Characterization of dog prenodal peripheral lymph lipoproteins. Evidence for the peripheral formation of lipoprotein-unassociated apoA-I with slow pre-β electrophoretic mobility

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Abstract Dog plasma and prenodal peripheral lymph apoA-I distribution was examined by nondenaturing gradient gel electrophoresis–immunoblot analysis. In control dogs, plasma apoA-I could be localized to two distinct populations of particles with modal diameters of 8.4 nm and 10.4 nm. The smaller sized population accounted for over 50% of plasma apoA-I. Peripheral lymph apoA-I distribution was significantly different. The percentage of apoA-I localized to the 10.4 nm population was reduced by 40% and the modal diameter of the smaller HDL apoA-I population was significantly decreased by 0.1 nm. Additionally, peripheral lymph apoA-I could be localized to particles smaller than albumin (lipoprotein-unassociated apoA-I). The presence of lipoprotein-unassociated apoA-I particles was confirmed by gel filtration chromatography. Immunoblots of column fractions subjected to agarose electrophoresis revealed that these particles had slow pre-beta electrophoretic mobility. In dogs fed an atherogenic diet, lipoprotein-unassociated apoA-I particles with slow pre-beta electrophoretic mobility could be found in both plasma and peripheral lymph. With increasing degree of hypercholesterolemia, the relative amount of plasma lipoprotein-unassociated apoA-I tended to increase. In peripheral lymph, an increasing degree of hypercholesterolemia was associated with a decrease in the relative amount of lipoprotein-unassociated apoA-I. Instead, a population of large apoA-I particles (11–25 nm) became increasingly prominent. — **Lefevre, M., C. H. Sloop, and P. S. Roheim.** Characterization of dog prenodal peripheral lymph lipoproteins. Evidence for the peripheral formation of lipoprotein-unassociated apoA-I with slow pre-β electrophoretic mobility. *J. Lipid Res.* 1988. **29:** 1139–1148.

Supplementary key words HDL • interstitial lipoproteins • reverse cholesterol transport • atherogenic diet

Low levels of plasma HDL are associated with increased incidence of cardiovascular disease (1). HDL are thought to exert their protective effects by facilitating reverse cholesterol transport (2), the delivery of cholesterol from peripheral tissue to the liver for excretion. Much of the information concerning the role of HDL in reverse cholesterol transport has been obtained from in vitro studies in tissue culture (3–6). Additionally, various models of interstitial fluid have been used to provide in vivo information on the initial events associated with reverse cholesterol transport (reviewed in reference 7). Previous studies in this laboratory have clearly shown that HDL obtained from dog prenodal peripheral lymph, an accepted model for interstitial fluid, differ in lipid and apolipoprotein composition and in physical characteristics from HDL obtained from plasma (8–10).

In this report, we extend our studies of dog interstitial fluid HDL to include a description of the differences between peripheral lymph and plasma apoA-I distribution as determined by nondenaturing gradient gel electrophoresis–immunoblot analysis (11). A major finding of this study is the identification of lipoprotein-unassociated apoA-I with slow pre-beta electrophoretic mobility in control dog peripheral lymph despite its absence in control dog plasma.

METHODS

Animals, diet and peripheral lymph collection

Mongrel dogs, free of heart worms, were fed either normal dog chow (five dogs) or dog chow supplemented with 10% sucrose, 0.3% cholic acid, 3% cholesterol, 20% lard, and 0.5% propylthiouracil (four dogs) for 30–40 days (8).

Abbreviations: HDL, high density lipoproteins; EDTA, ethylenediamine tetraacetic acid; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); CS, calf serum; BSA, bovine serum albumin; apo, apolipoprotein; PBS, phosphate-buffered saline; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; PBST, phosphate-buffered saline containing 0.05% Tween 20.

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Both control and cholesterol-fed dogs were fasted overnight prior to the surgery for lymph collection. The surgical procedure for acute peripheral lymph collection was carried out as previously described (8). Lymph samples were collected into chilled tubes containing EDTA, sodium azide, and DTNB to provide final concentrations of 0.1%, 0.1%, and 1.4 mM, respectively. Lymph was collected over an 8-hr period at an average lymph flow of 2 ml/hr per leg. On occasion, peripheral lymph was collected from unanesthetized chronically canulated dogs (Sloop, C. H., manuscript in preparation). In these instances, the first hour's lymph collection was discarded and the second hour's collection was used for the studies. Blood samples were drawn at the end of the experiment (or at the end of the second hour in chronically canulated dogs) into tubes containing EDTA, sodium azide and DTNB. Plasma was obtained by centrifugation at 1500 *g* for 20 min at 4°C.

Gradient gel electrophoresis–immunoblot analysis

The distribution of apoA-I as a function of lipoprotein size in both plasma and peripheral lymph was determined by gradient gel electrophoresis–immunoblot analysis (described in detail in reference 11). Briefly, 16 μl of plasma or 80 μl of peripheral lymph were mixed with 0.25 vol of sample buffer (90 mM Tris, 80 mM boric acid, 40% sucrose, 0.01% bromphenol blue; pH 8.35) and applied across the top of a 2–30% polyacrylamide gradient gel (concave gradient) prepared in our laboratory. The top of each gel was vertically partitioned by placing a single 25-μl plastic well-former (obtained by cutting up a 12-place Pharmacia plastic well-former) in the center of each gel. This allowed us to electrophorese the plasma and peripheral lymph samples from each dog on the same gel. Pharmacia high molecular weight standards were applied in the 25-μl well. The samples were electrophoresed at 220 V for 24 hr at 10°C. After electrophoresis, a strip of gel containing the molecular weight standards was excised from the center of the gel and stained for protein. The remaining two sections of the gel were incubated for 3 × 20 min in transfer buffer (20 mM Tris, 150 mM glycine; pH 8.4). The proteins in the gels were electrophoretically transferred onto charge-modified nylon membranes (Bio-Rad) at a field strength of 3 V/cm for 24 hr at 10°C. Following 1-hr fixation with 0.03% glutaraldehyde, the transfers were blocked overnight at 50°C with 10% calf serum (CS), 3% bovine serum albumin (BSA), 0.01% sodium azide diluted in 50 mM sodium phosphate, 0.15 M NaCl, 0.05% Tween 20; pH 7.4 (PBST). ApoA-I was localized by 1-hr incubation with goat anti-dog apoA-I antisera diluted appropriately in PBST with 10% CS. (The antisera were monospecific as judged by immunoblots against both total lipoproteins and plasma subjected to SDS-PAGE.) This was followed by five 10-min washes in PBST, 1-hr incubation with ^{125}I-labeled affinity-purified rabbit anti-goat IgG (10^6 cpm/tube diluted in PBST with 10% CS), and a final wash sequence. The dried transfers were autoradiographed overnight at −70°C (Kodak XRP-5) and subsequently scanned with a Bio-Rad Model 620 video densitometer interfaced to a microcomputer. The autoradiographic intensity was converted to relative apoA-I mass through comparisons with a standard containing known relative concentrations of transferred apoA-I. These data were then reduced to provide a plot of percent apoA-I distribution as a function of R_f and/or particle size. Treatment of the glutaraldehyde-fixed transfers with 1% NP-40 for 1 hr prior to immunolocalization had no significant effect on the subsequent calculated distribution of apoA-I, indicating that all epitopes were expressed equally within the different lipoprotein populations.

Gel filtration chromatography

Plasma and peripheral lymph were fractionated on 1.5 × 100 cm columns of Bio-Gel A-0.5m. Prior to application, samples of peripheral lymph were concentrated approximately tenfold under vacuum in a Micro-Confilt (Biomolecular Dynamics) apparatus. Two-ml samples were applied to the column and eluted with 50 mM sodium phosphate, 0.15 M NaCl; pH 7.4 (PBS) with 0.01% EDTA and 0.01% sodium azide at a flow rate of approximately 12 ml/hr.

Agarose immunoblots

The electrophoretic mobility of the apoA-I-containing particles was determined by agarose electrophoresis (12) followed by immunoblotting. Samples of plasma, peripheral lymph, column fractions, or purified apoA-I were electrophoresed in 0.5% agarose containing 0.35% BSA. Electrophoresis was carried out in a buffer of 24 mM Tricine, 80 mM Tris, 0.01% calcium lactate; pH 8.6. Following electrophoresis, the agarose strips were press-blotted onto nitrocellulose. The transfers were blocked with 3% BSA in PBS for 1 hr at 37° C and then incubated for 1 hr with monospecific goat anti-dog apoA-I antisera diluted in PBS containing 1% BSA (PBS-BSA). The transfers were washed three times with PBST and then incubated for 1 hr with horseradish peroxidase-coupled rabbit anti-goat IgG diluted in PBS-BSA. After three additional washes, the transfers were developed with 4-chloro-1-napthol.

Analytical methods

Total cholesterol was determined enzymatically (13) employing a commercially available kit (Behring Diagnostics). ApoA-I concentrations were determined by electroimmunoassay (14) as previously described (15). Data are presented as mean ± SEM. Statistically significant differences were evaluated by Student's *t*-test or paired *t*-test where appropriate.

RESULTS

Gradient gel electrophoresis–immunoblot analysis was employed to identify differences in apoA-I distribution between plasma and peripheral lymph of control, chow-fed dogs. Examples of autoradiograms produced by this method are shown in **Fig. 1** and the normalized scans appear in **Fig. 2.** Plasma apoA-I could be localized to at least two distinct populations of particles within the HDL size range with modal diameters of 10.4 ± 0.09 nm and 8.4 ± 0.04 nm (n = 5). Half of the plasma apoA-I (51.6 ± 3.6%) could be localized to particles between 8.0 and 9.0 nm **(Fig. 3).**

Peripheral lymph apoA-I distribution was significantly different from that of plasma. The majority of HDL-associated apoA-I could still be localized to a single peak between 8.0 and 9.0 nm. However, in peripheral lymph, this major apoA-I peak was shifted to a significantly smaller modal diameter (8.3 ± 0.04 nm; $P<0.005$ by paired *t*-test). When compared to plasma, relatively less peripheral lymph apoA-I was associated with larger HDL particles between 10.0 and 12.0 nm (Fig. 3).

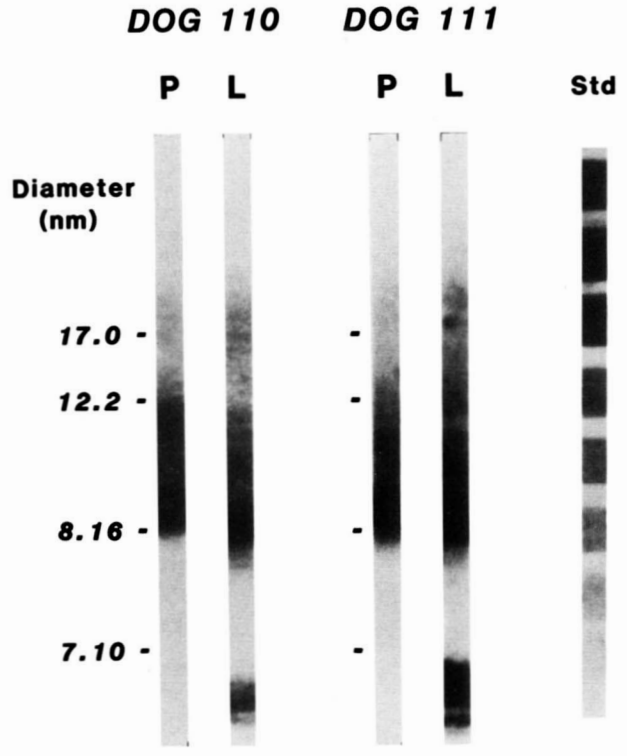

Fig. 1. Autoradiograms showing apoA-I distribution in plasma (P) and peripheral lymph (L) of two control dogs. Plasma cholesterol levels were 105 mg/dl and 108 mg/dl for dogs 110 and 111, respectively. (–) Indicates the relative migrations of protein size standards for each set of gels. Numbers to the left are the Stokes' diameters for the protein size standards. The standard lane (Std) on the right was used to convert autoradiographic optical density to relative apoA-I concentration.

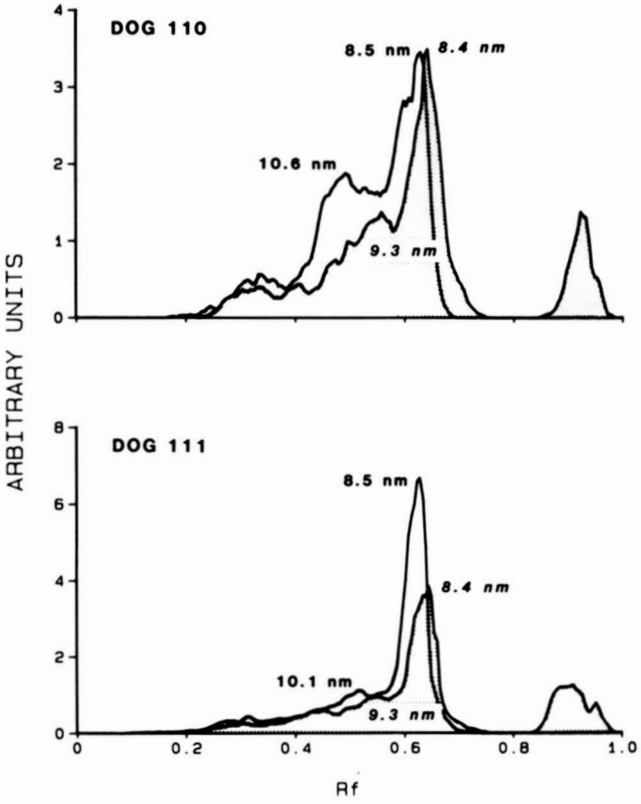

Fig. 2. Normalized apoA-I distribution in plasma (open area) and peripheral lymph (shaded area) of the two control dogs depicted in Fig. 1. Autoradiograms were scanned and the absorbances were converted to relative mass of apoA-I by procedures described in detail in reference 11. The distribution of apoA-I in both plasma and peripheral lymph for each dog was corrected to provide identical areas under each curve. Data are plotted as a function of relative migration of the gel (R_f) with the smallest particles exhibiting the largest R_f. The calculated modal diameters for the major peaks are displayed with values for peripheral lymph appearing in italics. The relative migrations of the protein size standards were: thyroglobulin (17.0 nm), 0.32; ferritin (12.2 nm), 0.43; lactate dehydrogenase (8.16 nm), 0.65; and human serum albumin (7.1 nm), 0.84.

Populations of apoA-I particles not evident in plasma were also identified in peripheral lymph. In the HDL region of the gel, an additional minor apoA-I-containing peak with a modal diameter of 9.3 ± 0.08 nm could be found (Fig. 2). Most striking, however, was the appearance of apoA-I particles in the small pore region of the gel in peripheral lymph samples. These particles, resolvable as one or sometimes two bands (one sharp and one broad), had apparent diameters smaller than both albumin and lipoprotein-unassociated apoA-IV. Collectively, these lipoprotein-unassociated (i.e., not associated with classical lipoproteins) apoA-I particles comprised an average of 14.7 ± 3.8% (range; 6.5%–26.5%) of peripheral lymph apoA-I.

The presence of lipoprotein-unassociated apoA-I was confirmed by gel filtration column chromatography (**Fig. 4**). Samples of peripheral lymph (Fig. 4, bottom), but not

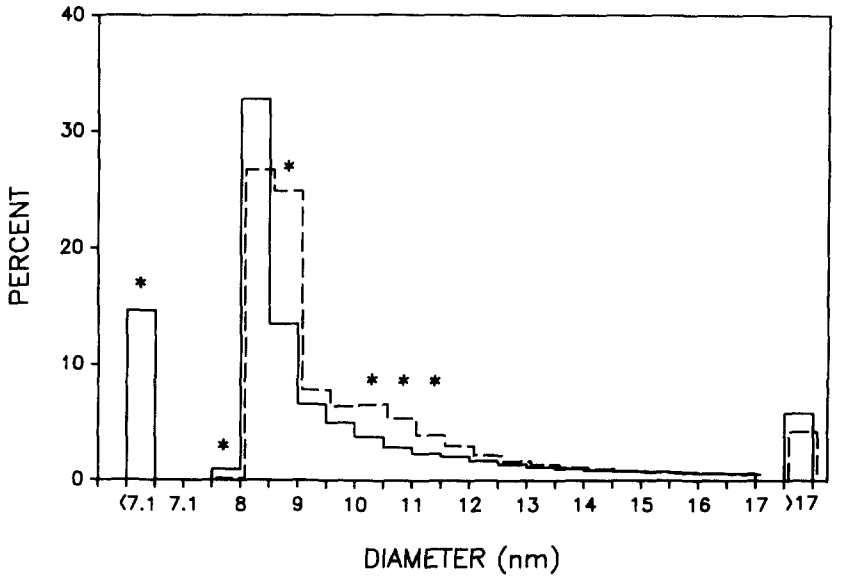

Fig. 3. Distribution of apoA-I as a function of apparent particle diameter in control dog plasma (dashed line) and peripheral lymph (solid line). Asterisks indicate significant differences ($P<0.05$ by paired t-test, n=5) between plasma and peripheral lymph percent apoA-I distribution for a given size interval.

plasma (Fig. 4, top), contained apoA-I particles eluting at volumes greater than those for HDL. Gradient gel electrophoresis–immunoblot analysis of these fractions confirmed that this population of particles was composed of lipoprotein-unassociated apoA-I (data not shown). Measurable amounts of cholesterol were not detected in the lipoprotein-unassociated apoA-I column fractions.

Samples of column fractions containing HDL and lipoprotein-unassociated apoA-I from plasma and lymph were subjected to agarose electrophoresis and then immunoblotted for apoA-I. ApoA-I from both plasma and lymph HDL column fractions migrated with alpha mobility (**Fig. 5**). In contrast, lipoprotein-unassociated apoA-I from peripheral lymph migrated between LDL and VLDL, i.e., with slow pre-beta mobility. The presence of slow pre-beta migrating apoA-I was also demonstrated in agarose immunoblots of unfractionated peripheral lymph, but not plasma. ApoA-I immunoblots of plasma, lymph, and column fractions subjected to isoelectric focusing did not reveal any significant differences in apoA-I isoform pattern (data not shown).

We next examined the effect of an atherogenic diet on the distribution of HDL-associated and lipoprotein-unassociated apoA-I (**Fig. 6** and **Fig. 7**). A substantial portion of plasma apoA-I was still localized to an HDL population between 8.0 and 9.0 nm in size. However, the modal diameter of this population was significantly larger (8.6 ± 0.03 nm; $P<0.05$) in dogs fed the atherogenic diet when compared to controls (8.4 ± 0.04 nm). As in control dogs, the corresponding apoA-I HDL population in peripheral lymph was significantly smaller (8.3 ± 0.03

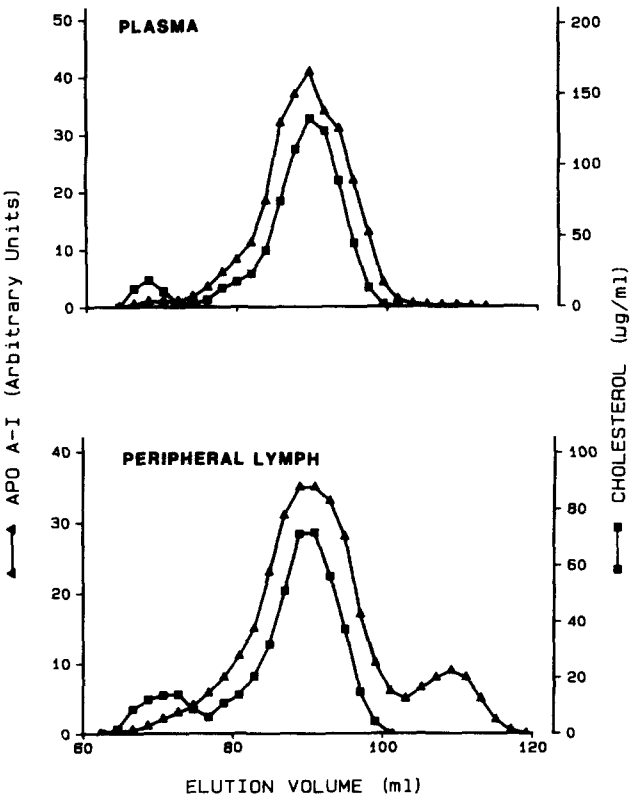

Fig. 4. Distribution of apoA-I (▲-▲) and cholesterol (■-■) in plasma and peripheral lymph of control dog 110 following gel filtration chromatography on Bio-Gel A-0.5m. HDL appear as a single peak eluting at volumes between 80 and 100 ml. Lipoprotein-unassociated apoA-I appears as a peak in peripheral lymph eluting at volumes between 100 and 120 ml.

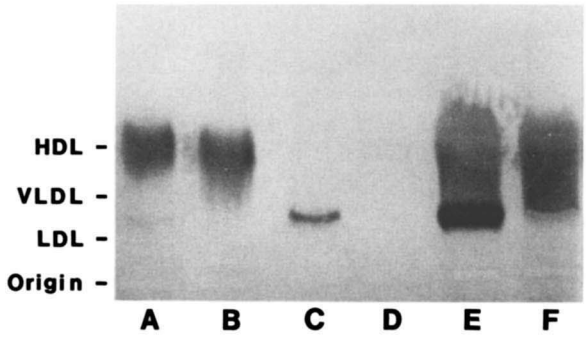

Fig. 5. Electrophoretic mobility of apoA-I particles in column fractions and unfractionated plasma and peripheral lymph of control dogs. A, Peripheral lymph HDL; B, plasma HDL; C, peripheral lymph lipoprotein-unassociated apoA-I; D, plasma lipoprotein-unassociated apoA-I; E, unfractionated peripheral lymph; F, unfractionated plasma. For HDL, column fractions eluting at a volume of 90 ml were analyzed. For lipoprotein-unassociated apoA-I, column fractions eluting at a volume of 110 ml were analyzed. Unfractionated plasma and peripheral lymph samples were overloaded and thus do not display clearly resolvable bands. Nonetheless, the presence of slow pre-beta migrating apoA-I is clearly present in peripheral lymph but appears to be absent or substantially reduced in plasma. The migration of human lipoproteins is indicated.

nm; $P < 0.005$ by paired t-test) than the corresponding plasma sample. Additionally, the difference in size of this population of apoA-I particles between plasma and peripheral lymph in dogs fed the atherogenic diet (0.3 ± 0.04 nm) was significantly greater ($P < 0.005$) than the difference found in dogs fed the control diet (0.1 ± 0.02 nm). In plasma, but not peripheral lymph, apoA-I particles substantially smaller (R_f 0.68–0.80; 7.3–8.0 nm) than the main plasma HDL peak could also be identified.

In contrast to dogs fed the control diets, lipoprotein-unassociated apoA-I was detected in both the plasma and peripheral lymph of all dogs fed the atherogenic diet. The appearance of lipoprotein-unassociated apoA-I in both plasma and peripheral lymph was confirmed by gel filtration chromatography (**Fig. 8**). Measurable amounts of cholesterol were not associated with lipoprotein-unassociated apoA-I in either plasma or peripheral lymph. Agarose immunoblots of the lipoprotein-unassociated fractions obtained from gel filtration chromatography again demonstrated that both the plasma and peripheral lymph apoA-I fractions migrated with slow pre-beta mobility (data not shown). Unfractionated plasma and peripheral lymph from cholesterol-fed dogs clearly contained apoA-I particles with slow pre-beta mobility (**Fig. 9**). These slow pre-beta apoA-I particles comigrated with the majority of purified dog apoA-I but had a slightly slower electrophoretic mobility than both human "pre-beta apoA-I" and purified human apoA-I.

The degree of hypercholesterolemia appeared to substantially influence the apoA-I profile in both plasma and peripheral lymph. In hyporesponding dogs (Fig. 7, dog 112), the population of larger apoA-I HDL particles (modal diameter, 10.9 nm) became quite prominent in plasma, while in hyperresponding dogs (Fig. 7, dog 106), this population was substantially reduced. Furthermore, the relative amount of plasma lipoprotein-unassociated apoA-I in hyperresponding dogs was elevated over that found in hyporesponding dogs. This, however, was not the case in peripheral lymph where the relative amount of lipoprotein-unassociated apoA-I tended to decrease in hyperresponding dogs. Instead, with increasing degree of hypercholesterolemia, a population of large particles, (R_f 0.20–0.50; 11–25 nm) accounted for an increasing percentage of the peripheral lymph apoA-I.

DISCUSSION

Prenodal peripheral lymph is a well-accepted model for the study of interstitial fluid and has been used in investi-

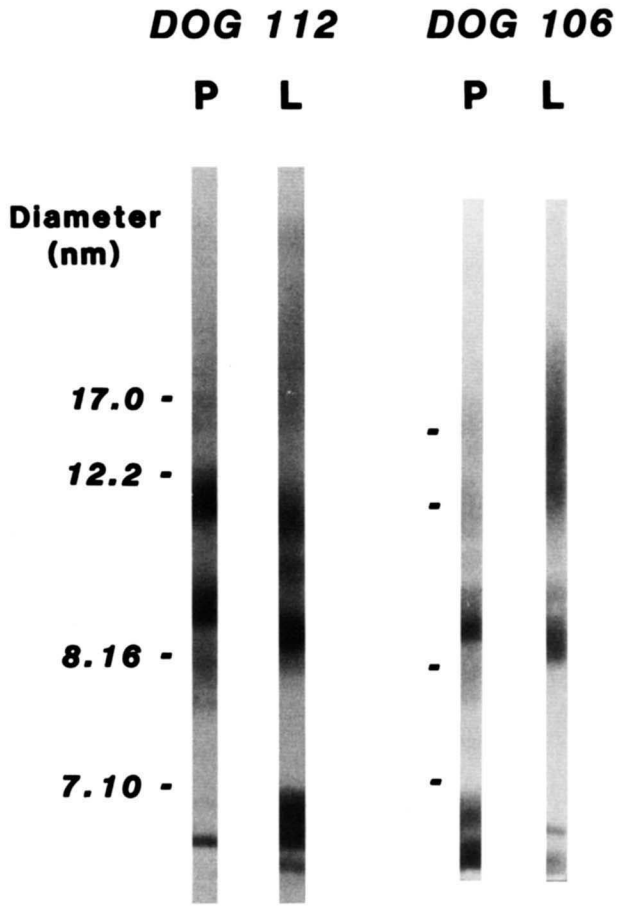

Fig. 6. Autoradiograms showing apoA-I distribution in plasma (P) and peripheral lymph (L) of two hypercholesterolemic dogs. Dog 112 was a hyporesponder with a plasma cholesterol value of 410 mg/dl. Dog 106 was a hyperresponder with a plasma cholesterol value of 2435 mg/dl. (–) indicates the relative migrations of protein size standards for each set of gels. Numbers to the left are the Stokes' diameters for the protein size standards.

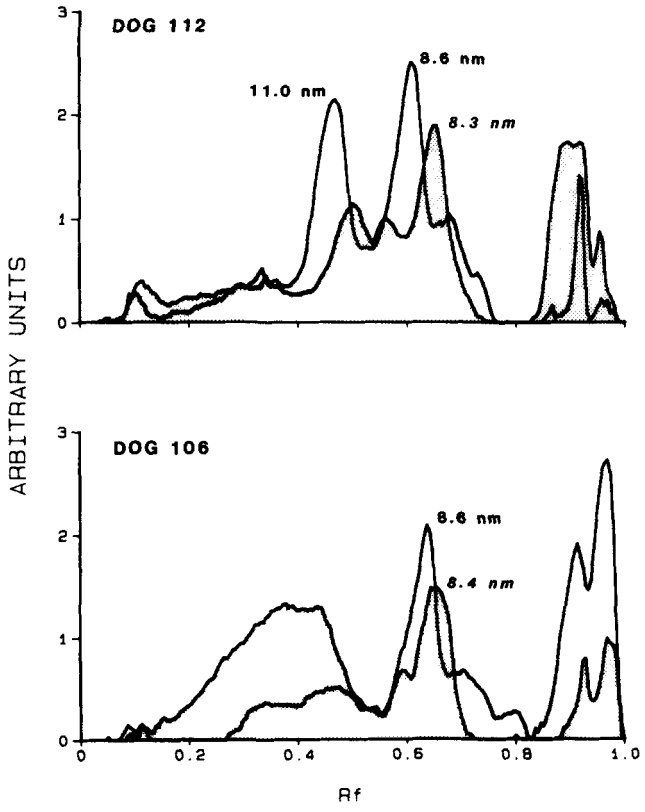

Fig. 7. Normalized apoA-I distribution in plasma (open area) and peripheral lymph (shaded area) of the two hypercholesterolemic dogs depicted in Fig. 6. Profiles were obtained as described in Fig. 2. The relative migrations of the protein size standards were: thyroglobulin (17.0 nm), 0.34; ferritin (12.2 nm), 0.44; lactate dehydrogenase (8.16 nm), 0.67; and human serum albumin (7.1 nm), 0.85.

gations concerning extravascular events associated with reverse cholesterol transport. Previously, we have shown that all lipoprotein classes and apolipoproteins present in dog plasma are also present in dog peripheral lymph, albeit at lower concentrations (8, 9). It was shown that significant differences exist between plasma and peripheral lymph with respect to lipoprotein composition and morphology which could not be explained by differential transport of plasma lipoproteins across the capillary endothelium (8, 9). Of particular note was the finding of a relatively elevated HDL apoE and apoA-IV content in peripheral lymph. The elevated HDL apoE was later shown to be due to peripheral apoE synthesis and assembly into discoidal HDL (16). Additionally, evidence had been previously presented suggesting that plasma apoA-I-containing HDL undergo extravascular modification through enrichment with unesterified cholesterol (10). In this report, we have examined more closely the extravascular modifications of apoA-I-containing HDL employing nondenaturing gradient gel electrophoresis–immunoblotting methodology.

Dog plasma apoA-I could be localized to two major populations of particles with modal diameters of 8.4 nm and 10.4 nm. The smaller population of particles accounted for the majority of HDL apoA-I and is comparable in size to human $(HDL_{3a})_{gge}$ while the larger population is comparable in size to human $(HDL_{2b})_{gge}$ (17). It is interesting to note that dog HDL, which does not contain significant amounts of apoA-II (18), exhibits a size distribution similar to that found for human HDL Lp (A-I without A-II) which display apparent diameters of 8.4 and 10.8 nm (19).

The apoA-I lipoprotein size distribution in peripheral lymph was considerably different when compared to that of plasma. Most interesting was the detection of apoA-I in the small pore region of the gel in samples of control dog peripheral lymph, but not in control dog plasma. Additionally, in peripheral lymph, the relative amount of apoA-I in the 10.4-nm population was decreased, a 0.1-nm decrease in the modal diameter of the smaller 8.4-nm (plasma) HDL population occurred, and an additional HDL peak appeared with a modal diameter of 9.3 nm. Several factors could account for these differences including: *1*) size-dependent differential transport across the

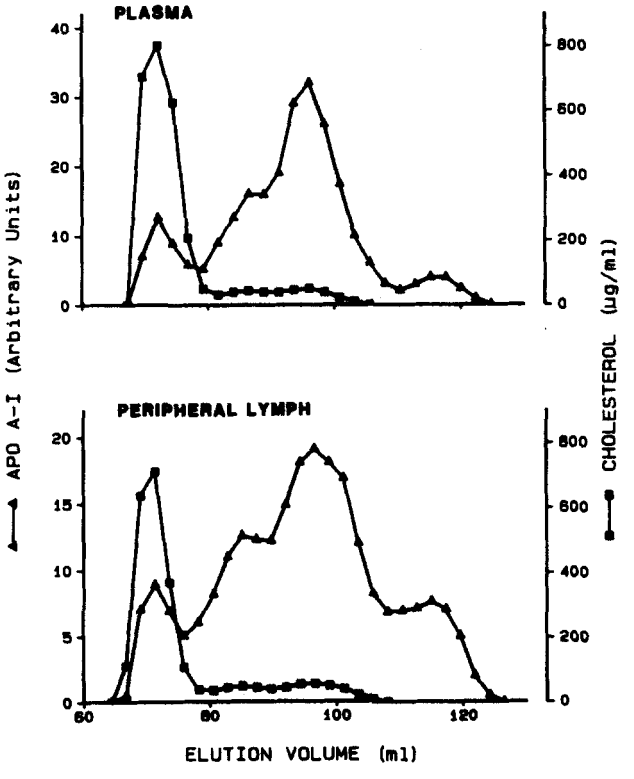

Fig. 8. Distribution of apoA-I (▲-▲) and cholesterol (■-■) in plasma and peripheral lymph of hypercholesterolemic dog 112 following gel filtration chromatography on Bio-Gel A-0.5m. HDL appear as a single peak eluting at volumes between 80 and 100 ml. Lipoprotein-unassociated apoA-I appears as a peak in plasma and peripheral lymph eluting at volumes between 100 and 125 ml.

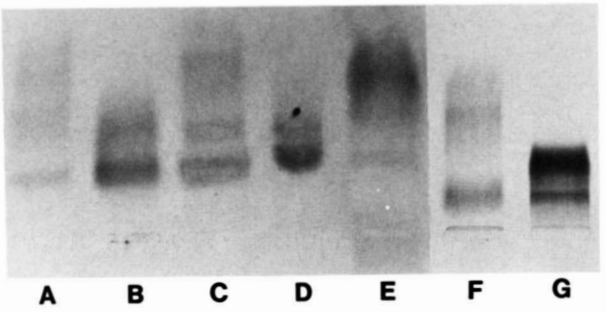

Fig. 9. Electrophoretic mobility of apoA-I particles in plasma and peripheral lymph of a hypercholesterolemic dog. Lanes A–E were subjected to immunoblot analysis to localize apoA-I. Samples are as follows: A, hypercholesterolemic dog peripheral lymph; B, purified dog apoA-I; C, hyperchlesterolemic dog plasma; D, purified human apoA-I; E, human plasma with demonstrable "pre-beta apoA-I." Lanes F and G were stained with oil red O. Samples are: F, normal human plasma; G, hypercholesterolemic dog plasma.

capillary endothelium; *2*) peripheral apolipoprotein synthesis; and *3*) extravascular modification of filtered plasma HDL via uptake of desorbed cellular lipids.

The relative decrease of the 10.4-nm particle in peripheral lymph can be partially explained by a decrease in filtration across the capillary endothelium due to its larger size. Carter, Joyner, and Renkin (20) demonstrated that an increase in effective protein radius from 8.5 nm to 10.5 nm can result in an approximate 25% reduction in a protein's lymph/plasma ratio. The relative decrease of the 10.4-nm population (taken as the interval between 10.0 and 12.0 nm) is on the order of 40%. Thus, while an increase in size can account for a majority of this decrease, we cannot at present exclude the possibility that other mechanisms, such as extravascular remodeling, are also operating to specifically reduce this HDL population in the peripheral lymph.

The decrease in particle size of the plasma 8.4-nm population to 8.3 nm in the peripheral lymph is not as easily explained. This small difference in particle diameter was extremely reproducible and did not appear to be due to methodological problems. Samples of plasma and lymph were electrophoresed side by side on the same gel, thus eliminating potential gel to gel variability. The difference in size was also evident on precast Pharmacia PAA 4/30 gels stained with oil red O (data not shown). This decrease in particle size appears to be at variance with our own previously published data (10) and that of others (21) which have shown that peripheral lymph lipoproteins in this size range (fraction "L-II" in reference 10; HDL_3 in reference 21) tend to be larger than their plasma counterparts. Differences in methodological approaches may partially account for this apparent discrepancy.

Assuming that the peripheral lymph 8.3-nm particle originates from the filtered plasma 8.4-nm particle, then the difference in size roughly translates to a molecular mass difference of 8000 Daltons. This difference is far too small to be acccounted for by the loss of an apoA-I molecule and is therefore most likely due to a loss of lipid moieties. Since the dog has no detectable cholesteryl ester transfer activity (22), loss of surface lipids, especially phospholipids (23) is a likely explanation for the reduction in size.

Within the HDL region of the gel, an additional population of apoA-I-containing particles with a modal diameter of 9.3 nm could be identified in peripheral lymph. Preliminary results (Lefevre, M., C. H. Sloop, and P. S. Roheim, unpublished observations) have shown that apoE can also be localized to a discrete population of particles which migrates to the same position on the gel as the 9.3-nm apoA-I particle. Whether in fact both apoE and apo A-I reside on the same 9.3-nm particles remains to be established.

As previously stated, of particular interest was the observation that a significant portion of peripheral lymph apoA-I was invariably present in the small pore region of the gel (lipoprotein-unassociated apoA-I). Gel filtration chromatography confirmed the presence of the lipoprotein-unassociated apoA-I particles in peripheral lymph and their absence in control dog plasma. The presence of lipoprotein-unassociated apoA-I in peripheral lymph is not unique to the dog model. In preliminary studies we have been able to demonstrate the presence of lipoprotein-unassociated apoA-I in rabbit prenodal peripheral lymph (Lefevre, M., C. H. Sloop, and P. S. Roheim, unpublished observation). Additionally, in human lymphedema fluid, Reichl and associates (21) demonstrated by gel filtration chromatography, the presence of small-sized (smaller than albumin) apoA-I particles.

On agarose electrophoresis, the lipoprotein-unassociated apoA-I fraction migrated with slow pre-beta mobility. Low molecular weight, pre-beta-migrating apoA-I-containing particles have also been identified in normolipidemic and hyperlipidemic human plasma (24–26). Similar to what was observed in dog peripheral lymph, human plasma pre-beta migrating apoA-I exhibited size heterogeneity on nondenaturing gradient gels (26). Dog slow pre-beta apoA-I was found to comigrate on agarose electrophoresis with the majority of purified dog apoA-I. Similarly, in our hands, human pre-beta apoA-I was also found to comigrate on agarose electrophoresis with purified human apoA-I. Taken together, the above observations suggest that dog slow pre-beta apoA-I and human pre-beta apoA-I are physiologically homologous.

In human plasma, pre-beta migrating apoA-I was found to contain approximately 10% lipid, including free cholesterol, esterified cholesterol, and phospholipids (24). We could not detect the presence of cholesterol (free or es-

terified) in association with the lipoprotein-unassociated apoA-I. However, this may be due to a lack of sufficient material for adequate analysis. Attempts at further lipid characterization following specific immunoprecipitation of the lipoprotein-unassociated apoA-I column fraction were not successful. We could not unequivocally demonstrate the presence of specific phospholipids or other lipids in association with the lipoprotein-unassociated apoA-I due to the presence of contaminating lipids in the IgG preparation. However, given the heterogeneous migration of lipoprotein-unassociated apoA-I on nondenaturing gradient gels, it is likely that this apoA-I fraction is complexed with minor amounts of lipids or possibly low molecular weight apolipoproteins.

Three mechanisms can be envisioned for the production of lipoprotein-unassociated apoA-I in the peripheral lymph: *1*) peripheral synthesis; *2*) fusion of apoA-I-containing HDL; and *3*) displacement of HDL apoA-I by phospholipids, free cholesterol, or other apolipoproteins derived from either peripheral cells or from the lipolysis of triglyceride-rich lipoproteins. Newly synthesized apoA-I has been found in the lipoprotein-unassociated fraction in cultures of Hep G2 cells (27, 28) and following transfection of human apoA-I gene into 3T3 cells (29). While apoA-I synthesis has been demonstrated in the peripheral tissue of avian species (30), there has been no evidence for peripheral apoA-I synthesis in mammalian species despite the presence of peripheral apoA-I mRNA (31).

Several studies have shown that lipoprotein-unassociated apoA-I can be produced following the fusion of model HDL particles containing two apoA-I molecules per particle to form a product HDL containing three apoA-I molecules per particle (23, 32). Fusion appears to take place following a reduction in the relative amount of surface components on an HDL particle as would occur following the LCAT-catalyzed production of core cholesteryl esters from the surface lipids, phosphatidylcholine and free cholesterol. However, in peripheral lymph, LCAT activity is quite low (33) and the lipoproteins are enriched rather than deficient in surface lipids (9).

The enrichment of HDL surface lipids argues for a displacement type of mechanism for the formation of lipoprotein-unassociated apoA-I in the peripheral lymph. Tall et al. (34), Nichols et al. (35), and Forte et al. (36) have demonstrated that phospholipids can transfer to HDL_2, resulting in the displacement of apoA-I from the surface. Additionally, it has been established that certain apolipoproteins, most notably apoA-II, can also displace apoA-I from the HDL surface (18). On the other hand, HDL appear to have a substantial capacity to incorporate free cholesterol without apparent disruption of particle integrity (37). These surface components have two possible origins. Following lipolysis of VLDL and chylomicrons, surface remnants are produced which are subsequently incorporated into HDL (38-40). Given the physical proximity between the sites of lipolysis and the interstitial fluid and the relatively low concentration of HDL in the interstitial fluid (leading to a high ratio of surface remnants to HDL), lipolysis of triglyceride-rich lipoproteins may provide sufficient surface components to lead to the displacement of peripheral lymph HDL apoA-I. Indeed, one report has demonstrated the production of plasma lipoprotein-unassociated apoA-I following heparin-enhanced lipolysis of plasma triglyceride-rich lipoproteins (25).

HDL can also become enriched in surface lipids after interaction with lipids desorbed or secreted from peripheral cells. This process, which is regarded as the initial step in reverse cholesterol transport, has been extensively studied in cell culture systems. It is well documented that HDL can take up cellular free cholesterol, leading to a net cholesterol efflux (3, 4). There are less extensive experimental data showing that HDL apolipoproteins can promote the net efflux of cellular phospholipids (3). Perhaps more important, peripheral cells also have the ability to secrete apoE which, when isolated, appears in the form of phospholipid-containing discoidal structures (41). These apoE-containing discoidal structures would be free to combine with peripheral lymph apoA-I-containing HDL (42) and possibly lead to apoA-I displacement. Conclusions similar to these have been made by Riechl et al. (21) with respect to the production of lipoprotein-unassociated apoA-I in human lymphedema fluid.

A previous study demonstrated that human plasma lipoprotein-unassociated apoA-I levels are elevated in hyperlipidemia (25). A more recent study showed similar results for human plasma pre-beta migrating apoA-I (26). We employed a high cholesterol, thyroid-suppressing diet to induce hypercholesterolemia in dogs. Consistent with the observations made in hyperlipidemic humans, hypercholesterolemic dog plasma contained lipoprotein-unassociated apoA-I with slow pre-beta mobility. Furthermore, the amount of plasma lipoprotein-unassociated apoA-I was elevated in hyperresponding dogs over that found in hyporesponding dogs. However, contrary to what was expected, peripheral lymph lipoprotein-unassociated apoA-I did not increase with increasing degree of hypercholesterolemia. Instead, the proportion of lipoprotein-unassociated apoA-I in peripheral lymph was lower in hyperresponding dogs than in hyporesponding dogs. The lower level of lipoprotein-unassociated apoA-I in the hyperresponding dogs appeared to coincide with the appearance of a new population of large-sized apoA-I particles.

These observations made in peripheral lymph of cholesterol-fed dogs can be partially explained by the findings of Nichols et al. (35) who found that the type of products formed following incubation of HDL with phospholipid vesicles is dependent on the ratio of the two components. At low phospholipid to HDL ratios, lipoprotein-unassociated apoA-I is produced; at high ratios, large discoidal

apoA-I complexes are formed at the expense of the lipoprotein-unassociated apoA-I product. In the hypercholesterolemic dog, plasma and peripheral lymph HDL apoA-I levels are less than 25% of control values (8). In the face of constant or elevated cellular phospholipid efflux (possibly in the form of apoE-containing discoidal particles), this reduction in peripheral lymph HDL levels would increase the phospholipid to HDL ratio and possibly result in the formation of large apoA-I discoidal structures. This would be consistent with the observation of discoidal lipoproteins containing apoA-I both with and without apoE in the peripheral lymph of hypercholesterolemic dogs (10). If the large apoA-I particles in the peripheral lymph are discoidal in nature, then their suitability as substrates for LCAT (containing both free cholesterol and apoA-I) would most likely result in their transformation into spherical particles with smaller apparent diameters. This would account for their absence in the plasma compartment.

It is clear from these studies that the relationship between plasma lipoproteins and their counterparts in the extravascular space is complex. Extravascular HDL do not just simply gain cholesterol, but undergo a number of transformations that may include lipid acquisition particle fusion, and loss of apoA-I. Because these particles are absent in the plasma, these peripherally modified HDL are either rapidly removed from the circulation or quickly modified by LCAT, LPL, lipid transfer proteins, and/or hepatic lipase. Given that the magnitude of cholesterol transport from the periphery to the liver has been estimated to be between 0.45 and 0.9 g/day in an adult male (43), the extravascular remodeling of HDL associated with reverse cholesterol transport is likely to have a significant impact on plasma HDL speciation and subclass distribution.

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