CHOLESTEROL CONTENT OF SMALL UNILAMELLAR LIPOSOMES CONTROLS PHOSPHOLIPID LOSS TO HIGH DENSITY LIPOPROTEINS IN THE PRESENCE OF SERUM

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Received 14 January 1980

1. Introduction

Successful application of liposomes as drug carriers [1,2] requires control of the extent of drug retention by its carrier in the biological milieu. Thus, although in many instances drugs must be transported quantitatively to where they are needed, in others their gradual release may be preferable, especially when membrane barriers prevent liposomes from reaching target sites [1]. However, work on the in vivo fate of a number of liposome-entrapped drugs [2,3] indicates that, after intravenous injection, there is an almost immediate release of drug in the blood circulation. The rate at which this occurs is much higher than that expected from solute diffusion through intact bilayers [4,5] and it has been attributed, at least in part, to the loss of the structural integrity of liposomes as a result of phospholipid removal by high density lipoproteins [6–8]. We have shown [4,5,9] that in the blood of injected animals or in vitro in the presence of whole blood, plasma or serum, stability (in terms of bilayer permeability to solutes) of small multilamellar [4,9] or small unilamellar [5] liposomes can be controlled by adjusting their cholesterol content. Greatest liposomal stability with total retention of entrapped solutes both in vivo and in vitro was achieved by using small unilamellar liposomes composed of equimolar amounts of phospholipid and cholesterol [5]. Here we show that cholesterol stabilises liposomes in the presence of serum by reducing the loss of their phospholipid component to high density lipoproteins.

2. Materials and methods

Sources and grades of egg phosphatidylcholine, cholesterol, [1-^{14}C]cholesteryl oleate (34 mCi/mmol), 6-carboxyfluorescein and Sepharose CL-6B have been described [4,5]. Egg [^{3}H]phosphatidylcholine (1.05 mCi/μmol) was radiolabelled by catalytic hydrogenation [10] of the fatty acid ester groups. Ultrogel AcA 34, bead size (swollen) 60–140 μm, was purchased from LKB, France. All other reagents used were of analytical grade.

Small unilamellar liposomes containing 6-carboxyfluorescein were prepared [5] from $CHCl_3$ solutions of 40 μmol egg phosphatidylcholine, tracers of the radiolabelled egg phosphatidylcholine (2.7 μCi) and cholesteryl oleate (0.08 μCi) and, when appropriate, 20 or 40 μmol cholesterol. The solvent was evaporated under a stream of O_2-free N_2 and the dried lipids were subsequently disrupted with 2 ml 0.1 M sodium phosphate buffer supplemented with 0.8% NaCl and 0.2% KCl (phosphate buffer, pH 7.4) and containing 0.25 M 6-carboxyfluorescein. The suspension was sonicated for 10 min (1 min sonication with 30 s cooling periods) at 4°C and then centrifuged at 100 000 × g for 60 min [5]. Separation of 6-carboxyfluorescein containing small unilamellar liposomes in the supernatant from the untrapped dye was carried out on a Sepharose CL-6B column (1 × 25 cm) equilibrated with phosphate buffer, the liposomes were then dialysed against the same buffer until their use, at most within 24 h following the gel filtration step. The diameter of such liposomes, measured by electron microscopy, was 30–60 nm [5]. 6-carboxyfluorescein in appropriately diluted liposomal samples was measured in the absence (free dye) and presence (total dye) of Triton X-100 (1% final conc.) on a Perkin-Elmer 204A fluorimeter using excitation and emission wavelengths of 490 and 520 nm, respectively. As little as 4 ng 6-carboxyfluorescein/ml could be

 Elsevier/North-Holland Biomedical Press

measured accurately. The % latent 6-carboxyfluorescein in the liposomal preparation was estimated from $100(Dye_t - Dye_f)/Dye_t$, where t and f denote total and free dye, respectively.

Fresh human serum (1.0 ml) obtained from healthy volunteers was incubated with 0.1 ml 6-carboxyfluorescein-containing doubly radiolabelled liposomes at 37°C for 30 min. On one occasion serum was heated at 55°C for 30 min to inactivate lecithin–cholesterol acyl transferase (LCAT) before mixing with liposomes. Subsequently, 0.50 ml mixture was passed at 20°C through an Ultrogel AcA 34 column (1 × 25 cm) equilibrated with phosphate buffer. The column was eluted with the same buffer at 0.4 ml/min flowrate and the 1.0 ml fractions obtained were analysed for free, total and latent 6-carboxyfluorescein as above and protein by spectrophotometry at 280 nm. For the assay of ^3H [11] and ^{14}C [5] double isotope counting conditions were set: ^{14}C was counted at 90% efficiency with no crossover of ^3H into the ^{14}C channel; efficiency for ^3H counting was 33% with 55% crossover of ^{14}C into the ^3H channel. This was taken into consideration in estimating ^3H radioactivity. In control experiments 1.0 ml serum mixed with 0.1 ml phosphate buffer or 0.1 ml liposomes mixed with 1.0 ml phosphate buffer were treated similarly. In other experiments, following incubation of 2.6 ml human serum with 0.26 ml liposomes as above, the mixture was fractionated [12] by sequential density centrifugation to chylomicrons, very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins using a 6 × 5 ml swingout rotor and a Superspeed 65 centrifuge. ^3H and ^{14}C radioactivities were measured in the fractions obtained. In one experiment individual lipoprotein fractions were passed through Ultrogel AcA 34 columns (1 × 25 cm).

3. Results and discussion

We have investigated the tentative suggestion [4,5] that cholesterol stabilises small unilamellar liposomes in the presence of serum by interfering with phospholipid loss to HDL. Changes in the structural integrity of liposomes were monitored by following the fate of two radiolabels ([^3H]phosphatidylcholine and [^{14}C]-cholesteryl oleate) incorporated into the lipid phase and of 6-carboxyfluorescein entrapped in the aqueous phase at a concentration (0.25 M) which quenches the dye fully. When liposomes become leaky because of changes in bilayer permeability to the solute, entrapped 6-carboxyfluorescein escapes into the medium to attain a concentration which allows the dye to fluoresce [13]. This offers a direct method for measuring changes in liposomal stability [4,5] and also the detection of intact (quenched dye-containing)-liposomes.

After incubation of the cholesterol-free liposomes with serum and the subsequent fractionation of the latter through an Ultrogel AcA 34 column, only a small portion (7.3% of total recovered) of the liposomal 6-carboxyfluorescein is eluted in a latent form (entrapped) with fractions 11–14 (fig.1). These fractions, obtained ahead of most serum proteins (fig.D), contain the eluate in which liposomes together with entrapped 6-carboxyfluorescein and the two radiolabels are recovered quantitatively when chromatographed in the absence of serum (legend to fig.1). Most (87.1%) of the dye initially associated with liposomes is eluted in its free form with fractions 26–34. This massive release of 6-carboxyfluorescein is paralleled by the loss of considerable proportions of the [^3H]-phosphatidylcholine and [^{14}C]cholesteryl oleate markers to the serum protein containing fractions with only 32.9% and 36.5%, respectively retained by liposomes in fractions 11–14 (fig.1A). Practically identical patterns of radioactivity (^3H and ^{14}C) elution were obtained when LCAT in the serum was inactivated before mixing with liposomes (not shown). Furthermore, when serum incubated with the cholesterol-free liposomes was subjected to sequential density centrifugation (table 1), most of the two radiolabels were found associated with the LDL (43.7% and 45.5%) and the HDL (43.9% and 36.0% for [^3H]phosphatidylcholine and [^{14}C]cholesteryl oleate, respectively). A similar association of the two liposomal lipid markers with serum HDL has been already observed [6] association of liposomal phosphatidylcholine with HDL shown [7,8] to represent net transfer of lipid which incorporates itself into the HDL lipid coat [14]. Phospholipid transfer also occurs when liposomes are incubated with purified HDL and apo HDL [8,15–17] but not with purified LDL and VLDL [8]. Since LDL both coelutes (legend to fig.1) and cosediments upon ultracentrifugation [8] together with small unilamellar liposomes, it is very likely that the recovery of some of the ^{14}C and ^3H labels with the LDL fraction (table 1) merely reflects cosedimentation of the remainder of the liposomal entity and the lipoprotein.

Incorporation of increasing amounts of cholesterol

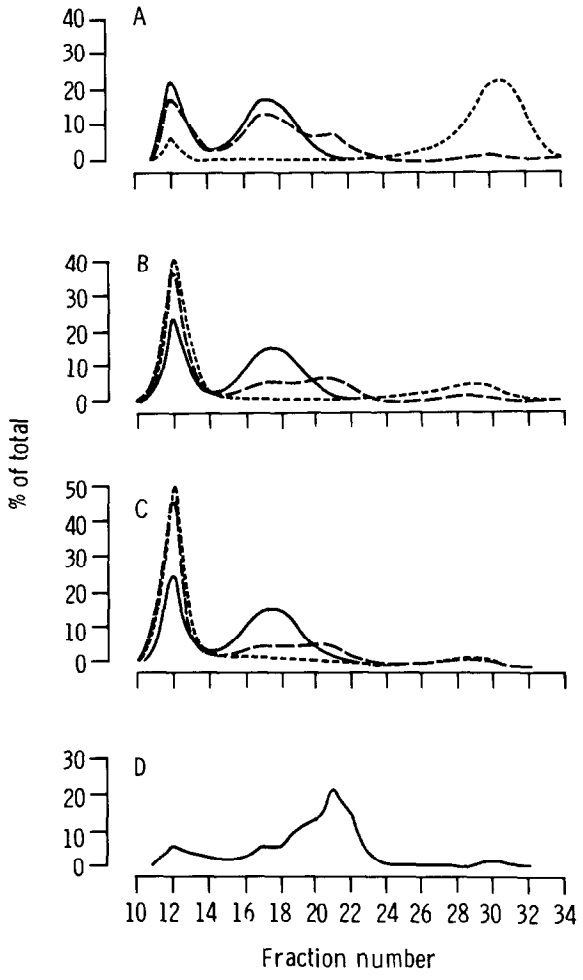

Fig.1. Small unilamellar liposomes (2.0 μmol phospholipid in 0.1 ml) composed of egg phosphatidylcholine [(A) alone or (B) supplemented with 1.0 or (C) 2.0 μmol cholesterol], radiolabelled with [1-^{14}C]cholesteryl oleate and egg [^{3}H]-phosphatidylcholine and containing 6-carboxyfluorescein were incubated with 1.0 ml human serum at 37°C for 30 min (see section 2). Subsequently, 0.5 ml of each mixture (7.4–7.9 × 10^{3} dpm, ^{14}C; 2.5–2.7 × 10^{5} dpm, ^{3}H; 8.2–32.0 μg 6-carboxyfluorescein) was passed through an Ultrogel AcA 34 column. Fractions were analysed for ^{14}C (solid line), ^{3}H (broken line) and 6-carboxyfluorescein (dotted line). Latency for 6-carboxyfluorescein was 95–101% in fractions 11–14 (denoting intact liposomes) and nil in all other fractions. In one experiment serum alone was chromatographed as above and fractions were analysed for protein (D). All values are expressed as % of total radioactivity, dye and protein recovered. Recoveries were 96–106% of the quantities applied. When 0.1 ml liposomes were incubated with 1.0 ml phosphate buffer (in the absence of serum) and 0.5 mixture was then passed through the column, 95–104% of ^{14}C, ^{3}H and 6-carboxyfluorescein were recovered in fractions 11–14. LDL and HDL purified [12] from human serum and then passed through the column were recovered in bulk in fractions 11–14 and 15–24, respectively.

(2.0 and 1.0 phospholipid:cholesterol molar ratio) into liposomes greatly diminished the release of entrapped 6-carboxyfluorescein in the presence of serum (fig.1B,C) and with a phospholipid:cholesterol molar ratio of 1.0, 83.0% of the dye recovered in all fractions remained entrapped, presumably in intact liposomes (fig.1C). Stabilisation in terms of solute

Table 1
Distribution of radioactivity in the lipoproteins of human serum incubated with liposomes radiolabelled with [^{14}C]cholesteryl oleate and egg [^{3}H]phosphatidylcholine

Liposomes	Molar ratio PC/CHOL	Chylomicrons		VLDL		LDL		HDL	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
PC		2.5	4.0	15.4	7.9	45.5	43.7	36.0	43.9
PC, CHOL	2.0	3.3	3.0	12.8	4.5	46.2	60.2	37.1	31.0
PC, CHOL	1.0	3.3	3.4	14.6	4.3	42.8	70.8	38.9	21.2

Small unilamellar liposomes (5.2 μmol phospholipid in 0.26 ml) composed of egg phosphatidylcholine (PC) alone or supplemented with cholesterol (CHOL) and radiolabelled with [1-^{14}C]cholesteryl oleate (6.4–8.2 × 10^{4} dpm) and [^{3}H]phosphatidylcholine (9.2 × 10^{5}–1.3 × 10^{6} dpm) were incubated with 2.6 ml human serum which was subsequently fractionated into chylomicrons, very low density (VLDL), low density (LDL) and high density (HDL) lipoproteins (see section 2). Radioactivity values in the fractions are expressed as % of the total recovered. ^{3}H and ^{14}C recoveries for the three liposomal preparations were 104–110%

release of liposomes with cholesterol was concurrent with their increased retention of phosphatidylcholine (up to 67.4%; fig.1B,C). This was consistent with the progressively increased recovery of the phospholipid in the LDL fraction (which coelutes and cosediments with liposomes, see above) and reduced association with the HDL fraction (down to 21.2% for liposomes with the higher cholesterol content). Interestingly, cholesterol incorporation into liposomes altered neither the pattern of cholesteryl oleate elution through the AcA 34 column (fig.1B,C) nor its distribution pattern upon ultracentrifugation (table 1).

A possible mechanism by which phosphatidylcholine from cholesterol-free small unilamellar liposomes is transferred to HDL in the presence of serum is that apoHDL loosely associated with the HDL particles attaches itself to liposomes subsequent to collision [8]. When a sufficient ratio of apoHDL to phospholipid is attained, the resulting apoHDL-liposome complex undergoes breakdown to smaller particles bearing a portion of the liposome-derived lipid and of a size similar to that of HDL [8]. The way by which cholesterol reduces such phospholipid transfer to HDL (fig.1, table 1) is not clear to us at present. It could be that packing of phospholipid molecules induced by the sterol [18,19] prevents the apoHDL from interacting with the liposomal surface. For instance, if such interaction entails insertion of hydrophobic regions of the protein into the bilayer, increased phospholipid packing could render this difficult. It is also possible that apoHDL and cholesterol-rich liposomes do form a complex but packing of phospholipid molecules by cholesterol or their binding to it [19] prevents them from being removed by the apoHDL. On the other hand, the failure of cholesterol to interfere with the loss of cholesteryl oleate HDL (table 1) as it does with phosphatidylcholine (table 1) is compatible with the notion [8] that cholesteryl oleate molecules tend to cluster in one region of the liposomal bilayer. In this way, the phospholipid–cholesteryl oleate complex becomes less stable than surrounding regions of phospholipid and, therefore, more susceptible to elimination by apoHDL [8]. It is of interest that loss of cholesteryl oleate from cholesterol-rich liposomes to HDL as observed in the present studies, does not appear to occur in vivo. Thus, when serum from mice killed 3, 30 and 60 min after intravenous injection of [^{14}C]cholesteryl oleate-labelled cholesterol-rich liposomes is chromatographed on an AcA 34 column, almost all radioactivity is retained by the carrier in fractions 11–14. In contrast, in similar in vivo work with cholesterol-free liposomes, the elution pattern of the cholesteryl oleate remains identical to that shown in fig.1 (unpublished). Although this discrepancy between the in vitro and in vivo behaviour of cholesteryl oleate is still under investigation, it would appear that for in vivo studies this lipid remains a valid marker for cholesterol-rich liposomes which it, apparently, follows to their destination [5].

These results and [4,5,9] indicate that by adjusting the cholesterol content of liposomes, the permeability of their bilayers to solutes in vivo and in vitro can be influenced accordingly. This is achieved through the control which cholesterol exerts on both phospholipid loss to HDL and the ensuing destabilization of the liposomal structure. In addition to cholesterol [4,5,9,20] sphingomyelin may also contribute towards liposomal stability [20], although not necessarily by a similar mechanism.

Acknowledgements

We thank Mrs Dorothy Seale for secretarial assistance. This work was supported in part by a NIH National Cancer Institute contract (NOI-CM-87171).

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