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Cholesterol mobilization and regression of atheroma in cholesterol-fed rabbits induced by large unilamellar vesicles

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Abstract

The antiatherogenic properties of repeated injections of egg phosphatidylcholine large unilamellar vesicles (LUVs) of 100 nm diameter were tested in an experimental model for atherosclerosis. Forty eight rabbits were divided into two diet groups fed standard rabbit chow or fed a cholesterol-enriched diet (0.5% by weight) to induce the formation of atherosclerotic lesions. Prior to the initiation of LUV therapy, the cholesterol diet was ceased and all animals were returned to standard rabbit chow. The treatment protocol consisted of a total of 10 bolus injections of vesicles, at a phospholipid dose of 300 mg/kg body weight or the equivalent volume of saline, with one injection given to each animal every 10 days. LUV injections brought about a large movement of cholesterol into the blood pool and resulted in a significant reduction in the cholesterol content as well as the degree of surface plaque involvement of aortic tissue in atherosclerotic animals. Most notably, the thoracic aorta of LUV-treated animals exhibited a 48% reduction in tissue cholesterol content per gram of protein compared to saline-treated controls. Histochemical analyses revealed that aortas from animals receiving the repeated injections of LUVs displayed less cholesterol deposits in lesions, and a moderate reduction in intimal-to-medial thickness. This regression of atheroma, induced by LUV therapy, was observed even though animals possessed persistent elevated plasma cholesterol levels after the cholesterol-enriched diet was ceased. These results suggest that repeated injections of

Abbreviations: CE, cholesteryl esters; C:P, cholesterol-to-phospholipid ratio; EPC, egg phosphatidylcholine; HDL, high density lipoprotein; Hepes, [4-(2-hydroxyethyl)]-piperazine ethane sulfonic acid; LDL, low density lipoprotein; LUV, large unilamellar vesicles; N.S., not significant; SUV, small unilamellar vesicle; UC, unesterified cholesterol

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LUVs, working with endogenous HDL, may be a useful therapy in the management of atherosclerosis. © 1998 Elsevier Science B.V.

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1. Introduction

Coronary heart disease, of which most cases are linked to atherosclerosis, remains one of the leading causes of death in the western world. Current preventive management of the disease focuses on the use of drugs along with dietary restrictions to induce favourable changes in plasma cholesterol levels. For example, when plasma low density lipoprotein (LDL) concentrations are kept to near normal levels [1] or antioxidant therapies [2] which suppress the formation and the uptake of modified LDL particles are used, there is evidence that the progression and accumulation of lipids in lesions can be halted. However, while these drugs which lower plasma cholesterol levels induce favourable changes that appear to slow the progression [3], or even promote the regression of atherosclerosis [4,5], the responses to treatment are varied. This is presumably because the primary effect of such therapies is to reduce serum cholesterol and not to directly cause the mobilization of cholesterol which forms the bulk of plaque that can occlude arteries. There is, however, increasing evidence that processes which stimulate the mobilization of peripheral cell cholesterol and promote its transport to the liver for catabolism may be important events in the prevention of atherosclerosis [6,7]. High density lipoprotein (HDL) is believed to be the physiological acceptor of tissue cholesterol and high levels of HDL cholesterol is inversely correlated with the development of heart disease. Consequently, this has led to searches for ways to raise HDL levels by drug intervention [6] or to stimulate the reverse transport of cholesterol by alternative means through the infusion of artificial HDL particles [7,8] or liposomes [9,10].

Many years ago, the repeated intravenous administration of phospholipid was shown to reverse experimentally induced atherosclerosis in a variety of animal models (see [9] for review). When hydrated in aqueous buffer these phospholipids self-assemble into spherical bilayers known as liposomes or vesicles. When these particles are administered intravenously at sufficient doses, they remain as distinct particles in the bloodstream and are capable of extracting cholesterol from lipoproteins and peripheral tissue [9,10]. Thus, circulating liposomes act as thermodynamic 'sinks' for sterol, reducing the chemical potential of plasma cholesterol, and as a result, sterol from peripheral tissues, including those present in the arterial wall are mobilized [9]. Liposomes because of their large size are confined mainly within the vasculature. In order to mobilize peripheral cholesterol stores, liposomes would likely have to interact endogenous small cholesterol acceptors in plasma [9,11], particularly HDL, which can readily access interstitium and cell surfaces. It had been proposed [9] and recently demonstrated in vitro [11] that two specific particle interactions, namely remodeling and shuttling, occur between HDL and phospholipid vesicles to promote the mobilization of cellular cholesterol [11]. During remodeling, the composition of HDL and liposomes are altered resulting in cholesterol-depleted phospholipid-enriched HDL that exhibited an increased capacity to mobilize cellular cholesterol. During shuttling, HDL acts to transport cholesterol back and forth from cells to liposomes. Presumably, as a result of these interactions in vivo, HDL that has been remodeled can scavenge more sterol from vascular and extravascular tissues [9–13]. Since intravenously administered liposomes are cleared mainly by the liver, liposomes are thought to enhance the capacity of HDL to promote the reverse transport of cholesterol [9,10,14].

Despite these seemingly striking results, the development of phospholipid therapy for use to rapidly regress of atherosclerotic plaques in humans, has largely been ignored. The reasons for this are not entirely clear, but might be attributed to the use of ill-defined lipid preparations whose cholesterol mobilizing properties were not well characterized. In addition, the intravenous administration of these early preparations were tedious and involved large doses of phospholipid infused over a period of 8 h [15] or intensive, often bi-weekly, bolus injections of smaller phospholipid doses [16,17]. Moreover, although the mechanisms by which circulating liposomes mediate

the massive removal of cholesterol deposits from peripheral cells were proposed over a decade ago, only recently have they been demonstrated [11]. Finally, recent evidence suggests that not all agents which promote the mobilization of cholesterol to the liver in vivo may be beneficial. For example, the delivery of cholesterol by an apoE-rich HDL fraction in rats [18] resulted in no change in biliary output of cholesterol or bile acids, but stimulated hepatic acyl-CoA:cholesterol transferase and caused increased VLDL secretion. In addition, the repeated intravenous injections of small unilamellar vesicles (SUVs) in rabbits suppresses LDL receptor and HMG-CoA reductase mRNA expression [14], and provokes a subsequent increase in apoB-rich atherogenic lipoproteins [13,14,19]. Also, the intravenous infusion of apolipoprotein A-I phospholipid disks in humans [8] causes a subsequent rise in plasma LDL levels. These potentially harmful side effects are more consistent with events leading to the promotion, not the prevention of atherosclerosis.

We have taken steps to overcome these hurdles by developing and testing a vesicle formulation suitable for clinical use. In a recent report, a variety of liposomal preparations were characterized to determine which was the most effective at mobilizing cholesterol in vivo [10]. These studies were conducted in mice and demonstrated that liquid crystalline, large unilamellar vesicles (LUVs) with a mean diameter of approximately 100 nm were capable of removing 0.5 mol of cholesterol to the liver for every mole of phospholipid injected. These vesicles are easily prepared in large quantities using pharmaceutically accepted procedures and are apparently nontoxic even at high doses [20]. LUVs composed of egg phosphatidylcholine (EPC) represent an ideal size and composition that maximizes the surface area capable of accepting cholesterol in vivo, and are capable of circulating with half-times much longer than multilamellar or small unilamellar preparations [10]. Most importantly, however, LUVs may represent a unique vesicle preparation because they appear to direct a net transport of peripheral tissue cholesterol to a distinct metabolic pool in the liver that does not provoke a subsequent rise in plasma LDL concentrations [14]. Thus, it was predicted on the basis of these criteria, that bolus injections of LUVs, given at reasonable phospholipid doses and using a manageable treatment regimen, would promote a significant regression of atheroma. Consequently, the ability of these optimized vesicles to remove cholesterol and cholesteryl ester deposits from atherosclerotic lesions in cholesterol-fed rabbits were tested.

2. Methods

2.1. Materials

EPC (> 97% purity) was purchased from Princeton Lipids (Princeton, NJ). The cholesterol supplemented diet (0.5% by weight) was obtained from Teklad Premier (Madison, WI). Blood collection tubes and butterfly needles (23 gauge) were from Becton–Dickinson (Missisauga, Ontario). Ketamine, xylazine, heparin, Innovar and Euthanyl were supplied by MTC Pharmaceuticals, Janssen Pharmaceutica and Organon Technika (Ontario). Bio-Gel A-15m was purchased from Bio-Rad. Prepacked Solid Phase silica gel columns were acquired from Burdick and Jackson (Muskegon, MI). All chemical and solvents were of analytical grade purchased from BDH Chemicals (Vancouver, BC).

2.2. Rabbits

Forty eight New Zealand White (NZW) rabbits were housed in wire cages at the Animal Unit of the Research Centre (Shaughnessy Site) conforming to guidelines set by the Canadian Council on Animal Care and the University of British Columbia. The animals were maintained in a controlled temperature environment with a 12 h dark/light cycle. Approximately 150 g of food were given per animal per diem while access to water was unlimited.

2.3. Experimental design

The correlation between hypercholesterolemia and the onset and progression of atherosclerosis in the rabbit is well established [21]. It is known that the response of rabbits to dietary cholesterol is varied with some animals behaving as hyper-responders (large increase in plasma cholesterol) and others classified as hypo-responders. Consequently, the rabbits used in this study were carefully screened so that an

equal distribution of hypo- and hyper-responders were included in each experimental group. The 48 NZW weanlings were screened by measuring their response to the cholesterol-enriched diet (Teklad diet 0533). Briefly, blood was drawn from animals to determine baseline plasma cholesterol concentrations then all animals were fed the 0.5% cholesterol-enriched diet for one week. Plasma cholesterol levels were monitored on a weekly basis until these returned to baseline. Animals were matched by the extent of the rise in their plasma cholesterol levels as well as the rate at which the levels returned to normal. Hyper- and hypo-responders were spread between two groups of 24 and were subsequently fed either standard rabbit chow or the cholesterol-enriched diet for 20 weeks to induce atherosclerotic plaque formation (see experimental outline in Fig. 1). During this time, plasma lipid levels were monitored on a monthly basis to facilitate the randomisation of animals into the respective treatment groups. Two animals were euthanized due to complications probably associated with stress resulting from handling and were not used in the final analyses. After the diet induction period, five animals per group were randomly selected by an outside observer, and sacrificed, to establish the formation of lesions, and serve as the standard or baseline against which the effectiveness of LUV treatment was assessed. Thereafter, all remaining animals were fed regular rabbit chow until the conclusion of the study.

The 18 remaining animals in each diet group were subsequently divided into two subgroups of nine (see Fig. 1) and were paired on the basis of their response to the diet phase of the protocol. The four treatment groups were: (1) Saline-treated cholesterol-fed animals, (2) LUV-treated cholesterol-fed animals, (3) saline-treated standard diet animals and (4) LUV-treated standard diet animals. Thereafter, 9 of the animals in each diet group were treated with bolus injections of LUVs given at a dose of 300 mg phospholipid/kg body weight or the equivalent volume of saline. Treatment was initiated 4 weeks after their return to standard rabbit chow and was given over a 100 day period. Treatment consisted of a total of 10 injections of LUVs (300 mg/kg body weight, ~ 10 ml volume) or saline, with one injection administered into the marginal ear vein every 10 days.

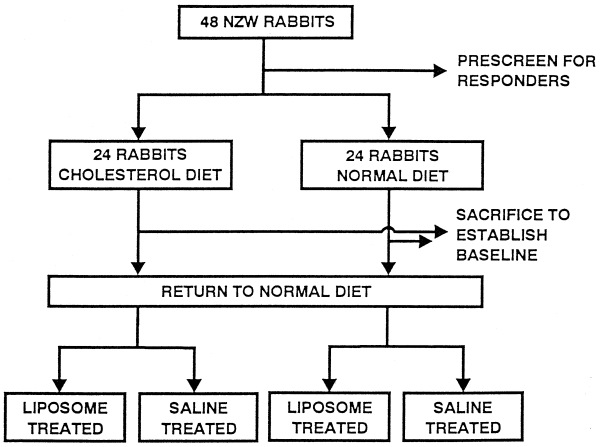

Fig. 1. Experimental design used to test the efficacy of liposomal therapy. Forty eight NZW rabbits were paired on the basis of their response to a 0.5% cholesterol-enriched diet and subsequently divided into two groups of 24 fed either the cholesterol-enriched diet or standard rabbit chow. After 20 weeks of feeding, 5 animals per group were sacrificed to establish baseline characteristics of atherosclerotic lesions. The 18 animals in each diet group were further divided into two subgroups of nine. Animals maintained on standard rabbit during the diet induction period were matched on the basis of the previous screening method described in the methods. The 18 animals fed the cholesterol-enriched diet for 20 weeks were paired on the basis of their response to the initial screening as well as the extent by which plasma cholesterol levels were elevated during the diet phase of the protocol. Treatment was initiated after all animals had been returned to standard chow for 4 weeks and consisted of a bolus injection of LUVs (300 mg phospholipid/kg body weight) or the equivalent volume of saline with one injection given every 10 days for a total of 10 injections.

2.4. Vesicle preparation

The rabbits ranged from 4–6 kg in weight and thus each treatment for the 18 rabbits receiving LUVs required the preparation of approximately 150 ml of LUVs that were prepared at a concentration of 200 mg/ml. Typically, 6 g aliquots of EPC were placed into sterile 50 ml polypropylene conical tubes, hydrated with 30 ml of sterile-filtered 150 mM NaCl, 20 mM Hepes, pH 7.4 (Hepes-buffered saline), vortexed and kept overnight at 4°C. This procedure generated multilamellar vesicles (MLVs) which were subsequently used to generate LUVs by extrusion. Ten ml portions of MLVs were forced through two stacked polycarbonate filters of 100 nm pore size using an 10 ml capacity Extruder (Lipex Biomem-

branes, Vancouver, BC), under medium pressures (300–500 psi). Each 10 ml portion was extruded through the device 10 times to yield LUVs of approximately 100 nm diameter, as previously described [10]. Vesicle diameters were determined by quasi-electric light scattering (QELS) [10]. The average diameter of the LUVs used for the 10 treatments was 114 ± 7 nm.

2.5. Collection of blood and tissue samples

Approximately 100 μl of Innovar was given to promote calmness and vessel dilation in animals to ease routine bleedings necessary for plasma lipid analyses. To anaesthesize the animals for the final blood collection, ketamine (40 mg/kg) and xylazine (8 mg/kg) were given intramuscularly to sedate the animals. Approximately 10 min later, 50 units of heparin (Hepalean) followed by a lethal dose of phenobarbitol (Euthanyl) were perfused into the marginal ear vein before laparotomy. Organs were removed, rinsed in saline and immediately frozen in liquid nitrogen. The heart, with the entire aorta attached, was collected and kept in ice cold saline. The animals were sacrificed in groups of 8–10 on alternate days. The organs and aortas were randomized and allocated for the different analyses by an outside observer prior to any processing. The treatment regimen of the aorta being processed or analysed was not known until all measurements were completed and the samples were decoded.

2.6. Preparation of aortas for analyses

Each aorta was separated from the heart at the aortic valve and was very carefully cleaned to remove any adherent adventitial fat. The aortas were then cut along the ventral surface, spread open, and photographed on a black surface. The developed photographs were later used in conjunction with the negatives to aid in the collection of digitization data as well as to facilitate the division of the aortas into three regions: arch, thoracic and abdominal segments as described by Rosenfeld et al. [22]. Six aortas from the nine animals per treatment group were stored at -20°C prior to lipid analyses. The remaining three aortas were immediately fixed in 10% neutral buffered formalin for at least 48 h, stained with Sudan IV [23], re-photographed, and were subsequently used for histological analyses. At the time of lipid analyses, aortas were pat dried, divided into three segments and a wet weight and length were determined prior to mincing the tissues in Hepes-buffered saline using a Polytron homogenizer. Two additional washes of the probe were carried out to ensure quantitative recovery. Lipid, histochemical and image analyses on all tissue samples were performed on randomized and independently coded samples.

2.7. Separation of vesicles from lipoproteins

Plasma samples were applied to 27×1.5 cm Bio-Gel A15m gel filtration columns equilibrated with 150 mM NaCl, 10 mM Tris, 0.1% EDTA, 0.3% NaN_3 (pH 7.4) to re-isolate LUVs from plasma lipoproteins, as previously described [10]. Briefly, plasma collected after LUV or saline injections were applied to columns and LUVs eluted in the void volume, and were well separated from the remaining lipoproteins. The cholesterol-to-phospholipid (C:P) ratios of LUVs, the void volume fractions of saline controls, and lipoproteins were determined following lipid extractions of pooled samples.

2.8. Digitization

Photographic negatives obtained from all unstained aortas (and the 12 stained aortas) were illuminated on a light box and a digitized image was generated using a Microcomputer Imaging Device (Imaging Systems). The % plaque involvement was calculated by dividing the area occupied by surface plaque by the area occupied by the entire aorta. These areas were easily distinguished by the computer due to distinct differences in the degree of shading between plaques areas and uninvolved aortic tissue. Moreover, computer generated values for % surface plaque involvement from negatives of Sudan IV-stained tissues and from the corresponding negatives of the same tissue unstained were identical. The assessment of the % atherosclerotic plaque involvement of the coded samples were performed by two observers and their results were averaged. Inter-observer variation was within ± 5%.

2.9. Lipid analysis

Cholesterol and phospholipid content of the aortas and livers were measured after Bligh and Dyer lipid extraction of the homogenates [24]. The lipid content of erythrocytes was determined after extraction using the procedure of Rose and Oklander [23]. Total cholesterol, unesterified cholesterol (UC), and cholesteryl esters (CE) were determined according to the method of Rudel and Morris [25]. Cholesterol and cholesteryl esters were separated by silica gel chromatography on pre-packed Solid Phase Silica Gel columns (200 mg silica per column) placed in 16 × 100 mm test tube carriers. Cholesteryl esters were eluted with 1 ml methylene chloride whereas cholesterol was collected following the transfer of the columns to a new carrier and the addition of 1 ml of methylene chloride/methanol (95:5, v/v). Phospholipid content was measured according to Fiske and Subbarow [26]. Lipoprotein lipid profiles were determined using enzymatic procedures after precipitation with phosphotungstic acid [27].

2.10. Protein analysis

Aliquots of aorta or liver homogenates were incubated overnight at 37°C with 1 ml of 1 N NaOH. Thereafter, sodium dodecylsulphate (SDS) was added to the mixture to make a 1% (v/v) solution necessary to solubilize remaining particulate matter. Protein content was quantified using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) after incubation for 1 h at 60°C and read at A_{562} against an albumin standard.

2.11. Histological analyses

Typically, 2–3 mm segments from the arch, thoracic, and abdominal aorta from each of the three animals within each treatment group were cut, further divided into left and right halves and embedded in paraffin blocks. Depending on the length of the aorta, at least 8 segments from each region were prepared as blocks. Alternate sections of 5 μm were adhered to gelatin-coated slides from paraffin blocks and visualized with haematoxylin and eosin (H and E) or Weigart's–van Gieson's stains. Photographs were taken from each paraffin block and due to the magnification used, three photographs of each block were needed to span the entire tissue on the slide. At three standard locations on each photograph, an intimal-to-medial ratio was determined, and from this an average intimal-to-medial ratio was calculated for that particular segment prepared as a block. A final mean ± standard deviation was determined for all the sections made for a particular region and represents the average obtained from the three animals in each treatment group.

The morphology of plaques from animals sacrificed after the diet induction period (but prior to any treatment) were also examined. Segments of aorta were held into place with tissue mount on wooden stages and quick frozen, first in isopentane then in liquid nitrogen. Subsequently, alternate sections of 5 μm were adhered to polylysine-coated slides and visualized with Sudan IV differentiated with Harris' haematoxylin, H and E or van Gieson's stains to highlight lipids and collagen.

2.12. Statistical analyses

Unless otherwise indicated values are presented as mean ± standard deviation. Statistical differences between treatment groups were assessed by analysis of variance using the two-sample *t*-test. Only values of $P < 0.05$ were considered significant.

3. Results

3.1. Establishment of lesions

The animals in this study were fed a 0.5% cholesterol-enriched diet for 20 weeks in order to induce intermediate lesions rather than fatty streaks. Indeed, chemical and histological analyses of the aortas obtained from animals after the diet induction period, but prior to treatment, revealed that the plaques formed were rich in lipid, surrounded by fibrous tissue, and consisted of almost equivalent amounts of UC and CE. The aortic phopholipid content was found to be 15 ± 4 μmol/g wet tissue and total cholesterol was 114 ± 28 μmol/g wet tissue, of which there was 61 ± 13 μmol unesterified cholesterol/g wet tissue and 53 ± 15 μmol CE/g wet tissue. Animals maintained on the standard diet had

aortic phospholipid levels of $4 \pm 0.3\,\mu mol/g$ wet tissue and total cholesterol levels, which were predominantly UC, of $10 \pm 1\,\mu mol/g$ wet tissue. The degree of surface plaque involvement in cholesterol-fed animals was $78 \pm 14\%$.

3.2. Cholesterol mobilization

Animals fed the cholesterol-enriched diet exhibited plasma total cholesterol concentrations that were 5–10 times higher than control animals fed the standard diet. Moreover, even after the cessation of the cholesterol-enriched diet and during the treatment period, plasma cholesterol levels remained elevated (2–5 times higher). This point is illustrated in a typical time course of cholesterol mobilization resulting from the injection of 300 mg LUV phospholipid/kg or the equivalent volume of saline and shown in Fig. 2. Animals previously fed the cholesterol-enriched diet (panel A, open circles) exhibit plasma cholesterol concentrations three times higher than control animals that were fed standard diet throughout the study (panel B, open squares) even though the cholesterol diet was ceased 10 weeks earlier.

The injection of LUVs resulted in a substantial 2.5-fold increase in plasma cholesterol concentrations for both cholesterol-fed and control animals (Fig. 2, solid symbols) when compared to their corresponding saline-treated controls. Note that the first time point was measured at 1 h. The rise in plasma total cholesterol concentrations observed was due solely to a rise in UC levels. No changes in plasma CE concentrations were detected following LUV injections (data not shown) and are consistent with prior work [14]. Plasma total cholesterol levels peak at 24 h before returning to baseline levels after 5 days which correlates with the removal of vesicles from the circulation. The clearance profiles of LUVs are illustrated in Fig. 3 and were determined by measuring plasma phospholipid concentrations. Note that atherosclerotic animals had slightly higher total phospholipid concentrations, nevertheless, similar clearance kinetics were seen for both groups.

The mass of cholesterol accumulated and removed by LUVs is a function of the rate of cholesterol uptake and the rate of vesicle clearance [10]. We can compute the rate of cholesterol uptake by LUVs knowing the C:P ratios in vesicles at specific intervals during the time course. This value was calculated from the cholesterol and phospholipid content of vesicles re-isolated from plasma by gel filtration. Lipid analyses revealed that the rise in plasma cholesterol concentrations in LUV-treated animals was entirely associated with the LUVs, and $> 90\%$ of the this cholesterol was UC with the remainder being CE (data not shown). Hence, C:P ratios of vesicles at any given time can be calculated as follows. The numerator is simply plasma cholesterol levels in LUV-treated animals after cholesterol levels in saline-treated controls are subtracted. This value is then divided by the amount of LUV phospholipid at each time point (this is the plasma phospholipid concentration of LUV-treated animals minus their respective saline-treated controls). The plasma volume of the rabbits is ap-

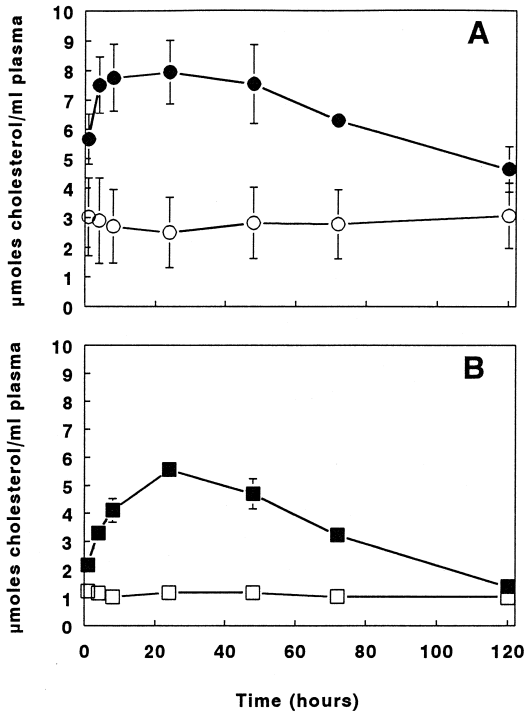

Fig. 2. Redistribution of cholesterol into plasma following a treatment with LUVs. Animals were injected with EPC LUVs at a dose of 300 mg phospholipid/kg or the equal volume of saline via the marginal ear vein. At the indicated time intervals, blood was drawn from the medial ear artery. Plasma total cholesterol concentrations for (A) saline-treated cholesterol-fed (○) and LUV-treated cholesterol-fed (●) animals; (B) saline-treated standard diet (□) and LUV-treated standard diet (■) animals. Each data point represents $n = 4$ randomly selected animals and were measured during treatment number 4.

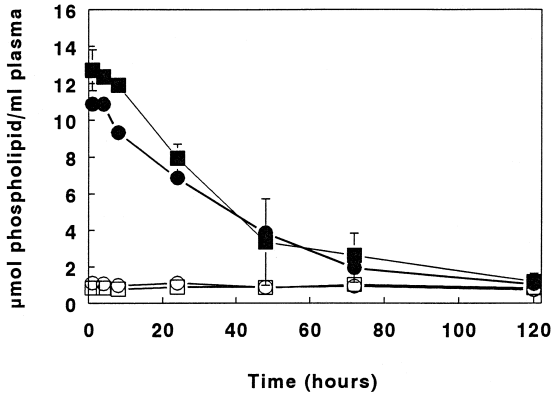

Fig. 3. Phospholipid clearance during a treatment. Animals were injected with LUVs at a dose of 300 mg/kg or the equal volume of saline via the marginal ear vein and at the indicated time intervals blood was drawn from the medial ear artery. Total phospholipid concentrations of plasma determined for saline-treated cholesterol-fed (○), LUV-treated cholesterol-fed (●), saline-treated standard diet (□) and LUV-treated standard diet (■) animals. Each data point represents the same 4 animals described in Fig. 2.

proximately 150 ml, consequently, the amount of phospholipid removed from the circulation at each time point can be estimated. Therefore, by multiplying C:P ratios by the amount of phospholipid removed at a given time interval, an estimate of cholesterol mobilized can be made and is shown in Fig. 4. The data is expressed as the cumulative mmoles of

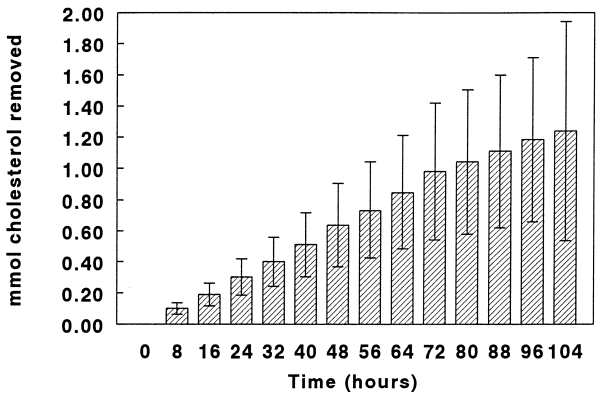

Fig. 4. The cummulative amount of cholesterol removed to the RES resulting from the injection of LUVs. The data presented represents the mean ± standard deviation of estimates made from treatments 1, 4, and 10 with a total $n = 12$ animals (i.e. $n = 4$ randomly selected animals for each treatment). The estimates shown at each time point were calculated as described in the Section 3.

cholesterol removed as a function of time in LUV-treated cholesterol-fed animals. Plasma cholesterol and phospholipid concentration obtained from serial bleeds taken during treatments number 1, 4 and 10 were used to estimate this value. The data represents the mean ± standard deviation. The analysis was not continued beyond the point where less than 10% of the initial phospholipid dose remains in the circulation, because below this level the measurement error was too large to determine accurate C:P ratios. After 104 h, approximately 1 mmol of cholesterol (387 mg) which represents approximately 50 mol% of the injected phospholipid dose, is removed from the circulation, and mobilized to the liver. Plasma cholesterol concentrations measured in LUV-treated animals 24 h post-injection showed similar increases (data not shown) suggesting that each of the LUV injections caused equally dramatic cholesterol mobilization. Using these estimates, the complete therapeutic regimen probably caused about 4 g of endogenous cholesterol to be mobilized from peripheral tissue pools.

3.3. Effects of repeated injections

The goal of this research is to obtain useful preclinical data that can support the development of repeated injections of LUVs as a means to manage atherosclerotic disease. Therefore, it was important to ascertain whether the phagocytic cells of the reticuloendothelial system (RES) were compromised in their capacity to remove LUVs from the circulation after multiple injections of phospholipid.

It is estimated at the dose of 300 mg phospholipid/kg, a transient 10-fold rise in plasma phospholipid concentrations was induced, and on the average, at the end of the 10 injections, each animal received a total of 10–15 g (12–20 mmol) of phospholipid. Although, there is evidence that large and repeated doses of phospholipid are non-toxic in mice [10,20], we are unaware of studies which have monitored the effects of repeated phospholipid infusions on normal clearance function in rabbits. The clearance profiles of several injections of LUVs in cholesterol-fed rabbits are shown in Fig. 5(A). No significant differences in the rates of vesicle clearance between the first, fourth, seventh and tenth injections were detected. The data in Fig. 5(B) also supports this observation. It shows that the concentrations of phospho-

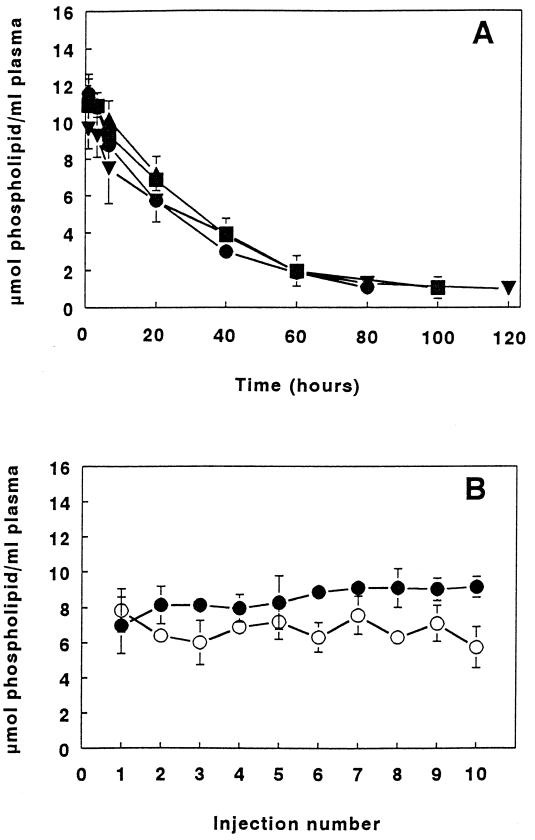

Fig. 5. Consequences of repeated injections of LUVs on clearance kinetics. (A) Clearance profiles of LUVs measured by plasma phospholipid concentrations over time. Data obtained from samples taken during treatment 1 (●), treatment 4 (■), treatment 7 (▲), and treatment 10 (▼) are shown. (B) Phospholipid concentrations remaining in the circulation 24 h-post injections in cholesterol-fed animals (●) and standard diet-fed animals (○). In Panels A and B, each data point represents $n = 4$ animals with four animals randomly selected each time.

lipid in the plasma 24 h post-injection, in both normal and cholesterol-fed animals were similar over the course of 10 injections. Furthermore, 5 days post-injection, plasma phospholipid and cholesterol concentrations always returned to baseline levels suggesting that LUV phospholipid is completely removed from the circulation. These observations suggests that normal clearance mechanisms do not appear to be compromised.

At the conclusion of the study, saline-treated cholesterol-fed animals still maintained elevated plasma cholesterol levels whereas vesicle-treated animals had levels comparable to animals maintained on the standard diet (data not shown). The decrease seen in plasma cholesterol concentrations in vesicle-treated cholesterol-fed animals was a result of a reduction in both plasma LDL and HDL cholesterol concentrations, although the relative proportions of HDL-to-LDL cholesterol were not affected. Also, at the end of the study, LUV-treated and saline-treated control animals maintained standard rabbit chow showed no differences between their lipid profiles (UC, CE, phospholipid or triglycerides, data not shown).

3.4. Source of liposomally accumulated cholesterol

Total erythrocyte cholesterol remained constant at approximately 150 nmol/10^9 cells throughout the injections and were similar to our previous results obtained in mice [10]. In contrast, the C:P ratio of lipoproteins at 1 h sampling was markedly reduced then gradually returned to normal levels after more than 48 h (see Fig. 6). This latter rise mirrors the time course of cholesterol accumulation by vesicles remaining in the circulation (see Fig. 2(A)). These results are consistent with our earlier observations with mice [10], and suggest that the lipoprotein pool of cholesterol in rabbits also rapidly equilibrates with vesicles.

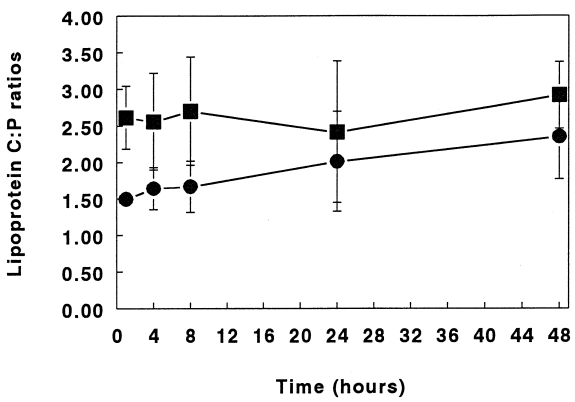

Fig. 6. Cholesterol-to-phospholipid ratios measured in lipoproteins during LUV injection. The plasma lipoproteins were separated from LUVs by gel filtration and pooled. Their lipid content was measured following Bligh and Dyer extraction. C:P ratios in four saline controls (■) and four LUV-treated animals (●) during treatment number 4 are shown. Values were statistically significant over the first 8 h.

3.5. Assessment of atherosclerotic plaque involvement

The extent of progression or regression of atherosclerotic lesions was assessed by three complementary methods: (1) chemical analysis of lipid and protein content which reflects lesion bulk; (2) assessment of the extent of plaque formation on the surface of aortas by image analysis; and (3) histochemistry to examine the morphology and intimal thickening of the lesions.

3.6. Aortic lipid content and digitization

Results presented in Fig. 7 summarize the extent of lipid infiltration and surface involvement in the whole aorta (WH), and the three segments, which were designated as the arch (AR), thoracic (TH), and abdominal (AB) regions. The lipid content is expressed per gram of protein. It was found that on average, the protein content of aortas from treated and untreated animals were not statistically different with values for saline-treated and LUV-treated animals being 0.08 ± 0.04 g protein/g wet tissue and 0.08 ± 0.03 g protein/g wet tissue, respectively. The data is expressed as lipid content per gram of protein weight rather than per gram of wet weight to minimize differences in the extent of tissue hydration. Although not shown, the same degree of statistical differences were observed between treatment groups when aortic lipid levels were compared on the basis of wet weight. Fig. 7(A) shows that LUV-treated cholesterol-fed animals exhibit a 30% reduction in the UC content of the whole aorta. The bulk of this loss, however, occurred in the thoracic region which exhibits a substantial 50% reduction of UC compared to their saline-treated controls. The lipid content for the abdominal segment were very variable and although mean values for LUV-treated animals were 60% lower than those of saline-treated controls, these differences were not statistically different ($P < 0.1$). A power analysis would suggest that increasing the number of animals (though not feasible in this study) might reveal a true difference. The arch region was least affected by LUV treatment, but nevertheless, cholesterol content of LUV-treated animals were reduced by approximately 10% at a significance level $P < 0.05$. Similar trends were seen for CE content

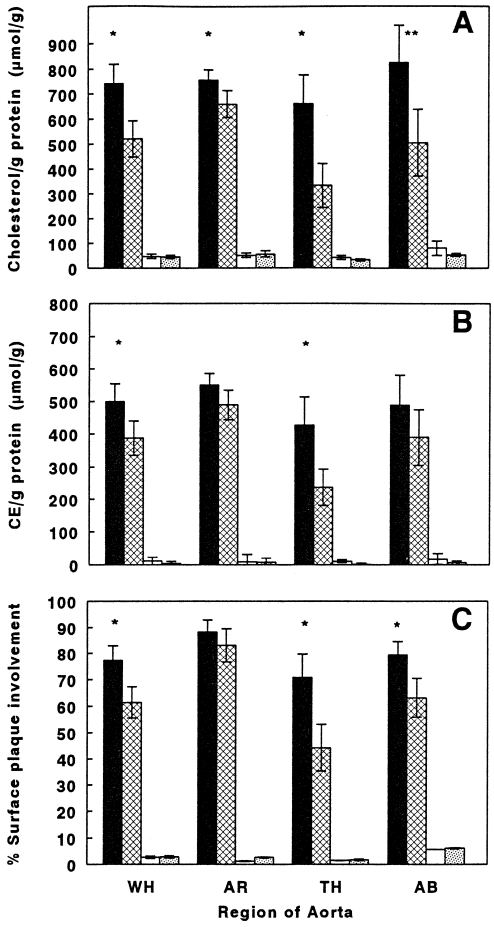

Fig. 7. Assessment of aortic lipid content and the degree of plaque involvement. (A) Cholesterol content (UC) of the whole (WH), arch (AR), thoracic (TH) and abdominal (AB) aorta expressed per g of protein weight. (B) Cholesteryl ester content. (C) Extent of plaque involvement quantitated by digitization. The percent plaque involvement was determined from the area of surface plaque observed and the total area of the aorta being analysed. (1) Solid bars represent saline-treated cholesterol-fed animals; (2) hatched bars represent LUV-treated cholesterol-fed animals; (3) empty bars represent saline-treated standard diet animals; and (4) dotted bars represent LUV-treated standard diet animals. Values shown are the mean ± SEM. $P < 0.05$ (*). Note that six animals were used for lipid analyses and data from nine unstained tissues were used for digitization.

(Fig. 7(B)) and for surface involvement (Fig. 7(C)). In all cases, the most significant and extensive reductions in lipid content were found in the thoracic region of LUV-treated animals. It is important to note that the cholesterol content of the lesions, after treatment with saline for 3 months, did not differ from levels measured in animals sacrificed prior to any

treatment. Prior to therapy, but after cessation of the cholesterol enriched diet, these aortas were found to have 61 ± 13 μmol UC/g wet weight tissue whereas even after saline-treatment and a return to normal diet for more than 12 weeks, aortic cholesterol levels were essentially unchanged and were found to be 58 ± 6 μmol UC/g wet tissue. In contrast, LUV-treated animals showed lower levels of 45 ± 4 μmol UC/g wet tissue. These data were not compared per g protein weight because the protein content of aortic tissue increased during the treatment phase of the diet. Nevertheless, these results suggest that there appears to be no progression or regression of disease during the treatment period in the absence of LUV therapy.

3.7. Histochemical analyses of aortic samples

Digitized photographic negatives were used to quantitate the extent of plaque involvement while histochemical analyses were used to determine the depth and nature of lesions. Extensive raised plaques (intimal thickening) were observed in the cholesterol-fed animals as expected from an inspection of gross surface morphology. Generally, plaques in cholesterol-fed animals exhibited extensive intimal thickening due to stratified lipid deposits that were surrounded by a collagenous network. The arch region was noted to display more advanced lesions and contained what appeared to be deposits of crystalline cholesterol as well as a few isolated necrotic foci as detected by H and E staining (data not shown). Representative sections of the thoracic aorta of control animals fed standard rabbit chow (Panel A), of saline-treated cholesterol-fed animals (Panel B) or LUV-treated cholesterol-fed animals (Panel C) are illustrated in Fig. 8. Note that lesions present in LUV-treated animals (Panel C) manifest less lipid deposits and show moderately reduced plaque thickening when compared to saline-treated cholesterol-fed animals (Panel B). This is also shown in Table 1 which summarizes the intimal-to-medial ratios obtained after measuring intimal and medial widths from photographs of sections derived from the arch, thoracic or abdominal areas. The arch and thoracic regions of LUV-treated animals, showed small reductions in intimal-to-ratios, however, no changes were detected in the abdominal aorta. It is important to

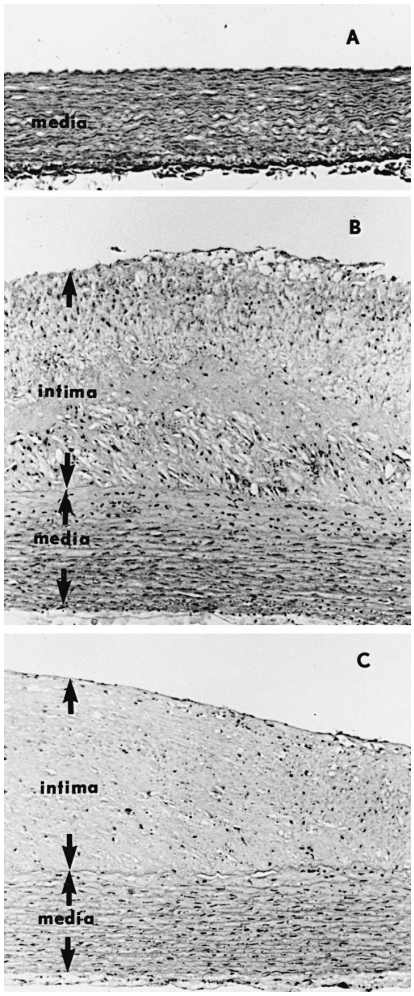

Fig. 8. Representative sections obtained from the thoracic aorta. Animals fed the standard diet only, regardless of whether they were treated with vesicles or saline, do not exhibit any sign of plaque formation (Panel A). In contrast, saline-treated cholesterol-fed animals had extensive intimal thickening and lesions were rich in lipid deposits that were surrounded by collagenous tissue (Panel B). Animals that received LUV treatments show reduced severity of the lesions with what appears to be less lipid and a reduction in plaque thickening (Panel C). Sections were stained with Weigart's-van Gieson's, magnification 10×.

note that these measurements are only approximates of intimal and medial widths since the tissues were not isolated under perfusion techniques. Nevertheless, using a limited number of animals, these results may suggest that the depletion of cholesterol from lesions by LUVs may reduce lesion bulk. No apparent differences were detected in the morphology of aortas between LUV-treated and saline-treated animals that

Table 1
Measurement of intimal-to-medial ratios in the different regions of the aorta of LUV-treated and saline-treated cholesterol-fed animals[a]

Portion of aorta	Intimal-to-medial ratios		Significance (P value)
	LUV-treated	Saline-treated	
Arch	1.51 ± 0.55	1.76 ± 0.94	N.S.
Thoracic	1.34 ± 0.73	1.93 ± 1.12	$P < 0.05$
Abdominal	1.84 ± 0.95	1.81 ± 1.25	N.S.

[a] Data collected from aortic segments obtained from $n = 3$ animals.

were maintained on the standard diet throughout the study.

3.8. Liver cholesterol content

Cholesterol feeding of rabbits often leads to the accumulation of cholesterol in a number of tissues including the liver. However, upon returning animals to regular rabbit chow, tissue cholesterol levels usually revert to normal within a month, with the exception of those found in arterial lesions [28]. We found that the cholesterol levels of LUV-treated cholesterol-fed animals were $8 \pm 3\,\mu mol/g$ wet liver weight and were comparable to control animals maintained on standard chow having levels of $8 \pm 1\,\mu mol/g$ wet weight, whereas saline-treated cholesterol-fed animals exhibited levels of $11 \pm 4\,\mu mol/g$ wet weight. Although these differences were not statistically significant, they results are consistent with those found by Adams et al. [29] who note that the infusion of polyunsaturated phospholipids alleviate fatty livers in the cholesterol-fed rabbit.

4. Discussion

Repeated injections of EPC LUVs, reduced the aortic cholesterol content, the degree of surface plaque involvement and lesion thickness in cholesterol-fed rabbits. Moreover, because lipid content and surface plaque involvement were not significantly different between saline-treated animals and animals sacrificed prior to treatment, it is most likely that LUV-treatment resulted in regression of atherosclerosis and not simply reduced progression. Our data is in general agreement with earlier work demonstrating that phospholipid infusions can promote the reversal of atheroma [15,17,21]. However, unlike previous studies, the data reported here was generated with a well characterized vesicle formulation, and prepared using materials and techniques validated for clinical use in the field of liposomal drug delivery.

The LUV formulation used in this rabbit study was administered as a bolus injection without visible discomfort to the animals. The circulating half-life was approximately 30 h (see Fig. 3), consequently, treating bi-weekly would have been possible. Under those circumstances, treatment might have resulted in even more removal of lipid from atherosclerotic lesions. The data indicated that the most extensive lipid removal occurred in the thoracic region of the aorta, which were observed to be lipid-rich, softer, and more malleable to the touch than lesions in the arch. In this regard, it is interesting to note that $> 80\%$ of fatal heart attacks are associated with the rupture of unstable plaque, where a lipid mixture composed of extracellular deposits of UC and CE is extruded into the vessel lumen through tears in the fibrous cap covering the lesion [30]. Lipid extruded in this way likely blocks critical coronary arterioles and the damaged lesions promote the formation of blood clots, which can further compound the occlusion. Only soft, lipid-rich plaque laden with foam cell macrophages, appear to be rupture-prone. It is this type of lesion that appears to be most responsive to LUV-induced lipid removal. Considering that the accumulation of UC and CE in lesions is a gradual process that occurs over many years, it is especially notable that liposome infusions can dramatically change cholesterol dynamics and may reverse years of cholesterol accumulation during weeks of therapy. The mobilization of cholesterol from arterial wall cells may also have other beneficial effects. It is known that vessel wall cells enriched with cholesterol or oxidized derivatives show substantial disfunction [31–34]. For example, cholesterol-enriched endothelial cells do not produce endothelial-derived relaxing factor [35,36], and cholesterol-enriched smooth muscle cells exhibit enhanced proliferation [37]. These effects can be reversed in vitro by HDL-mediated removal of excess cellular UC [38]. It is tempting to speculate that treatment with LUVs should accomplish the same in vivo quickly and directly.

The mechanisms that surround LUV-induced cholesterol mobilization are not fully understood. However, it is likely that in vivo, there is a co-operation between LUVs and plasma lipoproteins. Moreover, the relative contributions of remodeling and shuttling in vivo are not known. Mechanistic work in vitro shows that LUVs alone were very inefficient at causing the mobilization of cellular cholesterol, although cells were fully exposed in monolayer culture [11,39]. This is likely compounded in vivo, because LUVs are too large to penetrate the vasculature and thus cannot enter the interstitium to access cellular cholesterol or extracellular deposits buried deep within lesions owing to the rupture of cells [40]. In contrast, HDL are capable of promoting the efficient mobilization of cholesterol from cells grown in culture ([39] and references therein) and are able to access lesions and localize throughout plaques [41]. Moreover, Badimon et al. [42] have recently claimed that the intravenous injection of HDL induces a regression of atherosclerosis in the cholesterol-fed rabbit. Several lines of evidence that suggest that remodeling and shuttling are important events for LUV-induced cholesterol mobilization in vivo are as follows. First, it is generally accepted that HDL rapidly gives up its cholesterol to acceptor membranes [10,43] and interestingly, following LUV treatment there is a transient drop in lipoprotein C:P ratios (see Fig. 6 and [10]). Although we do not have direct evidence, it is reasonable to predict that because LUVs present an enormous surface area of (initially) cholesterol free bilayer they can sequester HDL UC, consistent with the idea that remodeling occurs. The prediction is that this remodeled lipoprotein can then scavenge more sterol from accessible tissue sites. Second, our data suggest that after the initial decrease in lipoprotein C:P ratio, these gradually return to normal, and mirror the rate at which LUVs accumulate cholesterol. Because UC entering the blood pool does not appear to be derived from circulating erythrocytes [10], and a net depletion of peripheral tissue cholesterol stores have been reported [44], it is likely that plasma lipoproteins are capable of extracting cholesterol from peripheral sources, consistent with idea of shuttling. The lipoprotein species responsible for this co-operation is not known. Recent evidence suggests that discoidal HDL particles exhibiting preβ mobility on an agarose gel are very efficient at promoting the removal of cholesterol from cell surfaces [45,46]. Although our in vitro studies [11] have demonstrated that the co-incubation of HDL and LUVs (at LUV-to-HDL phospholipid ratios of 10:1) results in the preservation of the integrity of both particles with no apparent generation of preβ-migrating species, the relative contribution of preβ-migrating acceptors in vivo remains to be determined.

It is well documented that LUVs are cleared mainly by the fixed macrophages of the liver and spleen, and to a lesser extent also by hepatocytes through endocytosis [47,48]. In fact, using radioisotopic tracers to track liposomal deposition, approximately 80% of the liposomal dose is cleared by the liver [10,19]. Moreover, at doses typically used in liposomal drug delivery, others have demonstrated that liposomal lipid can be converted into bile acids by the liver and then excreted [48,49]. However, the ways by which the liver disposes of excess peripheral tissue cholesterol delivered by LUVs remains to be determined. Studies aimed at examining the interactions between Kupffer and parenchymal cells may provide useful insights into a unique catabolic pathway especially since LUV-delivered cholesterol appears to be directed to a distinct regulatory pool in the liver that does not provoke a subsequent rise in plasma LDL concentrations [14].

A major goal for the treatment of atherosclerotic disease is to stabilize rupture-prone areas. Future management and possible reversal of atherosclerotic disease, probably lies in pursuing therapies which enhance the removal of arterial wall lipid to stabilize lesions whilst suppressing LDL levels [6]. Numerous experimental and clinical studies document the benefits of aggressive lipid-lowering therapies [50] or antioxidant treatments to decrease LDL concentrations and prevent oxidized LDL formation [2], which in turn, presumably can remodel plaque content. Moreover, there is also strong epidemiological evidence that low levels of HDL are associated with increased atherosclerotic risk while drug therapies and lifestyle changes which increase HDL concentration reduce the risk of developing atherosclerosis [51]. The amounts of HDL required in vitro for the synergistic removal of cellular cholesterol with HDL are remarkably low [11]. For this reason, it is anticipated that LUV therapy should be capable of mobiliz-

ing tissue cholesterol even in low-HDL states and could represent a more general form of therapy for the large number of patients at risk of developing atherosclerosis due to low levels of HDL [52]. Although the phospholipid dose of 300 mg/kg employed in this study is considerably higher than is presently used in liposomal drug delivery formulations, these lipid levels are well below the amounts of total lipid given to patients receiving daily parenteral nutrition in the form of Intralipid, for example. Moreover, the bolus injection of EPC LUVs given to rabbits in this study were very well tolerated, and it is anticipated that LUVs could be administered in the clinic to humans using similar rapid infusion times, thus avoiding the need for hospitalization. The results presented here are comparable to those reported following LDL apheresis [53]. This involves the ex vivo removal of LDL from plasma when blood from a patient is passed through an affinity column that selectively binds apoB-rich lipoproteins. The removal of LDL from the circulation radically reduces plasma cholesterol levels and apparently promotes the removal of cholesterol from peripheral tissues. Although LDL apheresis can promote significant regression, the procedure is highly invasive and requires long and costly dialysis times.

Finally, phospholipid therapy does not compete with existing drugs currently used to lower plasma cholesterol levels [1,54]. For example, bile acid binding resins promote the excretion of bile salts, which in turn directs more cholesterol to bile synthesis and stimulates expression of LDL receptors. Another class of drugs common in this area are the statins, which inhibit HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis. Recent clinical trials have demonstrated that in combination, these two types of drugs act additively to reduce plasma cholesterol levels in many patients (reviewed in [6]). However, this is still often insufficient to bring levels within a low risk, normal range which can be expected to prevent progression of disease. The ability of LUVs to bring about a reduction in the cholesterol content of atheroma inspite of persisting elevated plasma cholesterol levels, suggests that LUVs could provide therapeutic benefits to hypercholesterolemic individuals via a mechanism that is additive and not competitive with currently available therapy.

In summary, the repeated injections of LUVs promote a significant reduction in the lipid content of atheroma in the cholesterol-fed rabbit. This lipid mobilization was observed inspite of persisting elevated plasma cholesterol concentrations in animals during the treatment period. These results suggests that phospholipid therapy using LUVs may be a useful for the treatment of atherosclerosis in humans.

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References

[1] G.A. Reynolds, Circulation 79 (1989) 1146–1148.
[2] G.M. Chisolm, Clin. Cardiol. 14 (1991) Suppl 1:I25-30.
[3] S.M. Grundy, JAMA 264 (1990) 3053–3059.
[4] G. Brown, J.J. Albers, L.D. Fisher, S.M. Schaefer, J.T. Lin, C. Kaplan, X.Q. Zhao, B.D. Bisson, V.F. Fitzpatrick, H.T. Dodge, N. Engl. J. Med. 323 (1990) 1289–1298.
[5] J.P. Kane, M.J. Malloy, T.A. Ports, N.R. Phillips, J.C. Diehl, R.J. Havel, JAMA 264 (1990) 3007–3012.
[6] G. Franceschini, J.P. Werba, L. Calabresi, Pharmacol. Ther. 61 (1991) 289–324.
[7] S. Moncada, J.F. Martin, A. Higgs, Eur. J. Clin. Invest. 23 (1993) 385–398.
[8] J.A. Kuivenhoven, P.N.M. Demacker, A.F.H. Stalenhoef, D. Parjkrt, S.J.H. van Deventer, J.E. Doroan, P.H. Pritchard, J.J.P. Kastelein, Circulation 94 (1996) I-343-344 (Abstract).
[9] K.J. Williams, V.P. Werth, J.A. Wolff, Perspect. Biol. Med. 27 (1984) 417–431.
[10] W.V. Rodrigueza, P.H. Pritchard, M.J. Hope, Biochim. Biophys. Acta 1153 (1993) 9–19.
[11] W.V. Rodrigueza, K.J. Williams, G.H. Rothblat, M.C. Phillips, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 383–393.

[12] L. Krupp, A.V. Chobanian, P.I. Brecher, Biochem. Biophys. Res. Commun. 72 (1976) 1251–1258.
[13] K.J. Williams, A.M. Scanu, Biochim. Biophys. Acta 875 (1986) 183–194.
[14] W.V. Rodrigueza, K.D. Mazany, A.D. Essenburg, C.L. Bisgaier, K.J. Williams, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 2132–2139.
[15] M. Friedman, S.O. Byers, R.H. Rosenman, Proc. Soc. Exp. Biol. Med. 95 (1957) 586–588.
[16] R.F. Altman, J.M. de Mendonca, G.M. Schaeffer, J.R. de Souza, J.G. Bandoli, D.J. da Silva, C.R. Lopes, Arzneimittelforschung 24 (1974) 11–16.
[17] A.N. Howard, J. Patelski, D.E. Bowyer, G.A. Gresham, Atherosclerosis 14 (1971) 17–29.
[18] B.G. Stone, D. Schreiber, L.D. Alleman, C.Y. Ho, J. Lipid Res. 28 (1987) 162–172.
[19] K.J. Williams, S. Vallabhajosula, I.U. Rahman, T.M. Donnelly, T.S. Parker, M. Weinrauch, S.J. Goldsmith, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 242–246.
[20] T.M. Allen, L. Murray, S. MacKeigan, M. Shah, J. Pharmacol. Exp. Ther. 229 (1984) 267–275.
[21] R.W. St Clair, Prog. Cardiovasc. Dis. 26 (1983) 109–132.
[22] M.E. Rosenfeld, A. Chait, E.L. Bierman, W. King, P. Goodwin, C.E. Walden, R. Ross, Arteriosclerosis 8 (1988) 338–347.
[23] R.L. Holman, H.C. McGill, J.P. Strong, J.C. Gear, Lab. Invest. 7 (1958) 42–47.
[24] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[25] L. Rudel, M.D. Morris, J. Lipid Res. 14 (1973) 364–366.
[26] C.H. Fiske, Y. Subbarow, J. Biol. Chem. 66 (1924) 375–400.
[27] M. Burstein, H.R. Scholnick, R. Morfin, J. Lipid Res. 11 (1970) 583–595.
[28] J.T. Prior, D.D. Ziegler, Arch. Pathol. 80 (1965) 50–57.
[29] C.W. Adams, Y.H. Abdulla, O.B. Bayliss, R.S. Morgan, J. Pathol. Bacteriol. 94 (1967) 77–87.
[30] E. Falk, Circulation 86 (1992) Suppl:III30-42.
[31] P. Lesnik, M. Rouis, S. Skarlatos, H.S. Kruth, M.J. Chapman, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 10370–10374.
[32] L.A. Bottalico, G.A. Keesler, G.M. Fless, I. Tabas, J. Biol. Chem. 268 (1993) 8569–8573.
[33] K.E. Watson, K. Bostrom, R. Ravindranath, T. Lam, B. Norton, L.L. Demer, J. Clin. Invest. 93 (1994) 2106–2113.
[34] K.J. Williams, I. Tabas, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 551–561.
[35] P.L. Ludmer, A.P. Selwyn, T.L. Shook, R.R. Wayne, G.H. Mudge, R.W. Alexander, P. Ganz, N. Engl. J. Med. 315 (1986) 1046–1051.
[36] P. Rubba, M. Mancini, Curr. Opin. Lipidol. 6 (1996) 348–353.
[37] T.N. Tulenko, FASEB J. 8 (1994) A401 (Abstract).
[38] M. Chen, R.P. Mason, T.N. Tulenko, Biophys. J. 66 (1994) A388 (Abstract).
[39] W.S. Davidson, W.V. Rodrigueza, S. Lund-Katz, W.J. Johnson, G.H. Rothblat, M.C. Phillips, J. Biol. Chem. 270 (1995) 17106–17113.
[40] C.J. Schwartz, A.J. Valente, E.A. Sprague, J.L. Kelley, R.M. Nerem, Clin. Cardiol. 14 (1991) Suppl 1:I1-16.
[41] Y. Stein, O. Stein, in: A.M. Scanu, R.W. Wissler, G.S. Getz (Eds.), Biochemistry of Atherosclerosis, Marcel Dekker, New York, 1979, pp. 313–344.
[42] J.J. Badimon, L. Badimon, V. Fuster, J. Clin. Invest. 85 (1990) 1234–1241.
[43] M.C. Phillips, W.J. Johnson, G.H. Rothblat, Biochim. Biophys. Acta 906 (1987) 223–276.
[44] M. Friedman, S.O. Byers, Am. J. Physiol. 186 (1956) 13–18.
[45] G.R. Castro, C.J. Fielding, Biochemistry 27 (1988) 25–29.
[46] G.H. Rothblat, F.H. Mahlberg, W.J. Johnson, M.C. Phillips, J. Lipid Res. 33 (1992) 1091–1097.
[47] G. Poste, R. Kirsh, T. Kustler, in: G. Gregoriadis (Ed.), Liposome Technology CRC Press Inc., Boca Raton, Florida, 1984, pp. 1–28.
[48] F. Roerdink, J. Dijkstra, G. Hartman, B. Bolscher, G. Scherphof, Biochim. Biophys. Acta 677 (1981) 79–89.
[49] C. Esnault-Dupuy, F. Chanussot, H. Lafont, C. Chabert, J. Hauton, Biochimie 69 (1987) 45–52.
[50] B.G. Brown, X.Q. Zhao, D.E. Sacco, J.J. Albers, Circulation 87 (1993) 1781–1791.
[51] A.C. Arntzenius, Acta Cardiol. 46 (1991) 431–438.
[52] A. Khachadurian, in: R.J. Cherry, P.J. Quinn (Eds.), Structural and dynamic properties of lipids and membranes, Portland Press, London, 1991, pp. 385–419.
[53] C. Keller, Atherosclerosis 86 (1991) 1–8.
[54] D.R. Illingworth, S. Bacon, Arteriosclerosis 9 (1989) Suppl:I121-34.