Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 206-212

INCORPORATION OF CHOLESTEROL INTO HIGH DENSITY LIPOPROTEIN RECOMBINANTS

Alan R. Tall and Yvonne Lange, Biophysics Section, Boston University School of Medicine, Boston, Mass. 02118

Received November 22, 1977

SUMMARY

Apo-A-1, the principal apoprotein of high density lipoprotein, was incubated with cholesterol containing liposomes of dimyristoyl lecithin, lecithin from high density lipoprotein or sphingomyelin. Conditions were chosen to give 100% conversion of cholesterol-free liposomes into recombinants which were isolated by density gradient ultracentrifugation. For all phospholipids, there was a progressive decrease in incorporation of lipid into recombinants with increasing cholesterol/phospholipid ratio. The cholesterol/phospholipid ratio of recombinants was ∿ 45% of unreacted liposomes, for all initial cholesterol/phospholipid ratios. The reduced cholesterol content suggests exclusion of cholesterol from a fraction of recombinant phospholipid, probably a boundary layer in contact with apo A-1.

INTRODUCTION

Incubation or sonication of lecithin liposomes with the apoproteins of plasma high density lipoprotein (HDL)[1] results in the formation of soluble lipoprotein complexes (1,2) which have been characterized as phospholipid bilayer discs by electron microscopy (3) and small angle x-ray scattering (4). In these discs, apoprotein probably forms an annulus around the perimeter of the phospholipid bilayer, protecting their hydrocarbon chains from interaction with water (5). There appears to be a non-melting boundary layer of phospholipid in contact with apoprotein at the edge of the disc (5). There is evidence that sarcoplasmic reticulum ATPase, an intrinsic membrane protein, excludes cholesterol from its boundary phospholipid (6). To explore the possibility that cholesterol is excluded from the boundary phospholipid of discoidal HDL recombinants, we have initiated a systematic study of the effect of variable cholesterol/phospholipid ratio on recombinant formation and composition.

MATERIALS AND METHODS: Human plasma HDL was prepared from the plasma of normal, fasting male donors, as described previously (2,5). Lipoproteins were delipidated with 2/1 chloroform/methanol (7), and apo A-1 isolated from apo HDL by Sephadex G-200 chromatography, as described previously (7,8). The purity of apo

1. The abbreviations used are : HDL, plasma high density lipoproteins; apo A-1, the principal apoprotein of plasma high density lipoprotein (MW, 28, 331); DML, dimyristoyl lecithin.

0006-291X/78/0801-0206$01.00/0

A-1 was confirmed by Na dodecyl sulfate polyacrylamide gel electrophoresis. Dimyristoyl lecithin (DML) was purchased from Serdary Company and purified to >99% purity by silicic acid chromatography. [^{14}C] DML was prepared as described previously (5). Beef brain sphingomyelin 99% purity was purchased from Lipid Products, Red Hill, Surrey, U.K.
[^{14}C] beef brain sphingomyelin was purchased from New England Nuclear Co., Boston, Mass. Lecithin was purified to > 99% purity from the total lipid extract of HDL by silicic acid chromatography. Cholesterol >99% purity was purchased from Nu-Check Prep (Elysian, Minn) and ^{3}H-cholesterol from New England Nuclear (Boston, Mass). Cholesterol was assayed by the technique of Parekh and Jung (9), and phospholipid by phosphate determination (10).

For preparation of recombinants, aliquots of stock solutions in chloroform of phospholipid and [^{3}H] cholesterol of known specific activity were mixed, the solvent was evaporated under N_2, and the lipids lyophilized from benzene, dispersed in buffer (.01M Tris, .15 M NaCl, pH 9.0) and incubated with apoA-I at a temperature above the phospholipid gel to liquid crystalline transition (27°C for DML and HDL lecithin and 43°C for sphingomyelin). HDL recombinants were isolated by equilibrium density gradient ultracentrifugation at 10°C as described previously (5). It was found that recombinants of sphingomyelin or DML could be separated from unreacted lipid by centrifugtation at 12,000g for 5 min in a Brinkmann model 3200 centrifuge. For HDL lecithin, recombinants were also prepared by sonication for 2 min using a Branson sonifier.

Electron Microscopy-Lipoproteins were negatively stained with 2% Na phosphotungstate, pH7.4, on Formvar-coated copper grids. Micrographs were obtained with an AEI-6B electron microscope calibrated with a catalase standard at magnification approximately x100,000.

RESULTS: DML/cholesterol/apo A-1: Employing a 2/1 w/w DML/apo A-1 ratio without cholesterol there was 98% incorporation of DML into recombinants after 24 hours incubation at 27°C. (Table 1). On density gradients, the recombinants were isolated as a sharp peak of density 1.11g/ml (Fig. 1) Using comparable incubation conditions with cholesterol containing liposomes, there was a marked decrease in incorporation of DML into recombinants (Fig, 1, Table 1). There was also a decrease in the mole ratio of cholesterol/DML of the recombinants, compared to the unreacted, turbid lipid found on the top of the gradients (Table 1). Similar results were obtained for both apoHDL and apoA-1 (Table 1). In a parallel set of experiments it was found that identical results for % incorporation of lipid into recombinants and for the cholesterol/DML ratio of recombinants were obtained after separation of unreacted lipid by centrifugation at 12,000g x 5 minutes. The soluble recombinants were recovered in the clear supernatant while the pellet contained only unreacted lipid. Using this technique to

TABLE 1

Density[1] and lipid composition of recombinants[2] of DML/cholesterol/apoA-1 and DML/cholesterol/apoHDL isolated by equilibrium density gradient ultracentrifugation

Liposomes (mole cholesterol/mole DML)	Peak Density (g/ml)	% Incorporation DML[3]	Recombinant cholesterol/DML[4]	Unreacted lipid cholesterol/DML[5]
DML/apoA-1				
0	1.110	98	0	0
.045	1.096-1.116	92	.04	.10
.24	1.134	23	.19	.25
DML/apoHDL				
0	1.110	100	0	0
.041	1.104	98	.04	.08
.24	1.136	25	.07	.29

1. Density of fraction containing the highest cpm of DML.
2. Recombinants prepared as described in Methods.
3. % DML under peak/total DML on gradient.
4. Average molar ratio of cholesterol/DML in peak.
5. Taken from turbid fractions on the top of the gradient.

study the time course of recombinant formation, it was found the equilibrium values for recombinant formation and composition were attained within 2 hours incubation for all mixtures. In a series of 20 different cholesterol/DML mixtures, ranging from 0 to 1.0 mole ratio with increasing cholesterol/DML ratio there was a progressive decrease in the equilibrium incorporation of DML into recombinants, while the cholesterol/DML ratio of recombinants was consistently ∿45% of that of the pellet of unreacted lipid.

Sphingomyelin/cholesterol/apo A-1: When incubated at 43°C for 5 hours there was 92% incorporation of sphingomyelin into sphingomyelin/apo A-1 complexes. These complexes formed a peak at density 1.15 g/ml on density gradients. For six different mixtures almost identical results for recombinant formation and composition were obtained by equilibrium density and low speed centrifugation. With increasing cholesterol content there

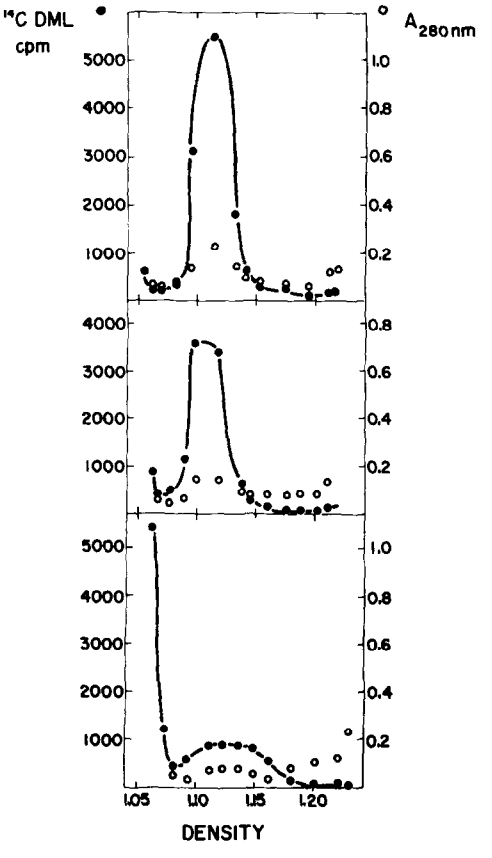

FIGURE 1 Equilibrium density gradients of apoA-1/DML/cholesterol recombinants, prepared from DML/cholesterol liposomes containing mole ratios of a) 0, b) .045, and c) .24. Two mg of DML was incubated with 1 mg of apoA-1 in a total volume of 2 ml at 27°C, with stirring under N_2 for 24 hours. Linear gradients of NaBr were generated with a 3 channel peristaltic pump, employing the incubation mixtures as the limiting buffers. The gradients were centrifuged for 5.4 x 10^6 g x h in a Beckman SW56 swinging bucket rotor. The density of each fraction was determined by weighing a known volume and aliquots were counted for ^{3}H and ^{14}C and the ultraviolet absorbance at 280 nm, determined by spectrophotometry. In a) and b) the top fraction and in c) the top 2 fractions of the gradient were turbid, indicating the presence of unreacted lipid. Recovery of counts from the gradient was > 90%. Density is in g/ml.

was a decrease in the equilibrium incorporation of sphingomyelin into recombinants (Table 2). Also, as shown in Table 2 the cholesterol/phospholipid mole ratio of the recombinants was consistently about 40-50% of the unreacted lipid (mean value = 42%, as determined by equilibrium density

TABLE 2

Lipid composition of recombinants of sphingomyelin/cholesterol/apo A-1 and HDL lecithin/cholesterol/apo A-1.[1]

Liposome (Mole cholesterol/ mole sphingomyelin)	% incorporation phospholipid	Recombinant mole cholesterol/ mole phospholipid
0	94	
0.02	93	0.01
0.04	93	0.02
0.07	86	0.03
0.14	76	0.05
0.61	9	0.33
(mole cholesterol/ mole HDL lecithin)		
0	90	
.29	39	.139

1. Sphingomyelin containing recombinants were separated from unreacted lipid by centrifugation at 12,000g x 5 minutes. HDL lecithin recombinants were separated from unreacted lipid by equilibrium density gradient ultracentrifugation.

gradient ultracentrifugation, and 47% by low speed centrifugation).

HDL lecithin/cholesterol/apo A-1: The formation of recombinants from HDL lecithin multilamellar liposomes and apo A-1 was relatively slow. After 24 hours incubation at 27°C, there was only ∿ 20% solubilization of lecithin. For .25 cholesterol/DML there was <10% incorporation of lecithin into recombinants and the cholesterol/DML ratio of the recombinants was 40% of the unreacted lipid. Incubation for >24 hours increased the incorporation of lipid into recombinants, but several days were required to achieve total solubilization. To increase the rate of recombinant formation HDL lecithin and .23 mole ratio HDL lecithin/cholesterol liposomes were co-sonicated for 2 minutes with apo A-1. Under these conditions 90% of the lecithin was incorporated into recombinants in the absence of cholesterol, while 39% was incorporated under identical conditions

in the presence of cholesterol. The recombinant contained 48% of the cholesterol content of the unreacted lipid (Table 2).

Electron Microscopy: Negative stain electron microscopy showed that for all phospholipids and all cholesterol concentrations, HDL recombinants were lipid bilayer discs of about 160-180 x 55A, resembling those described previously. (3,5)

DISCUSSION

Although apo HDL does not bind cholesterol (11), the effect of cholesterol on recombinant formation has not been investigated previously. We documented a marked reduction of recombinant formation in the presence of cholesterol, especially with higher cholesterol/phospholipid ratios. This may reflect decreased permeability of the lipid bilayer caused by cholesterol (12). Given a final arrangement of apoprotein around the edge of the recombinant disc, spanning the lipid bilayer (5), recombinant formation probably involves an initial stage of penetration of the membrane by segments of apoprotein, a process apparently inhibited by cholesterol.

The cholesterol/phospholipid ratio of recombinants formed from apo A-1 and DML, sphingomyelin or HDL lecithin was consistently $\sim$ 45% of that of the unreacted liposomes, suggesting exclusion of cholesterol from a constant fraction (55%) of recombinant phospholipid. The cholesterol free fraction of phospholipid approximates the estimated size of the boundary layer of phospholipid of DML/apo A-1 discs, representing the outer one to two molecular layers of phospholipid of 160 Å diameter discs (5). Thus, cholesterol in the nonboundary phospholipid is in equilibrium with cholesterol in liposomes, while cholesterol is excluded from the boundary lipid. The similar results obtained for sphingomyelin/apo A-1 or HDL lecithin/apo A-1 discs suggest that they also have a boundary layer of phospholipid which excludes cholesterol.

Nascent HDL is secreted by the liver (13) and small intestine (14) in discoidal form containing lecithin, sphingomyelin and a small amount

of unesterified cholesterol. It has been postulated that these discs derive further unesterified cholesterol from cell membranes and lipoproteins upon exposure to the plasma (13). In our study, results obtained with DML have been confirmed using HDL lecithin and a biological sphingomyelin. Since cholesterol is probably excluded from a major portion of the lipid bilayer of discoidal HDL, regardless of phospholipid composition, nascent HDL may have a limited capacity for dissolving unesterified cholesterol.

REFERENCES

1. Assmann, G., and Brewer, H.B. (1974) Proc. Nat'l. Acad. Sci. USA 71, 989-993.

2. Morrisett, J.D., Jackson, R.L. and Gotto, A.M. (1975) Annu. Rev. Biochem. 44, 183-207.

3. Forte, T.M., Nichols, A.V., Gong, E.L., Lux, S. and Levy, R.I. (1971), Biochim. Biophys. Acta 248, 381-386.

4. Atkinson, D., Smith, H.M., Dickson, J. and Austin, J.P. (1976) Eur. J. Biochem. 64, 541-547.

5. Tall, A.R., Small, D.M., Deckelbaum, R.J. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4701-4711.

6. Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature, 255, 684-687, 1975.

7. Scanu, A.M. and Edelstein, C. (1971) Anal. Biochem. 44, 576-588.

8. Scanu, A.M., Toth, J., Edelstein, C., Koga, S. and Stiller, E. (1969) Biochemistry, 8, 3309-3316.

9. Parekh, A.C. and Jung, D.H., (1970) Anal. Chem. 42, 1423-1427.

10. Gomori, G., (1942) J. Lab. Clin. Med. 27, 955-960.

11. Sodhi, H.S. and Gordon Gould, R. (1967), J. Biol. Chem. 242, 1205-1210.

12. Blok, M.C., van Deenen, L.L.M. and deGier, J. (1977), Biochim. et Biophys. Acta 464, 509-518.

13. Hamilton, R.L., Williams, M.C., Fielding, C.J. and Havel, R.J. (1976) J. Clin. Invest. 58, 667-680.

14. Green, P., Tall, A., and Glickman, R.G. (1977), Circulation 56, III-56.