Age-Related Changes in Populations of Aortic Glycosaminoglycans
Species With Low Affinity for Plasma Low-Density Lipoproteins, and Not Species With High Affinity, Are Preferentially Affected

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Abstract—Glycosaminoglycans were extracted from the intima and media layers of normal human thoracic aortas from donors of different ages. The arterial segments were devoid of macroscopically visible lesions obtained from patients who had no clinically evident cardiovascular disease. Total glycosaminoglycan content increases during the first 40 years of life. Changes in the content of hyaluronic acid and heparan sulfate are less noticeable. The content of chondroitin sulfate (mainly the 6-isomer) increases, whereas dermatan sulfate remains constant. Plasma LDL-affinity chromatography of dermatan sulfate+chondroitin 4/6-sulfate fractions allowed the separation of LDL high- and low-affinity glycosaminoglycan species. Remarkably, only glycosaminoglycan species with low affinity for plasma LDL increase with age in the disease-free areas of human thoracic aortas studied. These results suggest that age-related changes in glycosaminoglycan composition of the arterial wall do not contribute to increased deposition of plasma LDL. However, the alternative explanation that individuals with arterial glycosaminoglycans that avidly bind LDL would develop early and severe cardiovascular disease and would thus be excluded from our analysis cannot be ruled out. (Arterioscler Thromb Vasc Biol. 1998;18:604-614.)

Key Words: glycosaminoglycans ■ chondroitin sulfate ■ dermatan sulfate ■ atherosclerotic risk factor ■ LDL

Glycosaminoglycan chains that project from proteoglycans of the arterial wall are responsible for the formation of complexes with plasma LDL.1-11 This is a step in the normal exchange of components between the circulating plasma and the arterial wall. However, during atherosclerosis, this process contributes to continuous focal deposition of cholesterol-rich lipoproteins, mainly LDL, in lesions.12-14 In turn, glycosaminoglycan-LDL complexes are more easily internalized by macrophages than LDL alone,15 thereby enhancing the formation of foam cells. Also, glycosaminoglycans induce structural alterations in LDL molecules that may potentiate their atherogenic effects.3 Therefore, the nature of the glycosaminoglycan-LDL interaction has been extensively studied. It is known that certain glycosaminoglycan species, including particular populations of a given species, have greater affinity for LDL.6,11,16

The occurrence of atherosclerotic lesions is associated with a number of risk factors, such as elevated serum lipoprotein levels, hypertension, smoking, gender, and family history.17 However, intrinsic factors of the arterial bed and its blood-carrying functions also influence the occurrence of lesions. The primary evidence for this influence comes from morphological studies of necropsy material, showing that the incidence and/or severity of atherosclerotic lesions varies as a function of anatomical location.18-21 We have demonstrated that glycosaminoglycans from different locations vary in composition and in binding affinity for plasma LDL.11 These results suggested that glycosaminoglycan composition could be one of several factors that determine the susceptibility of a given artery to atherosclerosis.

The process of ageing is accompanied by important modifications in the extracellular matrix and in the way tissues respond to injury. It is known that ageing increases the severity22 and modifies the distribution19 of atherosclerotic lesions. However, assessment of both total arterial glycosaminoglycan content,23,24 and changes in individual glycosaminoglycan species with age gave conflicting results.22-27 These contradictory data possibly result from the difficulty in extracting arterial glycosaminoglycans, as well as from the different techniques used for identification of the various species. Many of these studies were undertaken two or three decades ago, when methods available for identification and characterization of the various glycosaminoglycans were less specific.

The purpose of the present study was to determine whether specific populations of arterial glycosaminoglycans, and/or their
ability to bind LDL, vary with age, therefore contributing to increased incidence and severity of atherosclerosis. To clarify contradictory data reported in the literature,22–27 we employed increased incidence and severity of atherosclerosis. To clarify the nature of the glycosaminoglycan species by a combination of several procedures, including a direct measurement of their interaction with plasma LDL. Our experimental approach consisted of (1) a comparison among glycosaminoglycans extracted from thoracic aortas of individuals of different ages; (2) analyses of glycosaminoglycan binding to plasma LDL; and (3) comparison between intact proteoglycans and their glycosaminoglycan chains in their ability to bind plasma LDL.

Methods

Human thoracic aortas were obtained at necropsy, from patients who had not died of cardiovascular disease. For proteoglycan extraction, the adventitial layer was removed and the intima-media layers of segments (3.0×1.0 cm) devoid of macroscopically visible lesions were kept at −20°C. For extraction of glycosaminoglycans, similar aortic segments were excised, fixed in acetone, and kept at 4°C. After their adventitial layer was stripped off, the segments were cut into small pieces and subjected to two changes of 10 volumes of chloroform:methanol (2:1, vol/vol) for 24 hours each. The final defatted powder was obtained by drying this material at 60°C. This methodology for extraction of glycosaminoglycan is the same used in our previous work.11 Standard chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, twice-crystallized papain (15 U/mg protein), and cyanogen bromide–activated Sepharose 4B were purchased from Sigma Chemical Co. Chondroitin AC lyase (EC 4.2.2.5) from Arthrobacter aurescens and chondroitin ABC lyase (EC 4.2.2.4) from Proteus vulgaris were from Seikagaku American Inc. Heparan sulfate was a gift from Dr Barbara Mulloy (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom).

Isolation of Total Arterial Glycosaminoglycans

One hundred milligrams of the acetylated-treated powder was rehydrated for 24 hours at 4°C in 3.7 mL of 0.1 mol/L sodium acetate buffer, pH 5.0, containing 5 mmol/L cysteine and 5 mmol/L EDTA. Papain (0.7 mg) was added to the mixture, followed by incubation at 60°C for 24 hours with gentle agitation. The incubation mixture was centrifuged (2000g, 20 minutes at room temperature), the supernatant was retained, and the pellet was resuspended in 3.7 mL distilled water and centrifuged. A 10% cetylpyridinium chloride solution was added to the combined supernatants to a final concentration of 0.5%, and the mixture was left to stand at room temperature for 24 hours. Next, the solution was centrifuged and the pellet washed with 15 mL of a 0.05% cetylpyridinium solution. This pellet, a glycosaminoglycan-cetylpyridinium complex, was then dissolved with 3.7 mL of a solution of 2 mol/L NaCl/absolute ethanol (100:15, vol/vol), and the glycosaminoglycans were precipitated with the addition of 7.5 mL absolute ethanol. After 24 hours at 4°C, the precipitates were collected by centrifugation and washed twice with 15 mL 80% ethanol and once with the same volume of absolute ethanol. The final pellet, which constituted the total tissue glycosaminoglycan preparation, was dried at 60°C for 30 minutes and dissolved in 1.0 mL distilled water. The total glycosaminoglycan contents in the aorta samples were determined by hexuronic acid assays28 on these solutions. Under these conditions, papain digestion completely solubilized all arterial samples, and controls using known amounts of glycosaminoglycans showed that recovery from the subsequent cetylpyridinium and ethanol precipitation was greater than 90%. Estimates of concentration and subsequent analyses of the arterial glycosaminoglycans were carried out separately for each individual.

Separation of the Arterial Glycosaminoglycans on a Mono Q-FPLC Column

Arterial glycosaminoglycans (200 μg, as hexuronic acid) were applied to a Mono Q-FPLC column (HR 5/5) from Pharmacia Biotech Inc, equilibrated with 20 mmol/L Tris/HCl buffer (pH 8.0). The column was developed by a linear gradient of 0.15 to 1.5 mol/L NaCl in the same buffer. The flow rate of the column was 0.45 mL/min, and fractions of 0.5 mL were collected. These were assayed by the metachromasia produced by sulfated glycosaminoglycans with 1,9-dimethylmethylen blue29 and by hexuronic acid using the carbazole reaction.30 The salt concentration was estimated by conductivity. Fractions containing glycosaminoglycans were pooled and precipitated with three volumes of absolute ethanol. The yield was approximately 85% with regard to the applied material.

Agarose Gel Electrophoresis

Glycosaminoglycans (~10 μg) were applied to a 0.5% agarose gel in 0.05 mol/L 1,3-diaminopropane/acetate buffer (pH 9.0). After electrophoresis (120 V for 1 hour), the glycosaminoglycans in the gel were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide in water, stained with 0.1% toluidine blue in acetic acid/ethanol/water (0.1:5.5, vol/vol/vol), and washed for about 30 minutes in acetic acid/ethanol/water (0.1:5.5, vol/vol/vol). The glycosaminoglycans on the agarose gels were quantified by densitometry with a model GS-690 imaging densitometer (Bio-Rad Laboratories). Three replicate densitometric profiles were obtained for each sample. In terms of peak height and position, the replicates did not vary more than 5% from the profiles shown in the figures. Quantification of materials represented by the peaks were made by estimating peak areas from these typical profiles.

Polyacrylamide Gel Electrophoresis

The molecular weights of the glycosaminoglycan chains were estimated by polyacrylamide gel electrophoresis. Glycosaminoglycan samples (~10 μg) were applied to a 6% 1-mm-thick polyacrylamide gel slab in 0.02 mol/L sodium barbital buffer (pH 8.6). After electrophoresis (100 V for 30 minutes), the gel was stained with 0.1% toluidine blue in 1% acetic acid, then washed for about 4 hours in 1% acetic acid. The molecular weight markers were the same as those used previously.28 In a previous study,3 we estimated the molecular weight of arterial glycosaminoglycans by gel filtration and polyacrylamide gel electrophoresis. The two methods have nearly identical accuracy. But we used the latter method in this work because it requires smaller amounts of sample.

Digestion of the Arterial Glycosaminoglycans with Chondroitin AC Lyase

Glycosaminoglycans (100 μg) were incubated with 10 μU chondroitin AC lyase or chondroitin ABC lyase in 100 μL of 50 mmol/L Tris/HCl buffer (pH 8.0) containing 5 mmol/L EDTA and 15 mmol/L sodium acetate. After incubation at 37°C for 12 hours, the mixtures were spotted on Whatman No. 1 paper and chromatographed in isobutyric acid/1 mol/L NH4OH (5:3, vol/vol) for 48 hours. The products were located by silver nitrate staining and quantified by densitometry.

Extraction of Aortic Proteoglycans

Fragments of intima-media layers of normal human thoracic aorta were cut in small pieces and immersed in 10 volumes of 50 mmol/L sodium acetate buffer (pH 6.0) containing 4 mol/L guanidine hydrochloride, 10 mmol/L EDTA, and the following protease inhibitors: 10 mmol/L 6-aminohexanoic acid, 1 mmol/L benzamidine hydrochloride, 1 mmol/L PMSF, and 10 mmol/L N-ethylmaleimide. Proteoglycans were extracted by stirring this mixture for 48 hours at 4°C. Extracts were clarified by centrifugation (2000g for 10 minutes at 4°C). The residue was washed twice with 10 volumes of distilled water to remove guanidine hydrochloride and protease inhibitors, digested with papain, and precipitated as described above for arterial glycosaminoglycan. The supernatant was dialyzed at 4°C against 6 L.
of 50 mmol/L sodium acetate buffer (pH 6.0) containing 7 mol/L urea. Guanidine hydrochloride extraction solubilizes ~80% of the total aortic proteoglycans, based on measurements of hexuronic acid in the residue and supernatant.

Purification of Aortic Proteoglycans on DEAE Cellulose

The dialyzed guanidine hydrochloride extract was applied to a DEAE-Sephaead column (18.0 x 1.5 cm, ~30 mL bed) equilibrated with 50 mmol/L sodium acetate buffer (pH 6.0) containing 7 mol/L urea and 0.1 mol/L NaCl. The column was washed with 240 mL of this same buffer and subjected to a linear gradient of 0.1 to 1.0 mol/L NaCl at a flow rate of 18 mL/h, and fractions of 3.0 mL were collected. These were assayed by the metachromasia produced by the sulfated glycosaminoglycans with 1,9-dimethylmethylene blue,29 by hexuronic acid using the carbazole reaction,29 and absorbance at 280 nm. Salt concentration was estimated by conductivity. Fractions containing proteoglycans, as indicated by positive tests for hexuronic acid and metachromasia, were pooled, dialyzed against distilled water, and lyophilized.

Analysis of Proteoglycans by Gel Filtration on FPLC Superose 6

Aortic proteoglycans previously purified on a DEAE-Sephaead column (50 μg as hexuronic acid) were applied on a Superose 6 FPLC (HR 10/30) column from Pharmacia Biotech Inc (Sweden) and eluted with 20 mmol/L Tris/HCl buffer (pH 8.0) containing 1.0 mol/L NaCl. Part of each sample was subjected to β-elimination before chromatography. The flow rate of the column was 0.2 mL/min, and fractions of 0.5 mL were collected and assayed by the metachromasia produced by sulfated glycosaminoglycans with 1,9-dimethylmethylene blue.29

Isolation of LDL From Plasma

LDL (d=1.020 to 1.050 g/mL) was purified by the method of Havel et al,30 using human plasma obtained from healthy donors. After sequential ultracentrifugation in KBr, the LDL preparation was dialyzed exhaustively at 4°C against 0.9% NaCl containing 0.01% EDTA and stored at 4°C. Purity of LDL in this preparation was assessed by agarose gel electrophoresis in barbital buffer. The LDL migrated as a single band. Typically, the concentration of LDL in this preparation was 1.8 mg protein per milliliter.

Interaction Between LDL and Glycosaminoglycans

Experiments on the interaction between human plasma LDL and arterial glycosaminoglycans were performed essentially as described elsewhere17,18 using LDL affinity chromatography.

The LDL affinity column was constructed by coupling the ligand to CNBr-activated Sepharose 4B according to the protocol supplied with the product (Pharmacia). The efficacy of the procedure used for binding LDL to Sepharose 4B was assessed by measuring cholesterol content of the resin. A control column was prepared in the same way but without the lipoprotein. To avoid natural variations in LDL obtained from different individuals, LDL obtained from a single donor was used for all affinity columns.

About 200 μg (as hexuronic acid) of glycosaminoglycan in 1 mL of buffer was applied to the LDL-Sepharose column (0.7 x 8.0 cm) preequilibrated with 5 mmol/L Tris/HCl buffer (pH 7.0) containing 10 mmol/L CaCl2. After the column was washed with 20 mL of the same buffer, the retained material was eluted using a linear NaCl gradient. Eluted fractions were analyzed by their metachromatic property and by their hexuronic acid content.28 On a control column, aortic glycosaminoglycans were not significantly retained. After three replicate analyses of the same glycosaminoglycan mixture on an LDL affinity column, replicates did not vary more than 10% in terms of the amounts of glycosaminoglycans retained by the column and of the chondroitin sulfate:dermatan sulfate ratio in the retained fraction.

Results

Effect of Donor Age on Glycosaminoglycan Content Expressed by Thoracic Aortas

Total glycosaminoglycan content of human thoracic aortas increases with age up to ~40 years and thereafter shows a tendency to decrease (Fig 1A). No difference was observed between samples from males and females.

A purified extract of total aortic glycosaminoglycans was resolved into three peaks by fractionation on a Mono Q-FPLC column. These were identified as hyaluronic acid, heparan sulfate, and dermatan sulfate plus chondroitin 4/6-sulfate (Fig 2). No variations were observed in the NaCl concentrations necessary for elution of these peaks in aortas at different ages, but the relative amounts in each peak (based on hexuronic acid content) varies slightly with age. From these data and the total glycosaminoglycan contents (Fig 1A), the concentrations of the three glycosaminoglycan species in human aorta were calculated.
The content of dermatan sulfate in thoracic aortas can be estimated (Fig 1B). It can be seen that the content of dermatan sulfate decreases with age (lower mobility band, Fig 3A and 3B). For each sample of human aorta, relative proportions of dermatan sulfate and chondroitin 4/6-sulfate were determined by densitometry of electrophoretic bands stained with toluidine blue. These ratios were applied to determinations of total dermatan sulfate+chondroitin 4/6-sulfate content (Fig 1B) to allow estimation of the concentrations of the two glycosaminoglycans in thoracic aortas (Fig 1C). It can be seen that the content of chondroitin 4/6-sulfate markedly increases with age, while that of dermatan sulfate remains constant. Similar proportions of dermatan sulfate and chondroitin 4/6-sulfate were previously obtained following analyses of unsaturated disaccharides in chondroitin AC and ABC lyase digestions.6,11

The molecular weights of the dermatan sulfate+chondroitin 4/6-sulfate chains were estimated by polyacrylamide gel electrophoresis and were comparable among human aortas at different ages (Fig 3C).

Digestion with chondroitin AC lyase yielded proportions of isomeric chondroitin 4-sulfate and chondroitin 6-sulfate (Fig 3D) and allowed estimation of the aortic content of these two glycosaminoglycans. Aortic chondroitin 6-sulfate increases and chondroitin 4-sulfate decreases with age (Fig 1D).

Thus, in human thoracic aortas without any evident atherosclerotic change, total glycosaminoglycan content varies with age (Fig 1A). Among the individual glycosaminoglycan species, there is a marked increase in chondroitin sulfate (mainly the 6-isomer), whereas the content of dermatan sulfate remains constant (Fig 1C and 1D). (We did not attempt to correlate the content of total or individual glycosaminoglycan species with some known cardiac risk factor, such as high blood pressure, smoking, diabetes, and lipoprotein concentrations. This type of study requires a larger number of affected patients. In addition, some of these clinical parameters were not available for the individuals included in our study.)

### Interactions Between Plasma LDL and Glycosaminoglycans Extracted From Thoracic Aortas at Different Donor Ages

Glycosaminoglycans were extracted from aortas at different ages, and a direct measurement of their interaction with plasma LDL was undertaken to obtain evidence concerning a role for this interaction in the effect of ageing on increased atherogenesis.

Interactions between aortic glycosaminoglycans and plasma LDL were analyzed by affinity chromatography. A column was prepared by coupling human LDL to Sepharose 4B, and to this column the purified dermatan sulfate+chondroitin 4/6-sulfate fractions were applied (Fig 4). (Amino groups of apoB lipoprotein might play an important role in the interaction with glycosaminoglycan, and some of these groups could be blocked on the LDL affinity column. We addressed this question previously with experiments in...
which glycosaminoglycan instead of LDL was linked to Sepharose 4B. LDL was eluted from this column at approximately the same NaCl concentration as that required to elute glycosaminoglycan from the LDL-Sepharose column. Thus, the blocking of amino groups on the apo-B lipoprotein does not appear to be detrimental to use of this affinity column for detecting formation of LDL-glycosaminoglycan complexes.

Aortic heparan sulfate is practically not retained on this column.2,6,11 To obtain a quantitative index of the interaction with LDL, we compared the amount of total dermatan sulfate1 chondroitin 4/6-sulfate retained on the affinity column with the total amount applied. The ratio of retained glycosaminoglycans varied from 24% to 50% of the total, and all glycosaminoglycans were eluted from the column with the same concentration of NaCl irrespective of age (Fig 4). Combining these data with those for total glycosaminoglycan contents (Fig 1) allows estimation of the concentrations of glycosaminoglycans with and without affinity for plasma LDL in thoracic aortas of different ages (Fig 5). Surprisingly, the content of dermatan sulfate + chondroitin 4/6-sulfate that interacts with LDL does not increase with age (Fig 5C).

The relative amounts of dermatan sulfate and chondroitin 4/6-sulfate in the fractions from the LDL affinity column were determined. The results are shown in the Table, from which the following observations can be made: (1) dermatan sulfate is constituted mostly of glycosaminoglycan chains with a comparatively high affinity for LDL, as a significant proportion of this compound is retained by the column; (2) the relative contents of chondroitin 4-sulfate and chondroitin 6-sulfate. After incubation of the dermatan sulfate + chondroitin 4/6-sulfate with chondroitin AC lyase (see “Methods”), the mixtures were spotted on Whatman No. 1 paper and subjected to descending chromatography in isobutyric acid/1 mol/L NH₄OH (5:3, vol/vol) for 48 hours. Products were located on the chromatograms by silver nitrate staining. Chromatographic migration of standard unsaturated disaccharides derived from chondroitin 4-sulfate (ΔGlcUA-GalNAC4S), chondroitin 6-sulfate (ΔGlcUA-GalNAC6S). C-4-S indicates standard chondroitin 4-sulfate and C-6-S, chondroitin 6-sulfate. Panels show typical electrophoretic and chromatographic profiles in terms of intensity and position of the bands for each group of age, as observed among glycosaminoglycan samples from 18 individuals.

Figure 3. Characterization of the dermatan sulfate + chondroitin 4/6-sulfate extracted from human thoracic aortas at different ages. A and B. Relative proportions of dermatan sulfate and chondroitin 4/6-sulfate. Samples (10 μg) of the dermatan sulfate + chondroitin 4/6-sulfate, purified by Mono Q-FPLC (see Fig 2) were applied to a 0.5% agarose gel in 0.05 mol/L 1,3-diaminopropane/acetate buffer (pH 9.0). After electrophoresis, the glycosaminoglycans in the gel were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide and stained with toluidine blue (see “Methods”). Standard glycosaminoglycans used were chondroitin 4-sulfate from whale cartilage (CS) and dermatan sulfate from pig skin (DS). Peaks were identified as chondroitin 4/6-sulfate and dermatan sulfate based on incubation with chondroitin AC and ABC lyases (see Reference 11). The glycosaminoglycans were quantified by densitometry, and typical profiles for glycosaminoglycans extracted from aortas of young and old individuals are shown in B. C. Molecular weight of the dermatan sulfate + chondroitin 4/6-sulfate. Samples of the dermatan sulfate + chondroitin 4/6-sulfate fractions from 8 aortas are compared with standard polysaccharides of different molecular weights (St1 through St4) (10 μg of each) on 6% polyacrylamide gels in sodium barbital buffer (pH 8.6), after staining with toluidine blue. St1 indicates chondroitin 6-sulfate from shark cartilage (average molecular weight, 60 kD); St2, chondroitin 4-sulfate from whale cartilage (average molecular weight, 30 kD); St3, dermatan sulfate from pig skin (average molecular weight, 20 kD); St4, dextran sulfate (average molecular weight, 8 kD). D. Relative proportions of isomeric chondroitin 4-sulfate and chondroitin 6-sulfate. After incubation of the dermatan sulfate + chondroitin 4/6-sulfate with chondroitin AC lyase (see “Methods”), the mixtures were spotted on Whatman No. 1 paper and subjected to descending chromatography in isobutyric acid/1 mol/L NH₄OH (5:3, vol/vol) for 48 hours. Products were located on the chromatograms by silver nitrate staining. Chromatographic migration of standard unsaturated disaccharides derived from chondroitin 4-sulfate (ΔGlcUA-GalNAC4S), chondroitin 6-sulfate (ΔGlcUA-GalNAC6S). C-4-S indicates standard chondroitin 4-sulfate and C-6-S, chondroitin 6-sulfate. Panels show typical electrophoretic and chromatographic profiles in terms of intensity and position of the bands for each group of age, as observed among glycosaminoglycan samples from 18 individuals.
has a higher apparent molecular weight than the fractions not retained by the column. The retained glycans are all similar in size, whereas the nonretained fractions are somewhat variable (Fig 6).

The proportions of chondroitin 4-sulfate and chondroitin 6-sulfate in the fractions from LDL affinity column were assessed by digestion with chondroitin AC lyase (the same methodology used in the experiment of Fig 3D). No difference was observed in the proportions of the two isomeric chondroitin sulfates among retained and nonretained fractions (not shown; see also Reference 2).

A possible criticism to the result in Fig 5 is that the amount of retained glycosaminoglycan did not vary because the affinity columns were saturated. This does not seem to be the case, since retained and nonretained fractions show a marked difference in molecular weight (Fig 6) and in the proportion of dermatan sulfate and chondroitin 4/6-sulfate (Table). In addition, the same proportion of retained and nonretained fractions was obtained when 100 mg rather than 200 mg of glycosaminoglycan was applied to the column.

Overall, the data shown in Figs 4 to 6 and the Table demonstrate that in human aortas the glycosaminoglycan species that increase with age have low affinity for plasma LDL. Surprisingly, glycosaminoglycans that interact with LDL (dermatan sulfate + chondroitin 4/6-sulfate with high molecular weight) remain constant.

Comparison Between Intact Aortic Proteoglycans and Their Constituent Glycosaminoglycan Chains in Interaction with Plasma LDL

The material extracted from aortas by 4 mol/L guanidine hydrochloride was separated by DEAE cellulose into a major
Relative Contents of Chondroitin 4/6-Sulfate and Dermatan Sulfate in Fractions of Different Affinities for LDL

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The proportion of chondroitin 4/6-sulfate (CS) and dermatan sulfate (DS) in each fraction obtained in the experiments shown in Fig 4 was determined by agarose gel electrophoresis (Fig 7A) and polyacrylamide gel electrophoresis (Fig 7C) of this fraction confirmed its high molecular weight and therefore excluded the action of proteases during the procedures of extraction and purification of the proteoglycans. Glycosaminoglycan chains were released after β-elimination of the proteoglycan and showed an average molecular weight similar to those observed for glycosaminoglycans extracted from the tissue by protease digestion.

A comparison between intact proteoglycans and their constituent glycosaminoglycans was carried out using the LDL affinity column (Fig 8). Compared with the amount initially applied, the amounts retained on the affinity column were slightly higher for the glycosaminoglycans than for the intact proteoglycan. We have no explanation for this observation.

The relative amounts of the glycosaminoglycans released by β-elimination from the proteoglycan fractions obtained on the LDL affinity column were determined by agarose gel electrophoresis (Fig 9). The proteoglycan fraction retained by LDL had a increase in the dermatan sulfate and a decrease in chondroitin sulfate compared with nonretained proteoglycans.

Discussion

Some Glycosaminoglycan Species Have Greater Affinity for Plasma LDL

It is well established that proteoglycans and their glycosaminoglycan chains contribute to pathogenesis of atherosclerosis by retaining and modifying LDL that insudates into the arterial wall. The nature of the glycosaminoglycan-LDL interaction has been extensively studied, and it is known that certain glycosaminoglycan species, including particular populations of a given species, have greater affinity for LDL.1–12 In addition, several stimuli related to known cardiac risk factors have been shown in vitro to alter glycosaminoglycan synthesis. These include shear stress,33 angiotensin II,34 hypoxia,35 certain cytokines,36 and native37 or modified38,39 lipoproteins.

Glycosaminoglycans can be extracted from aortas by proteolysis and partially purified by precipitation with a cationic detergent. After ion exchange chromatography, total glycosaminoglycan extracts yield three peaks, identified as hyaluronic acid, heparan sulfate, and a mixture of dermatan sulfate +chondroitin 4/6-sulfate (Fig 2, References 11 and 40). Arterial heparan sulfate and hyaluronic acid do not bind LDL, whereas dermatan sulfate and chondroitin 4/6-sulfate form both soluble and insoluble complexes with this lipoprotein.2,6,11

A preferential binding of dermatan sulfate to LDL was observed by Iverius,41 and a role for this glycosaminoglycan in the development of the atherosclerotic lesions was postulated.41,42 These results contrast with other studies in which chondroitin sulfate was shown to interact with plasma LDL.1,43 These apparently contradictory reports are reconciled by the observation that high-molecular-weight chains of both glycosaminoglycans can interact with LDL.2,6 Unlike proteins, glycosaminoglycans occur naturally as polydisperse polymers—that is, their degree of polymerization, and hence their molecular weights, vary considerably. This parameter affects binding of dermatan sulfate and chondroitin 4/6-sulfate chains to LDL.2,6 The fact that aortic dermatan sulfate constitutes most of the glycosaminoglycans with a high affinity for LDL can be ascribed to their comparatively longer glycan chains compared with chondroitin 4/6-sulfate.2,11

A similar conclusion was reached in investigations on synthesis of glycosaminoglycans by arterial smooth muscle cells under different experimental conditions. In these cells, the shift from the resting to proliferating condition has been implicated in atherogenesis. This shift is accompanied by an increase in the synthesis of proteoglycans with higher affinity for plasma LDL, due essentially to an increase in size of their chondroitin sulfate chains.44 Among the complex array of mediators taking part in atherogenesis, growth factors are capable of inducing phenotypic changes in arterial smooth muscle cells and also of increasing the synthesis of proteoglycans.45 More important, the proteoglycans synthesized under these conditions have longer glycosaminoglycan chains.46
Overall, these results indicate that aortic chondroitin sulfate and especially aortic dermatan sulfate chains with high molecular weight have higher affinity for plasma LDL.

Total Glycosaminoglycan Content in Apparently Disease-Free Human Aortas Increases With Age: Identification of Affected Species

Previous studies have demonstrated that dermatan sulfate and dermatan sulfate chondroitin 4/6-sulfate-rich aortic proteoglycans (see Fig 7A) and the glycosaminoglycan chains released by β-elimination (B) were applied on an LDL-Sepharose column (0.7×8.0 cm) and eluted using a linear NaCl gradient. Eluted fractions were assayed for metachromasia and NaCl concentration (broken line). Fractions corresponding to retained and nonretained proteoglycans or glycosaminoglycans were pooled as indicated (horizontal brackets). The numbers above the bracket indicate the relative amounts, in percent of the total.

Overall, these results indicate that aortic chondroitin sulfate and especially aortic dermatan sulfate chains with high molecular weight have higher affinity for plasma LDL.

Molecular weight of the aortic proteoglycans and of the released glycosaminoglycan chains. Purified aortic proteoglycans, before (lane 1) and after (lane 2) β-elimination (~10 μg of each) were run on 6% polyacrylamide gel electrophoresis, as described in the legend of Fig 6. Vertical brackets at right indicate the mobilities of polysaccharides of different average molecular weights.
The proportions of chondroitin 4-sulfate and chondroitin 6-sulfate are the same in retained and nonretained fractions from the LDL affinity column. Thus, it is not clear whether modifications in the proportions of these two isomeric chondroitin sulfates are significant for atherosclerosis.

**Interaction of Glycosaminoglycan Chains With Plasma LDL Does Not Require Their Attachment to the Protein Core**

The experiments shown in Figs 4 to 6 and the Table on interaction with plasma LDL were conducted with free glycosaminoglycan chains obtained after protease digestion. This raises the question of whether our results are relevant to events that occur in situ, where glycosaminoglycans are covalently linked to a protein backbone as proteoglycans. Previous studies comparing interaction of LDL with proteoglycans and with free glycosaminoglycan chains gave contradictory results. 2–6,47 We used proteoglycans extracted with a chaotropie solute in the presence of protease inhibitors. Both intact proteoglycans and free glycosaminoglycan chains interacted similarly with LDL (Fig 8). Therefore, the results obtained with free glycosaminoglycan chains are probably representative of the interactions of the intact molecules with LDL.

Very high concentrations of glycosaminoglycans are found on the arterial walls. 56 Thus, if plasma LDL binds arterial glycosaminoglycan avidly at normal salt concentration in vivo, we would expect a rapid accumulation of the lipoprotein on the arterial walls. Perhaps this means that both intact proteoglycans and free glycosaminoglycan chains require low salt concentrations for binding LDL in vitro. At physiological salt concentrations, in vivo binding of arterial glycosaminoglycans to plasma LDL probably requires additional factors, such as fusion of LDL particles 48 or certain lipases known to enhance the association of lipoproteins with smooth muscle cells and extracellular matrix. 49,50

**Does an Increase in Arterial Glycosaminoglycan–Plasma LDL Interaction Account for the Higher Incidence of Atherosclerosis With Ageing?**

Comparison of glycosaminoglycan species from arterial segments with and without atherosclerotic lesions would be an important extension of our present observation. However, this is not a simple procedure. Alteration of glycosaminoglycan may be a very early event in atherosclerosis, even preceding LDL trapping or retention 51,52 on vessel walls. On the other hand, once the lesion starts, alterations in glycosaminoglycan composition may reflect secondary modifications of the arterial wall induced by the disease. Thus, it is not simple to find the appropriate stage of the lesion to study the glycosaminoglycan modification. In addition, the initial atherosclerotic lesion is usually restricted to a small region of the arterial wall, raising limitations to a complete analysis of the glycosaminoglycan species.

Previously we demonstrated that glycosaminoglycans may be important in determining the susceptibility of a given artery to atherosclerosis. 53 Here we show that the glycosaminoglycan composition of a specific artery is unlikely to contribute to an increase in deposition of plasma LDL on the
vessel wall with ageing. Among glycosaminoglycans extracted from aortic segments without macroscopically visible lesions, it would not be possible to detect small changes located within an initial focal lesion. Although we cannot exclude a role for altered glycosaminoglycan composition in restricted areas, there is certainly no overall modification of glycosaminoglycan composition that favors an increase in binding of LDL with age. Thus, it is unlikely that the increased risk of developing lesions in older arteries results from altered glycosaminoglycan composition. As the data in the present study suggest, increasing atherosclerosis with age may simply reflect the longer time available for lipid accumulation to occur, and not changes in glycosaminoglycan composition in older arteries that favor an increased binding of LDL with age.

An alternative explanation for our results would be a survivor effect: individuals with arterial glycosaminoglycans that avidly bind LDL would develop early and severe cardiovascular disease, and would thus be excluded from our analysis. In this case, the vessels from older individuals included in this study would come from individuals resistant to developing atherosclerosis. A similar explanation has been proposed for plasma cholesterol levels. Thus, in men, plasma cholesterol levels rise until age 45 to 50, but after this age, the rate of the rise tends to slow and mean cholesterol levels tend to decline, probably as a result of a survivor effect. Further work involving large sample sizes and comparison with patients suffering from active cardiovascular disease will be required to better understand the role of arterial wall glycosaminoglycans in the atherosclerotic process and other aspects raised by this study.

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Aortic Glycosaminoglycans, Ageing, and LDL Binding


Age-Related Changes in Populations of Aortic Glycosaminoglycans: Species With Low Affinity for Plasma Low-Density Lipoproteins, and Not Species With High Affinity, Are Preferentially Affected

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