Specificity of the Low Density Lipoprotein–Glycosaminoglycan Interaction

T. Sambandam, J.R. Baker, J.E. Christner, and S.L. Ekborg

There is ample documentation of the binding of chondroitin sulfate/dermatan sulfate proteoglycans to low density lipoprotein (LDL) both in vivo and in vitro. The interaction of these two species may be an early and important step in atherogenesis. Therefore, there is interest in defining the features of both molecules that are critical for their interaction. We employed a recently described competitive microassay that measures initial binding of proteoglycan to immobilized LDL. We confirmed the work of others that it is the apolipoprotein B component and, at least in part, a heparin-binding domain of LDL that are responsible for binding chondroitin sulfate/dermatan sulfate proteoglycans. The principal thrust of our study was concerned with the effects of a glycosaminoglycan's degree of sulfation on the binding to LDL. Initial experiments comparing dermatan sulfate and chondroitin sulfate proteoglycans indicated that the former was more efficient at binding LDL than the latter and that oversulfation, rather than chain length or iduronate content, was the preeminent feature involved. Additional binding studies with dermatan sulfate, chemically sulfated chondroitin-4-sulfate, and naturally occurring oversulfated chondroitin sulfates indicated that the degree of sulfation, not the position of sulfation, determined affinity for LDL. These results suggest that studies should be undertaken to determine whether oversulfated segments of glycosaminoglycans are especially involved in associations with LDL, leading to lipid accumulation, in the artery wall. (Arteriosclerosis and Thrombosis 1991;11:561–568)

Evidence for the existence, in vivo, of low density lipoprotein (LDL)–proteoglycan complexes has been obtained by biochemical and histochemical studies of aortic lesions. Studies of fixed tissue sections with histochemical stains, immunofluorescence, and autoradiography have shown glycosaminoglycans (GAGs) and LDL in close association in atherosclerotic lesions. Also, complexes containing chondroitin-6-sulfate, hyaluronic acid, cholesterol, and immunoreactive apolipoprotein B (apo B) have been isolated from fatty streaks and fibrous plaques.

Positive correlations between proteoglycan content and increasing degrees of atherosclerosis in the artery wall have been demonstrated by many workers. For example, during progression of atherosclerosis in hypercholesterolemic monkeys, the involved arteries exhibited increased GAG content. In postmortem samples from humans, it has been shown that chondroitin sulfate increases preferentially during fatty streak formation. Other studies have indicated that dermatan sulfate exhibits the greatest increase in human atherosclerotic lesions. At present, there is little understanding of the factors that control these different responses.

The in vitro interaction between LDL and aortic proteoglycan has been demonstrated and studied by many workers. It is clear that chondroitin sulfate/dermatan sulfate proteoglycans from aortas form both soluble and insoluble complexes with LDL. Free GAGs bind LDL more weakly than do their parent proteoglycans when the interaction is moni-
tored by a precipitation assay. By contrast, the GAG is seen to be the major determinant of binding when the initial binding of GAG-proteoglycan to immobilized LDL is assayed. Desulfation of the GAG abolishes the interaction. Clearly, sulfate ester groups are important for the interaction.

Not only are there different types of GAGs (e.g., chondroitin sulfate, dermnan sulfate, and keratan sulfate), but also their chains are rarely composed of identically substituted disaccharide repeats. A major source of variation is the extent and pattern of sulfation, and in some cases, a unique sulfation pattern will allow a given GAG to perform a very specific function (e.g., antithrombin III binding by a region of the heparin molecule). The present study was undertaken to better define the features, especially the sulfation of proteoglycan–GAGs, which is important for LDL binding.

Methods

Materials

N-hydroxysuccinimimidobiotin, bovine serum albumin (catalog No. A-7030), alkaline phosphatase–conjugated avidin, and p-nitrophenyl phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Formamid, chlorosulfonic acid, and pyridine were from Aldrich Chemical Co., Milwaukee, Wis. The pyridine was kept over Drierite (Hammond Drierite Co., Xenia, Ohio). Anti-apo B (sheep anti-human apo B, globulin fraction) and anti-apo A-II (sheep anti-human apo A-II, globulin fraction) were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

A preparation of human plasma LDL was isolated as described. Briefly, serum was centrifuged in a three-step density gradient of KBr in the presence of a protease–antioxidant mixture. Recovered fractions that contained LDL were pooled and recentrifuged in a shallower gradient. The final preparation was dialyzed exhaustively at 4°C against Dulbecco's phosphate-buffered saline (with 1 mM EDTA and without Ca2+ and Mg2+). Analyses of such preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed a single band with a mobility coincident with that of apo B-100. Lipoprotein preparations typically contained approximately 2 mg protein/ml. A synthetic hexadecapeptide (residues 3,144–3,159 of LDL) was a generous gift from G. Anantharamaiah, University of Alabama at Birmingham.

Proteoglycan monomers from bovine nasal cartilage and from rat chondrosarcoma were isolated as described previously. Free chondroitin sulfate chains were isolated from bovine nasal cartilage and rat chondrosarcoma proteoglycans after treatment with papain. A preparation of proteoglycans from human aorta was obtained by a previously outlined procedure. The aortic proteoglycans were further fractionated by gel chromatography on Sepharose CL-2B to give a partial separation of chondroitin sulfate and dermatan sulfate proteoglycans (see Figure 2 and Table 1). Dermatan sulfate, isolated from porcine mucosa, was obtained from Calbiochem Corp. (San Diego, Calif.). Chondroitin sulfate D and chondroitin sulfate E were purchased from Seikagaku America, Inc. (Rockville, Md.). Heparin (catalog No. H-3125) was from Sigma. For the preparation of buffers, reagent-grade chemicals were used.

Biotin-Conjugated Proteoglycans

The preparation from bovine nasal cartilage proteoglycan was as described previously. The proteoglycan–biotin, at approximately 10 mg/ml in 0.14 M NaCl/0.01 M NaH2PO4, pH 7.4, was divided into aliquots and stored at –20°C.

Assay for Low Density Lipoprotein–Proteoglycan Interaction

The method employed has been published recently. In outline, LDL was immobilized in the wells of a 96-well microtitration plate, and the plate was blocked by incubation with 1% nonfat dry milk. Potential inhibitors of the interaction were allowed to compete with a limiting amount of proteoglycan–biotin for binding to the LDL. Subsequent incubation with alkaline phosphatase–conjugated avidin was followed by addition of the phosphatase substrate, p-nitrophenyl phosphate. The enzyme reaction was stopped by addition of 5 M NaOH when color development in wells showing maximum color had reached an absorbance of approximately 0.8 at 405 nm (usually after 30–40 minutes).

Chemical Sulfation

Both rat chondrosarcoma proteoglycan and chondroitin sulfate were chemically sulfated by the method of Inoue et al. The proteoglycan or chondroitin sulfate (20 mg) was dissolved in dry formamide (1 ml). Then, pyridine (200 μl) and chlorosulfonic acid (41 μl) were added, the vessel was closed, and the mixture was stirred at room temperature. After 8 hours, methanol (7.5 ml) at 0°C was added with stirring to precipitate the pyridinium salts of sulfated proteoglycans or GAGs. Precipitation was completed by leaving the mixture at 0°C for 2–4 hours before centrifugation. After the supernatant was discarded, the precipitate was dissolved in H2O (2.5 ml), and sodium acetate was added to bring the pH to 8.0. A small amount of insoluble material was removed by centrifugation before ethanol was added (three volumes) to precipitate the sodium salts or proteoglycan–GAG. The precipitating mixture was left at 0°C overnight before collection of the precipitate by centrifugation. The precipitate was washed with ethanol/0.1 M aqueous sodium acetate (9:1, vol/vol) and then absolute ethanol before it was dried and stored in a desiccator. Typically, the yield was 12–15 mg.

Established procedures were employed for the estimation of uronate and sulfate in proteoglycan and for protein in lipoprotein preparations.
Results

In previously published work,\textsuperscript{22} it was shown that LDL and lipoprotein (a) but not high density lipoprotein could inhibit the LDL/proteoglycan-biotin interaction. From these findings, one can reasonably suggest that apo B may be essential for the interaction. It is difficult to test this probability directly owing to the insolubility of apo B, so instead, we chose to determine the ability of an anti-apo B antibody to inhibit binding of proteoglycan-biotin. As shown in Figure 1A, binding was inhibited 50% by a 1:200 dilution of this antibody. At a comparable concentration of an anti-apo A-II, inhibition was very low (=5%). Further evidence for the binding of apo B to proteoglycan-biotin was obtained through studies with a synthetic hexadecapeptide. This peptide has a sequence identical to that of residues 3,144–3,159 of human apo B (Figure 1, legend) and encompasses a heparin-binding sequence.\textsuperscript{33} When employed as an inhibitor of the LDL/proteoglycan-biotin interaction, it was more effective than LDL on the basis of protein concentration (Figure 1B). Therefore, the same kind of sequence that is involved in heparin binding, which includes many basic residues, may also be required for chondroitin sulfate proteoglycan binding. Other evidence in support of this conclusion has been reported.\textsuperscript{34}

The assay we employed for measuring LDL–proteoglycan binding can be used for examining the inhibitory effects of different proteoglycans or GAGs, as well as lipoproteins, on the interaction. At present, it is not clear, and there are conflicting results concerning the features of proteoglycans that are necessary for LDL binding. When proteoglycans from human aortas were fractionated according to size on a column of Sepharose CL 2B, they were (as...
TABLE 1. Analyses of Human Aortic Proteoglycans

<table>
<thead>
<tr>
<th>Protoglycan</th>
<th>Chondroitin-6-sulfate (%)</th>
<th>Chondroitin-4-sulfate (%)</th>
<th>Dermatan Sulfate (%)</th>
<th>Sulfate/uronic acid (molar ratio)</th>
<th>Molecular weight (kd)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human aortic PGs</td>
<td>55</td>
<td>26</td>
<td>19</td>
<td>0.83</td>
<td>ND</td>
</tr>
<tr>
<td>Pool 1</td>
<td>65</td>
<td>31</td>
<td>4</td>
<td>ND</td>
<td>687</td>
</tr>
<tr>
<td>Pool 2</td>
<td>36</td>
<td>24</td>
<td>40</td>
<td>ND</td>
<td>288</td>
</tr>
</tbody>
</table>

PG, proteoglycan; ND, not determined.

*Molecular weights of PG preparations were estimated from their elution behavior (Kav) from Sepharose CL-2B as described by Ohno et al.*

measured by a Dimethylmethylene Blue assay) broadly included across the fractionation range of this gel (Figure 2). There was little indication of separation between chondroitin sulfate proteoglycan and the class of smaller dermatan sulfate proteoglycans present in the aortas. Assay of fractions for inhibitory activity in the LDL–proteoglycan binding assay gave an activity peak more included than that of total proteoglycan (Figure 2). This finding is certainly suggestive of greater binding by the smaller, relatively dermatan sulfate–rich proteoglycan. Fractions were pooled: pool 1, fractions 23–28; pool 2, fractions 33–39 and the proteoglycans recovered by precipitation with ethanol (three volumes). Analyses of the pools to determine their GAG contents are reported in Table 1. It is clear that the proteoglycans of pool 1 are relatively enriched in chondroitin-6-sulfate and that those of pool 2 are enriched in dermatan sulfate. Assay of dilutions of each pool for inhibition of LDL/proteoglycan–biotin binding confirms much greater inhibition (on the basis of uronate content) by the dermatan sulfate proteoglycan–rich fraction (i.e., pool 2, Figure 3). The aortic proteoglycans are, in turn, more effective than cartilage proteoglycans as inhibitors of LDL–proteoglycan interaction. Even the aortic chondroitin sulfate proteoglycan, unlike cartilage proteoglycan, contains some dermatan sulfate. Possession of this GAG or some sequence within it may be an important factor for inhibition of LDL–proteoglycan binding.

Further work has had the purpose of revealing the features of dermatan sulfate structure that are important for LDL binding. Thus, dermatan sulfate was fractionated by ion-exchange chromatography on DEAE-Sephadex with a salt gradient. The dermatan sulfate eluted as a broad peak, as monitored by the carbazole assay for uronic acid (Figure 4A). Aliquots of fractions after digestion with chondroitinase ABC were assayed for ΔDi-4S (Figure 4B). This disaccharide, which represents the major repeat disaccharide of dermatan sulfate, was measured in a broad peak but had a maximum concentration in fraction 13. Oversulfated disaccharides, principally ΔDi-diS, were also measured and were present mainly in later fractions (i.e., maximally in fraction 16). It was expected that fractionation of dermatan sulfate on DEAE-Sephadex would proceed primarily according to charge (i.e., later elution with increased negative charge). The elution of oversulfated segments of dermatan sulfate, as measured by the concentration of ΔDi-diS, at later times than most ΔDi-4S is consistent with this expectation. Assay of fractions for inhibition of LDL–proteoglycan interaction revealed high activities in fractions 14–19 but with maximal activity (i.e., minimum IC50) in fraction 16 (Figure 4C). This is also the fraction containing dermatan sulfate chains with the highest level of oversulfated residues. This could also be the fraction with the maximal iduronate/glucuronate ratio, as oversulfation of dermatan sulfate occurs at residues...
we examined the effect of chemically further sulfating the chondroitin sulfate chains of rat chondrosarcoma proteoglycan. The sulfation procedure and the method of product recovery are outlined in "Methods." Disaccharide analysis of the product, termed proteoglycan_{sx}, after digestion with chondroitinase ABC indicated that 6% ΔDi-diS residues had been introduced, whereas the untreated proteoglycan from this chondrosarcoma contained 86% ΔDi-4S and 14% ΔDi-0S but no oversulfated disaccharides\(^3\)7 (see also Figure 5). When proteoglycan_{sx} was tested as an inhibitor of the LDL–proteoglycan interaction, its activity was found to be dramatically enhanced over that of the parent proteoglycan (Figure 6). Likewise, chemical sulfation of the free chondroitin sulfate chains from rat chondrosarcoma caused a considerable increase in their inhibitory activity. These results clearly support the notion that the level of sulfation of a GAG is an important determinant of its ability to bind LDL.

Whether the positions of sulfate substitution along the GAG chain are critical for LDL binding is uncertain. Comparing the LDL binding of some GAGs with different positions of sulfate substitution can suggest an answer to this question. When inhibition of the interaction by chondroitin sulfate, chondroitin sulfates D and E, and heparin was examined (Figure 7), one major difference was seen among them. Inhibition by bovine nasal cartilage chondroitin sulfate (1_{50}=8.5 μg/ml) was less than that produced by the other more highly sulfated GAGs (1_{50}=0.075–0.15 μg/ml). Despite differences in disaccharide repeat structure (chondroitin sulfates D and E compared with heparin; see Figure 5) and positions of sulfate substitution (chondroitin sulfate D compared with E), the oversulfated GAGs show similar high degrees of inhibition of LDL–proteoglycan binding (Figure 7).

It is worthy of note that similar levels of inhibition in this system are given by bovine nasal cartilage proteoglycan and bovine nasal cartilage chondroitin sulfate (Figure 7). Using this binding assay, we have consistently seen little difference between the binding of a GAG and its parent proteoglycan. It appears that the initial event in proteoglycan binding is association of GAG chains with LDL and only subsequently a less reversible interaction involving the proteoglycan’s protein core. Essentially, our assay measures the initial GAG binding event.

**Discussion**

Previous studies have indicated that binding of proteoglycans to LDL is via the protein moiety of the
Figure 5. Sketch showing disaccharide repeat structures of chondroitin sulfates, dermatan sulfate, and heparin. Panel A: Disaccharide repeats of chondroitin sulfates. Disaccharides \(\Delta Di-4S\) and \(\Delta Di-6S\) are major components of most chondroitin sulfates. Only low levels of oversulfated disaccharides (\(\Delta Di-diS_D\) and \(\Delta Di-diS_e\)) are generally present, the exceptions being chondroitin sulfates D and E. Panel B: Major and minor disaccharide repeats of dermatan sulfate are \(\Delta Di-4S\) and \(\Delta Di-diS_B\), respectively. Panel C: Only the characteristic trisulfated disaccharide repeat of heparin, termed \(\Delta Di-triS\), is shown.

The relatively smaller human aortic dermatan sulfate proteoglycan was found to compete more efficiently than chondroitin sulfate proteoglycan from the same source in the LDL-proteoglycan interaction. In earlier work,²² it was found that dermatan sulfate was considerably more effective than chondroitin sulfate as an inhibitor in this system. Dermatan sulfate possesses regions of oversulfation,³⁹ and these may be responsible for the binding. In a subsequent experiment, chondroitin sulfate from rat

Figure 6. Line plot showing inhibition (%) of low density lipoprotein/proteoglycan-biotin binding by oversulfated rat chondrosarcoma proteoglycan and chondroitin sulfate. Curves are shown for inhibition of binding by rat chondrosarcoma proteoglycan (○), rat chondrosarcoma proteoglycan \(m\) (●), rat chondrosarcoma chondroitin sulfate (■), and chondroitin sulfate \(m\) (□). Disaccharide compositions of proteoglycan preparations were as shown in Table 2. SX, product after sulfation.
chondrosarcoma, which contains no oversulfated disaccharide sequences\(^3\) and has little or no ability to bind LDL (Figure 6), was further sulfated chemically. The product showed considerably enhanced competition in the LDL-proteoglycan binding assay. Therefore, the degree of sulfation is important for LDL binding by proteoglycan.

Three GAGs with levels of sulfate substitution of more than two per repeat disaccharide occur naturally: chondroitin sulfates D and E and heparin. These same three GAGs bind LDL more effectively than does a GAG of lesser sulfation (Figure 7). Although most chondroitin sulfate chains bear only a low proportion of oversulfated residues (\(\approx 1-2\%\)), such residues could be disproportionately involved in LDL binding. By contrast, the chondroitin sulfate from rat chondrosarcoma, which possesses no oversulfated disaccharides,\(^3\) exhibits no capacity for binding to LDL (Figure 6).

GAGs isolated from aortas have been reported to contain oversulfated disaccharide repeat sequences.\(^4\)

Their cellular origin has not been investigated, although the macrophage must be a candidate, as some macrophage cell lines synthesize an oversulfated chondroitin sulfate, chondroitin sulfate E.\(^41\) Neither is it known how the oversulfated species are distributed in the artery wall. The increasing availability of antibodies with specificities for particular oversulfated segments of GAGs\(^42,43\) will facilitate future investigations of this question. It is quite probable that regions of relatively high concentration of proteoglycans that bear such GAGs will tend to bind LDL most avidly and could be the foci of fatty streak formation.

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