Aortic Glycosaminoglycans in Genetically Selected WC-2 Pigeons with Increased Atherosclerosis Susceptibility

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A genetically selected line of White Carneau pigeons (WC-2) was studied in an attempt to relate changes in composition and content of aortic glycosaminoglycan (GAG) to increased atherosclerosis susceptibility. The WC-2 pigeons were fed an atherogenic diet for 3 months and, when compared to randomly bred White Carneau (RBWC) controls, they showed similar plasma cholesterol concentrations but significantly greater aortic atherosclerosis. In the WC-2 pigeons, 35% of the aortic surface was covered with plaque compared with only 12% in RBWC pigeons; WC-2 birds showed cholesterol contents of 3.3 mg/aorta/500 g body weight, while the RBWC birds had only 0.9 mg/aorta/500 g body weight. After papain treatment of delipidated dried artery, the aortic GAG were isolated, purified using cetylpyridinium chloride, and identified and quantitated by a combination of procedures including selective enzymatic digestion and electrophoresis. In both pigeon groups, aortic GAG included 8% hyaluronic acid, 11% dermatan sulfate, 15% heparan sulfate, and 66% chondroitin sulfate. For the entire group, total aortic GAG content was 35% greater in WC-2 pigeons. Since we did not know if this increase in GAG was simply due to increased atherosclerosis in the WC-2 birds, we sorted the pigeons into matched groups representing minimal, moderate, and severe atherosclerosis on the basis of aortic cholesterol content. At all levels of cholesterol, all GAG contents were greater in the aortas of WC-2 pigeons. The accumulation of dermatan sulfate was 30% higher than in RBWC birds in the minimal arteriosclerosis group, 101% higher in the moderate group, and 53% higher in the severe group. Hyaluronic acid tended to decrease as aortic cholesterol contents increased in WC-2 pigeons. Reduced hyaluronic acid and increased dermatan sulfate may suggest the presence of an altered hyaluronic acid-dermatan sulfate-containing proteoglycan aggregate in the intercellular matrix of the WC-2 pigeon aorta. Possible consequences include increased artery permeability and a binding and retention of lipoproteins in the artery wall. These factors may explain why atherosclerosis develops at an increased rate in the WC-2 pigeon.

(Arteriosclerosis 1981; 1:192-201)
osclerosis. We therefore assumed that atherosclerosis was predominately influenced by genetic factors operative at the level of the arterial wall. In an earlier report, we described a possible site of genetic regulation involving aortic glycoproteins. In that study, WC-2 pigeons showed altered aortic glycopeptide-sugar contents when compared to RBWC pigeons, and the progression of atherosclerosis was associated with increases in the absolute amount of aortic glycopeptide-sialic acid only in the WC-2 pigeons.

In this study we examined the aortic composition and content of the glycosaminoglycan (GAG) moiety of the other major family of arterial wall complex carbohydrates, the proteoglycans (PG). Recent evidence indicates that proteoglycans may bind low density lipoproteins (LDL) and play a role in either the initiation or progression of atherosclerosis. Binding of the GAG and lipoproteins in vitro is well known, and there have been reports on the extraction of GAG-lipoprotein complexes in fatty streaks and fibrous plaques. The presence of lipoprotein-PG complexes in vivo has been suggested by studies that provided histologic evidence of a close association between GAG and lipid within the atherosclerotic plaque. Recent findings have demonstrated that apo-B-containing lipoproteins are usually found in the atherosclerotic plaque, which suggests that retention of LDL and very low density lipoprotein (VLDL) is related to atherosclerosis development.

The purpose of this study was to identify the types of GAG present in the thoracic aortas of WC-2 pigeons and to determine any changes occurring in the GAG during the progression of atherosclerosis. In each case we compared the WC-2 pigeon with the RBWC pigeon from which it was genetically selected. We attempted to implicate changes, mainly of dermatan sulfate, to the increased atherosclerosis susceptibility of the WC-2 pigeon.

**Methods**

The pigeons used in the study were described in a report on aortic glycopeptides. Briefly, the design of the study was as follows. All pigeons were produced at the Bowman Gray School of Medicine Research Farm. A group of 100 WC-2 pigeons was age-matched with 115 5- to 9-month-old RBWC pigeons. Each group had an equal number of male and female pigeons (1:1). For 3 months all pigeons were fed an atherogenic diet consisting of 89.5% (by weight) Purina pigeon pellets (Ralston Purina Company, Checkerboard Square, St. Louis, Missouri) coated with 10% melted lard which contained 0.5% crystalline cholesterol (Nutritional Biochemical Corporation, Cleveland, Ohio).

Plasma cholesterol concentrations were determined before the beginning of the atherogenic diet and at monthly intervals thereafter. Isopropanol extracts of serum were prepared and cholesterol concentrations determined according to the method of Rush et al.

At necropsy the thoracic aorta extending from the left common carotid artery to a point 2 mm below the origin of the celiac artery was removed, cleaned of extraneous connective tissue, opened longitudinally, and flattened. The aorta contained the atherosclerosis-prone area located at the origin of the celiac artery. The extent of atherosclerosis was determined by visually estimating the percentage of intimal surface covered with fatty streaks or plaques. Fatty streaks were defined as nonraised white to white-yellow intimal flecks or streaks, and plaques were defined as yellow lesions raised above the plane of the intimal surface. The aortic length, width at midpoint, and wet weight were recorded, and the vessels were frozen at —20°C.

**Aortic Cholesterol Determinations**

Individual aortas were minced into 2 to 3 mm pieces, delipidated and dehydrated with acetone:absolute ethanol 1:1 (v/v) at 55°C for 1 hour; acetone:absolute ethanol 1:1 (v/v) at 26°C for 18 hours; and acetone at 26°C for 2 hours. All lipid extracts were pooled and used to determine the cholesterol concentration by the method described for plasma. The dehydrated aortic residue was dried over Silica gel in a vacuum desiccator and multiple measurements made to determine the dry weight.

**Preparation of Aortic GAG**

GAG were released from individual aortas enzymatically by treatment with papain (Worthington Biochemical Corporation, Freehold, New Jersey) (1 μg/mg dry aorta) in versene buffer (0.1 M sodium acetate; 0.05 M cysteine hydrochloride; 0.01 M disodium EDTA; 7:1:1 v/v/v) pH 6.0 at 65°C for 5 hours.

The optimum time for hydrolytic release of aortic GAG was determined in a study in which lipid-free aortic minces from rhesus monkeys were digested with papain as described above. At various times up to 48 hours, duplicate samples were removed and cold TCA added to a final concentration of 5%. The supernatants were diazylized and the uronic acid concentration measured. We determined that the maximum GAG release occurred following 3 to 5 hours of digestion.
**Purification of Aortic GAG**

After digestion, the samples were centrifuged at 1200 × g. The supernatant fraction was treated with cold trichloroacetic acid (TCA) at a final concentration of 5%. Following centrifugation, the supernatant fraction was exhaustively dialyzed to a final concentration of 0.5% with cetylpyridinium chloride (CPC). To insure complete CPC-GAG complex formation, the samples were maintained at 26°C for 24 hours. The CPC-GAG complexes were collected following centrifugation at 1400 × g. The GAG were isolated from CPC by precipitation following the addition of two volumes of absolute ethanol. The samples remained at 26°C for 24 hours, and the GAG were collected following centrifugation at 1400 × g. The GAG were washed sequentially in 90% ethanol, 95% ethanol, and absolute ethanol, then dried and dissolved in 1 ml water for subsequent chemical analyses. No corrections for GAG losses were made in calculating the total GAG isolated. These potential losses throughout the procedure are described in the following section.

**Measurements on the Recovery of GAG**

Because it was difficult to precisely quantitate the losses of GAG from single pigeon aortas, we used 14 additional 1-year-old RBWC pigeons in a study designed to assess GAG recovery throughout the procedures employed. Each pigeon was radiolabeled by i.v. injection (0.5 mCi) of Na$_2$SO$_4$ (New England Nuclear, Boston, Massachusetts) to enable us to monitor small losses during the isolation and purification procedure. Twenty-four hours after labeling, the left and right brachiocephalic arteries were collected, washed with 0.05 M Na$_2$SO$_4$, and minced to prepare a normal artery pool. Duplicate 25, 50, 100, and 200 mg wet weight samples were delipidated and the GAG isolated and purified according to the procedures described. The loss of $^{35}$S-GAG was monitored at several steps throughout the procedure. Where possible, uronic acid concentrations were also measured on the samples. In the eight samples examined, the final radioactivity of the purified GAG averaged 52,500 ± 3300 cpm/100 mg dry artery.

The lipid extract was dried completely, dissolved in and dialyzed against 0.05 M Na$_2$SO$_4$. No radioactivity was detected in the lipid extract. Based upon detection limits, the amount of sample examined, and the total GAG radioactivity, the potential losses were estimated to be <0.2%.

The residue (4% to 5% of the dry artery weight) that remained after papain treatment and centrifugation was dissolved in KOH; losses of GAG were calculated to be <0.4%. Following TCA treatment, the precipitate was dissolved in KOH and losses at this step averaged 2.5% ± 0.5% (mean ± SEM) on the basis of radioactivity. After the GAG were precipitated and removed as CPC-GAG complexes, significant amounts of radioactivity remained in the supernatant. Based upon radioactivity measurements, this represented a potential loss of 9.2% ± 1.0%. Some of the supernatant material was made to 2.0 M with NaCl to dissociate any CPC-GAG complexes not removed following centrifugation. The samples were centrifuged to remove the salt-precipitated protein that contained no radioactivity. The supernatant was dialyzed against 2 M NaCl for 6 hours and distilled water for 18 hours. Uronic acid analyses, based upon the levels determined in the final purified GAG, indicated potential losses of only 2.7%. The discrepancy between losses calculated using radioactivity and uronate concentrations were not resolved. The ethanol-salt supernatant remaining after the CPC-GAG complexes were dissociated and the GAG precipitated had radioactivity that indicated a loss of 0.3% ± 0.18%. The 90%, 95%, and absolute ethanol washes contained no detectable radioactivity. These potential losses were calculated to be <0.4%.

**Electrophoretic Separation of Individual GAG**

Electrophoresis of the GAG were carried out as described previously using a procedure based upon methods described by Seno et al. and Curwen and Smith. Our modifications enabled us to separate GAG from a single pigeon aorta. Quantitation is based on measurements of uronic acid concentration and calculation of the percentage of individual GAG separated on cellulose acetate. Cellulose acetate electrophoresis membranes (Beckman Instruments, Incorporated, Fullerton, California) were used as the support media. A multisample applicator and electrophoresis chamber (Model 373) were purchased from Instrumentation Laboratory, Lexington, Massachusetts. To adequately separate the individual GAG, it was necessary to use two different buffer systems. Calcium acetate (0.3 M, pH 10.0) was used to separate dermatan sulfate (DS) from chondroitin sulfate (C4-S and C6-S). Hyaluronic acid (HA) and heparan sulfate (HS) migrate to positions that overlap, while C4-S and C6-S are not effectively separated in either of the buffer systems. In this study, both are reported as chondroitin sulfate (CS). In the calcium acetate buffer system, the strips were
run at 8.0 mA/strip for 3 hours; in the cadmium acetate buffer system, the average was 4.4 mA/strip and the duration of the run was 1 hour. The strips were stained in 0.5% Alcian blue (in methanol/water/acetic acid, 5:5:1, pH 5.8) for 5 minutes, destained in several changes of methanol/water/acetic acid (10:9:1), and cleared in absolute ethanol/acetic acid (3:1). The percentages of HA, HS, DS, and CS (C4-S plus C6-S) were calculated from peak areas of the densitometric scans of the electrophoreograms using a Gelman ACD-18 densitometer (Gelman Instrument Company, Ann Arbor, Michigan) at a wavelength of 615 nm.

Since determination of the percentages of the individual GAG by densitometry is dependent upon the staining affinity of each GAG for the dye, we calculated the differences in dye binding as follows. Standard solutions of HA, HS, DS, C4-S, and C6-S (2 mg/ml) were applied to different positions of a cellulose acetate membrane. The membrane was stained, decolorized, and scanned as described above. When the five standard GAG solutions were compared, C4-S and C6-S were similar and had the greatest dye binding. When the integrated areas of C4-S and C6-S were both taken as 1.0, the relative dye binding of equal amounts of HA was 0.68, HS was 0.86, and DS was 0.91. Standard solutions at 1.5 and 1.0 mg/ml had similar dye-binding affinities. After mathematically combining the relative percentages from both buffer systems, the concentrations of individual GAG were calculated according to the general formulas: \( \mu g \) individual GAG per thoracic aorta equals (the total \( \mu g \) uronic acid per thoracic aorta multiplied by the percentage of GAG) and divided by 3%; the percentage of GAG equals the percent area from densitometric scans divided by the dye binding correction factor for the specific GAG.

**Identification of Individual GAG**

Individual GAG were identified by their similar electrophoretic mobilities as authentic GAG standards and their susceptibility to specific degradative enzymes. Standards of HA, DS, C4-S, and C6-S were purchased from Miles Laboratories, Incorporated, Elkhart, Indiana; HS was a generous gift from J. A. Cifonelli, University of Chicago. The enzymatic digestion of individual GAG for identification purposes was done by the method of Saito et al.\(^{28}\) using chondroitinase ABC (\textit{Proteus vulgaris}), chondroitinase AC-II (\textit{Arthrobacter aurescens}), hyaluronidase (bovine testicular and \textit{Streptomyces hyalurolyticus} nov. sp.) obtained from Miles Laboratories, Incorporated, Elkhart, Indiana. The digested samples were subjected to electrophoresis and examined as described.

**Analytical Methods**

Uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen.\(^{29}\) Glucuronic acid (2 to 100 \( \mu g/ml \)) was used as a standard, and unknowns were run in duplicate using one without m-hydroxydiphenol to monitor for interfering impurities. No interferences were detected, and absorbances of unknowns were used to calculate uronic acid concentration. The method of Blumenkrantz and Asboe-Hansen\(^{26}\) was used to measure the total hexosamine concentration. In the differential assay for glucosamine and galactosamine, we used the modified procedure of Wagner.\(^{31}\) Standards of 2 to 40 \( \mu g/ml \) (glucosamine HCl and galactosamine HCl, Sigma Chemical Company, St. Louis, Missouri) were used in both assays.

**Expression of Data and Analysis of Results**

All GAG chemical data were expressed both on the basis of concentration (mass/entire aorta) and content (mass/entire aorta). We used values for content consistently throughout the report to provide a measure of the absolute amount of GAG per aorta. The absolute amount was normalized to 500 gm of body weight to adjust for small individual differences in pigeon body weights. The expression is particularly useful in atherosclerosis research where one deals with arterial thickening. In expressing the data in these units, the numbers will not be affected by changes in other arterial components (e.g., collagen) that increase during atherosclerosis. This can occur if data are expressed in a relative or concentration manner (mass/unit weight, e.g., mg/g). However, since many investigators have previously reported data expressed on a concentration basis, for comparison we have included these numbers where appropriate.

**Results**

As we have seen in other studies of WC-2 and randomly bred White Carneau pigeons, the extent of atherosclerosis, as measured by visually estimating the surface of the aorta covered with raised atherosclerotic plaque, was significantly greater (p < 0.01) in WC-2 pigeons (figure 1). In addition, the diet-aggravated atherosclerotic plaques in the WC-2 pigeon were more severe, based upon a significantly greater accumulation of cholesterol. In WC-2 birds, the extent of atherosclerosis was 2.9 times greater and their cholesterol accumulation was 3.4 times greater than in RBWC pigeons. In most species of experimental animals, increased plasma cholesterol concentrations are associated with

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increased atherosclerosis. Both WC-2 and RBWC pigeons had similar levels of plasma cholesterol concentrations throughout the study (figure 2). Before the atherogenic diet was begun, the plasma cholesterol concentration in WC-2 birds was 304 ± 8 mg/dl (mean ± SEM), and in RBWC birds, 293 ± 7 mg/dl. Both groups showed increased plasma cholesterol concentrations after 1 month. Both WC-2 and RBWC pigeons showed a positive relationship (p < 0.001) between plasma cholesterol concentration (mean value for Months 2 and 3) and atherosclerosis severity (measured by aortic total cholesterol concentration, mg/gm wet aorta). The correlation coefficients were 0.533 for the 97 WC-2 birds and 0.520 for the 113 RBWC pigeons.

Significantly greater amounts of uronate and total hexosamine were measured in the aortas of WC-2 birds than in RBWC aortas (figure 3). Assuming that an average of 38% of the weight of the GAG is hexuronic acid,25 we determined the total GAG content of the WC-2 pigeon aortas to be 35% greater than that for RBWC pigeon aortas. In both groups of pigeons, uronate-to-hexosamine ratios were similar, about 1:1. The majority of the hexosamine in both groups of pigeons was galactosamine, indicating a predominance of GAG types such as chondroitin sulfate and dermatan sulfate (figure 3).

To compare aortic GAG content in WC-2 and RBWC pigeons in a manner that would adjust for the differences in extent and severity of atherosclerosis, we sorted the animals in each group on the basis of the content of their aortic cholesterol. From each population three groups were formed: animals with minimal, moderate, or severe atherosclerosis as measured by cholesterol accumulations (table 1). The procedure for forming these categories was to select three groups of WC-2 pigeons, then match each pigeon with a RBWC pigeon having a similar aortic cholesterol concentration. Restrictions included few WC-2 aortas with very low aortic cholesterol concentrations and few aortas from RBWC with high aortic cholesterol concentrations. About 35% of the available pigeons were successfully matched for comparisons. The pigeons with minimal atherosclerosis had about 2 to 3 times the level of cholesterol found in the White Carneau pigeon without the presence of grossly visible atherosclerosis.32 After the pigeons were grouped, no major differences in
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Table 1. Atherosclerosis Characteristics of White Carneau (WC-2) and Randomly Bred White Carneau (RBWC) Pigeons Formed from the Entire Experimental Group

<table>
<thead>
<tr>
<th>Atherosclerosis severity</th>
<th>WC-2</th>
<th>RBWC</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/aorta/500 g body weight</td>
<td>4.0 ± 1.2</td>
<td>10.6 ± 1.0</td>
</tr>
<tr>
<td>mg/g wet aorta</td>
<td>15.8 ± 1.0</td>
<td>25.3 ± 1.1</td>
</tr>
<tr>
<td>% intimal surface covered</td>
<td>4.1 ± 1.1</td>
<td>7.3 ± 1.5</td>
</tr>
</tbody>
</table>

All values are means ± SEM.

Table 2. Aortic Glycosaminoglycan Content in White Carneau (WC-2) and Randomly Bred White Carneau (RBWC) Pigeons

<table>
<thead>
<tr>
<th>GAG content</th>
<th>WC-2 (n = 18)</th>
<th>RBWC (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mg lipid-free dry wt</td>
<td>µg/aorta/500 g body wt</td>
<td>% Total</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>1.06 ± 0.02*</td>
<td>16.5 ± 1.9</td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>1.52 ± 0.11*</td>
<td>23.5 ± 1.7*</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>2.04 ± 0.20*</td>
<td>31.7 ± 3.1*</td>
</tr>
<tr>
<td>Chondroitin sulfate (C4-S + C6-S)</td>
<td>7.70 ± 0.45</td>
<td>119.4 ± 7.0</td>
</tr>
</tbody>
</table>

Values are means ± SEM of WC-2 and RBWC pigeons selected to have low aortic cholesterol contents of about 0.45 to 0.60 mg/aorta/500 g body wt.

*p < 0.05 between WC-2 and RBWC.
The changes in aortic GAG in WC-2 and RBWC pigeons with minimal, moderate, and severe atherosclerosis are illustrated in figure 5. At any given level of aortic cholesterol, larger amounts of GAG were found in all WC-2 aortas compared to RBWC aortas. Statistically significant (p < 0.05) differences between the two groups were seen for DS at all levels of atherosclerosis. In addition, HS was higher in WC-2 birds than in RBWC pigeons with minimal atherosclerosis. Significant increases were seen in aortic DS as the aortic cholesterol content increased in WC-2 birds but not in RBWC pigeons. The HA content decreased significantly in RBWC pigeon aortas as the cholesterol content increased. A similar trend was suggested in WC-2 pigeons. These differences were equally evident when the data were expressed as concentration (μg/mg lipid-free dry artery). The DS concentrations for the minimal, moderate, and severe groups were respectively 1.52 ± 0.11, 1.94 ± 0.26, and 1.62 ± 0.19 for WC-2 birds as compared with 1.07 ± 0.09, 1.05 ± 0.08, and 1.21 ± 0.15 (mean ± SEM) for RBWC birds. The HA concentrations were 1.06 ± 0.02, 0.59 ± 0.13, and 0.59 ± 0.13 for WC-2 birds compared with 0.77 ± 0.07, 0.60 ± 0.07, and 0.41 ± 0.04 for RBWC. Very small changes occurred in aortic HS and CS in both groups as their aortic cholesterol content increased.

Discussion
We previously reported that WC-2 pigeons have no demonstrated differences in the commonly known risk factors for the development of atherosclerosis, and we assumed that the atherosclerosis was influenced by genetic factors operative at the arterial wall level. Since recent evidence has implicated arterial wall proteoglycans in atherosclerosis development, we examined the aortic GAG in the WC-2 pigeon in an attempt to define a possible mechanism by which the exacerbated atherosclerosis of the WC-2 birds might be explained. The PG macromolecules are the major constituents of the intercellular matrix within which collagen, elastic fibers, and the smooth muscle cells are embedded. These macromolecules not only occupy a large physical domain and influence the flux of macromolecules through the arterial intercellular space, but they also behave as polyelectrolytes and thus can ionically bind plasma constituents such as lipoproteins.

In this study, the content of aortic total GAG was significantly greater in WC-2 pigeons than in RBWC pigeons. Several investigators have reported alterations in arterial GAG during the progression of atherosclerosis in several species, including Macaca mulatta, pigeons, and humans, and it was not surprising to have differences between WC-2 and RBWC birds due to the
increased severity of atherosclerosis in the WC-2 pigeon. We saw no major differences in the types of GAG present in the aortas of the WC-2 pigeon as compared to human aortas\textsuperscript{36, 40-42} or to rhesus monkey aortas\textsuperscript{24} where the major constituent is CS with lesser amounts of DS, HS, and HA.

Since we did not know if the increased GAG in the WC-2 pigeon was simply a result of increased atherosclerosis, we sorted the pigeons into several matched groups having equivalent aortic cholesterol contents. Following this subdivision, both groups showed decreased levels of HA and increased levels of the sulfated GAG with the progression of atherosclerosis. The contrasting point that distinguished the changes in WC-2 aortas was a significantly greater accumulation of GAG in animals at any level of atherosclerosis.

The levels of DS were 30\%, 101\%, and 53\% higher than in RBWC birds with minimal, moderate, and severe atherosclerosis. These findings are consistent with the reports of increases in DS in the monkey and human atherosclerotic plaque.\textsuperscript{24, 38, 39}

A possible mechanism involving DS may be through a binding and retention of plasma lipoproteins. It has been reported that DS binds lipoproteins, specifically LDL and VLDL.\textsuperscript{11, 43} Using conditions of physiologic pH and ionic strength, Iverius\textsuperscript{11} investigated the binding of plasma lipoproteins to different GAG attached to agarose gels and found that VLDL and LDL were bound to gels containing heparin, DS, HS, and C4-S under physiologic (low ionic) conditions. In contrast, HDL did not bind to the gels at any ionic strength. The binding affinity for each of the GAG with lipoprotein was assessed by measuring the ionic strength necessary to release the lipoproteins from their association with the GAG. For the release of half the amount of VLDL or LDL bound to heparin, DS, HS, and C4-S, respective ionic strengths of 0.26, 0.15, 0.09, and 0.08 were required. Iverius suggested that because of its strong binding affinity to LDL, DS could be one of the factors responsible for lipid deposition in artery walls during the process of atherogenesis.\textsuperscript{11, 33}

Although Iverius\textsuperscript{11} indicated that the binding affinities of GAG for lipoproteins were dependent on the type of GAG and not on the presence of divalent cations, other studies by Srinivasan et al.\textsuperscript{43} have shown that divalent cations increase the stability of the LP-GAG complex. In these studies, preferential binding occurred with L-iduronate-containing GAG. The order of affinities were heparin > DS > HS > CS > HA.

In addition to the ionic binding of LDL to GAG, other effects of proteoglycans on lipoprotein behavior such as a molecular sieving and steric exclusion effect have been described.\textsuperscript{30} These two effects on lipoproteins are dependent upon the total extent of the molecular network formed by the proteoglycan in the intercellular matrix. Our present study demonstrates that aortas in the WC-2 pigeon have greater amounts of each GAG when compared to RBWC aortas. This increased GAG content may provide for a greater sieving and exclusion phenomenon and could result in changed physical properties of plasma-derived lipoprotein within the artery wall.\textsuperscript{33}

Although in vitro studies have implicated DS as a potential binder of plasma lipoprotein, studies of lipoprotein-GAG complexes extracted from fatty streaks and fibrous plaques have not borne this out.\textsuperscript{12-15} In one study, Srinivasan et al.\textsuperscript{14} identified the individual GAGs that were associated with the extracted LDL-GAG and VLDL-GAG complexes of fibrous plaques. The GAGs were largely HA (90,000 MW), C4-S, and a very small amount of heparin. It is possible that if DS-LDL complexes were present they may not have been extracted under the conditions employed by the authors. In further studies using normal bovine aorta, Radhakrishnamurthy et al.\textsuperscript{44} have shown that DS is usually extractable after the use of more drastic measures such as elastase treatment.

An interesting finding in the present study was the decreased content of aortic HA associated with increased aortic cholesterol content in both RBWC and WC-2 pigeons. One of the functions of HA in connective tissues is its ionic association with PG monomers to form high molecular weight complexes or aggregates.\textsuperscript{45-48} Although these complexes have been mostly studied in cartilage, reports suggest that this type of macro-molecular structure is also common to artery.\textsuperscript{49, 50} The in situ significance of this structure is speculative but it has been suggested that the PG-HA aggregate is needed to retain PG monomers within the artery wall. It has not been determined whether the PG monomer containing DS in pigeon aortas is capable of associating with HA. However, if this does occur, it suggests that, with reduced HA and increased DS, the HA-DS-containing PG aggregate has been altered in WC-2 pigeons when they are compared with RBWC pigeons. If this modified PG is responsible for increased permeability of the artery and increased binding of plasma LDL, it may be an important factor in determining the rate at which atherosclerosis progresses in WC-2 pigeons.

Acknowledgments

The authors acknowledge the assistance of J Connor and L. Ellis in preparing the manuscript, and the gift of heparan sulfate by Dr. J. A. Cifonelli, University of Chicago (NIH Contract NO1-AM-5-2205).
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Index Terms: glycosaminoglycans • atherosclerosis • WC-2 pigeons • cholesterol
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doi: 10.1161/01.ATV.1.3.192

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