as follows:

a. Large Interstitial PGs. These are found in both cartilage and soft tissues. These PGs from different tissues (named aggrecan, versican etc.) have some common features. They have a molecular weight of 1-4 million. Cartilage PG has > 100 CS chains as well as

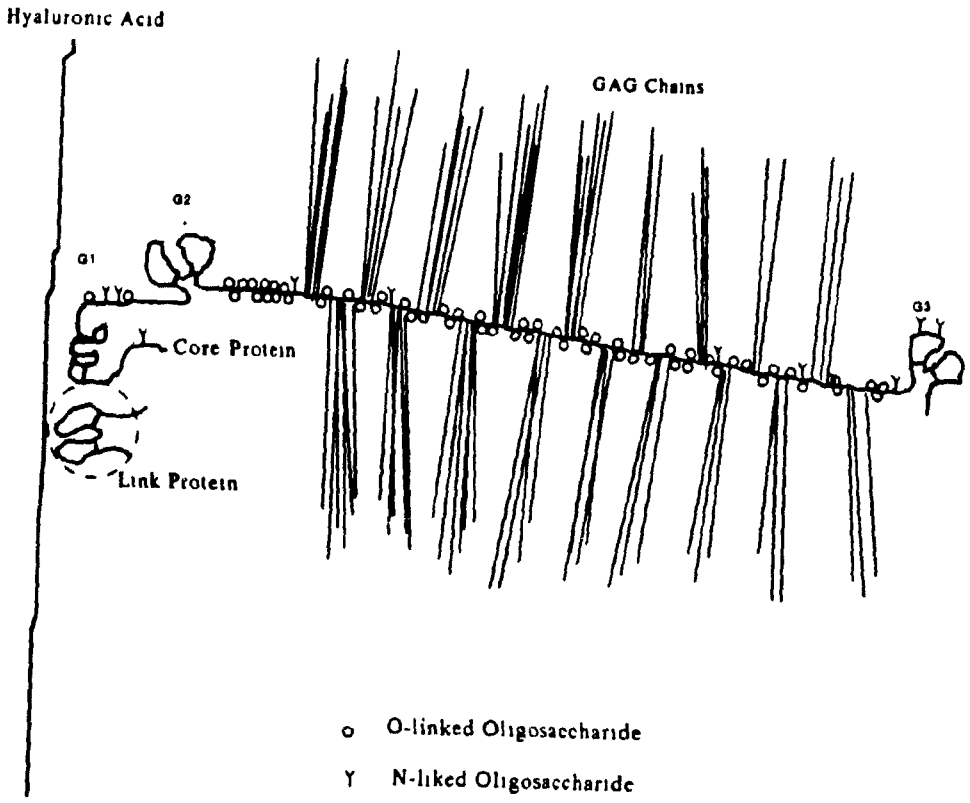


Figure 3

A large interstitial CSPG from the cartilage.

Similar PG from the aorta bears fewer GAG chains and no G2 domain.

many KS chains. Soft tissue PG has less than 30 CS and DS chains. These PG have a common feature of having a globular domain at the N-terminal of the core protein (G1) which interacts with HA and link protein to form large supramolecular aggregates (fig. 3). This is the major type of PG found in aortic tissues and has been found to be associated with the soluble part of matrix (Volker et al., 1987). Protein content of this PG has been reported to be 20-30 % by weight.

b. Small Interstitial PGs. These are much smaller PGs and have a molecular weight of about 100,000 and contain only 1-2 chains of CS or DS. They have been further identified as biglycan and decorin etc. They have been found to be closely associated with collagen fibres in the arterial tissues.

c. HSPG. This group of PG is found predominantly on cell surface and in basement membrane. In aorta they are also found close to elastic fibres. They are also of small size.

In addition, many other PGs have been characterized in different tissues and the list is increasing every day.

1.1.2.5. Core Protein:

Core proteins of different PGs differ in their length and primary structure. With the advent of recombinant DNA technology core proteins of some of the PGs have been sequenced. Molecular weight of the core protein of large CSPG from cartilage has been found to be 200,000-300,000 while that from aorta 150,000-200,000 daltons. They have been found to have an essentially extended conformation (fig. 3) with 3 globular domains (G1, G2, G3). G1 domain (N-terminal) binds to a decapeptide of HA and this interaction

is stabilized by another protein called link protein. The G2 domain is absent from the soft tissue PG. The C-terminal domain (G3) is apparently a lectin with the capacity to bind to fucose and galactose. The core protein of smaller PGs are around 30,000-40,000 daltons.

1.1.3 Biosynthesis:

Biosynthesis of PGs follows the same pattern as that of glycoproteins and involves the following steps (Heinegard et al., 1984; Kjellen et al., 1990; Schwartz et al., 1989):

a. Translation of mRNA for core protein in rough endoplasmic reticulum.

b. N-linked oligosaccharides are attached in rough endoplasmic reticulum during or immediately after the translation.

c. Extensive glycosylation occurs in the Golgi body. This involves transfer of monosaccharide units, one by one, from the respective high energy nucleotide sugars catalyzed by the glycosyltransferases. There is a specific enzyme for each type of sugar acceptor, donor and linkage formed.

d. The chain is initiated by the transfer of xylose to specific serine residues of the core protein (except in the case of KS). This is followed by addition of two galactose residues to form Ser-Xyl-Gal-Gal linkage region.

e. The chain is elongated by the addition of monosaccharides specific to the group i.e. GlcUA & GalNAc for CS & DS and GlcUA & GlcNAc for HS & Hep.

f. Chain modification occurs concomitant with or shortly after polymer synthesis. It involves epimerization at C-5 of GlcUA residues to form iduronic acid, sulfation, deacetylation and phosphorylation, depending on the type of GAG.

Sulfation requires the presence of specific sulfotransferases and active sulfate, phosphoadenosine 5′ phosphosulfate (PAPS).

g. Little is known about the initiation of HA chain, which is synthesized without any protein core.

Although our knowledge of protein synthesis has grown to maturity, our understanding of PG biosynthesis is still in its infancy. Various questions need to be answered:

a. What determines selection of a protein as a PG? There is no structural similarity and specificity of different core proteins.

b. To which serine residue will xylose attach? Although a Gly-Ser assembly has been found in some cases, it is by no means universal. The role of conformation has been proposed.

c. How are the length and compositional specificities of a GAG chain attained? A multienzyme-complex theory has been proposed, which is more speculative than factual.

1.1.4 Functions:

While some of the functions of PG are clearly known, most others are postulated on the basis of indirect evidence and in vitro experiments (Hascall 1988; Ruoslahti 1989; Wight 1989).

a. Structure Stability: PGs interact with HA, with each other, and with structural proteins, like collagen and elastin, in a specific manner which gives strength and stability to the tissues.

b. Turgor and Resilience: PGs attract cations due to their polyanionic nature. It

results in osmotic swelling which can resist compression by a load and is responsible for the resilience in cartilage tissues.

c. Filtration: PGs act as molecular sieves, restricting passage of macromolecules into the extracellular matrix while allowing free passage of smaller molecules.

d. Anticoagulant: Hep. and HS bind to antithrombin III which greatly increase the ability of the latter to inactivate serine proteases (e.g., thrombin).

e. Lipolysis: Hep. and HS bind to lipoprotein lipase present on the capillary endothelium and release it into circulation.

f. Permeability: HS and Hep. in the basement membrane of the glomerulus participate in charge selective filtration.

g. Cell Adhesion: PGs may facilitate or inhibit cell adhesion to substratum, probably by modulating the activities of primary adhesion molecules, e.g., fibronectin. They may also have an influence on cell-cell interaction.

h. Cell Migration: Cell migration is an important phenomenon which is involved in events like morphogenesis, angiogenesis and atherosclerosis. PGs have been found to be involved in this phenomenon, e.g., Hep. inhibits smooth muscle cell migration from the vascular media to the intima, and migrating endothelial cells show quantitative and qualitative changes in their PG synthesis.

i. Cell Proliferation: Proliferation of arterial smooth muscle cells has been found to be associated with an increase in the synthesis of PG and other matrix components. In particular, HS and Hep. have been proven to be antiproliferative for arterial smooth muscle cells.

j. PGs may be found as an integral protein of plasma membrane and act as receptors for certain ligands, such as, transforming growth factor ß and thrombospondin.

k. PGs permit corneal transparency.

1.2 Atherosclerosis:

Atherosclerosis is a disease of arteries and is characterized by plaque like lesions, developed as a result of smooth muscle proliferation associated with accumulation of lipid and extracellular matrix. The disease starts at a young age but does not become manifest until middle age or later in the form of ischemic heart disease, ischemic encephalopathy or cerebral infarction, and peripheral vascular disease etc. It must be stressed that whereas atherosclerosis is the primary and often sole pathology behind these clinical entities, a complex network of other risk factors (e.g., hemodynamic imbalances, thromboembolism, etc.) is also commonly involved.

1.2.1 Risk Factors:

Atherosclerosis has been associated with 4 important and unequivocal risk factors; hyperlipidemia, hypertension, cigarette smoking and diabetes. This has been proved by a number of epidemiological, morphological and experimental studies (Kannel et al., 1976; Averbrook et al., 1989; Getz, 1990). Immense attention has been paid to hyperlipidemia and currently, increased levels of plasma low density lipoprotein (LDL) (cholesterol and/or apoliporotein B) with decreased levels of plasma high density lipoprotein (HDL) are considered as the most important parameters in predicting atherosclerosis risk in the general population. Recently, lipoprotein(a), which exists as a complex of apolipoprotein B100 linked by a disulfide bridge to a unique apolipoprotein(a), has been found to be another important risk factor (Loscalzo 1990). There is also increased prevalence of atherosclerosis or its complications with increasing age, male gender, and in families and in certain geographic locations (Tejada et al.,

1968). A number of other minor risk factors have also been incriminated, e.g., type A personality, obesity, insufficient physical activity etc.

1.2.2 Morphology:

Atherosclerosis lesions always start in the intima of arteries and later compress and encroach upon the media on one side and protrude in the lumen on the other (Haust 1981; Adams et al., 1989; Cotran et al., 1989).

a. Atherosclerosis Plaques: Plaque like lesions are the hallmark of fully developed atherosclerosis. These are raised lesions, white or whitish yellow in color. Typically they consist of a fibrous cap and a central necrotic and lipid core. These lesions usually start in the third decade and increase in extent and severity with age. Although very common, they are not an invariable consequence of life. Histologically they are composed of:

i. Cells: Smooth muscle cells (SMCs), macrophages and few lymphocytes.

ii. Extracellular matrix: Mainly collagen with elastin and PGs.

iii. Lipid: Both extracellularly and intracellularly (foam cells) and is primarily cholesterol and cholesteryl ester, most probably derived from plasma LDL and possibly VLDL. Foam cells are derived from both SMCs and macrophages.

These components are present in varying composition in different plaques, giving rise to a spectrum of lesions.

b. Early Lesions: While plaque is a well defined and accepted hallmark of atherosclerosis, some controversy exists concerning the earlier or precursor lesions (Cotran et al., 1989). These include fatty streaks, gelatinous elevations, microthrombi, and intimal cushions or cell masses. Of these fatty streak is more common and is actually universal in

geographical distribution. They are found in infants and children throughout the world. The fatty streak appears as a yellow discoloration of flat intima and microscopically consists of lipid laden foam cells. The topographic distribution of fatty streaks is similar to plaques in the coronaries but is quite different in the aorta (McGill, 1984; Stary, 1987). Now there seems to be an agreement that some of them probably develop into plaques. Our view, then, towards the pathogenesis of atherosclerosis should be how fatty streak changes into plaque and not how fatty streaks are formed.

c. Complicated Lesions: As the disease progresses, plaques invariably undergo a series of changes, viz., calcification, ulceration, thrombosis, and hemorrhage. These changes generally lead to clinical manifestations.

1.2.3 Pathogenesis:

Our knowledge of the pathogenesis of atherosclerosis is still in the theoretical stages. Starting in the mid-nineteenth century, many theories were presented, but none succeeded in explaining the phenomenon completely. Currently, the modified response to injury hypothesis is the most widely accepted by most of the biologists in this field (Clowes, 1989; Fuster et al., 1992). A recent variant of this hypothesis incriminates lipid peroxidation as an important initiating and promoting factor in the pathogenesis of atherosclerosis The following is the brief introduction of the major hypotheses:

a. Imbibition Hypothesis: It was originally proposed by Virchow in 1856 who suggested that lipid accumulation was due to increased infiltration of plasma protein and lipid from the blood. The cellular reaction was considered to be a low grade inflammatory reaction to injurious plasma components.

b. Encrustation hypothesis: This was originated by Rokitansky in 1852 and later modified by Duguid. It was proposed that small thrombi were collected over foci of endothelial injury; subsequent organization resulted in plaque formation.

c. Monoclonal Hypothesis: Benditt and Benditt observed that smooth muscle cells of some human plaques appeared to be mono- or oligoclonal in nature and proposed that it might be equivalent to benign neoplastic growth in response to mutagens (Benditt et al., 1973).

d. Response to injury hypothesis: This was formally presented by Ross and Glomset in 1976 and an updated version was published by Ross in 1986 (Ross et al., 1976; Ross, 1986). It had already been shown that a continued injury to arterial endothelium could lead to atherosclerosis like lesions (Moore, 1973). Endothelial injury can be produced experimentally in a number of ways and has been detected in conditions like, hypertension, cigarette smoking, homocysteinuria, diabetes, and hyperlipidemia. Moreover, they have been produced iatrogenically by embolectomy catheters and angioplasty etc. (Moore, 1981). Today the major issues are what type of injury is actually responsible for atherosclerosis inside the human body, what is the actual sequence of events following an injury, and what molecules are the key players?

The current understanding of the pathogenesis of atherosclerosis can be summarized as follows:

a. Following nondenuding endothelial injury there is adherence of monocytes to endothelium and migration to subendothelium where they change into foam cells.

b. Sometimes after nondenuding injury (due to the exposure of the subendothelial tissues as a result of endothelial retraction) or immediately after a denuding injury, platelets adhere to subendothelial tissues and release growth factors.

c. SMC migration from the media into the intima and subsequent proliferation is the key event in atherogenesis. This can be induced by an interplay of growth factors secreted by the 4 key cell types (macrophages, platelets, endothelial cells, and SMCs) in both paracrine and autocrine fashion. These include growth promoting factors (Platelet derived growth factor, Basic fibroblast growth factor, Endothelium derived growth factor), growth inhibiting factors (Heparin, Transforming growth factor-ß) and cytokines (Tumor necrosis factor, Interleukin-1, τ-Interferon) (Davies, 1986).

d. Proliferating SMCs change their phenotype from myofilament-rich "contractile" to rough endoplasmic reticulum-rich "synthetic" and secrete large amounts of collagen, elastin and PGs (Campbell et al., 1985). Some of them accumulate lipid to form foam cells.

e. Lipid in the lesion is derived from plasma apoB containing lipoproteins (LDL, VLDL, LPa)(Smith, 1974b; Yomantas, 1984). Lipoproteins (LPs) form complexes with PGs and other components of the matrix which cause entrapment of lipid within the arterial wall. These complexes are taken up avidly by macrophages which lead to the formation of foam cells (Berenson et al., 1985; Wight, 1989). HDL_3, a subfraction of HDL, has been suggested to bear antiatherogenic properties by providing a means for reverse cholesterol transport

from tissues to other LPs and eventually to liver (Glomset, 1968).

f. Recently, largely as a result of the work of Steinberg and coworkers, oxidatively modified LP is gaining great interest. It has been found that in the presence of different cell types LPs undergo oxidative modifications which change their properties. Oxidized LDL are taken up avidly by macrophages by the way of the acetyl LDL receptor and probably an additional oxidized LDL receptor. In addition, it is cytotoxic, chemoattractant for monocytes, and inhibits the release of resident macrophages (Steinberg, 1987 and 1990).

1.2.4 GAG changes in Atherosclerosis:

The involvement of PG in atherosclerosis was first shown a few decades ago by histological techniques (Berenson et al., 1971). An increase in metachromasia was observed by many investigators at the site of atherosclerosis lesions during the early twentieth century. In 1951 Rinehart and Greenberg emphasized metachromic staining of arterial tissue during the initial stages of experimental atherosclerosis, caused by pyridoxine deficiency. Since then a number of studies have presented evidence of GAG changes in atherosclerosis. Basic dyes, such as, toluidine blue, alcian blue, Azure A etc. were used to demonstrate metachromasia. Quantification of GAGs by histological methods was not successful due to inherent problems in the method. Recently, electron microscopy and immunocytochemistry are being used as useful tools to study GAGs in the lesions (Volker et al., 1987 & 1989; Richardson et al., 1989).

The isolation of arterial GAGs was first performed in 1895 by Morner but the actual quantitative work started after the middle of this century. At the same time investigators started isolating GAGs from human atherosclerotic lesions. Extensive studies have been performed in the laboratories of Berenson (USA), Buddecke (FRG), Murata (Japan) and others. The results of the studies on arterial GAGs are compiled in table 4. Due to differences in the criteria used for the selection of samples and in the techniques used, the results of these studies failed to agree. The major inference we can draw is that GAGs increase during the early period of atherogenesis, but decrease as the lesion advances. There are also changes in GAG distribution within the lesions. Common findings are an increase in DS and CS and a decrease in HS and HA proportions. Changes similar to these have also been found in experimental atherosclerosis using either injury or a hypercholesterolemic model (Wagner et al., 1978; Alavi et al., 1985; Salisbury et al., 1985). Metabolic studies have confirmed that the increase in GAGs during the early lesion is due to increased synthesis by intimal tissues and especially by SMCs (Li et al., 1990).

Isolation of intact PGs from human lesions has provided further information on this issue. CSPG has been found to be of smaller size along with longer but fewer GAG chains in atherosclerotic lesions (Wagner et al., 1986). Again a decrease in PG has been detected in plaques (Dalferes et al., 1987; Cherchi et al., 1990). In a study of a Chinese population a low total GAG with a change in distribution to a lower HS and a higher DS proportion have been found in the area of higher atherosclerosis prevalence (Ying-Shan et al., 1991).

Table 4 (a,b,c). *Changes in GAGs in human atherosclerotic lesions.*

4a. Aorta *FS = Fatty Streak*

References	Changes Observed
Buddecke (1962)	Total GAG: ↑ GAG Distribution: HA ↓ , HS ↓ , CS a small ↑
Smith (1965)	Total GAG: ↑ in FS, ↓ in Plaques, ↓↓ in Calcified Plaques GAG Distribution: Not performed
Klynstra (1967)	Total GAG: ↑ in FS, ↓ in Plaques GAG Distribution: Difficult to interpret
Kumar (1967)	Total GAG: ↑ in FS, ↓ in Plaques GAG Distribution: Complicated and difficult to interpret.
Nakamura (1968)	Total GAG: ↓ in Plaques GAG Distribution: HS ↓ more as compared to other GAGs.
Dalferes (1971)	Total GAG: ↑ in FS GAG Distribution: Not much changed in FS.
Murata (1971)	Only Chondroitin sulfates studied GAG Distribution: CS ↓ , DS not changed

Stevens (1976)	Total GAGs: ↓ with progression of lesions GAG Distribution: DS ↑ in absolute amounts
Hollman (1989)	Total GAG: ↑ with cholesterol content. GAG Distribution: HS ↓ , CS+DS ↑ (with cholesterol)

4b. Coronaries

References	Changes Observed
Tammi (1978)	Total GAG: ↓ GAG Distribution: HA ↓ , HS ↓ , DS ↑
Murata (1982)	Total GAG: ↓ GAG Distribution: HA ↓ , HS ↓ , DS ↑ , CS ↑
Yla-Herttuala (1986)	Total GAG: Initially ↑, later ↓ GAG Distribution: HS ↓ , HA ↓ , DS ↑ , CS ↑

4c. Cerebral

References	Changes Observed
Murata (1989)	Total GAG: ↓ GAG Distribution: HS ↓ , DS ↑ , CS ↑

1.2.5 Role of PGs in Atherosclerosis:

The fact that GAGs can form complexes with LPs was first observed during the 1950s by Bernfeld and coworkers and Burstein and colleagues. They observed the precipitation of LPs by heparin and other polyanions. Later work of Amenta and Waters, Bihari-Varga, Iverius, Berenson & coworkers, Camejo and associates, and Alavi & Moore confirmed the findings (Berenson et al., 1972; Camejo, 1982; Wight, 1989). At the same time work has been started to perform LP-GAG interaction in a quantitative way. These in vitro interactions are best performed in the presence of a divalent cation (Ca^{++} and Mg^{++}) at low ionic strength: other variables like temperature, pH etc. also seem to be important (Vijayagopal et al., 1981; Steele et al., 1987). The most important factors in this interaction are the reactants; different GAGs interact with different avidity and so do different types of LP. PGs isolated from animals under conditions of experimental atherosclerosis have shown greater interaction with LPs (Alavi et al., 1989). Similarly LDL from individuals with clinically significant atherosclerosis has been found to interact more avidly with PGs (Linden et al., 1989). It is important to note that interaction is specific with apolipoprotein-B containing LPs, and HDL does not interact with GAGs. Molecular weight and charge on GAGs are critical, as HA which lacks sulfate does not bind to LPs and intact PGs have been found to have much higher strength of interaction as compared with deproteinized GAGs. Further studies have proved that it is primarily an electrostatic interaction involving negatively charged GAG groups and basic amino acids of apolipoprotein B.

The point that LP-PG complexes are found in atherosclerosis in vivo is underscored by

studies involving both human and experimental atherosclerosis (Srinivasan et al., 1972; Mawhinney et al., 1978). These studies have demonstrated the presence of LP-PG complexes in the vessel wall. Isolation of intact complexes require a more gentle method of extraction to avoid breaking of ionic and other noncovalent bonds. These studies prove that PG-LP interaction is not just a test tube phenomenon but is present in vivo also.

Incubation of macrophages with LP-PG complexes results in intracellular accumulation of cholesteryl ester leading to foam cell formation (Salisbury et al., 1985b; Vijayagopal et al., 1985). These complexes are taken up by receptor mediated endocytosis involving a pathway distinct from the apoB,E or acetyl-LDL receptors. Like acetyl-LDL receptors there seems to be no feedback down regulation (Vijayagopal et al., 1991) which results in intracellular cholesterol accumulation. Further work is required to elucidate this phenomenon clearly as both the receptor and the ligand involved in this process are not known.

These findings suggest that PGs provide a very effective mechanism by which LDL may be trapped inside the arterial intima and may then be transported to macrophages and smooth muscle cells thus leading to both extracellular and intracellular accumulation of lipid.

Lipid binding is not the only property of PGs which may be important in atherosclerosis. As mentioned earlier, with the advent of modern technologies PGs have been found to be actively involved in cell biology. There is growing evidence that PGs take part in SMC migration and proliferation. These two events have been found to be critical in atherogenesis.

Hep. and HS have been found to be growth inhibitory for SMC's (Castellot et al., 1987; Wight, 1989). They have been shown to inhibit intimal proliferation after endothelial injury in vivo and SMC proliferation in tissue culture. Other GAGs do not have this quality. Growth inhibitory activity of these GAGs depends upon an enzyme heparitinase which has been found in serum and arterial tissues. Enzymatic degradation of these GAGs generate fragments which are bound and internalized by SMCs through receptor mediated endocytosis. These in turn inhibit DNA and RNA synthesis; overall protein synthesis is not effected but synthesis of specific proteins may be induced or repressed.

PGs have also been found to be active in cell adhesion and migration (Wight, 1989). Specifically HSPG has been found to be involved in cell adhesion. HSPG has been found to be associated with elements of cytoskeleton which are involved in cell adhesion. PGs may interact with specific adhesion proteins, such as fibronectin, and help in stabilizing the interaction (Ruoslahti et al., 1985). Other PGs, i.e., DSPG and CSPG have been found to inhibit attachment of cells to matrix elements. Similarly, there are some reports which implicate PGs in cell migration. Heparin has been found to inhibit arterial SMC migration in vitro (Majack et al., 1984).

PGs are therefore associated with all three elements of atherosclerotic lesions, i.e., matrix, lipid and cells. They are involved in organization of matrix, binding and trapping of lipid, and cellular migration and proliferation. Since different types of PGs have different and often opposing actions, it is important to find out the exact nature of changes in GAG component in atherosclerosis and their relation to risk factors.

1.3 Diabetes Mellitus (DM):

DM is one of the most common diseases, and one of the top ten leading causes of death in the western world. It is characterized by a relative or absolute deficiency of insulin and results in hyperglycemia associated with disorders in the metabolism of glucose, protein and lipid. Clinically two distinct types of DM were recognized which were later found to be the result of different pathogenic mechanisms. According to the classification developed by the National Diabetes Data Group of the National Institute of Health there is a Insulin dependent DM, also called type I DM (juvenile onset) and a non-insulin dependent DM or type II DM (maturity onset) (Bennet, 1983). About 90 % of cases belong to type II and usually do not require insulin therapy. These patients are often obese and the mechanism responsible for diabetes is insulin resistance at the cellular level. Consequently, they are usually found to have hyperinsulinemia in the presence of a relative insulin deficiency. The underlying cause is unknown but is probably genetically related.

Type I DM is more severe, patients require regular insulin therapy and it becomes manifest at younger age. Here the disease is caused by a totally different mechanism of beta-cell damage which is probably immune-mediated and subsequently leads to insulin deficiency. Interestingly, insulin therapy may lead to hyperinsulinism even in type I patients. In spite of the difference in their etiologies and severity, their long term complications are almost the same. Both types of patients are similarly affected by accelerated atherosclerosis, microangiopathy, nephrosis etc. Probably these are the result

of metabolic derangements which are present in both types, rather than an independent phenomenon, related to genetic or environmental variables (Cotran et al., 1989).

1.3.1 Atherosclerosis in Diabetics:

Involvement of arteries in both type I and type II DM has been documented both epidemiologically and morphologically. As a result DM is well recognized as a risk factor for atherosclerosis. Epidemiological studies (Kannel et al., 1978; Pirart, 1978) have demonstrated that stroke, coronary heart disease, and especially peripheral vascular disease are more common in diabetics and they become manifest at an earlier age. Female diabetics seem to have the same prevalence of atherosclerosis manifestations as do male diabetics. In fact, atherosclerosis is the principal cause of mortality (75%) in diabetics in the western world. The mortality rate of type I patients with proteinuria is several-fold that of type I patients without proteinuria (Borch-Johnsen et al., 1987). Diabetes has been found to be an independent risk factor for coronary heart disease acknowledging other associated risk factors (Kannel et al., 1979; Butler et al., 1985). In fact, the degree of coronary ischemia and atherosclerosis have been related to the severity and duration of hyperglycemia and metabolic control (Pirart, 1978; Dupree et al., 1980; Lemp et al., 1987). Surprisingly, it is still not clearly known whether a tight control of hyperglycemia would be helpful for protection against coronary heart disease (Raskin et al., 1986).

The International Atherosclerosis Project was a very extensive morphological study performed in the 1960s and it established unequivocal proof of exaggerated atherosclerosis in diabetes (Robertson et al., 1968). This study has clearly demonstrated an increased presence of raised atherosclerosis lesions in diabetics of different age and

location groups when different arteries were examined. However, in the aorta no difference was found in the quantity of fatty streaks, while that in coronaries did show an increase. While atherosclerosis lesions in diabetics and nondiabetics are morphologically indistinguishable, there is a predilection for peripheral arteries.

1.3.2 Atherogenic Factors:

The cause and mechanism of accelerated atherosclerosis in diabetes is not clearly known. The problem is accentuated by the fact that although we know a lot about the different theories and models of atherosclerosis the real mechanism is still elusive. Many factors have been implicated as being atherogenic in DM but they are either controversial, inconclusive or present in only a fraction of patients (Ganda, 1980; Ruderman, 1984). We can infer that this phenomenon depends on the interplay of a host of factors acting synergistically. Some of these are discussed in the following sections.

1.3.2.1 Lipid Abnormalities:

The greatest emphasis has been given to the wide spectrum of lipid changes present in many (20-50 %) but not all of the diabetics. The subject is complicated by different types of the abnormalities found in the two types of DM. Common changes are (Betteridge, 1989; Ginsberg, 1991):

a. The most frequent finding is hypertriglyceridemia, which is considered a risk factor for atherosclerosis but not unequivocally (Austin, 1990). This metabolic defect is the result of either lower lipoprotein lipase activity, increase production of triacylglycerol & VLDL, or both. Moreover, VLDL is more triacylglycerol enriched and larger in size.

b. Lower HDL-cholesterol levels are also frequently found in type II diabetics, but are normal or elevated in type I.

c. Higher LDL-cholesterol is found in many cases.

d. Glycosylated LDL or LDL isolated from diabetics increases cholesterol accumulation in macrophages by a pathway not involving classic or acetyl LDL receptors.

e. There is evidence of increased peroxidation of LDL in diabetics (Baynes, 1991).

f. Lp(a) levels have been found to be increased in type I diabetes with nephropathy (Jenkins et al., 1991; Levitsky et al., 1991).

1.3.2.2 Hematologic Disturbances:

Following alterations have been detected and may bear atherogenic potentials.

a. Increase platelet adhesiveness and aggregation and elevated levels of von Willibrand factor activity.

b. Increase levels of factors I, V, VII, and VIII.

c. Increase in the ratio of thrombaxane to prostacyclin synthesis.

1.3.2.3 Obesity and Hypertension:

These are found in many cases of type II diabetes, and especially hypertension is a well defined risk factor for atherosclerosis. These accompanying factors accentuate the risk caused by diabetes itself.

1.3.2.4 Non-enzymatic Glycosylation:

Hyperglycemia leads to nonenzymatic glycosylation of various proteins. Glucose reversibly combines with an amine group to form a Schiff base at first and later a more

stable Amadori product (Brownlee et al., 1988). Over a long period of time there is further rearrangement to form irreversible, cross-linking, advanced glycosylation end products (AGE). These will continue to accumulate on stable tissue proteins, like collagen, in chronic diabetes. AGE can be atherogenic due to:

a. It can cross link collagen to plasma lipoproteins, thus favouring their accumulation.

b. AGE cross-linked products are resistant to enzymatic degradation.

c. AGE-linked protein binding to a novel receptor on macrophages induces secretion of cytokines.

1.3.2.5 Polyol Pathway Disturbance:

Various tissues, such as , blood vessels, lens , kidney and nerves, do not require insulin for glucose transport across the plasma membrane. Hyperglycemia leads to an excess of intracellular glucose in these tissues which is then converted to sorbitol and eventually to fructose. This results in depletion of specific myo-inositol pool leading to decreased diacylglycerol and protein kinase C activity and eventually to deranged Na^+/K^+ ATPase regulation. This increases intracellular osmolarity, leading to swelling and injury to the cells (Greene, 1987; Weingrad et al., 1987; Simmons et al., 1989).

1.3.2.6 Hyperinsulinemia and Insulin Resistance:

Hyperinsulinemia, which can be found in both types of diabetes, has been incriminated as an independent risk factor for atherosclerosis in both diabetics and nondiabetics. This has been suggested by both epidemiological and experimental studies (Welborn et al., 1979; Ducimetier et al., 1980; Ronnemaa et al., 1991). Insulin, when used in excess, has

been found to stimulate arterial smooth muscle cell proliferation and enhance cholesterol synthesis (Sato et al., 1989). In fact, insulin, like most growth factors, has been found to be involved in cellular proliferation, growth regulation, tissue repair, and fetal organogenesis. It is also found to be associated with hypertension, low HDL cholesterol, and high VLDL cholesterol levels (Zavaroni et al., 1989; Laws et al., 1991).

More recent studies implicate insulin resistance rather than hyperinsulinemia per se to be the origin of problems. Insulin resistance has independently been found with atherogenic factors, like, abnormal lipid and lipoprotein levels and hypertension in type II diabetics. Recently, insulin resistance has been found to be associated with atherosclerosis in nondiabetics also (Laakso et al., 1990 and 1991).

1.3.2.7 Other Hormones:

Many other hormones have been implicated and include:

a. Growth Hormone: Raised growth hormone levels in diabetes have been found and may induce cell proliferation through insulin-like growth factor (somatomedin C).

b. Glucocorticoids: Raised in poorly controlled type I diabetes. May be atherogenic.

c. Catecholamines: Also raised in poorly controlled type I diabetes and have atherogenic potential.

d. Sex Hormones: The role of sex hormones is quite ambiguous.

1.3.2.8 Genetic Factor:

A genetic marker for atherosclerosis has been described. A specific , highly polymorphic DNA fragment flanking the 5′ end of insulin gene (U allele Vs L allele) has been shown to be more prevalent in both type II diabetics and nondiabetics with atherosclerosis. The

nature of the atherogenic factor in individuals with the U-allele is not known (Owerbach et al., 1982; Tybjaerg-Hansen et al., 1990).

1.3.2.9 Endothelial Injury:

Injury to the endothelium is now recognized as the most important event in atherogenesis. Alterations to the endothelial cells have been reported in a number of studies in both human and experimental diabetes (Dolgov et al., 1982; Arbogast et al., 1984; Hadcock et al., 1991; Raij 1991). Proof of endothelial injury is both morphological (non-denuding endothelial injury, adhesion of white blood cells, platelets and fibrin-like material, smooth muscle hyperplasia) and functional (increased plasma levels of von Willibrand factor, decreased prostacyclin production, decreased plasminogen activator production, impaired response to agonists of endothelium dependent relaxation). Factors which might be responsible for endothelial injury in diabetes are: hyperlipoproteinemia, hypertension, polyol pathway disturbance, lipid peroxides, fibrinogen, Insulin, free fatty acids, immune mechanism, and viruses.

1.3.3 GAG Changes:

The importance of GAGs in atherosclerosis has already been described. Changes in arterial GAGs prior to or concomitant with the development of lesions may be an important factor in accelerated atherogenesis.

There are many changes in the extracellular matrix which have been detected in various organs in both human and experimental animals (Sternberg et al., 1985). Changes have been found in collagens, elastin, PGs and other glycoproteins both in matrix proper and

Table 5. *Changes in Arterial GAGs in Experimental Diabetes.*

References	Species	Type of Diabetes	Duration (Days)	Findings
Ichida 1968	Rabbits Rats	Alloxan	90-360	Total GAG: Not changed HA ↑
Cohen 1970	Rats	Pancreat-ectomy	90	Total GAG: ↑ All GAGs except HS ↑
			180	Total GAG: ↓ All GAGs ↓
Malathy 1972	Rats	Alloxan	15	Total GAG: ↑ CS and DS proportion ↓, HA ↑
			60	Total GAG: ↓ CS and DS proportion ↓, HA ↑
Sirek 1980	Dogs	Alloxan	100	Coronaries: Total GAG: ↑ DS ↑ Thoracic Aorta: Total GAG: ↓ HA ↓ , HS ↓

basement membrane. Many studies are on basement membrane, specially glomerular, due to the very common association of microangiopathy with diabetes.

Changes in GAG moiety have been found in arterial tissues but all of these studies are confined to experimental animals. Their findings are summarized in table 5. As evident from the table there is no general agreement on the type of GAG changes. The reason for such versatility is probably due to different species used, different methods of producing diabetes, duration of diabetes, and different methodology for GAG extraction and quantification. We feel that there is a need of such a study in human diabetes which would provide more reliable and authentic results. As a result we started this project to furnish information on the actual situation of diabetes, present for a long time, which none of the existing studies could have provided.

2. METHODS

2.1 Subjects:

The source of tissues in my project was human aortas. They were kindly provided by pathologists of the Royal Victoria Hospital at the time of the autopsies. Fresh tissues were received and immediately frozen at -20 C until processed. A maximum time interval of 24 hours between death and autopsy was allowed in this study. It is to be noted that GAGs are much more resistant to degradation than proteins and no change in aortic GAG composition has been detected up to 90 hours of postmortem interval (Manley, 1965). As most of the cases were of old age, no specimen was included from young individuals. Table 6 and 7 shows different information regarding those subjects whose aortas were used. Male and females were not separated, nor was segregation done on the basis of concomitant diseases. Any such attempt, although desirable, would lead to narrowing of the spectrum and difficulty in obtaining specimen of precise interest. All of the diabetics suffered type II disease as this type is much more common. Duration of diabetes ranged from 3 years to decades.

2.2 Tissues:

All tissues for the isolation of GAGs were selected from the arch and thoracic part of aorta. This part of arterial tree was chosen as it is easy to remove and usually contains sufficient quantities of both normal looking and atherosclerotic areas. Only those aortas were included in the study which had sufficient amount of both normal looking and uncomplicated plaque areas. An area of about 3-4 cm^2 would give enough tissue for

Table 6. *Basic Information about Nondiabetic Group.*

No.	Sex	Age	Major Chronic Problem
1	F	61	Metastatic Parotid Carcinoma
2	F	68	Motor Neuron Disease
3	F	79	Liver and Breast Carcinoma
4	F	70	Primary Biliary Cirrhosis
5	M	78	Adenocarcinoma Stomach
6	F	67	Squamous Cell Carcinoma Lung
7	M	66	Carcinoma Colon
8	F	76	Parkinsonism
9	F	58	Metastatic Uterine Sarcoma
10	F	72	Metastatic Adenocarcinoma Lung

Table 7. *Basic Information about Diabetic Group.*

No	Sex	Age	Duration of Diabetes (Years)	Other Major Chronic Problem
1	F	92	> 15	Adenocarcinoma Cecum Chronic Cholecystitis
2	F	67	3	-
3	F	73	> 15	Hypertension
4	M	68	> 15	-
5	M	70	12	Carcinoma Rectum
6	M	57	5	Idiopathic Cirrhosis

processing. As the disease starts in the intima, we tried to use only the superficial layer of the arterial wall. The innermost layer (intima) which was easily separable from underlying adventitia-media and was peeled off and used for the isolation of GAGs. Normal areas were those which were grossly normal, thin, translucent and without any visible elevations (fig. 4). Meticulous search for early lesions, such as, fatty streak, dot, intimal thickening was not performed. Areas selected as lesion contained definitely elevated white to yellow opaque plaques (fig. 4). Only those plaques were selected which looked uncomplicated i.e. they did not have gross ulcers or calcification. Doubtful areas were not included in any of the groups. No differentiation was made on the basis of amount of lipid in the lesion or the ratio of fibrous to fatty tissues.

Selection of tissues was confirmed in a few cases by histological examination. Small pieces of selected tissues were immediately fixed in 10 % buffered formalin and subjected to routine histological processing and staining in hematoxylin & eosin (H & E). Fig. 5 gives examples of sections from normal and lesion areas of the selected tissues. It can be seen that normal tissue contained only a few layers of cells in the intima while plaque showed several-fold thickening and other characteristics of atherosclerotic lesions such as fibrous cap, necrotic core and cholesterol clefts and abundant matrix. It should be noted that a few sections cannot map the entire area selected but they provided the general confirmation that gross and microscopic picture did correspond. Histologic examination also confirmed that tissues used for isolation of GAGs contained mainly intima with little or no media attached (fig. 5).

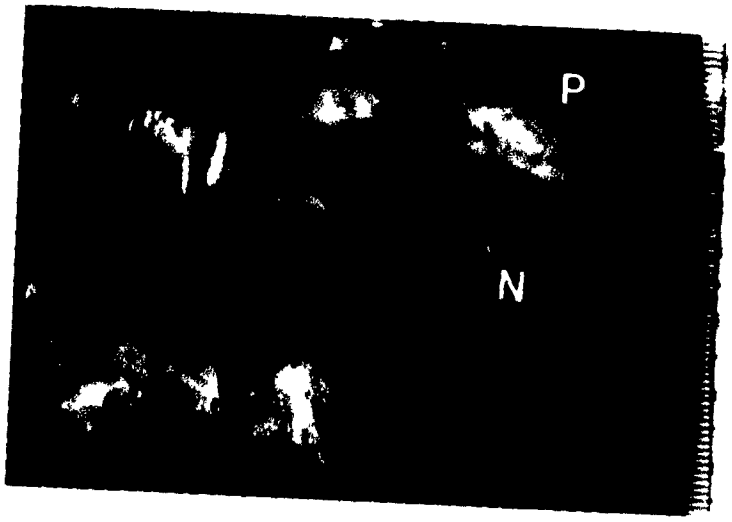
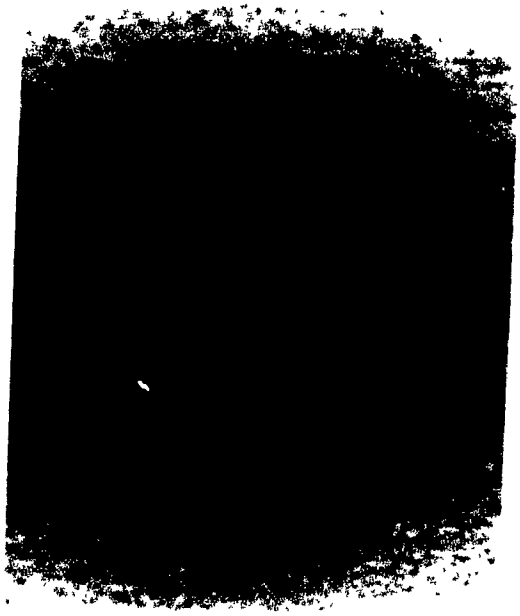


Figure 4

A photograph of the peeled out intima from the human thoracic aorta.

Please note many elevated opaque plaques (P) and thin translucent normal area (N).



a

b

Figure 5 (a, b, c)

c

Figure 5 (a, b, c)

Low-power micrographs from sections of normal intima-media (a) and peeled out intima from normal area (b) and plaque (c). All at same magnification (x156)

2.3 Isolation of GAGs:

The methodology used for isolation of GAGs has been successfully employed by many investigators with some minor variations (Roden et al., 1972; Wagner et al., 1978; Alavi et al., 1985; Yla-Herttuala et al., 1986). Fig. 6 gives an outline of the steps used in this study.

Every time, 1-3 aortas were processed depending upon the availability of tissues. After peeling and segregating the two types of tissues, their wet weight was determined. This provided an idea about the quantity of tissue required for further processing. Usually a wet weight 5-7 times that of required dry defatted tissue weight was found to be adequate. Subsequent steps are as follows:

2.3.1 Delipidation:

Lipid was removed from the tissues as the first step in the isolation of GAGs. For this purpose a commonly used method developed by Folch was used (Folch et al., 1957). Pieces of tissues were homogenized in a mixture of chloroform (2 parts) and methanol (1 part) using an electric homogenizer. This process was repeated 2-3 times until all the tissue was finely divided. Lipid solvents were removed by passing through a Whatman filter paper while tissues were separated from the filter paper and allowed to dry. Tissues were left for few hours and weighed repeatedly until no decrease in their weight was found. The final weight called dried defatted tissue (DDT) weight was used as the denominator in comparison of GAG yields.

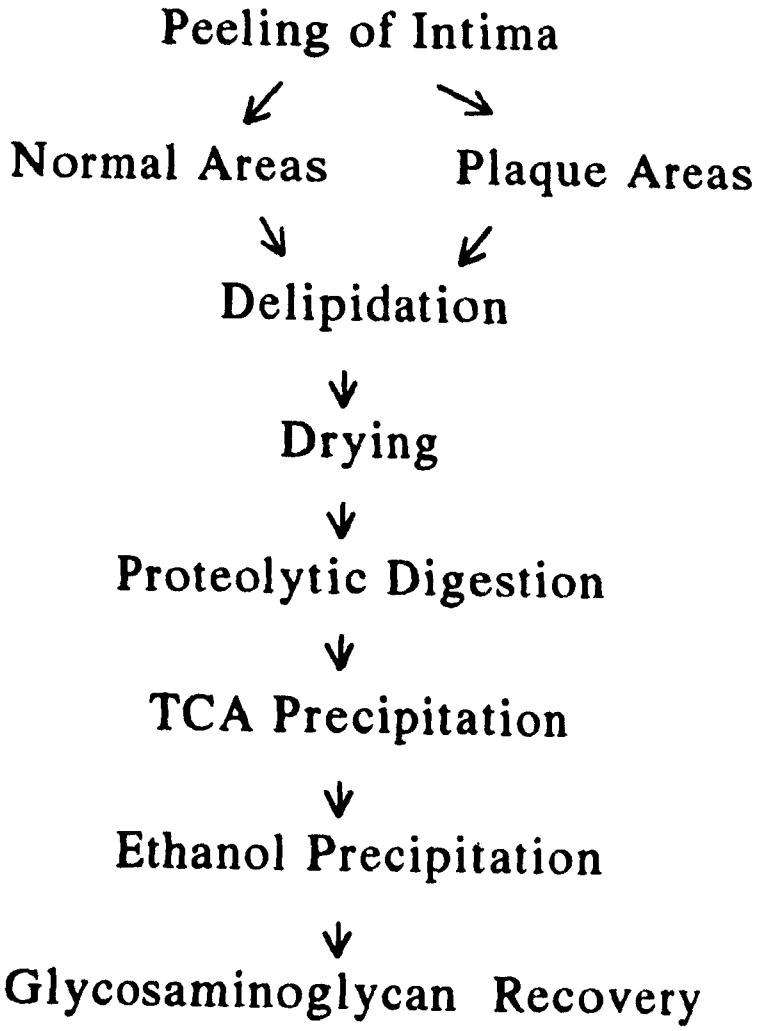


Figure 6

Steps involved in isolation of GAGs.

2.3.2 Proteolytic Digestion:

To liberate GAGs from the tissues proteolytic digestion by papain was performed next. Papain has a low specificity for the type of peptide bound and digests proteins to almost the amino acid level. Papain has been widely used for the liberation of GAG chains from the core and other matrix proteins. Less commonly treatment with alkali is used for this purpose, usually in the presence of borohydride. Papain has been found to be active in a wide range of pH (4-9) and is relatively resistant to heat (60-65 °C is commonly used). It is activated by sulfhydryl groups and inhibited by metal ions therefore buffer containing cystein and EDTA is used with papain (Arnon, 1970). It has been suggested that the ratio of papain and DDT weight should be 1:200 or higher. We used a ratio of 1:100 to ensure complete digestion as diabetic tissues have been reported to be somewhat resistant to proteolytic digestion.

For digestion with papain, a buffer containing 0.1 M Na acetate, 10 mmol cystein, 10 mmol EDTA and pH adjusted to 6.5 with 1N NaOH was used. It was made x2 strength. Fifty mg of tissues were placed in 1.5 ml tubes and enzyme (0.5 mg = 50 μl), buffer and distilled water were added to make a total of 0.8 ml of solution. Incubation was performed at 60 °C in a shaking water bath for about 20 hours. An extra 250 μg papain in 25 μl distilled water plus 25 μl buffer was added during the incubation. All tissues were digested into very fine particles. Following incubation tubes were centrifuged for 10 minutes and supernatants were collected in separate tubes. Pellets were washed with 100 μl of water once which was added to the supernatant.

2.3.3 Trichloroacetic Precipitation:

Precipitation with trichloroacetic acid (TCA) was performed to remove partially or undigested proteins and nucleic acid from the samples. Collected supernatant and washings were mixed with 60 % TCA to make a final concentration of 5%. Tubes were left at 4 °C for a few hours which resulted in the development of whitish precipitates. Centrifugation was performed for 5 minutes and Pellets were washed once with 0.1 ml water. Supernatants were collected in 15 ml centrifuge tubes along with washings.

2.3.4 Ethanol Precipitation:

GAGs were isolated from supernatants of TCA precipitation by precipitation with 67 % ethanol. Four volumes of ethanol and 1 volume of saturated solution of sodium acetate were added to the tubes. Samples were kept at 4 °C overnight. Precipitates were pelleted by centrifugation for 10 minutes. Pellets were washed with pure ethanol once. After removing ethanol, precipitates were dissolved in distilled water and the whole process of precipitation was repeated. Finally, precipitates containing purified GAGs were allowed to dry and dissolved in 200 μl of distilled water.

2.3.5 Losses:

To ascertain that extraction of GAGs was complete and there were no losses in tissues, TCA precipitates or during the extraction following experiments were performed.

Tissues: In some cases digested tissues were further digested with papain to find out if GAGs were still incorporated. Tissues were reincubated with 250 μg of papain in a total of 0.4 ml buffer. Incubation was performed under conditions already described for 24 hours. Digested materials were further processed similarly to the original ones.

TCA precipitate: Since incompletely digested PGs may be precipitated by TCA, we tried to extract GAGs from TCA precipitates. This was performed by dissolving the TCA precipitates in 0.1 M NaOH, neutralizing the pH with 0.1 M HCl and then digesting the solubilized material with papain. The rest of the steps for GAG isolation have already been described.

Isolation Process: Experiments were also performed to detect losses during the whole isolation process other than those due to incomplete digestion. For this purpose, a known quantity of standard GAGs was aliquoted and the process of GAG isolation was performed starting from the papain digestion. Losses were calculated by comparing GAG weight before and after the process.

2.4 Characterization of GAGs:

Isolated GACs were assayed by alcian blue method and their distribution was determined by densitometry following electrophoretic separation.

2.4.1 Measurement of Total GAG:

GAGs were measured by a method involving complex formation with alcian blue on cellulose acetate membrane (Hronowsky et al., 1979). This dye has a high affinity for GAGs and is more or less specific for them. This method has been found to be fairly simple, reliable, specific and sensitive. The membrane was marked by a pen to make 1 cm x 0.8 cm rectangles. Samples (4.5 μl) were spotted inside the marked areas. After drying, strips were cut and stained in a solution of 50 % ethanol containing 0.2 % alcian blue 8 GX, 0.03 M $MgCl_2$, and 0.1 % glacial acetic acid. Half an hour later strips were removed and destained in a similar solution but lacking alcian blue. Destaining was

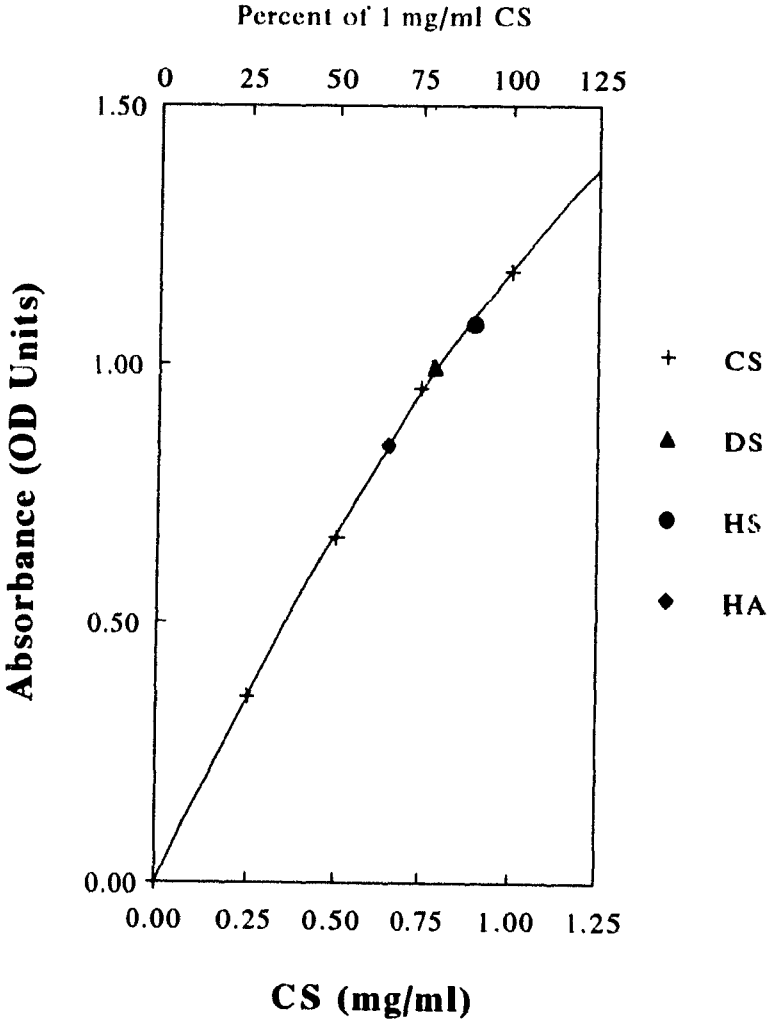
performed for a total of 30 minutes involving 3 changes of solution and under gentle shaking. Later, strips were dried and divided into pieces (1 cm x 0.8 cm). These were placed in test tubes and 1 ml of dimethyl sulfoxide containing 0.5 % concentrated sulfuric acid was added to each tube. Tubes were shaken at various intervals using a vortex. Membranes were completely dissolved within a few minutes. Twenty minutes later reading was performed in a spectrophotometer at 678 nm. Absorbance was found to be stable for more than 30 minutes. A blank containing spots of distilled water and standards containing 0.2-1 mg/ml solution of CS-6 (shark cartilage) were included in every run. CS-6 was used as it is the major GAG in the intima. All measurements were done in triplicates. Duplicate determinations were performed in some cases to assess the precision of results. These values are tabulated in the results section.

Standard curves were made as in fig. 7 and it can be seen that they were not linear. This was not a problem and results were reproducible. Data in fig. 7 is fitted by second order polynomial curve. Comparisons were made between standard GAGs (DS, porcine kidney; HS bovine kidney; HA, human umbilical cord) and CS-6 to find out their relative binding capacities for alcian blue. This was done for the final adjustment of GAG weight. For this purpose absorbance for 1 mg/ml solutions of different GAGs were compared with CS-6 by alcian blue assay (fig. 7). This method was performed to obviate nonlinearity of the curve.

Figure 7

A typical standard curve for GAG assay with CS-6.

Alcian blue binding capacity of other standards was determined by comparing values for 1 mg/ml solutions.



2.4.2 Distribution of GAGs:

For this purpose electrophoresis was performed on cellulose acetate membrane followed by staining with alcian blue and densitometry.

2.4.2.1 Electrophoresis:

Initially a calcium acetate buffer was tested for electrophoresis but later cadmium acetate was found to be better for this purpose. For calcium acetate we repeatedly tried either a 0.3 M or a 0.2 M solution of pH 7.0 or 8.0 for 3 hours at a current of 8 mamp/strip. This buffer failed to resolve HS and HA as distinct bands. With cadmium acetate buffer a good separation of these two GAGs was obtained (fig. 8a). Both types of buffers failed to resolve the two types of CSs (CS-6 & CS-4). In both cases standards of DS showed a distinct band but resolution of DS from the CS band was not complete in the samples. To overcome this problem every sample was digested with chondroitinase AC which resulted in digestion of CS leaving behind a distinct DS band (fig. 8b).

The bands were further identified with the help of specific glycolytic enzymes. These enzymes have the following specificities (Heinegard et al., 1987):

Chondroitinase AC (*Arthrobacter aurescens*) : CS & HA

Chondroitinase ABC (*Proteus Vulgaris*) : DS, CS & HA(low activity)

Hyaluronidase (*Streptomyces hyaluroticus*) : HA only

HS was digested by treatment with nitrous acid. Following methodology was used for digestion of different GAGs (Heinegard et al., 1987).

Chondroitinase AC and ABC: Small quantities of samples were placed in Eppendorf tubes and incubated with enzyme in a buffer containing 0.1 M tris pH 8.0 at 37 °C for 3-4

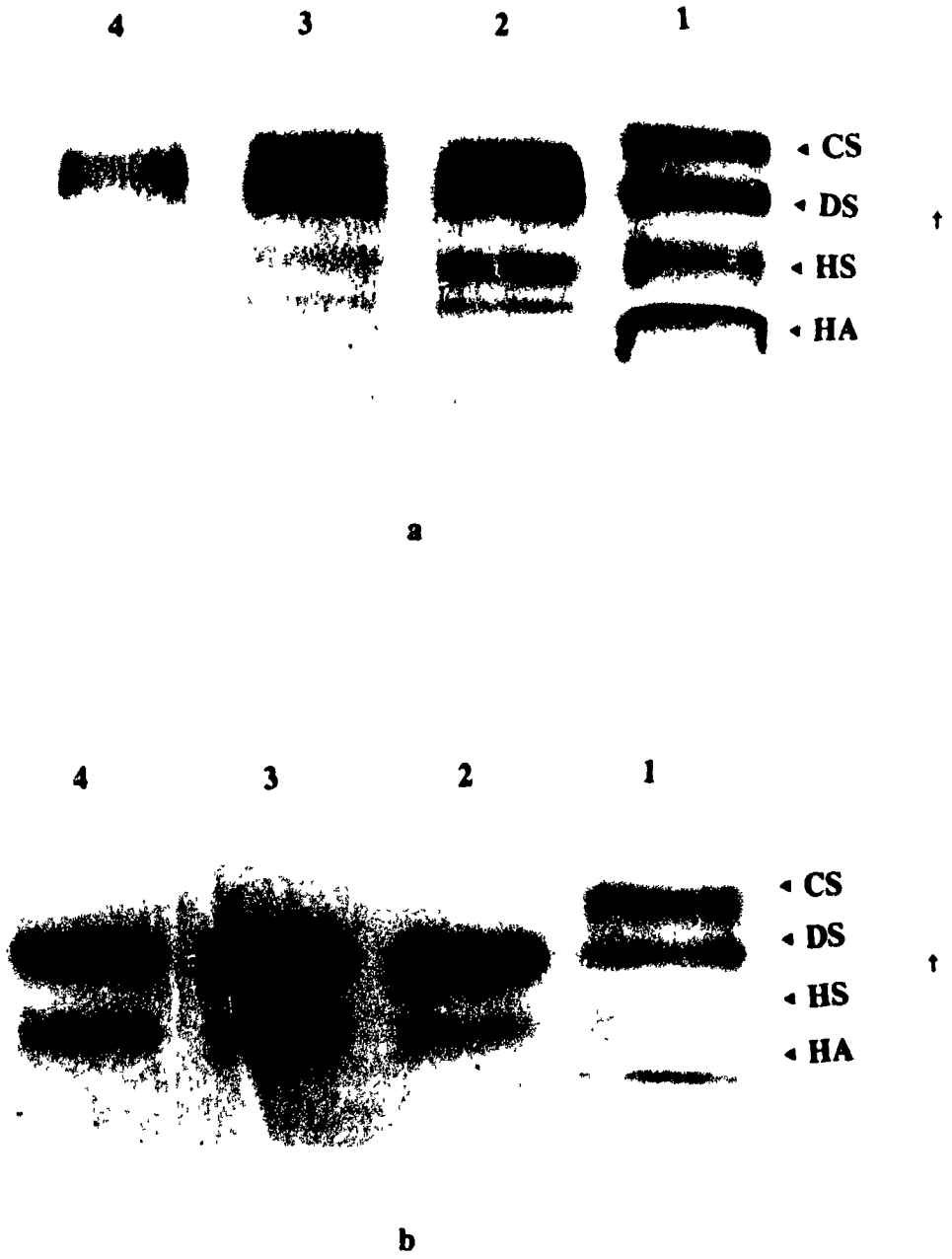


Figure 8 (a, b)

Electrophoretograms of standards (lane 1)

and samples (lanes 2-4) before (a) and after (b) digestion with chondroitinase AC. ,

Arrow depicts the direction of migration

hours in a shaking water bath. The ratio between quantity of total GAGs (mg) and enzyme (units) was 1:2 for chondroitinase AC and 1:1 for ABC. A higher amount of ACase was used because it is inhibited by DS (Yamagata et al., 1968).

Fungal HAase: Samples were incubated with enzyme in a 0.1 M tris buffer pH 7.4 at 37 °C for 3-4 hours.

Nitrous acid treatment: 1 M nitrous acid was freshly prepared by combining 1 M HCl and 1 M sodium nitrite. Nine parts of this solution were incubated with 1 part of sample at 37°C for 48 hours.

Treatment with these enzymes abolished specific bands. Once the bands have been identified with confidence and buffer has been chosen the following methodology was used routinely. For every sample digestion with chondroitin ACase was performed before the electrophoresis. This procedure separated the band of DS which then could be compared with the HS band. Electrophoresis was performed on the Gelman semi-micro electrophoresis system. Cellulose strips (Sepraphore-I., Gelman) were soaked in the electrophoresis buffer for a few minutes, gently blotted and loaded on the bridge. Samples and standard were pipetted to the wells on the application block and then applied on the membrane with a special applicator. A fraction of a μl was applied with each application. Electrophoresis was then performed in 0.3 M cadmium acetate, pH 4.1 at a current of 4.5 mamp/strip for 1 hour. Strips were stained with alcian blue at the end of the run.

For staining of bands, initially a solution similar to that used in GAG assay was tried, but an immediate precipitation of dye was observed. This was probably due to cadmium

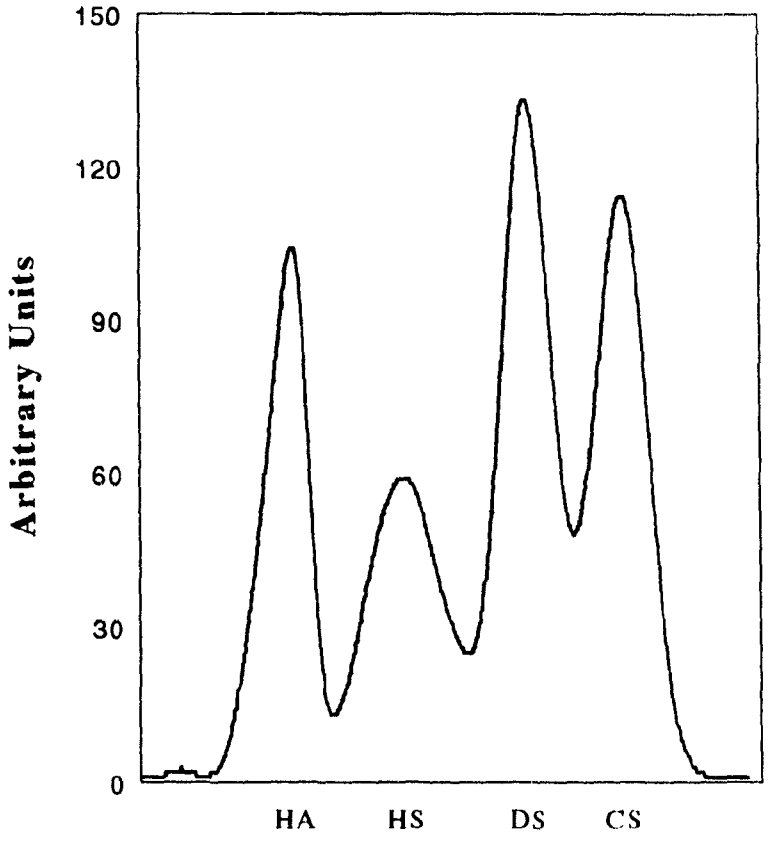
acetate in the electrophoresis buffer. To solve this problem, we tried lower concentrations of ethanol as some investigators have used 10 % ethanol solution for GAG assay and band staining for other reasons. Finally a solution containing 10 % ethanol was found suitable for our use. Other components were similar to that used in GAG assay. The only drawback of using 10 % ethanol in place of 50 % was a little higher background. Staining was performed for 30 minutes and was followed by destaining in a similar solution lacking alcian blue. Destaining was performed for 45 minutes under gentle shaking and solutions were changed twice. Following destaining, strips were allowed to dry and placed in glycerol to make them transparent for densitometry. Samples were run in duplicates in a few cases to assess reproducibility.

2.4.2.2 Densitometry:

For densitometry, GS 300 densitometer (Hoefer) and its software was used. Each band was scanned in triplicate and the curves obtained were saved in the computer. The software was used to calculate the area under the curves corresponding to the optical density of the bands. The values were then mathematically converted to percentage or proportion of different GAGs in a sample. ACase digested samples were used to compare the relative proportion of DS compared with HS. This value was then adjusted in the proportion occupied by the CS+DS band. Fig. 9 (a,b,c) shows examples of densitometry curves obtained for samples (before and after chondroitinase AC digestion) and a mixture of standard GAGs.

Figure 9 (a, b, c)

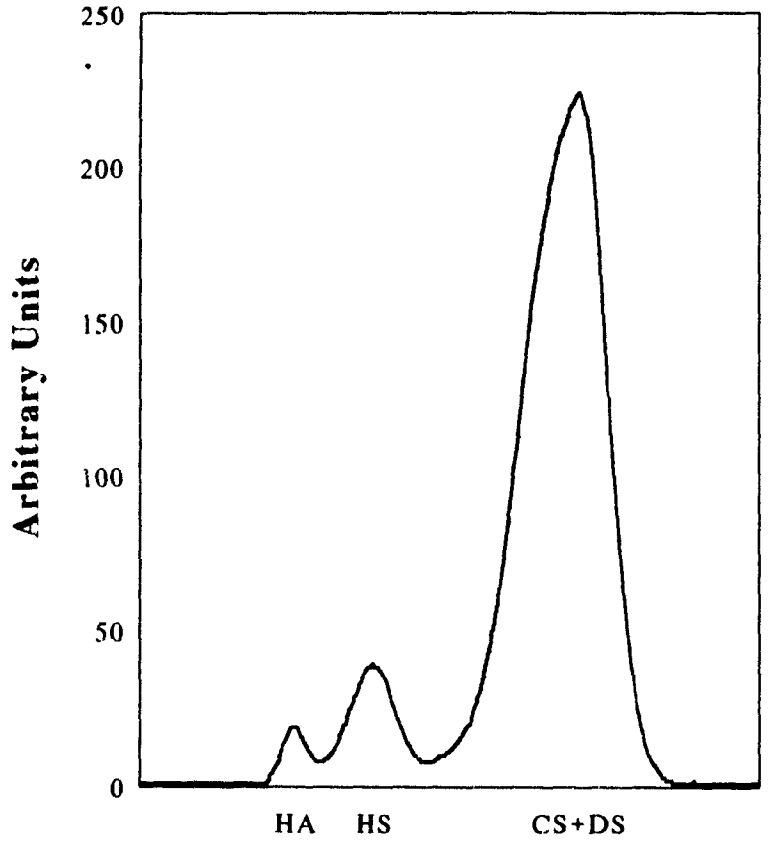
Densitometric scans of a mixture of standard GAGs (a) and a sample before (b) and after (c) chonroitinase AC digestion.



a

Figure 9 (a, b, c)

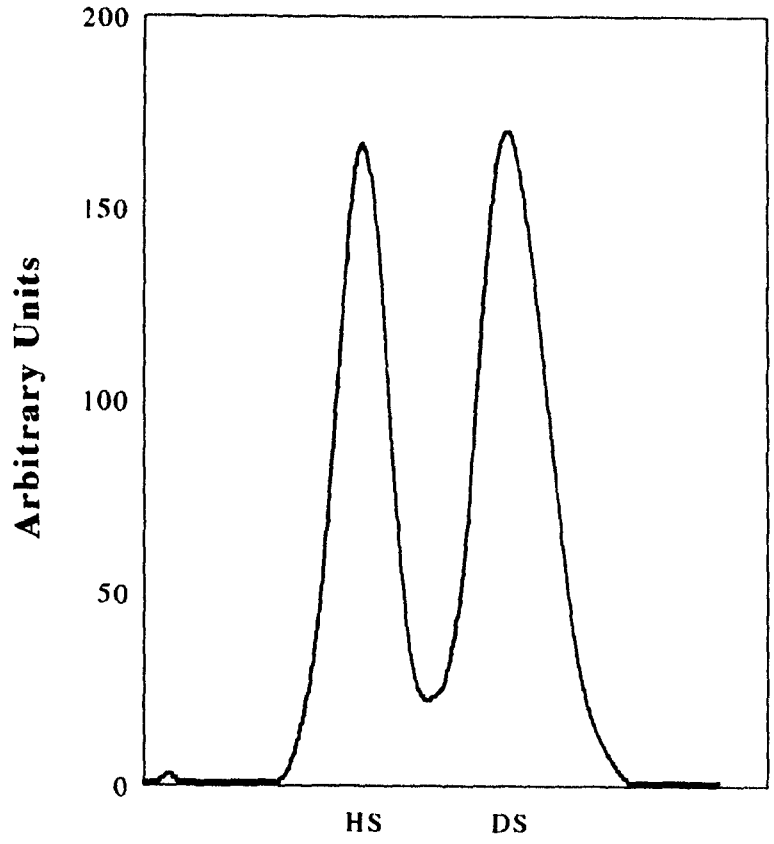
Densitometric scans of a mixture of standard GAGs (a) and a sample before (b) and after (c) chonroitinase AC digestion.



b

Figure 9 (a, b, c)

Densitometric scans of a mixture of standard GAGs (a) and a sample before (b) and after (c) chonrotinase AC digestion.



c

Different GAGs have different strengths of staining by alcian blue as described in the GAG assay. This was separately determined for this part of the GAG characterization. This was performed by running mixtures of equal quantities of standard GAGs (described earlier) and finding out their relative staining capacity. Fig. 10 shows the result of such an experiment where the area under the curve from densitometry is plotted against the number of applications of GAG on membrane. Linear curves were made, their slopes (b) were calculated mathematically (by $Y=a+bX$) and compared with that of CS. Distribution of GAGs in samples was adjusted according to their strength of staining.

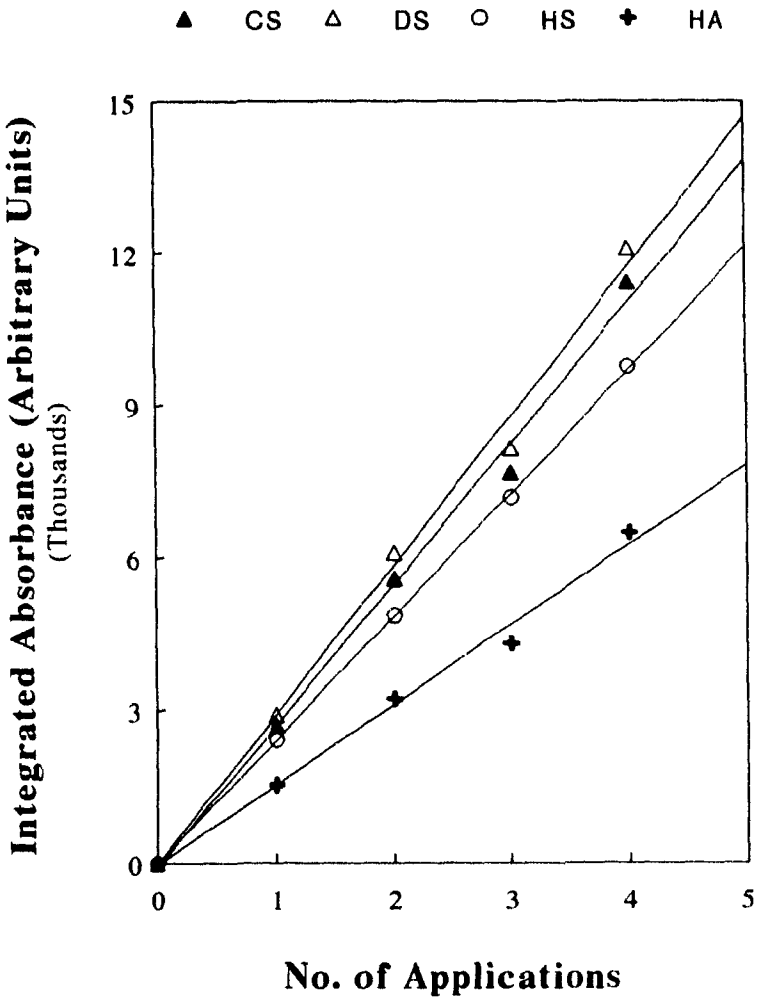
2.5 Statistical Analyses:

For this purpose a software (Systat 5.01) was used. Means and standard deviations (SD) of sample groups were calculated. Data from different groups were compared by independent (unpaired) student t test and the significance (p) of difference determined.

Figure 10

Standard curves for densitometric readings of standard GAGs.

Slopes were compared with CS-6 slope to estimate alcian blue binding capacity



3. RESULTS

Results include a comparison of the mean age of the two groups. It will be followed by assessment of losses during the procedure, alcian blue binding capacity and precision of determinations. Finally values from each sample will be presented and their means will be compared statistically.

3.1 Age:

Age of the two groups is expressed as mean $\pm$SD.

n=10 for nondiabetic and n=6 for diabetics

Nondiabetic group	=	69.5 ± 7.0
Diabetic group	=	71.2 ± 11.5
Significance	=	0.72

This comparison indicates that there was no significant difference between the mean age of nondiabetic and diabetic groups. A difference in age could result in differences in GAG quantity and distribution due to age factor alone.

3.2 Losses:

Losses were assessed in both tissues and TCA precipitates and during isolation process separately.

a. Losses in tissues: Six experiments were performed. Values are expressed as the percentage of total GAG in the sample.

i.	1.4 %
ii.	1.5 %
iii.	2.0 %
iv.	1.3 %
v.	1.5 %
vi.	1.1 %
Mean	1.47 %

b. Losses in TCA precipitate: Six experiments were performed. Values are expressed as the percentage of total GAG in the sample.

i.	1.5 %
ii.	1.8 %
iii.	2.0 %
iv.	1.4 %
v.	1.7 %
vi.	2.3 %
Mean	1.78 %

c. Losses during isolation of standard GAG. Six experiments were performed. Results are expressed as percent recovery.

i. 102 %

ii. 97 %

iii. 105 %

iv. 98 %

v. 101 %

vi. 95 %

Mean 99.7 %

These data indicate that there were very little losses during the whole extraction process. Only a little GAG was lost in tissues and TCA precipitates while losses during different stages of isolation were undetectable. Since losses were negligible no correction were made for them.

3.3 Alcian Blue Binding Capacity:

This was determined for standard GAGs for the purpose of correction of total GAG values and the values for GAG distribution.

a. For GAG assay: This was performed by comparing values for 1 mg/ml solution of standard GAGs with CS-6. Values are expressed as percentage of CS-6 binding capacity.

HA = 65 %

HS = 90 %

DS = 79 %

b. For GAG distribution: This was performed by making linear standard curves for different GAGs and comparing values of their slope with that of CS-6. Values are expressed as percentage of CS-6 binding capacity.

HA = 57 %

HS = 76 %

DS = 108 %

3.4 Precision:

Precision of values was determined for both total GAG and GAG distribution by running assays in duplicates.

a. For GAG assay: Eight experiments were performed. Values are expressed as mg GAG/100 mg DDT and are followed by values showing percent difference from their means.

	Values		% Difference (±)
	.....		.....
i.	1.12	1.07	2.28 %
ii.	1.20	1.15	2.13 %
iii.	1.60	1.50	3.22 %
iv.	1.80	1.78	0.56 %
v.	1.95	1.86	2.36 %
vi.	0.88	0.92	2.22 %
vii.	1.24	1.39	5.70 %
viii.	1.82	1.72	2.82 %

			Mean 2.66 %

These results show that on the average the duplicate results of GAG assay are only about 2.66 % different from their means. This shows that our readings of total GAGs are fairly precise and reliable.

b. For GAG distribution: Following are the duplicate values for GAG distribution after separate electrophoretic runs and densitometric evaluations. All values are percent compositions.

i.	3.6	11.6	84.8
	4.1	11.5	84.4
ii.	2.8	9.1	88.1
	2.7	9.8	87.4
iii.	4.6	13.1	82.8
	4.0	12.8	83.1
iv.	3.8	10.3	85.9
	4.8	10.7	84.4
v.	3.3	15.0	81.6
	2.5	16.0	81.4
vi.	20.6	79.4	
	21.7	78.3	

These data provide evidence that duplicate determinations of GAG distribution were only slightly different from each other and our results are quite precise.

3.5 Sample Values:

Raw data obtained from GAG assay and densitometry was treated mathematically to obtain true values for total and individual GAGs for each sample. Adjustments were made to accommodate different binding capacity of different GAGs. After adjustment we obtained about 7-8 % greater values for total GAG.

Data obtained from 10 nondiabetic samples and 6 diabetic samples are presented in tables 8 to 11. The values for total GAG are expressed as mg/100 mg of DDT while those for individual GAGs as percent of total GAG. It is evident from the tables that total GAG is decreased markedly in plaques. For this reason to get a true picture of GAG distribution, data are expressed as percent of total GAG value for each sample. We also observed that there was a trend of decreasing HS and increasing DS in both atherosclerotic plaques and in diabetes and thus it would be interesting to express results in the form of a ratio of HS to DS.

Table 8. *GAG Values from Normal Areas of Nondiabetic Group.*

Sample No.	Total GAG mg/100mg DDT	CS (%)	HS (%)	DS (%)	HA (%)	HS:DS
1	1.90	59.66	22.84	10.45	7.05	2.18
2	1.67	55.25	22.97	15.75	6.20	1.47
3	2.56	61.92	19.35	12 09	6 64	1.60
4	1.64	64.90	19.89	10 91	4 30	1 82
5	1.91	65.34	15.77	13.15	5.74	1.20
6	2.01	62.51	19.69	11 92	5.88	1.65
7	1.77	57.84	23.20	13.67	5 28	1 70
8	2.06	71.77	14.05	9.49	4.68	1.48
9	2.14	65.67	19.21	10 24	4.87	1 88
10	2.40	60.50	17.90	12.02	9.58	1.49
Mean	2.01	62.53	19.49	11 95	6.02	1 65
SD	0.30	4.67	3.04	1.82	1.52	0.27

Table 9. *GAG Values from Plaques of Nondiabetic Group.*

Sample No	Total GAG mg/100mg DDT	CS (%)	HS (%)	DS (%)	HA (%)	HS:DS
1	1.69	56.40	18.70	19.25	5.64	0.97
2	1.53	56 19	15.74	23 63	4.44	0.67
3	2.15	62.01	16.24	15.20	6.55	1.07
4	1.27	62.71	11.64	19.34	6.30	0.60
5	1.20	55.97	14.89	19.08	10 05	0.78
6	1.72	63.62	11.99	18.83	5 55	0.63
7	1.50	70.54	12.67	12.53	4.25	1.01
8	1.41	63.64	11.98	20 58	3.80	0.58
9	1.48	61.85	18.62	13.91	5.62	1.33
10	1.22	54.22	12.74	19.54	13.49	0.65
Mean	1.52	60.72	14.52	18.19	6.57	0.83
SD	0.28	4.99	2.72	3.33	2.99	0.25

Table 10. *GAG Values from Normal Areas of Diabetic Group.*

Sample No.	Total GAG mg/100mg DDT	CS (%)	HS (%)	DS (%)	HA (%)	HS:DS
1	1.31	57.64	16.18	16.36	9.81	0.99
2	2.03	55.68	18.39	18.19	7.74	1.01
3	1.20	55.35	17.36	17.90	9.39	0.97
4	2.47	67.04	16.38	10.26	6.31	1.60
5	1.55	56.05	15.85	21.56	6.54	0.74
6	2.01	54.73	18.78	18.94	7.53	0.99
Mean	1.76	57.75	17.16	17.20	7.89	1.05
SD	0.49	4.66	1.22	3.80	1.44	0.29

Table 11. *GAG Values from Plaques of Diabetic Group.*

Sample No.	Total GAG mg/100mg DDT	CS (%)	HS (%)	DS (%)	HA (%)	HS:DS
1	1.62	57.80	12.04	23.10	7.06	0.52
2	1.53	57.18	12.30	22.96	7.55	0.54
3	2.02	67.73	11.43	15.75	5.08	0.66
4	1.19	60.78	10.96	19.65	8.60	1.10
5	1.53	53.11	13.30	26.35	7.23	0.50
6	1.94	51.63	11.72	30.85	5.80	0.38
Mean	1.64	58.04	11.96	23.11	6.89	0.62
SD	0.30	5.79	0.80	5.23	1.26	0.25

3.6 Statistical Comparisons:

Comparisons were made between selected groups by student t test and consisted of values for total GAG, proportion of each GAG, and the ratio of HS to DS. We were specifically interested in comparing, at first, data from normal areas of nondiabetics with those from plaque areas of nondiabetics. This comparison will mark the significant changes in GAGs in atherosclerotic plaques of nondiabetics and is expected to reflect changes in atherosclerotic lesions in general.

The other comparison will be between normal areas from diabetics and the same from nondiabetics. This will be helpful in distinguishing significant changes found specifically in diabetic intima not involved in lesions. This should reflect GAG changes due to diabetes alone.

These two comparisons should be important in finding if GAGs serve as a link between atherosclerosis and diabetes.

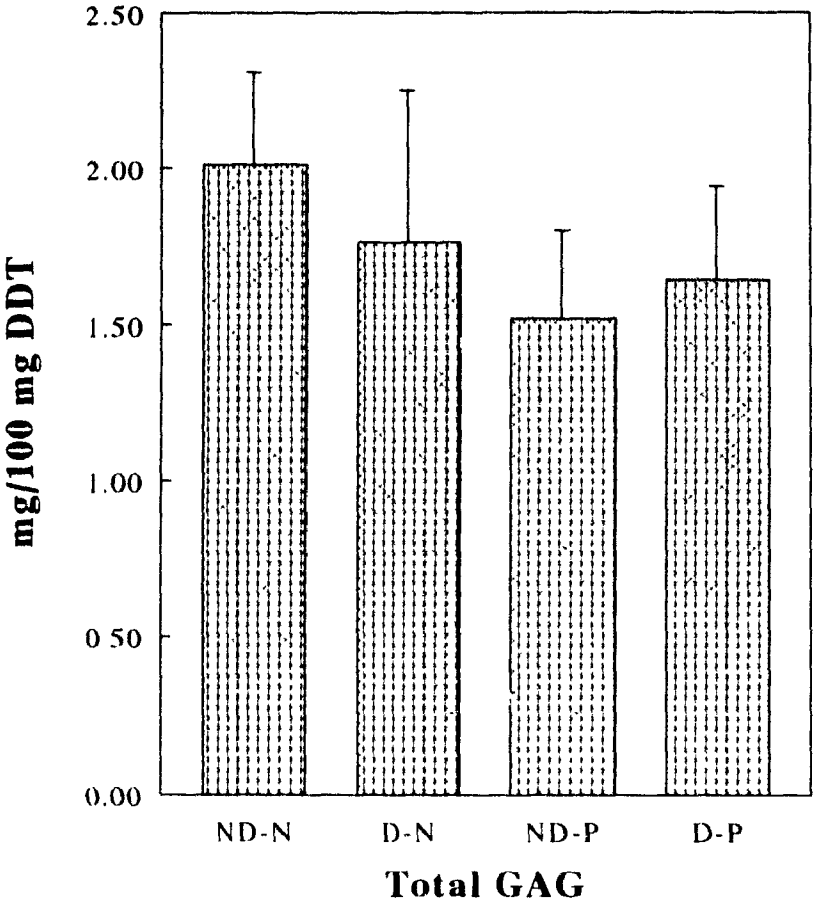
To give a visual impression of these comparisons they are expressed in graphic form in figs. 11 to 16. Results from all 4 groups are depicted in each graph to provide a way of comparing all the groups simultaneously for a given variable. Significance of selected comparisons are given with each graph.

Figure 11

Comparisons of total GAG values.

ND-N Vs ND-P p= 0.0015

ND-N Vs D-N p= 0.2



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

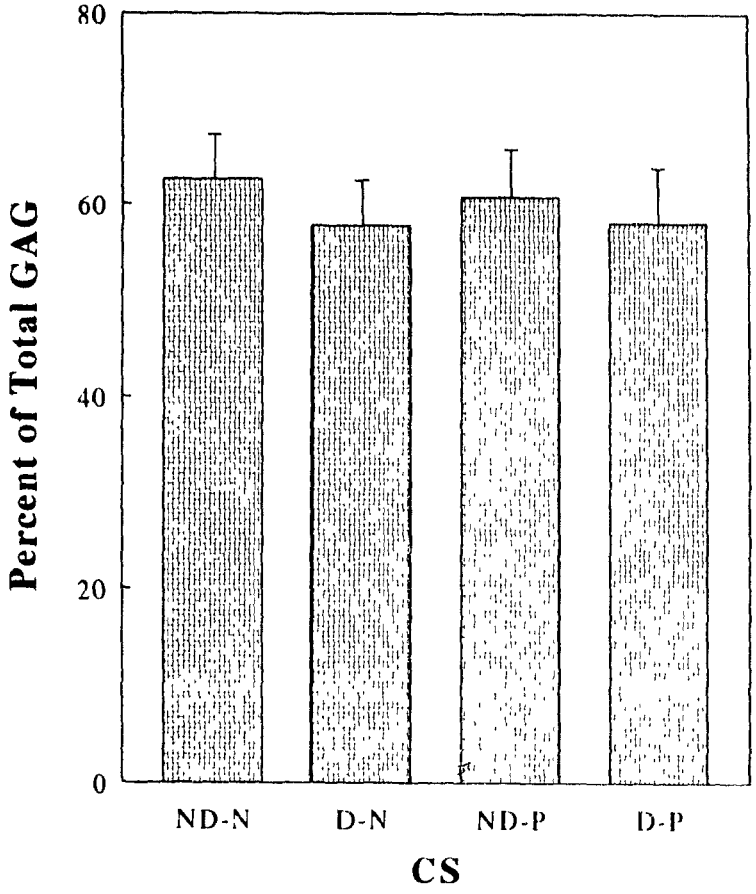
D-P = Diabetic Plaque

Figure 12

Comparisons of CS values.

ND-N Vs ND-P p= 0.41

ND-N Vs D-N p= 0.07



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

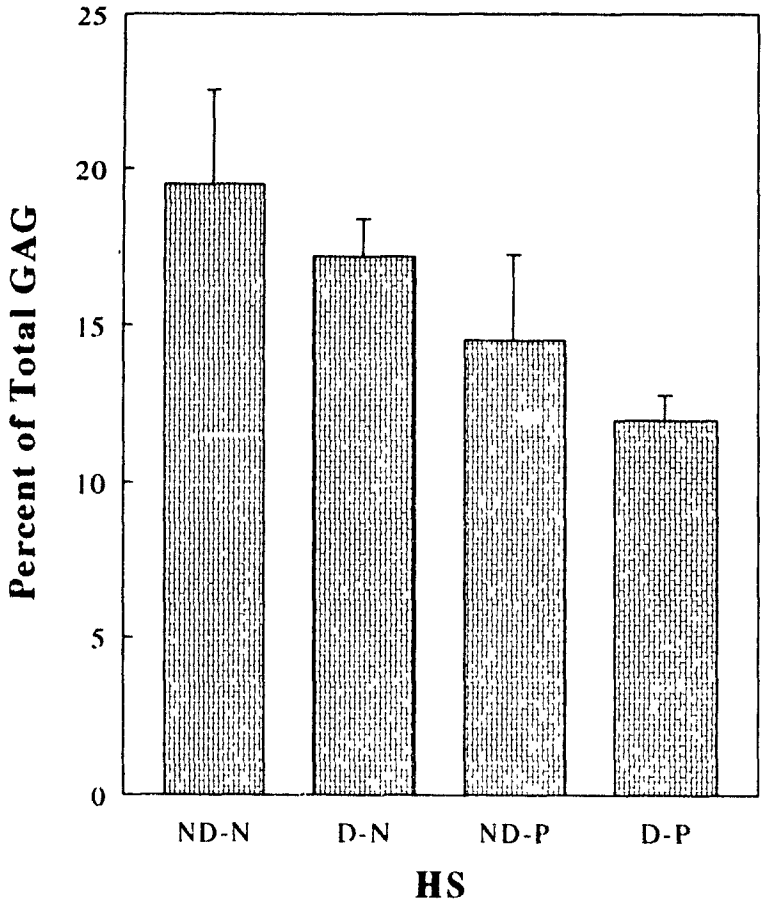
D-P = Diabetic Plaque

Figure 13

Comparisons of HS values.

ND-N Vs ND-P p= 0.001

ND-N Vs D-N p= 0.1



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

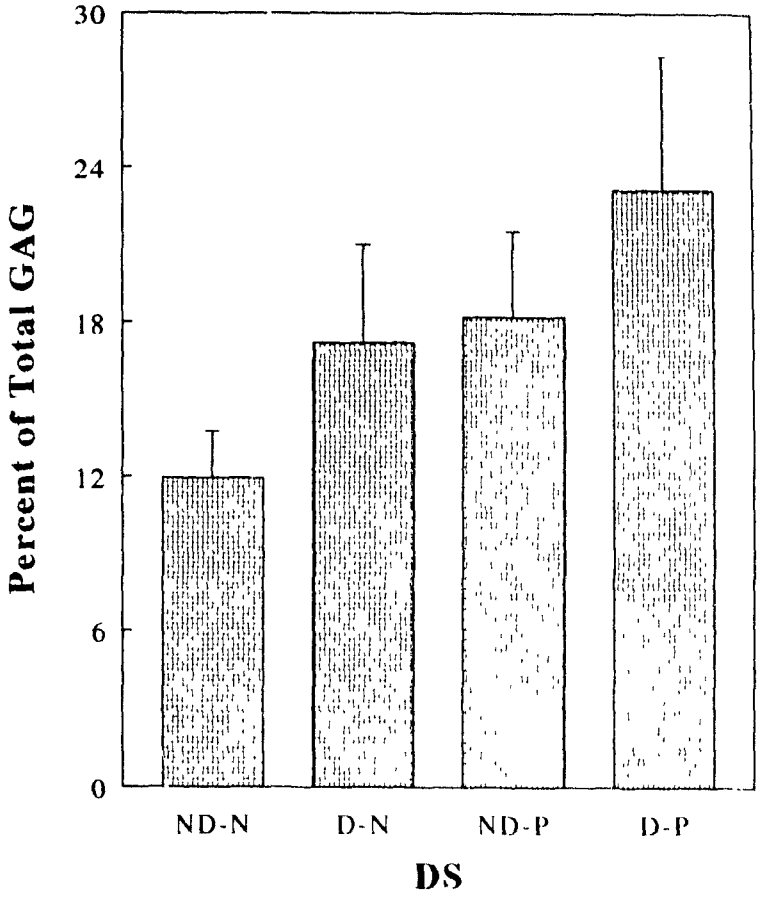
D-P = Diabetic Plaque

Figure 14

Comparisons of DS values.

ND-N Vs ND-P p= 0.00006

ND-N Vs D-N p= 0.002



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

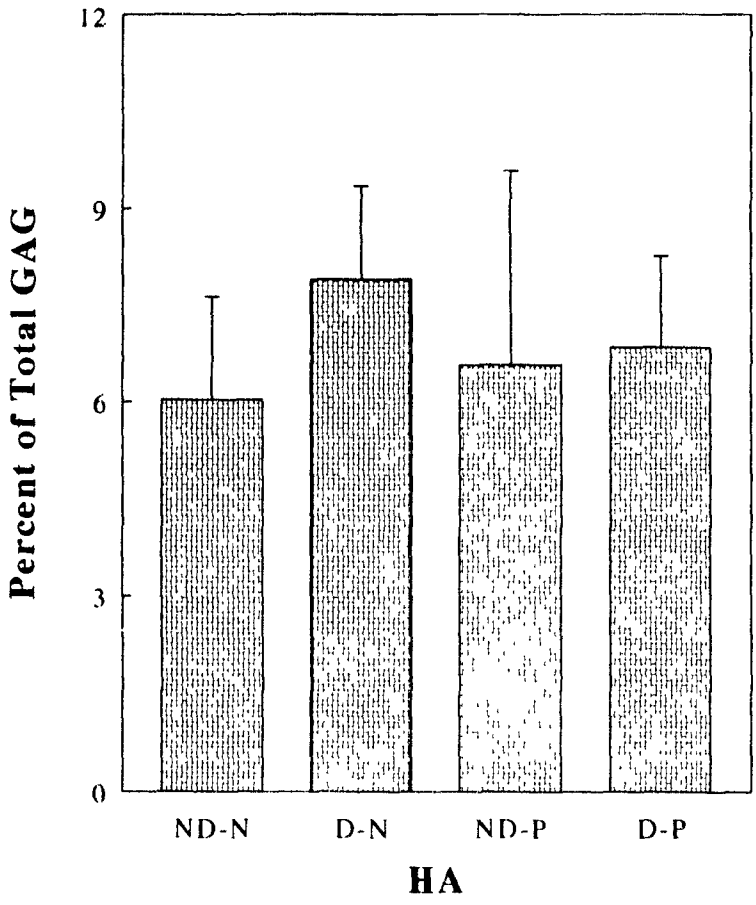
D-P = Diabetic Plaque

Figure 15

Comparisons of HA values.

ND-N Vs ND-P p= 0.6

ND-N Vs D-N p= 0.03



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

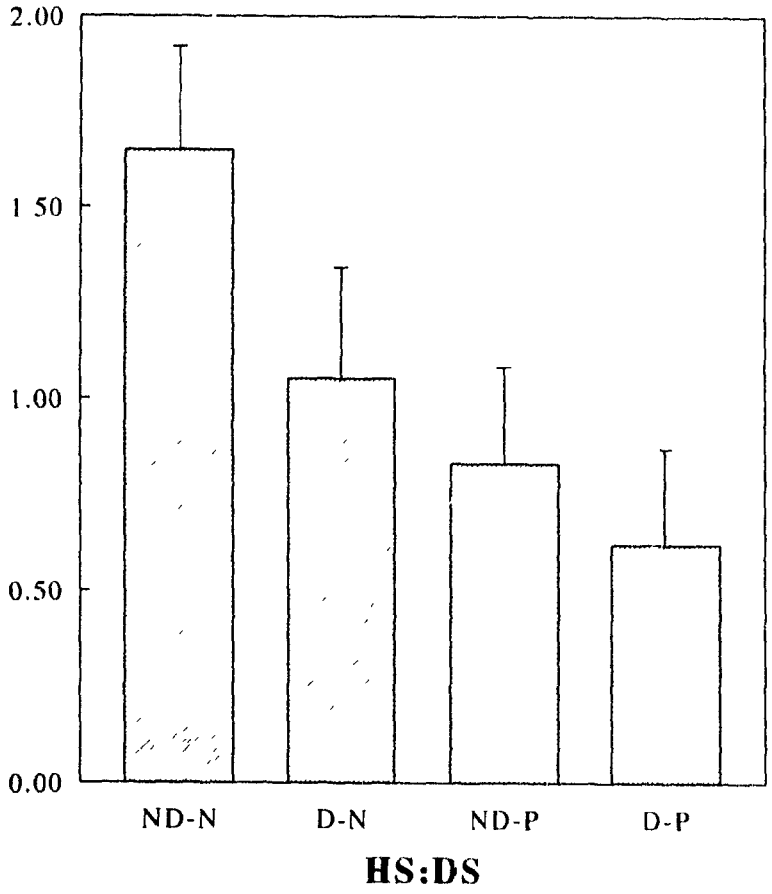
D-P = Diabetic Plaque

Figure 16

Comparisons of HS:DS values.

ND-N Vs ND-P p= 0.000002

ND-N Vs D-N p= 0.0009



ND-N = Nondiabetic normal area

D-N = Diabetic normal area

ND-P = Nondiabetic plaque

D-P = Diabetic Plaque

The results of these comparisons indicate that there is a significant decrease in total GAG along with a highly significant change in GAG distribution in atherosclerotic plaques. There is a highly significant decrease in the proportion of HS, an increase in the proportion of DS and a decrease in the ratio of HS to DS.

Comparison of results from normal area of diabetics with that of nondiabetics exhibits no significant change in total GAG but a significant change in GAG distribution. There is a non-significant decrease in HS but a significant increase in DS and a significant decrease in HS:DS. It is evident that changes in GAG distribution are similar to those found in plaques of nondiabetics but are of lesser magnitude. Data from plaque areas of diabetics clearly show similar changes of a greater extent.

For comparison of normal and atherosclerotic areas we utilized data only from nondiabetics. To get a better view of the situation, data from nondiabetic and diabetic groups can be pooled. Table 12 displays the results expressed in pooled form. Comparisons between data from normal areas and plaques were estimated with student's t test and are shown in the same table. It is evident that there is a significant decrease in total GAGs and significant changes in GAG distribution (a decrease in HS proportion, an increase in DS proportion, and a decrease in HS:DS). These changes are exactly the same as when the nondiabetic group was employed alone. This observation confirms our findings of changes in GAGs found in atherosclerosis.

Table 12. *Comparison of Pooled (Nondiabetic + Diabetic) Data.*

Variable	Normal	Plaque	p
Total*	1.92 ± 0.38	1.56 ± 0.29	0.007
CS (%)	60.74 ± 5.10	59.71 ± 5.28	0.6
HS (%)	18.61 ± 2.72	13.56 ± 2.51	0.000006
DS (%)	13.92 ± 3.70	20.04 ± 4.67	0.0003
HA (%)	6.72 ± 1.72	6.69 ± 2.43	1.0
HS:DS	1.42 ± 0.40	0.75 ± 0.27	0.000004

** = mg/100mg DDT*

All values are mean ± SD; n=16 for each group.

Considering the findings in atherosclerotic lesions and in diabetes, we can conclude that changes in GAG distribution were present in diabetic intima before the appearance of atherosclerotic lesions. As these changes showed the same pattern as observed in atherosclerotic lesions they might have implications for atherogenesis. The most significant change observed in both atherosclerosis and diabetes was a decrease in the ratio of HS to DS.

4. DISCUSSION

The following discussion is composed of critical and objective views on the methodology used and the results obtained. This will be followed by rationalization and consideration of relevance of the findings.

4.1 Methodology:

4.1.1 subjects:

Subjects in both diabetic and nondiabetic groups were random samples from the general population. Selection was only performed to exclude samples from younger subjects or when sufficient normal or lesion areas were not available for the study. It was important to have samples from the same age group as some studies have shown a change in GAG concentration and distribution with age (Smith, 1974; Murata, 1985). In our study the difference between mean age of the two sample groups was found to be non-significant by the student's t test.

As no report is available on the effect of gender on GAG content, no selection was made on that basis. Similarly, the presence of concomitant diseases, though undesirable, could not be avoided in our study. One way to avoid this could be to study juvenile diabetics versus a younger normal population of the same age group. Not only would it have been difficult to obtain samples of interest but we could not have obtained any data from atherosclerotic plaques. These limitations are usually present when using human samples. Results in this scenario, however, represent a true picture of human findings. Although any study in experimental animals provides undoubtedly a better chance of controlling

different variables, the findings need to be confirmed in humans.

4.1.2 Materials:

We used the intimal layer of the thoracic part of the aorta as the source of tissue. Histological examination confirmed that the material we used contain mainly intima. This was important because medial GAG composition has been found to be different from intima and as media is many times thicker than intima the presence of media may obscure the results. Distinction between normal and lesion areas was based on gross examination. It was very easy to separate plaques. Thin and transparent areas were considered normal and grossly doubtful areas were excluded. Microdissection was not performed to separate early lesions. Histological examination of some samples confirmed the gross findings. We can confidently say that areas we used as normal were normal for the age group we studied and were definitely free of atherosclerotic plaques.

4.1.3 GAG Isolation:

The methods we used for isolation and characterization of GAGs are the standard methods and have previously been used by different investigators for this purpose. For isolation of GAGs different authors report a few minor variations but the overall methodology involves delipidation, proteolytic digestion, and precipitation of GAGs. The method used for delipidation is the most common one and has become a standard procedure. For proteolytic digestion alkali with or without borohydride or papain with or without pronase are commonly used. Papain alone has been found to be very effective for this purpose. For precipitation of GAGs we used ethanolic precipitation on TCA supernatant. GAGs are insoluble in ethanol provided they are in sufficient quantity and are accompanied by

a salt, e.g., sodium acetate. Another method employed for this purpose is a more selective precipitation of GAGs from proteolytic digest or TCA supernatant with cetylpyridinium chloride. Many investigators include a step of dialysis after proteolytic digestion but we tried to avoid this as it might have resulted in loss of some GAGs. Since GAGs exist as PG, some investigators have attempted to first extract PG from the tissues and then determine their GAG composition. This procedure is the method of choice when an intact PG molecule is to be studied. For isolation of total GAGs, problem can occur because extraction of PG using different types of buffer is not complete. A commonly used technique of extraction with 4 M guanidine hydrochloride has provided only 50% to 70% extraction from aortas (Salisbury et al., 1981; Dalferes et al., 1987; Cherchi et al., 1990). Moreover, we also found out that it is difficult to control extraction efficiency and losses from one experiment to other (Unpublished Observations). Since we needed to have a complete picture of GAGs in tissues we employed the time honoured technique of GAG isolation directly from the tissues.

4.1.4 GAG Assay:

The assay we used for quantitation of GAGs depends upon alcian blue binding capacity of GAGs. This assay system is relatively new and is very sensitive and specific for GAGs. Other negatively charged molecules, like nucleic acids and glycoproteins, have very little dye binding capacity as compared with GAGs (Hronowsky et al., 1980). Many investigators prefer to employ an assay for uronic acid and/or hexosamine. Although these assays are good for biochemical analysis of GAGs, it is easier and more straight-forward to measure the GAG weight directly. These assays also are not free of

problems, such as, specificity problems and most importantly a different chromogenicity of uronic acid and iduronic acid. Recently, a sensitive and reliable, but very expensive, method employed is digestion of GAGs and elution and detection of sugars in HPLC system. Alcian blue assay is becoming commoner and many investigators employ this method either directly for total GAG or for GAG quantification after electrophoresis. We found it easier and more reliable to first quantitate the total GAG in each sample and use electrophoresis to determine the relative proportion of each GAG. Comparing GAG bands within each sample is much more reliable than comparing with standard as the latter will depend upon precision on the part of amount of sample applied every time. In total GAG assay it is easier to control the amount of sample applied as few microliters were spotted every time with the help of a pipette. Triplicate readings were close to each other. To assess the precision, some samples were assayed in duplicates. Readings were only about 2.7 % higher or lower than the mean results.

Alcian blue binding also depends upon charge density of GAGs. A change in degree and pattern of sulfation may result in a difference in the binding capacity of a type of GAG. This, however, should be associated with a change in electrophoretic mobility which is also sensitive to charge density. Since mobility of samples from different groups was essentially the same, we can speculate that their charge density did not change effectively. We realize that we are using CS-6 as standard and other GAG have different dye binding capacity. As other GAGs have lower absorbance values as compared with CS-6 our GAG assay was showing an underestimation. To overcome this problem we corrected the values after the corrected results of electrophoresis.

4.1.5 GAG Distribution:

For GAG distribution electrophoresis on cellulose acetate membrane is one of the most reliable and accepted methods. Alcian blue is the dye most often used to detect the bands. Quantitation is done by densitometry or by elution of bands and spectrophotometric evaluation. A number of buffers have been used by different investigators for this purpose and include calcium acetate, cadmium acetate, barium acetate, and formic acid-pyridine etc. at different pH (Beeley, 1985). A better but more complicated way is two-dimensional electrophoresis (Stevens et al., 1976). We used different buffers and finally found cadmium acetate at pH 4.1 to be very good for the separation. Bands were identified with the help of enzymatic digestion and GAG standards. Difficulty was observed in quantitating DS and CS as their bands were not completely separated. Digestion with chondroitinase AC removed CS and evaluation could be performed with more confidence and accuracy. Densitometric evaluation was very precise and triplicate reading of each band provided very similar readings. Similarly duplicate analysis of samples by electrophoresis and densitometry furnished similar readings. To obtain a more accurate distribution, binding capacity of each GAG in this system was determined and corrections were made in the proportion occupied by each GAG. We can confidently say that GAG distributions provided in this study are accurate.

4.1.6 Standard GAGs:

It should be noted that it is difficult to find an ideal standard for any type of GAG. This is because they are polydisperse and may differ in chain length and charge density depending on the source. Nevertheless, they have the same basic units and structure and

it is expected that values provided by standard GAGs can be extrapolated to samples. All investigators have used similar standards in their studies. Similar problems are faced in most of the protein assays where bovine serum albumin is usually used as a standard but chromogenicity of standard and sample may be quite different (Peterson, 1985). Due to the same reasons it can be seen in electrophoretograms that mobility of GAGs in samples is slightly different from that of standards. But again, due to properties inherent in the basic structure, the order of mobility of different GAG types remains essentially the same. Since all the groups in our study were treated in the same way, these problems will not affect the overall result.

Another difference found between standards and samples was the difficulty to completely separate DS from CS in samples. The same phenomenon was observed using different electrophoresis buffers. This property can be attributed to copolymeric nature of some GAGs. It has been found that chains of DS contain repeating units of CS types (Kjellen, 1991). It is expected that their mobility would be somewhere in between that of DS and CS. This problem was solved by routinely using chondroitinase AC which would break up CS types of units. Therefore in this study DS measurement is related only to iduronic acid containing units of DS.

4.1.7 Recovery and Losses:

We monitored both extraction efficiency and losses during further isolation of GAGs. Tissues were digested with sufficient quantities of enzyme and further digestion resulted in a gain of only a small fraction ($\sim$ 1.5 %). Since papain is expected to liberate all the GAGs from tissues we can conclude that extraction in our study was almost complete.

Other means of losing GAGs could be during proteolytic digestion, TCA precipitation, and precipitation with ethanol. Using standard GAG we found that there was no detectable loss during the whole process. An almost complete recovery was observed (~ 99.7 %)

A way in which loss might have occurred and still have been missed is during TCA precipitation Since GAGs might still be attached to incompletely digested proteins, they could have been precipitated by TCA. Recovery of a standard GAG might have provided incorrect information. Further digestion with papain and subsequent isolation and assay of GAG detected that there was a small loss during this process (~ 1.8 %)

Since all the losses combined were still very little (< 4 %), no corrections were made for them. We believe that we recovered almost all the GAGs present in the tissues. Based on these and other observations it can be concluded that values presented in this study fairly accurately represent actual tissue values.

4.2 Results:

4.2.1 GAGs in Normal Intima:

Normal Tissues: We found the total GAG concentration in our normal samples to be about 2 % of DDT. In most of the studies results are expressed in terms of DDT as it is a reliable variable, but occasionally total protein, surface area, or decalcified DDT have also been used. Comparing our result with other studies (table 2) in humans we find that our result is not different from others. Since these studies have presented quite diverse results we cannot compare our findings with any single study. Many of the studies have reported values lower than ours. At least two studies have reported results very close to

ours (Bottcher et al., 1963; Dalferes et al , 1971). Values reported by Smith (1965), is described in terms of protein but seems to be close to ours Stevens et al., reported a little higher value than the rest of the studies but their denominator was decalcified DDT. Two of the studies on coronary arteries described values a little lower than ours (Murata et al., 1982; Yla-herttuala et al., 1986) These differences are probably the result of difference in methods used for isolation and assaying of GAGs and not due to difference in samples themselves. Since our values agree well with some of the studies and are on the higher side of the spectrum we can confidently rely on our results

It is even more difficult to compare GAG distribution in our study with other studies. As evident from table 3, diverse results have been reported; for example HA proportion varies from 4.1 % to 33.3 % but except in one study (Nakamura et al , 1968) values are less than 15 %. For HS, values vary from 10% to 40%, but more commonly are between 16 %-26 %. In many studies DS has not been separated from CS while in others values from 6 % to 30 % have been described For CS alone there is at least one consensus that its proportion is greatest; values vary from 33 % to 66 % but more commonly are greater than 50 %. In view of this background we can only say that our values are generally in those ranges which have been more commonly reported. Our values are closer to those of Klynstra et al. (1967), Stevens et al. (1976), and Tammi et al. (1978).

4.2.2 GAGs in Atherosclerosis:

We found a significant decrease in total GAG in fully developed atherosclerotic lesions. This is in full agreement with the results of the most of the studies. Almost all of the studies have described an increase in GAG concentration in fatty streaks and a progressive decrease with the advancement of lesions. Indirect isolation of GAGs from extracted PGs have also shown the same result (Dalferes et al., 1987; Cherchi et al., 1990). This looks contradictory to the usual belief that there is an increase in matrix components in atherosclerosis. In fact this is due to the way we express our results. In most of the studies the result is expressed in relation to DDT which is composed of dead and living cells and matrix components like collagen, elastin and PG. Collagen and elastin form a major portion of DDT (> 40 %) and it has been reported that there is a substantial increase in collagen content of atherosclerotic plaques (Smith, 1974; Tammi et al., 1978). There is also an accumulation of necrotic debris in the centre of atheroma. Due to a concomitant increase in the major constituents of DDT, accumulation of GAGs gets overshadowed and in spite of the increased synthesis by smooth muscle cells of lesions their concentration is decreased. Since the intima of plaques is greatly thickened, there is accumulation of GAGs, and overall GAG content is increased. This could be clearly evident if data were to be expressed in relation to surface area and this has been documented by Wagner et al. (1978). An increase shown in fatty streaks and related earlier lesions in both humans and experimental animals is due to the fact they present very little distortion of normal matrix structure and an increase in GAG content is truly represented. With the passage of time and advancement of lesions, other more

pronounced changes in matrix obscure the increase in GAG content.

We detected highly significant changes in GAG distribution in atherosclerotic plaques. These were a decrease in HS proportion and an increase in DS proportion. Results were much more significant when we used HS:DS for comparison. No difference was found in the HA and CS portions. Other studies have reported results which are not much different. Our results of increased DS and decreased HS distribution agree well with a number of studies including those of Tammi et al.(1978), Murata et al.(1982), Yla-Herttuala et al.(1986), Hollman et al. (1989), and Murata et al. (1989). Some of these studies have also reported an increase in CS with or without a decrease in HA. Other studies partially agree, such as Stevens et al. (1976) who detected only an increase in DS while Buddecke (1962) and Nakamura et al. (1968) agree on a lower HS proportion. We cannot speculate on why our results differed in CS proportion from those of recent studies.

Studies involving PG extraction have also reported results agreeing with our findings. Dalferes et al. (1987) have reported a decrease in HS in plaques but the overall interpretation of GAG distribution was difficult while Cherchi et al. (1990) have described an increase in DS proportion. Data shown by Ying-Shan et al. (1991) completely agree with our findings. They detected an increase in DS and a decrease in HS proportion in aortic PGs from a population with a higher prevalence of atherosclerosis.

Studies in experimental animals have also described very similar results. Wagner et al. (1978) detected an increase in DS of monkeys' aortas after cholesterol feeding. Alavi et

al. (1985) have reported changes in GAG distribution in the neointima of rabbits developed after a balloon catheter injury. They found a marked decrease in the proportion of HS parallel with a similar increase in the CS. In a similar study Salisbury et al. (1985) described a decrease in HS proportion and an increase in DS.

It is not clearly known how these changes in GAG distribution develop nor do we know about the significance of these changes in atherosclerosis. It is known that intimal GAGs are produced by both endothelium and smooth muscle cells. Endothelium provides predominantly a HSPG while PGs from smooth muscle cells are rich in CS and DS. This has been documented in a number of studies using cell culture (Wight, 1989). Endothelial injury has been described as the key event in atherosclerosis and a large body of evidence has indicated the presence of endothelial injury with risk factors for atherosclerosis. With a disturbed endothelial behaviour, synthesis of PGs is also expected to be affected. Since HS is the major GAG type synthesized by endothelium, a decrease in intimal HS proportion can be explained by continued endothelial dysfunction.

The decrease in HS is expected to have a bearing on SMC proliferation. As discussed earlier, both heparin and HS have been found to be antiproliferative for SMC. Since the rate of growth depends upon the interplay of growth promoting and growth inhibitory factors a decrease in HS may then result in increased proliferation of SMCs. In this way endothelial dysfunction may directly effect the behaviour of intimal SMCs. These changes in HS may be actively involved in atherogenesis. In addition HS and heparin have been found to be associated with cell adhesion and inhibition of cell migration. A decrease in HS may therefore cause/enhance endothelial detachment and facilitate SMC migration

from media to intima. All these events will be helpful in the promotion of atherosclerosis. Some studies have reported an increase in DS accompanied with an increase in CS but we and other (Stevens et al., 1976; Tammi et al., 1978; Wagner et al., 1978; Salisbury et al., 1985; Cherchi et al., 1990; Ying-Shan et al., 1991) have described an increase in DS only. Atherosclerotic lesions are characterized by proliferation of smooth muscle cells which are derived from both intima and media. These cells have been reported to change their phenotype and secrete a greater amount of GAGs and other matrix elements. Enzymes concerned with the synthesis of CS type of GAGs have been found to be more active during proliferation in arterial SMCs (Hollman et al., 1985).A combined increase in CS and DS can easily be explained by this phenomenon. A selective increase in DS is more difficult to explain. DS has been found to be selectively associated with collagen fibres in the extracellular matrix (Volker et al., 1986). They have been observed to play a part in the process of formation and arrangement of collagen fibres (Ruoslahti, 1988; Wight, 1989). Interestingly, arterial SMCs when cultured on collagen gels modulated their PG synthesis to produce predominantly DSPG (Lark et al., 1986). Since there is a marked increase in collagen component in atherosclerotic lesions, an increase in DS may be related to that. It is not known whether there is a cause and effect relation between these two. We think this scenario represents a concomitant increase in collagen and DS which are both parts of collagen-rich matrix, and probably the same mechanism is underlying the increased synthesis of these two elements. The increase in DS may be necessary for the maintenance of the extracellular matrix of the lesions.

These changes in GAG distribution may result in alterations in the PG-LP interactions.

DS have been found to have the highest affinity for LP among the GAG types found in human aorta (Iverius, 1972) but histochemical studies have detected very little lipid associated with collagen-rich matrix and mostly within the soluble matrix associated with CSPG (Volker et al., 1989). In another study collagenase treatment of arterial wall liberated the major proportion of LP-GAG complexes but GAG content was limited to HA and CS (Srinivasan et al., 1979). In view of these contradictory findings we cannot speculate with confidence if these changes will result in a more avid LP-GAG interaction.

4.2.3 GAGs in Diabetes:

In this study we present the first report of the changes in arterial GAGs in human diabetes. Before this there were a few studies in experimental animals but their results presented a wide variety of changes and it is difficult to apply them to humans. In this project we detected a decrease in total GAG which was not statistically significant. Although the mean value was less than that of the nondiabetic group, sample values covered a wide range. It looks as if in some cases it is decreased while in others it is not. We do not know the cause of this variability. Results from the experimental studies have also reported both higher and lower values in diabetes. Since we are more interested in changes in GAG distribution we will concentrate more on that part of our results.

Changes in GAG distribution in diabetes were more or less similar to those present in atherosclerotic lesions but they were of lesser degree. There was a significant increase in DS and a small and nonsignificant decrease in HS. The ratio of HS to DS was significantly lower than that in nondiabetics but was still higher than that in the lesions.

Interestingly, changes detected in atherosclerotic plaques in diabetics were similar but more marked than that found in nondiabetic lesions. Even if we pool nondiabetic and diabetic samples the difference between normal and plaque areas remain essentially the same and highly significant. These results indicate that changes in GAGs are present in diabetic intima before the appearance of lesions and become pronounced with or after their appearance. This provides additional proof of the importance of GAGs in atherosclerosis.

Changes in arterial GAGs reported in experimental diabetes are diverse and no two studies agree on the type of changes. Our findings partially conform to the results of Sirek et al. who used dogs for alloxan diabetes. They detected an increase in DS in coronaries and a decrease in HA and HS in thoracic aortas.

Our findings provide one more proof that matrix structure and composition undergo pronounced changes in diabetic tissues. Changes have been detected in various tissues of the body in both human and experimental animals and include, in addition to GAGs, collagen, elastin, fibronectin, and laminin etc. Collagen content is generally increased, it is more resistant to degradation and is heavily glycosylated (Ruderman et al., 1984; Sternberg et al., 1985). In fact, the presence of advanced glycosylation endproducts on collagen is now considered as an important mechanism of lipid binding and atherogenesis (Brownlee et al., 1988). GAG content is generally decreased with reports of diverse changes in their distribution. These changes in basement membrane have been implicated in microangiopathy, another common complication of diabetes. These findings point towards a common factor which may be responsible for changes in the matrix. Insulin

either directly or indirectly (through hyperglycemia or other metabolic disturbances) has been incriminated. In fact, insulin has been found to alter the metabolism of GAGs and collagen along with a general effect on sugar, protein and lipid metabolism (Breton et al., 1988). In view of these findings it can be suggested that changes in GAGs may be the result of insulin deficiency or resistance.

These changes in GAGs can be explained from another point of view. We have found that changes in diabetic intima are similar to those found in atherosclerosis. It is tempting to speculate that a similar mechanism would be working in both cases. This mechanism would be expected to occur in diabetes in a more general way, while in atherosclerotic lesions this mechanism should be more or less localized. This mechanism should be able to explain the risk of atherosclerosis not only in diabetes but also in other conditions with atherogenic potential. One such mechanism which is involved in atherosclerosis and has been detected in diabetes and can explain the link is endothelial injury.

Endothelial injury has been recognized as the most important mechanism for the initiation of atherosclerosis. Both denuding and nondenuding injury have been found to result in atherosclerosis like lesions. Endothelial injury has been detected in conditions of atherosclerosis risk, like hyperlipidemia, hypertension, cigarette smoking, and diabetes. A number of factors have been suggested to be involved in injury in diabetes and have already been discussed in the introduction. Most of them are the result of metabolic disturbance. Whatever the mechanism, it has been established in a number of studies that both morphological and functional parameters of endothelial injury exist in diabetes. It can be speculated that persistent injury to endothelium would increase the risk of

atherosclerosis and would result in the development of gross lesions in areas of arterial tree where other local factors might also be involved.

The changes in arterial GAGs in diabetes may be the result of continued but mild endothelial dysfunction. A decrease in HS can be explained directly on the basis of endothelial injury while the increase in DS may be involved with a more complex sequence of events. In this case these changes represent a similar type of events as those occurring in atherosclerosis, but probably of a lesser strength and greater generality. Changes in GAGs can then be viewed as innocent bystanders, merely representing the injury phenomenon, or they may be expected to be actively involved in atherogenesis. We support the second view, as a large body of evidence is in favour of active participation of GAGs in lipid binding, foam cell formation, calcification, cell adhesion, and cell proliferation. These events are of the greatest significance in atherogenesis.

In this study we have detected significant changes in intimal GAGs in atherosclerosis and diabetes. We found a decrease in HS:DS in atherosclerosis and a similar but less marked decrease in diabetes. The significance of this ratio is not fully known but it may be viewed as an indicator of atherosclerotic risk. Extrapolating our finding in diabetes, we can speculate that persons with a lesser ratio of HS to DS may be at an increased risk of atherosclerosis. This finding agrees well with that of Ying-Shan et al. (1991) who detected similar changes in arterial GAGs in a population at increase risk of atherosclerosis. These findings will be important in understanding the role of GAGs in atherosclerosis, especially in diabetics.

4.4 Conclusion:

In this study we detected the changes in intimal GAGs in thoracic aortas of nondiabetics and diabetics. We found a significant decrease in total GAG and HS:DS in atherosclerosis. Our findings agree with a number of other studies in both human and experimental atherosclerosis.

This is the first report of changes in arterial GAGs in human diabetes. We detected again a significant decrease in HS:DS. This change was of lesser magnitude than that in atherosclerosis.

These findings indicate that changes in intimal GAGs precede the development of atherosclerotic plaques in diabetes and suggest that a decrease in HS:DS may be important in atherogenesis. These findings may be extrapolated to other conditions of atherosclerotic risk.

The results of our study will help in understanding the role of GAGs in atherogenesis and will provide a new perspective of viewing HS:DS as a possible factor in the development of atherosclerosis.

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