The Interaction between Human Plasma Lipoproteins and Connective Tissue Glycosaminoglycans

PER-HENRIK IVERIUS
From the Institute of Medical Chemistry, University of Uppsala, S-751 22 Uppsala, Sweden

SUMMARY

The interaction between human plasma lipoproteins and glycosaminoglycans has been studied by use of gels consisting of cross-linked hyaluronic acid and of sulfated glycosaminoglycans covalently attached to agarose (Sepharose 4B). When chromatographed on a column of granulated hyaluronic acid gel at physiological pH and ionic strength, very low density (VLDL), low density (LDL), and high density (HDL) lipoproteins were shown to emerge with the void volume, indicating that no significant binding to the polysaccharide had occurred. Equilibration of the same lipoproteins with glycosaminoglycan-substituted agarose gels at pH 7.4 showed that VLDL as well as LDL were bound to gels containing heparin, dermatan sulfate, heparan sulfate, and chondroitin 4-sulfate, provided the ionic strength was sufficiently low. In contrast, HDL and acetylated specimens of VLDL and LDL did not bind to the gels at any ionic strength. The observed interactions have been interpreted as an ionic binding of positively charged amino groups on apolipoprotein B to negatively charged groups on the glycosaminoglycans.

The range of ionic strength at which each lipoprotein was released from a polysaccharide was relatively narrow and dependent on the type of polysaccharide. For the release of half the amounts of VLDL, or LDL, bound to gels of heparin, dermatan sulfate, heparan sulfate, and chondroitin 4-sulfate, ionic strengths of 0.26, 0.15, 0.09, and 0.08, respectively, were required. The results were interpreted in terms of an electrostatic binding between polyvalent anionic and cationic sites with the charge density of the polysaccharide being an important parameter for the strength of the ionic bond. At equal charge density, polysaccharides containing L-iduronic acid seemed to interact more strongly than those containing D-glucuronic acid.

Although the genesis of atherosclerosis is far from understood, certain causative factors as well as prominent features of the pathological process are known. Thus, the accumulation of lipids in the arterial intima seems to be a crucial step (1). The origin of the lipids is unclear, but it seems probable that they derive from plasma in which they are constituents of the lipoproteins. Histochemical evidence, obtained by the immunofluorescence technique, suggests that β-lipoprotein antigens are present in areas of the arterial intima, where extracellular lipid can be detected by fat stains (2, 3).

When it was discovered that β-lipoproteins could form complexes with sulfated polysaccharides (see e.g. Reference 4), interest was also directed towards the sulfated glycosaminoglycans and their possible role in the lipid accumulation. Most studies hitherto performed have been based on electrophoretic or turbidimetric methods (5–8). The biological significance of the cited work is questionable since physiological ionic conditions were not used. Furthermore, fractionated connective tissue glycosaminoglycans were studied only to a limited extent.

In the present study the interaction between glycosaminoglycans and human plasma lipoproteins has been investigated in more detail, and with particular regard to the effect of physiological pH and ionic strength. A novel method for interaction studies has been employed.

EXPERIMENTAL PROCEDURE

Materials—Fresh human blood, to which had been added 20% (v/v) of acid citrate dextrose (Acedex, Pharmacia AB, Uppsala, Sweden) as anticoagulant, was supplied by the courtesy of the Blood Center at the University Hospital in Uppsala. No selection was made with regard to blood groups.

Bovine serum albumin was purchased from Poviet Producten N. V., Amsterdam, Holland. Acetic anhydride was obtained from E. Merek AG, Darmstadt; agarose and antisera were obtained from Behringwerke AG, Marburg-Lahn in West Germany. Polyethylene glycol (PEG 20M) was supplied by Kebo AB, Stockholm, Sweden and a high molecular weight dextran fraction (FDR 1197, weight average molecular weight 12 × 10^6) by Pharmacia AB, Uppsala, Sweden.

Solid phase glycosaminoglycans were obtained in two different ways. The sulfated glycosaminoglycans, which have a serine or a peptide residue in their link region and thus a free amino group (9), were covalently attached to agarose gels (Sepharose 4B) activated by cyanogen bromide (10). The batches used in the present work have been described previously (11). They contained pig intestinal mucosal heparin (Hep-II-Sep. 4B-V), human aortic heparan sulfate (HS II Sep. 4B I), bovine aortic dermatan sulfate (DS-III-Sep. 4B-II), and bovine...
aortic chondroitin 4-sulfate (CS-I, Seph. 4B-III). A reference
gel contained covalently linked ethanolamine. Some analytical
data of the gels and their constituent glycosaminoglycans are
presented in Table I. Hyaluronic acid, which has no demon-
strable link region (9), was provided as a cross-linked, granulated
gel by Dr. K. Hellsing of this Institute. The polymer con-
A. Gustafson, personal communication.

The LDL was isolated at d 1.063 and recentrifuged consecu-
tively at d 1.006 followed. Plasma was obtained from blood by centrifugation at 3500 X g for 15 min at 4°C. After further separation at d 1.20 for 24 hours in rotor 50.1, the top fraction containing the lipoproteins was concentrated in a dialysis bag by submersion in dry polyethylene glycol. Thus, the lipoproteins from 1.6 liters of blood were concentrated in a dialysis bag by submersion in dry polyethylene glycol. Thus, the lipoproteins from 1.6 liters of blood were

* The abbreviations used are: LDL, low density lipoproteins (d 1.019 to 1.063 g cm⁻³); VLDL, very low density lipoproteins (d < 1.006 g cm⁻³); HDL, high density lipoproteins (d 1.063 to 1.215 g cm⁻³); HDEL, high density lipoproteins (d 1.125 to 1.20 g cm⁻³).

** Critical density lipoprotein.

A. Gustafson, personal communication.
TABLE II
Chemical composition of lipoprotein fractions

Values are expressed as percentage of the sum of all constituents analyzed.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Phospholipid</th>
<th>Triacylglycerol</th>
<th>Cholesterol-ester</th>
<th>Free cholesterol</th>
<th>Total cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>7</td>
<td>20</td>
<td>51</td>
<td>16</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Acetylated VLDL</td>
<td>5</td>
<td>19</td>
<td>69</td>
<td>6</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>LDL</td>
<td>15</td>
<td>24</td>
<td>9</td>
<td>43</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>15</td>
<td>28</td>
<td>12</td>
<td>37</td>
<td>8</td>
<td>43</td>
</tr>
<tr>
<td>HDL₄</td>
<td>42</td>
<td>30</td>
<td>8</td>
<td>18</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

a Determined by the Lowry method against a standard of bovine serum albumin and corrected for the lower color yield of this standard as compared to that of the lipoprotein (17).

b Calculated as lipid phosphorus × 25.
c Calculated as triolein.
d Calculated as cholesteryl oleate.

The HDL₄ remained in solution after removal of HDL₃ by centrifugation at d 1.125 for 24 hours. The former fraction was finally purified by another centrifugation for 24 hours at d 1.20.

The lipoprotein fractions were stored at 4°C after dialysis against 0.15 M NaCl containing EDTA (0.1 mg per ml). Thimerosal was also added (0.1 mg per ml) after the chemical analysis of the fractions had been performed.

Acetylation of LDL and VLDL—The acetylation procedure of Fraenkel-Conrat (18) was adopted. LDL (190 mg in 2.5 ml) or VLDL (183 mg in 3 ml) were mixed with equal volumes of saturated sodium acetate and chilled in an ice bath. Acetic anhydride (50 μl) was added four times at 15-min intervals. Fifteen minutes after the last addition, the lipoproteins were dialyzed against the appropriate buffer for the experiments.

Gel Chromatography—A granulated gel of cross-linked hyaluronic acid was packed into a column (1.1 x 18.5 cm) and equilibrated with 3 mM veronal-1 mM EDTA-HCl buffer (pH 7.4) containing 0.151 M NaCl (ionic strength 0.160). The void volume (V₀) and the total volume of the column were determined after the elution pattern of dextran and tritiated water, respectively (27, 28). VLDL (4.6 mg), LDL (7.6 mg), or HDL₄ (7.1 mg) were applied in a volume of 0.1 ml and were then eluted at a flow rate of 5.2 ml per hour. Effluent fractions of 0.6 to 0.7 ml were collected and analyzed for protein by measuring the absorbance at 280 nm after dilution with buffer (1:1).

Equilibration of Lipoproteins with Glycosaminoglycan-substituted Agarose Gels—Agarose gel, suspended in distilled water, was distributed in a series of small test tubes. After centrifugation (135 x g for 15 min), the supernatants were carefully removed and the gel contents of the tubes were adjusted to 0.50 g. The gels were then equilibrated with buffers of ionic strength varying between 0.01 and 1.00 by washing four times with 2 ml of 3 mM veronal-HCl (pH 7.4) containing varying amounts of NaCl. In addition, the buffers contained either 1 mM EDTA or 2.5 mM CaCl₂-1 mM MgCl₂. After the last washing, the supernatants were carefully removed and all of the tubes were charged with 0.25 ml of lipoprotein solution dialyzed against buffers containing either EDTA (ionic strength 0.01) or divalent cations (ionic strength 0.02). Finally, 0.25 ml of buffer containing varying amounts of NaCl was added to each tube in order to obtain the desired ionic strength of the equilibration mixture. The contents were carefully mixed four times within 1 hour, left at 4°C overnight, then mixed again and finally centrifuged before the supernatants were subjected to assays for total cholesterol.

RESULTS

Properties of Native and Acetylated Lipoprotein Fractions—The chemical compositions of the lipoprotein fractions are given in Table II.

The extent of acetylation of LDL and VLDL was estimated by...
performing the ninhydrin reaction on acetylated as well as on nonacetylated samples. The color yield in the ninhydrin reaction of each sample was compared to the corresponding lipoprotein concentrations as calculated from cholesterol determinations. The acetylated specimens of LDL and VLDL yielded only 10.3 and 4.4%, respectively, of the color produced by equivalent amounts of nonacetylated lipoproteins.

Electrophoresis (Fig. 1) of HDL₃, VLDL, and LDL indicated pure fractions with α₁-, pre-β-, and β-mobilities, respectively. The acetylated specimens of VLDL and LDL moved considerably faster than their nonacetylated counterparts, indicating that positively charged groups had been blocked.

Immunodiffusion experiments showed that the LDL and VLDL fractions reacted strongly with anti-β-lipoprotein and very faintly with anti-α₁-lipoprotein. The HDL₃ fraction gave a strong reaction with anti-α₁-lipoprotein and a very faint reaction with anti-β-lipoprotein. Albumin was not detected in any of the fractions.

Chromatography of Lipoproteins on Hyaluronate Gel—The chromatograms in Fig. 2 indicate that VLDL, LDL, and HDL₃ emerged with the void volume. It was, therefore, concluded that no significant binding of the lipoproteins to the hyaluronate gel occurred under the conditions of physiological pH and ionic strength.

Ionic Interactions between Lipoproteins and Sulfated Glycosaminoglycans—After the equilibration of a lipoprotein species with a polysaccharide- or ethanolamine-substituted gel at varying ionic strength, the lipoprotein concentration in the supernatant was estimated by total cholesterol assay. The supernatant concentration was then plotted against the ionic strength. The lipoprotein concentration recorded in the supernatant at the highest ionic strength (1.00) was set to 100%. Due to exclusion of soluble lipoprotein from the gel phase, it was generally in the order of 1.2 times higher than the total concentration of lipoprotein in the equilibration mixture.

The experiments performed with VLDL and LDL in the presence of EDTA, shown in Figs. 3 and 4, respectively, can be discussed together since both lipoprotein classes behaved almost identically. Three different ionic strength-intervals could be distinguished with regard to the effect on the lipoprotein concentration in the liquid phase. In a broad interval at high ionic strength, fairly constant lipoprotein levels were recorded. Here, presumably no lipoprotein was bound. Within a narrow ionic strength interval, which was characteristic of each polysaccharide, the extent of binding changed from negligible to maximal. Finally, at the very lowest ionic strength the lipoprotein concentrations in the liquid phase increased again. This decrease in binding capacity may tentatively be ascribed to an increased exclusion of macromolecules from the gel, due to steric as well as ionic factors. The former should be important since charged polysaccharide chains attain an expanded configuration at low ionic strength (29). The latter may cause an increased repulsion between negative charges on the polysaccharide and the lipoprotein, at least in the case of LDL, which should bear an over-all negative charge at pH 7.4 since its isoelectric point is lower (4). The three intervals of ionic strength,
mentioned above, were also traced in the experiments with the ethanolamine-substituted gel. However, only a minor reduction of lipoproteins in the liquid phase was observed when the ionic strength was lowered. It is therefore concluded that at appropriate conditions of ionic strength the sulfated glycosaminoglycans, bound to the gel matrix, possessed a strong capacity to bind VLDL and LDL. The binding capacity, although small, of the ethanolamine-substituted gel might be ascribed to the presence of small amounts of sulfate in the agarose (11).

The ionic strength dependence of the binding of VLDL and LDL in the intermediate interval has a striking similarity to the reaction between glycosaminoglycans and cationic detergents (30). The salt concentration in the middle of such a narrow interval, in the case of detergents, has been denoted a critical electrolyte concentration. In the experiments depicted in Figs. 3 and 4, the CEC's were intrapolated from the curves at the levels halfway between the highest and the lowest concentrations of the curves. VLDL as well as LDL showed CEC's of 0.26, 0.15, 0.09, and 0.08 with heparin, dermatan sulfate, heparan sulfate, and chondroitin sulfate, respectively. Ideally, when the supernatant concentration varies between 0 and 100%, the CEC is the ionic strength at which the supernatant concentration attains 50%. However, since nonideal conditions prevailed, the adopted method of evaluation seems satisfactory.

Equilibration of LDL with the various gels in buffers containing 2.5 mM CaCl$_2$-1 mM MgCl$_2$ instead of 1 mM EDTA resulted in CEC's identical with those obtained in the presence of EDTA.

When HDL$_3$ was equilibrated with gels at various ionic strengths (Fig. 5A), the curves obtained with the four polysaccharide-substituted gels were similar to the curve obtained with the ethanolamine-substituted gel. Thus, there was no significant binding of HDL$_3$ to the sulfated glycosaminoglycans at any ionic strength.

The acetylated specimens of VLDL and LDL were only equilibrated with the dermatan sulfate- and heparin-substituted gels (Fig. 5, B and C). Since the curves were similar to those obtained with nonacetylated lipoproteins and ethanolamine-substituted gel (Figs. 3 and 4), it is concluded that the acetylation procedure blocked the binding of VLDL and LDL to the sulfated glycosaminoglycans.

**DISCUSSION**

**Nature of Cationic Sites on Lipoproteins**—The interaction demonstrated between the sulfated glycosaminoglycans and VLDL or LDL was strongly influenced by salt concentration and should, therefore, be ionic (30). Treatment of the lipoprotein with acetic anhydride, which is known to block free amino groups (18, 31), inhibited the interactions completely. Hence, the ionic bonds are probably established between the negatively charged groups of the polysaccharide and positively charged amino groups of the lipoprotein.

Free amino groups in lipoproteins are provided by lysine residues, amino-terminals of polypeptide chains, phosphatidylserine, and phosphatidylethanolamine. The last two substances account for less than 5% of all positive charges of the phospholipids in LDL (17). The involvement of phospholipids in the interactions observed with VLDL and LDL seems improbable since HDL$_3$ did not bind to the glycosaminoglycans despite its higher phospholipid contents. The alternative interacting component is the apolipoproteins. LDL contains almost exclusively apolipoprotein B, while VLDL in addition has two others, named A and C. The latter two are also constituents of HDL$_3$ (32), which did not bind to the polysaccharides. Therefore, it seems likely that the binding of VLDL as well as LDL to the glycosaminoglycans is mediated by apolipoprotein B. The finding of identical CEC's for LDL and VLDL with each particular polysaccharide lends further support to this statement.

The present work, performed by a new technique, corroborates conclusions recently drawn by others. Levy and Day (33), as well as Nishida and Cogan (34), studied the interaction between...
hyaluronic acid. Although the experiments with hyaluronic
dermatan sulfate > heparan sulfate = chondroitin sulfate >
gels, should be comparable since the molar disaccharide con-
centration? were of the same order in all gels (Table I). The
following order of affinities may, therefore, be stated: heparin >

FIG. 6. Calculation of the Hill coefficients for the binding of
LDL to heparin (Hep), dermatan sulfate (DS), heparan sulfate
(HS), and chondroitin sulfate (CS). The straight lines were cal-
culated on a desk-top computer according to a least squares pro-
gram. The slopes (z), which equal the Hill coefficients, and the
correlation coefficients (r) are indicated in the figure.

LDL and semisynthetic polyanions. Both groups found that
chemical blocking of positively charged groups of the lipoprotein
inhibited complex formation. In contrast, procedures directed
towards the lipid moiety, such as partial delipidation (33) or
treatment with phospholipase C (34), did not negatively affect
the interaction between the lipoprotein and the polyanion.

The concept of divalent cations being necessary for the inter-
action between lipoproteins and polyanions (4, 8, 35) is strongly
contradicted by the present work, since interactions were ob-
served despite the presence of EDTA instead of such ions (Figs.
3 and 4). Furthermore, the mode of interaction with LDL was
not altered when physiological concentrations of calcium and
magnesium were substituted for EDTA-4 instead of such ions (Figs.

There are two exceptions to the importance of the charge
density in the establishment of different affinities for a lipop-
protein. First, the dermatan sulfate shows significantly higher
CEC and z values than the chondroitin sulfate, although both
acid were performed at a physiological ionic strength only, and
with a technique different from the other experiments, it seems
reasonable to add this polysaccharide at the end of the sequence.
The above order differs from that recently suggested by Srin-
vasan et al. (35). Since that work was carried out by a different
technique, using incompletely characterized polysaccharides, a
detailed comparison with the present work is precluded.

If the experimental values obtained at the lowest ionic
strengths are disregarded (see under “Results”), the interaction
curves obtained with LDL and VLDL have approximately a
sigmoid shape, indicating that the binding and the dissociation
of the lipoproteins is a cooperative phenomenon (36). The
curves may therefore be described by the Hill equation (36)

\[
\log \frac{\alpha}{1 - \alpha} = z \log [M^{+}Q^{-}] - \log K
\]

where \(\alpha\) is the fraction of unbound lipoprotein, \(z\) is the Hill
coefficient, \([M^{+}Q^{-}]\) is the concentration of monovalent salt and
\(K\) is a constant.\(^4\) Assuming that all lipoprotein is free \((\alpha = 1)\)
at an ionic strength of 1.0, the Hill coefficient \(z\) was calculated
for the various experiments with LDL (Fig. 6). The consecu-
tive order of the polysaccharides with regard to their \(z\) values
turned out to correspond to the order with regard to their
CEC’s, which implies that also the \(z\) values can be used as a
measure of the affinity between the lipoprotein and the poly-
saccharide. Ideally, the various \(z\) values may reflect the number
of ion pairs cooperating to form a polyvalent binding site be-
tween the polysaccharide and the lipoprotein. Alternatively,
the \(z\) values may represent the number of mono- or polyvalent
cooperating binding sites. Although the nature of the coopera-
tive mechanism cannot be elucidated by the present experiments
and, in addition, the \(z\) values certainly are subject to experimental
error, the present findings, nevertheless, may give clues to how
different affinities for a certain lipoprotein may be achieved.

With two exceptions, discussed below, the affinity between a
polysaccharide and a lipoprotein seems to be dependent on the
charge density of the polysaccharide. The latter parameter may
exert its influence in different ways. First, the charge density
can affect the binding properties of each particular negative
group. Second, a polymer of high charge density should offer a
greater number of binding positions along the chain than one of
low charge density and thus influence the binding by the law of
mass action. Third, the charge density may influence the degree
of cooperativity in the binding. If the binding site on the lipoprotein
consists of a small cluster of positive charges, a
polysaccharide of high charge density should cover more of these
charges than one of low, provided that the charge density on the
lipoprotein equals or exceeds that of the highly charged poly-
saccharide. This last alternative is presumably the most im-
portant one.

There are two exceptions to the importance of the charge
density in the establishment of different affinities for a lipop-
protein. First, the dermatan sulfate shows significantly higher
CEC and \(z\) values than the chondroitin sulfate, although both

\(^4\) Scott has given the reaction between free polysaccharide
chains and anion exchangers a mathematical treatment (30). If
the law of mass action is applied, in a slightly modified way, to
the reaction between \(z\) positive charges on a free molecule with
an equal amount of negative charges on a matrix-bound polysac-
charide chain, the number of ion pairs \((z)\) in the ionic bond can be
shown to appear as the Hill coefficient in a Hill equation.
polysaccharides contain equal amounts of sulfate (Table I). Second, the CEC and z values of the heparan sulfate are of the same order as those of chondroitin sulfate, despite a lower degree of sulfate substitution (Table I). Since dermatan sulfate and chondroitin sulfate differ only with respect to the uronic acid composition (9), it seems valid to assume that the presence of L-iduronic acid in dermatan sulfate accounts for the higher reactivity of this polysaccharide. The possibility that also heparan sulfate contains L-iduronic acid (9) might explain the anomalous behavior of this polysaccharide. If a cooperation between ion pairs accounts for the establishment of different affinities, the steric arrangement of the charges on a polysaccharide chain is certainly important. Thus, the local charge density in a certain direction from the polysaccharide chain may be higher in polysaccharides containing L-iduronic acid than in those containing D-glucuronic acid, at equal chemical charge densities.

Aspects on Atherosclerosis—As shown above, two lipoproteins (VLDL and LDL), known to be atherogenic (37), bind at physiological pH and ionic strength to one of the glycosaminoglycans (dermatan sulfate) found in the arterial wall. This observation demands some remarks concerning arterial polysaccharides. The extracellular glycosaminoglycans hitherto identified in human aorta are hyaluronic acid, dermatan sulfate, heparan sulfate, chondroitin 6-sulfate (38), and chondroitin 4-sulfate (39). There is, however, no convincing evidence for the presence of heparan and keratan sulfate. Unfortunately, chondroitin 6-sulfate has not been included in this work. Since it is similar to chondroitin 4-sulfate in many respects (40), it is not expected to behave much differently with regard to its interaction with lipoproteins. Data in the literature, concerning the polysaccharide composition of the arterial wall in various states of age and health, are conflicting. The discrepancies may be due to differences of methodology as well as sample selection. However, the maturation of an individual means that the polysaccharides digestible with testicular hyaluronidase, i.e. hyaluronic acid and chondroitin sulfate, are more or less replenished by the hyaluronidase-resistant ones, namely heparan sulfate and dermatan sulfate (38). An interesting finding in this context was made by Kumar et al. (41), who analyzed the glycosaminoglycan composition in human arterial intima and externa in different stages of atherosclerosis. The dermatan sulfate was almost exclusively confined to the fatty streaks and pre-stages of this lesion. If lipoprotein deposition in the arterial wall is related to the polysaccharide composition, there may be two alternative explanations to the phenomenon cited. Dermatan sulfate may appear in the fatty streaks as part of a tissue reaction, due to the presence of lipid. According to the other alternative, which is favored by the present work, lipoproteins accumulate and form fatty streaks where dermatan sulfate is abundant.

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The Interaction between Human Plasma Lipoproteins and Connective Tissue Glycosaminoglycans
Per-Henrik Iverius


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