Canine Lipoproteins and Atherosclerosis

I. ISOLATION AND CHARACTERIZATION OF PLASMA LIPOPROTEINS FROM CONTROL DOGS

By Robert W. Mahley and Karl H. Weisgraber

ABSTRACT

Canine plasma lipoproteins were fractionated into four distinct classes by ultracentrifugation combined with Geon-Pevikon block electrophoresis and characterized with respect to physical and chemical properties. The distribution of plasma lipids and lipoproteins was quite unlike that in man, the dog having approximately five to six times as much high density as lower density lipoproteins. Despite the marked difference in distribution, human lipoprotein equivalents were present. Very low density lipoproteins (VLDL) isolated at density less than 1.006 g/ml were triglyceride-rich particles ranging in size from 260 to 900 Å in diameter. The density range from 1.006 to 1.063 g/ml contained two classes of lipoproteins: one closely resembled a low density lipoprotein (LDL) with β mobility and a particle size of approximately 200 Å and the other (called HDL) was closely related to the high density lipoproteins with respect to immunochemical reactivity, electrophoretic mobility, and apoprotein content. The HDL₂ particles ranged in size from 100 to 350 Å in diameter and appeared to be unlike any of the commonly described human lipoproteins. High density lipoproteins called HDL₃, isolated in the density range from 1.087 to 1.21 g/ml were protein-rich particles ranging in size from 55 to 85 Å. The apolipoprotein patterns of VLDL, LDL, and HDL₃ on polyacrylamide gel electrophoresis were similar to those of the corresponding lipoproteins of man.

KEY WORDS

low density lipoproteins
very low density lipoproteins
apolipoproteins

Methods

FRACTIONATION BY ULTRACENTRIFUGATION

Plasma was obtained from fasted purebred foxhounds and mongrel dogs, and 0.01% ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. Fractionation of the plasma lipoproteins into various density (given throughout in g/ml) classes was accomplished by adding sodium chloride and potassium bromide to the plasma (1) and centrifuging in a Beckman L2-65B ultracentrifuge with a 60 titanium rotor. Fractions were removed by the tube-slicing method. Very low density lipoproteins (VLDL) were isolated at 1.006 by centrifugation at 60,000 rpm for 12 hours and washed in saline with a density of 1.006. Other lipoprotein fractions were isolated by sequentially raising the plasma density to 1.063, 1.087, and 1.21. The 1.063 and 1.087 fractions were centrifuged for 18 hours at 60,000 rpm. The 1.21 fraction was centrifuged for 24 hours at 60,000 rpm and then washed in a salt solution with a density of 1.063 in the same manner. Other lipoprotein fractions were isolated by sequentially raising the plasma density to 1.063, 1.087, and 1.21. The 1.063 and 1.087 fractions were centrifuged for 18 hours at 60,000 rpm. The 1.21 fraction was centrifuged for 24 hours at 60,000 rpm and then washed in a salt solution with a density of 1.21 in the same manner. Fractions were concentrated using a Diaflo concentrator (Amicon Corp.).

GEON-PEVIKON BLOCK ELECTROPHORESIS

Purification of the lipoproteins in the density ranges described in the preceding section was performed by Geon-Pevikon block electrophoresis based on the method described by Barth et al. (2). The electrophoresis apparatus was composed of a 38 x 29-cm tray and two buffer chambers, each holding approximately 4 liters of barbital buffer (pH 8.6, ionic strength 0.08). Geon (Goodrich...
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Chemical Co.) and Pevikon (Mercer Consolidated Corp.) (300 g of each) were washed in distilled water and then equilibrated in 500 ml of the barbital buffer. Various ratios of Geon to Pevikon were tried, but optimal separation occurred with a 1:1 mixture. Several types of Geon were tried, but optimal resolution was obtained with type 101, which was used for all studies in this paper, although Geon type 427 also provided good resolution. The slurry was poured into the tray; Telfa surgical dressing pads (Kendall) were used as wicks to connect the block to the buffer chambers. Excess buffer was allowed to drain from the slurry through the wicks onto towels. After approximately 15 minutes, the mixture was such of a consistency that a slit for application of the sample could be made in it with a narrow spatula. After the sample of up to 100 mg of lipoprotein protein had been applied to the block, a constant current of 50 mg was applied for 18 hours at ~6°C.

The lipoprotein bands could be directly visualized in most cases by placing the block on an X-ray view box. However, in all cases, location of the bands was verified by running a small sample of prestained lipoproteins alongside the unstained sample. Staining was accomplished by mixing 0.2-0.4 ml of dilute Sudan black B containing Tween 20 with an equal volume of the lipoprotein fraction containing 0.5-2 mg of protein. The Sudan black B solution was made fresh daily from a stock solution (1 g/100 ml diethylene glycol) by diluting it 1:20 with distilled water. Diluted stain (1 ml) was added to 0.2-0.4 ml of dilute Sudan black B containing Tween 20 (1:100). At the end of the electrophoresis procedure, the purified lipoprotein bands were cut from the block, transferred to a coarse sintered glass funnel, and eluted with 0.15M sodium chloride. Eluates were centrifuged in the cold at low speed to remove polyvinyl particles.

CHEMICAL DETERMINATIONS

Protein was determined by the method of Lowry et al. (3) with a bovine albumin standard. Lipid analyses included triglyceride (4), total cholesterol (5), cholesteryl ester (6), and phospholipid (7) determinations.

PAPER AND AGAROSE ELECTROPHORESIS

Paper electrophoresis was performed according to the method of Hatch and Lees (8), and the patterns were stained with Oil red O. Agarose electrophoresis was carried out using kits prepared by Bio-Rad.

IMMUNOCHEMISTRY

Antisera to native canine lipoproteins or their apoproteins were prepared as previously described (9). Ouchterlony immunodiffusion and immunoelectrophoresis were performed according to published procedures (10).

NEGATIVE-STAINING ELECTRON MICROSCOPY

Negative stains of lipoproteins were prepared by placing a drop of 2% sodium phosphotungstate (pH 7.4) on top of a drop of a dilute lipoprotein fraction on an electron microscopic grid (11). Electron micrographs were taken of random areas of several grids, and then the diameters of 500-2000 particles were measured from the photographs. An AEI electron microscope was used.

RESULTS

Plasma lipid levels in dogs fed Argo dog chow and fasted overnight prior to analyses are presented in Table 1. Since no significant differences were detected between the foxhounds and the mongrel dogs, all results were combined. Lipoprotein electrophoresis on paper or agarose revealed three distinct bands (labeled LDL, HDL1, and HDL2 in Fig. 1). A distinct band in the pre-/S position (VLDL) was never seen in normal dogs. A chylomicron band at the origin was occasionally seen in dogs fasted less than 12 hours.

FRACTIONATION OF CANINE PLASMA LIPOPROTEINS BY ULTRACENTRIFUGATION

To determine the flotation density of canine lipoproteins, plasma from fasted dogs was sequentially raised to higher salt concentrations by adding sodium chloride and potassium bromide to obtain densities ranging from 1.006 to 1.21. The lipoproteins from numerous density cuts were concen-

<table>
<thead>
<tr>
<th>Triglycerides</th>
<th>Total Cholesteryl Ester</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>36</td>
<td>127</td>
</tr>
<tr>
<td>Range</td>
<td>15-70</td>
<td>60-185</td>
</tr>
</tbody>
</table>

Results are from duplicate determinations on plasma from control dogs.

* Expressed as cholesterol equivalents.
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CANINE PLASMA LIPOPROTEINS

FIGURE 1
Paper electrophoretograms of canine plasma, density fractions, and purified lipoproteins. 1.063 = ultracentrifugal density fraction from 1.006 to 1.063 g/ml, 1.21 = ultracentrifugal density fraction from 1.087 to 1.21 g/ml, VLDL = very low density lipoproteins, LDL = low density lipoproteins, and HDLi and HDL2 = high density lipoproteins.

FIGURE 2
Density ranges of canine plasma lipoproteins. d = density; see Figure 1 for definition of other abbreviations.

initial flotation of VLDL. A second flotation by ultracentrifugation at a density of 1.006 or electrophoresis on a Geon-Pevikon block removed the albumin reactivity. The composition of VLDL washed by ultracentrifugation was essentially identical with that of VLDL purified by block electrophoresis.

To prepare canine LDL or HDLi, the density range from 1.006 to 1.063 was isolated by ultracentrifugation and then purified by Geon-Pevikon block electrophoresis. The density range from 1.006 to 1.063 contained only LDL and HDLi (Fig. 1). This density range was chosen to eliminate all of the HDL2. On Geon-Pevikon block electrophoresis there was good resolution between the LDL and HDL bands, making it possible to recover approximately 70–80% of the total protein in the 1.006–1.063 fraction without contamination. The

FIGURE 3
Diagrammatic representation of pattern of migration of canine plasma lipoproteins on Geon-Pevikon block electrophoresis. d = density; see Figure 1 for definition of other abbreviations.

trated and analyzed by paper electrophoresis and immunoelectrophoresis using antisera prepared to whole plasma lipoproteins (density < 1.21). There was considerable overlap in the density of different lipoprotein classes, making it impossible to isolate pure classes by ultracentrifugation in the density range of 1.006 to 1.21. The four classes of lipoproteins in the plasma of normal fasted dogs had densities extending over the ranges shown in Figure 2.

Purification by Block Electrophoresis

Preparation of pure classes of lipoproteins required ultracentrifugation combined with Geon-Pevikon block electrophoresis. A typical pattern of migration for the plasma lipoproteins on Geon-Pevikon block electrophoresis is diagramed in Figure 3. The VLDL class was obtained by ultracentrifugation at a density of 1.006. Albumin was detected immunochemically after the
LDL and HDL₂ obtained after Geon-Pevikon block electrophoresis were judged to be pure classes of lipoproteins on the basis of paper electrophoresis (Fig. 1) and immunoelectrophoresis (Fig. 4).

In four experiments to determine the chemical composition of canine lipoproteins, comparison of the total protein in the 1.006-1.063 fraction with the protein recovered in purified LDL and HDL₁ revealed an average recovery of 76%. The assumption was made that this recovery represented an equal loss of approximately 12% of the LDL and 12% of the HDL₁ in this fraction. Analysis of the 1.063-1.087 fraction revealed that approximately 1.5% of the total plasma LDL and approximately 2% of the total plasma HDL₁ were present in this density range. Approximately 15% of the total plasma HDL₂ was also present in the 1.063-1.087 fraction. It was impossible to purify completely lipoproteins from this fraction, because resolution between HDL₁ and HDL₂ was not complete on block electrophoresis when large concentrations of HDL₂ were present. All calculations were corrected for losses as shown in Table 2.

To prepare canine HDL₂, lipoproteins in the density range of 1.087 to 1.21 were isolated, washed at a density of 1.21, and then purified by block electrophoresis. As shown in the electrophoretic patterns in Figure 1, this density range contained primarily HDL₂ with only traces of HDL₁. No LDL was detected by immunoelectrophoresis. Approximately 85% of the total plasma HDL₂ was in this density range. Raising the density higher than 1.087 rapidly decreased the amount of HDL₂ that was recovered. The HDL₂ eluted from the starch block was shown to be free of HDL₁ by paper electrophoresis and negative-staining electron microscopy. Comparison of the total protein in the 1.087-1.21 fraction with the protein recovered from block electrophoresis as HDL₂ revealed a 15% loss.

**TABLE 2**

<table>
<thead>
<tr>
<th>Percent of Total Lipoprotein Lost during Isolation</th>
<th>Block electrophoresis of density fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.006-1.063</td>
</tr>
<tr>
<td>LDL</td>
<td>12</td>
</tr>
<tr>
<td>HDL₁</td>
<td>12</td>
</tr>
<tr>
<td>HDL₂</td>
<td>15.0</td>
</tr>
</tbody>
</table>

**DISTRIBUTION AND COMPOSITION OF CANINE PLASMA LIPOPROTEINS**

Lipid and protein determinations were performed on the four ultracentrifugal density cuts and the purified lipoproteins as described earlier in this
paper. Recovery of all lipid classes in the various density ranges compared with total plasma lipids ranged from 80 to 95%. In the density range greater than 1.21, only phospholipid was measurable; it accounted for no more than 5% of the plasma phospholipid. The distribution of plasma lipoprotein protein (Table 3) revealed that the normal dog was primarily an “HDL_2 animal;” this lipoprotein accounted for approximately 146 mg protein/100 ml plasma. Plasma VLDL (1.5 mg protein/100 ml), LDL (6 mg protein/100 ml), and HDL_1 (3.0 mg protein/100 ml) were present in much lower concentrations. The distribution of lipids in the purified plasma lipoproteins is also shown in Table 3. Approximately 85% of the total plasma cholesterol was carried by HDL_2, and approximately 50% of the plasma triglyceride was carried by VLDL.

The percent composition of each of the purified lipoprotein classes is presented in Table 3. The VLDL class was composed of about 60% triglyceride, 15% cholesterol, 16% phospholipid, and 10% protein. Each of the constituents of the LDL class represented approximately one quarter of the total composition. The HDL_1 class was phospholipid-rich (41%) and contained 35% cholesterol. The HDL_2 class was protein- and phospholipid-rich (43% and 36%, respectively).

**IMMUNOCHEMICAL CHARACTERIZATION**

Ouchterlony immunodiffusion and immunoelectrophoresis were performed on ultracentrifugal fractions and purified plasma lipoproteins. Characterization of the antisera prepared to native canine lipoproteins and to the apoproteins of purified lipoproteins is shown in Table 4. Immunodiffusion lines of identity were formed between LDL and VLDL with antisera prepared to native LDL; this reaction was interpreted as indicating the presence of apo-LDL or the B-apoprotein in these two lipoproteins. The lack of reaction of LDL antisera with HDL_1 and HDL_2 was interpreted as indicating that apo-LDL was absent from these classes.

Antisera prepared to the apoproteins of VLDL reacted with all classes of lipoproteins. As established later by polyacrylamide gel electrophoresis, this reaction suggests that VLDL, HDL_1, and HDL_2 have major apoproteins in common. Antisera to native HDL_2 reacted only with HDL_1 and HDL_2, which were later shown to have similar apoprotein patterns. Immunoelectrophoresis (Fig. 4) revealed the characteristic precipitin arcs produced by each purified lipoprotein.

**NEGATIVE-STAINING ELECTRON MICROSCOPY**

Negative-staining electron microscopy of the purified lipoproteins revealed distinct particle sizes and morphology (Fig. 5). The distribution of particle sizes is presented in Figure 6. VLDL particles were roughly spherical and ranged in size from 260 to 900 Å in diameter. LDL particles were spherical and ranged in size from 160 to 250 Å in diameter. HDL_1 particles overlapped LDL particles in size but had a greater range of diameters (100 to 350 Å). HDL_2 particles were relatively uniform in size and ranged from 55 to 85 Å in diameter.

**APOPROTEINS OF PLASMA LIPOPROTEINS**

The apoprotein patterns of the purified lipoproteins on polyacrylamide gel electrophoresis were characteristic of the specific lipoproteins and resembled the apoprotein patterns of human and rat lipoproteins (9, 14, 15). The characteristic apoprotein patterns on polyacrylamide gel electrophoresis in Tris buffer (pH 8.9) are shown in Figure 7. The patterns of VLDL and LDL revealed a large

**TABLE 3**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Triglyceride (mg/100 ml plasma)</th>
<th>Cholesterol (mg/100 ml plasma)</th>
<th>Cholesteryl esters* (mg/100 ml plasma)</th>
<th>Phospholipid (mg/100 ml plasma)</th>
<th>Protein (mg/100 ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>9.0 ± 1.7</td>
<td>2.3 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>2.5 ± 1.0</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>LDL</td>
<td>7.6 ± 1.8</td>
<td>5.7 ± 1.9</td>
<td>4.2 ± 0.2</td>
<td>6.4 ± 1.8</td>
<td>6.1 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>(38.8)</td>
<td>(29.4)</td>
<td>(22.1)</td>
<td>(24.8)</td>
<td>(23.6)</td>
</tr>
<tr>
<td>HDL_1</td>
<td>0.3 ± 0.05</td>
<td>4.6 ± 1.4</td>
<td>3.4 ± 0.2</td>
<td>5.4 ± 1.3</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>(2.2)</td>
<td>(34.6)</td>
<td>(22.1)</td>
<td>(40.6)</td>
<td>(22.6)</td>
</tr>
<tr>
<td>HDL_2</td>
<td>2.2 ± 0.3</td>
<td>7.0 ± 12.0</td>
<td>51.5 ± 1.3</td>
<td>124.6 ± 10.3</td>
<td>145.8 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>(0.6)</td>
<td>(20.4)</td>
<td>(36.4)</td>
<td>(42.6)</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± SE from duplicate determinations on plasma from four dogs. The percent composition is given in parentheses.

*Expressed as cholesterol equivalents.

*Circulation Research, Vol. 35, November 1974
Table 4
Characterization of Antisera

<table>
<thead>
<tr>
<th>Antiserum prepared to</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL₁</th>
<th>HDL₂</th>
<th>Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Native HDL₂</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Apo-VLDL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Apo-LDL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Apo-HDL₁</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Apo-HDL₂</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Recent studies have demonstrated that the dog develops atherosclerosis closely resembling the human disease subsequent to the development of hyperlipoproteinemia (16). Attempts to characterize the hyperlipoproteinemia have been unsuccessful because of the lack of detailed information concerning normal canine lipoproteins. Previous studies have indicated that dogs have high levels of high density lipoproteins compared with those of the lower density lipoproteins (1, 17-19). In most studies, fractionation of the plasma has been performed by preparative ultracentrifugation using density ranges established for man. However, it has been reported that the use of conventional density ranges does not result in purification (18, 19). Sakagami and Zilversmit (18) detected a low density (β) and a high density (α) lipoprotein throughout the fraction with a density less than 1.063.

In this report, we showed that the dog has four distinct classes of plasma lipoproteins which can be isolated and purified by ultracentrifugation combined with Geon-Pevikon block electrophoresis. Three of the classes of canine lipoproteins appear to correspond to the VLDL, LDL, and HDL classes of man. The canine VLDL class consists of triglycer-
eride-rich particles ranging in size from 260 to 900 Å in diameter. This size range is similar to that described for VLDL particles from man (20), rabbit (21), and rat (22). By agarose and paper electrophoresis, the VLDL particles have pre-β mobility. The canine VLDL and LDL classes appear to share a major apoprotein which has been referred to as apo-LDL or B-apoprotein.

The canine LDL class contains less of the total plasma cholesterol and has a higher content of triglyceride than has been reported for the LDL class in man (23). However, canine LDL particles are approximately 200 Å in diameter and morphologically identical to negatively stained preparations of LDL particles from man (20) and from the rat (22). Immunochemically, canine LDL can be detected over a wider density range (1.006 to 1.087) than has been described for man.

The canine high density lipoproteins referred to in this paper as HDL₂ have α, mobility on electrophoresis and can be detected immunochemically in the density range from 1.070 to 1.21. Chemically, they are protein-rich lipoproteins, but they contain most of the total plasma cholesterol. Negative-staining electron microscopy showed that the HDL₂ particles of the dog are approximately 80 Å in diameter and closely resemble the high density lipoproteins of man (20) and the rat (22).

The fourth class of canine plasma lipoproteins referred to in this paper as HDL₄, is unlike any of the commonly described lipoproteins of man but may in fact be similar to human HDL₂, about

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**Figure 6**

Percent distribution by particle size of the purified canine lipoproteins. n = number of particles measured; see Figure 1 for definition of other abbreviations.

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which little is known. HDL\(_1\) particles have \(\alpha\) mobility and migrate as a sharp band on agarose, paper, and Geon-Pevikon block electrophoresis. However, they have a broad size distribution from 100 to 350 \(\text{Å}\) in diameter and are detectable over a wide density range from approximately 1.025 to 1.10. It is impossible to separate LDL and HDL\(_1\) by ultracentrifugation. The HDL\(_1\) class contains nearly as much cholesterol as does the LDL class and becomes a major cholesterol carrier in the hypothyroid cholesterol-fed dog (24).

Polyacrylamide gel electrophoresis of the apoproteins of canine plasma lipoproteins revealed that lipoproteins of different classes share many of the same apoproteins based on the distance migrated into the gel. However, the relative amounts of these apoproteins differ in each class. There are striking similarities between the apoprotein patterns of canine lipoproteins and the corresponding lipoproteins in man (for review, see ref. 25) and in the rat (9, 15). Comparisons have previously been made between rat and human apolipoproteins (15).

A tentative identification of the canine apolipoproteins is possible based on studies in other species. The fast-moving apoproteins present in zone 4 of VLDL, HDL\(_1\), and HDL\(_2\) may be equivalent to human C-II (apo-Glu), C-III (apo-Ala\(_\alpha\)), and C-IV (apo-Ala\(_\beta\)). The bands in zone 3 of VLDL, HDL\(_1\), and HDL\(_2\) may correspond to the arginine-rich protein (top band), A-I (apo-Gln I), and A-II (apo-Gln II). The band tentatively identified as A-II in the dog migrates farther than does human A-II, as does the A-II of the Macacus rhesus (26). Precise comparisons await isolation and characterization of these apoproteins.

Acknowledgment

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