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Remodeling and Shuttling:

Mechanisms for the Synergistic Effects Between Different Acceptor Particles in the Mobilization of Cellular Cholesterol

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Abstract

In normal physiology, cells are exposed to cholesterol acceptors of different sizes simultaneously. The current study examined the possible interactions between two different classes of acceptors, one large (large unilamellar phospholipid vesicles, LUVs) and one small (HDL or other small acceptors), added separately or in combination to Fu5AH rat hepatoma cells. During a 24-hour incubation, LUVs of palmitoyl-oleoyl phosphatidylcholine at 1 mg phospholipid (PL) per milliliter extracted $\approx 20\%$ of cellular unesterified cholesterol (UC) label and mass in a slow, continuous fashion (half-time [$t_{1/2}$] for UC efflux was ≈ 50 hours) and human HDL₃ at 25 μg PL per milliliter extracted $\approx 15\%$ cellular UC label with no change in cellular cholesterol mass ($t_{1/2}$ of ≈ 8 hours). In contrast, the combination of LUVs and HDL₃ extracted over 90% of UC label ($t_{1/2}$ of ≈ 4 hours) and $\approx 50\%$ of the UC mass, indicating synergy. To explain this synergy, specific particle interactions were examined, namely, remodeling, in which the two acceptors alter each other's composition and thus the ability to mobilize cellular cholesterol, and shuttling, in which the small acceptor ferries cholesterol from cells to the large acceptor. To examine remodeling, LUVs and HDL were coincubated and reisolated before application to cells. This HDL became UC depleted, PL enriched, and lost a small amount of apolipoprotein A-I. Compared with equivalent numbers of control HDL particles, remodeled HDL caused faster efflux ($t_{1/2} \approx 4$ hours) and exhibited a greater capacity to sequester cellular cholesterol over 24 hours ($\approx 38\%$ versus $\approx 15\%$ for control HDL), consistent with their enrichment in PL. Remodeled LUVs still extracted $\approx 20\%$ of cellular UC. Thus, remodeling accounted for some but not all of the synergy between LUVs and HDL. To examine shuttling, several approaches were used. First, reisolation of particles after an 8-hour exposure to cells revealed that HDL contained very little of the cellular UC label. The label was found almost entirely with the LUVs, suggesting that LUVs continuously stripped the HDL of cellular UC. Second, bidirectional flux studies demonstrated that LUVs blocked the influx of HDL UC label into cells, while the rate of efflux of cellular UC was maintained. These kinetic effects explained the massive net loss of cellular UC to LUVs with HDL. Third, cyclodextrin, an artificial small acceptor that does not acquire PL and hence does not become remodeled, exhibited

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substantial synergy with LUVs, supporting shuttling. Thus, the presence of large and small acceptors together can overcome intrinsic deficiencies in each. Small acceptors are efficient at extracting cellular cholesterol because they approach cell surfaces easily but have a low capacity, whereas large acceptors are inefficient but have a high capacity. When present simultaneously, where the small acceptor can transfer cholesterol quickly to the large acceptor, high efficiency and high capacity are achieved. The processes responsible for this synergy, namely, remodeling and shuttling, may be general phenomena allowing cooperation both during normal physiology and after therapeutic administration of acceptors to accelerate tissue cholesterol efflux *in vivo*.

Keywords

HDL; large unilamellar vesicles; shuttling; remodeling; cholesterol flux

The maintenance of cholesterol homeostasis in extrahepatic tissues involves a delicate balance between synthesis, cholesterol delivery, and the removal of excess UC from nonsteroidogenic cells, which cannot catabolize cholesterol. RCT is the process whereby cholesterol deposits in peripheral tissues are mobilized to the liver for disposal.¹ HDL is thought to be the natural mediator, which may explain why elevated plasma concentrations of this lipoprotein are associated with a reduced incidence of atherosclerosis.^{1–3} However, the precise mechanisms by which HDL acquires excess peripheral tissue cholesterol are not fully understood.

Recently, small particles (rich in PL and containing only apoA-I), termed pre- β -HDL owing to their electrophoretic mobility through agarose gels, have been demonstrated to be the most efficient cholesterol acceptors in fresh plasma.^{4–7} These particles may be related to the HDL species that have been detected in intestinal lymph, liver perfusates, and interstitial fluid⁸ and are believed to be crucial for initiating RCT *in vivo*. Cellular UC acquired by pre- β -HDL eventually appears on the more abundant, α -migrating HDL species,^{4,9,10} and it has been suggested that this occurs through transformation of pre- β particles into α -HDL^{7,10} and possibly by the transfer of some UC from pre- β particles to α -HDL.⁷

Studies using cholesterol-free PL vesicles, a large artificial acceptor of cellular cholesterol, also indicate a complex process for RCT. After intravenous injection *in vivo*, initially cholesterol-free PL vesicles can remain intact in the circulation for hours, during which they accumulate tissue cholesterol^{11–13} (see also Reference¹⁴). Because the liver is the predominant organ that clears vesicles from the circulation, these injected particles thereby act as synthetic mediators of RCT *in vivo*.¹¹ The ability of these vesicles to mobilize cholesterol *in vivo*, however, is puzzling. Large particles penetrate poorly into the interstitial space,¹⁵ where mobilization must occur. Furthermore, recent work has indicated that LUVs are ineffective at removing cholesterol from cultured cells.¹⁶ Evidently, large particles are unable to approach the cell surface, where cholesterol molecules are released (see Reference¹⁷), even when the cells are fully exposed in monolayer culture.¹⁶ In 1984, Williams et al¹¹ proposed that for liposomes to mobilize cholesterol *in vivo*, they would have to interact with small cholesterol acceptors in plasma, such as HDL, through two processes. The first process is remodeling, in which the HDL would become PL enriched

and cholesterol depleted, thereby enhancing its efficacy as a cholesterol acceptor.^{11,18} The second process is shuttling, in which the HDL would diffuse back and forth between cells and liposomes, picking up cellular cholesterol, delivering it to the liposomes, and then returning to the cells to begin the cycle again.¹¹

In the current study, the roles of remodeling and shuttling as mechanisms for the mobilization of cellular cholesterol have been investigated by using mixtures of two types of well-defined acceptor particles, one large (LUVs) and one small (HDL or other small acceptors). Our results indicate that efflux of cellular cholesterol to these mixtures is greater than expected from the efflux observed to the two types of particles separately. The interactions between the large and small acceptors responsible for this synergistic effect are defined.

Methods

Materials

POPC was purchased from Avanti Polar Lipids (+99% grade). [1,2-³H]Cholesterol and [1,2-³H]CHE were obtained from DuPont-New England Nuclear. [8-³H]Adenine was bought from Amersham. Sepharose CL-4B was obtained from Pharmacia, and 0.1 μ m polycarbonate filters were purchased from Poretics Corporation. Sodium cholate and BSA were purchased from Sigma Chemical Co. MEM and FBS were from GIBCO. Media were supplemented with 50 μ g per mL of gentamicin (Sigma). ACAT inhibitor 58035 was a generous gift from Dr John Heider (Sandoz Corporation). All chemicals and solvents were of analytical grade and purchased from Fisher Scientific.

Preparation of Vesicles

POPC taken from a stock in chloroform was placed into a test tube and all solvent was removed, first under a stream of N₂ and then by placing the material under high vacuum for 2 hours. The dry lipid film was hydrated in TBS (NaCl 150 mmol/L, Tris 10 mmol/L, and EDTA 1 mmol/L; pH 7.4) to a concentration of 100 mg PL per milliliter and vortexed to generate MLVs. LUVs were prepared from MLVs by extrusion using a water-jacketed thermobarrel extruder (Lipex Biomembranes) as previously described.¹⁹ LUVs of $\approx$ 100 nm diameter were prepared by passing MLVs 10 times through two stacked polycarbonate filters of 0.1- μ m pore diameter. The vesicles generated had an average diameter of 119 $\pm$ 25 nm (mean $\pm$ SD, as determined by quasi-electric light-scattering analyses using a Nicomp Model 370 submicron laser particle sizer, Pacific Scientific).¹³ LUVs with trace amounts of [1,2-³H]CHE, a lipid marker that does not move between particles in the absence of cholesteryl ester transfer protein, were also generated by this procedure. These labeled LUVs were coincubated with HDL and then used to determine optimal conditions for complete separation of LUVs from HDL by column chromatography.

Purification of Human Lipoproteins and Apolipoproteins

The different lipoprotein species VLDL, LDL, and HDL₃ were isolated from fresh plasma of normolipidemic subjects by sequential ultracentrifugation as described previously.²⁰ HDL was delipidated in ethanol:diethyl ether as described by Scanu and Edelstein,²¹ and purified

apoA-I was isolated by anion exchange chromatography on Q-Sepharose²² and stored in lyophilized form at -70°C . Before each use, apoA-I was resolubilized in 6 mol guanidine HCl per L and dialyzed extensively against standard TBS. POPC-apoA-I discoidal complexes were prepared as described previously.²³

Characterization of Native and “Remodeled” LUVs and Lipoprotein Particles

The size and homogeneity of the lipoprotein particles were estimated by electron microscopy and by nondenaturing PAGE.²³ The hydrodynamic diameter of HDL₃ was estimated by Phast electrophoresis by using precast, 8% to 25% Polyacrylamide gels (Pharmacia) and known protein standards¹⁶ after digitizing gels with a Hewlett Packard desk scanner followed by computer image analysis (Sigma Scan/Image, Jandel Scientific). HDL₃ used for bidirectional flux studies was radiolabeled by an overnight coincubation at 37°C with [^{14}C]cholesterol complexed to celite and then filtered as previously described.²⁴ LUVs and HDL preparations were chemically analyzed for protein,²⁵ PL,²⁶ and sterol contents.²⁷ The electrophoretic mobilities of lipoproteins were measured on Beckman Paragon preformed 0.5% agarose gels as described by Sparks and Phillips.²³ LUVs and HDL used to create remodeled particles were coincubated overnight at 37°C in the absence of cells (ratio of LUV PL to HDL PL was 10:1, unless otherwise indicated). After coincubation, LUVs and HDL were reisolated on a Sepharose CL-4B column ($90\times 1.5\text{ cm}$), run at 0.75 mL/min at 25°C , that was equilibrated with TBS. Fractions corresponding to each repurified particle were pooled and concentrated (Dialflo Ultrafiltration System, 10K membrane, Amicon Corporation) before characterization and use in measurements of cell cholesterol efflux. Prior column calibration with [^3H]CHE-labeled LUVs indicated that $<0.01\%$ of the CHE label eluted in the HDL size range. Moreover, assays of EC, a form present in HDL but not LUVs, showed no detectable EC in the repurified LUVs. Thus, the repurified particles were completely separated from each other. Structural and compositional alterations in the repurified LUVs and HDL were detected by (1) native PAGE (8% to 25%), (2) assays for PL and cholesterol content, and (3) changes in protein content by protein assays²⁵ and protein composition by SDS-polyacrylamide gel electrophoresis.

Cholesterol Flux Studies

Cell Labeling—Five days before each experiment, Fu5AH rat hepatoma cells were plated in 12-well plastic dishes in MEM containing 5% FBS. After 3 days, the cells were washed and labeled with [^3H]cholesterol in the presence of $1\text{ }\mu\text{g/mL}$ Sandoz 58035, an inhibitor of intracellular cholesterol esterification,²⁸ during an overnight incubation in MEM containing 2.5% FBS. ACAT inhibitor compound 58035 was present at $1\text{ }\mu\text{g/mL}$ of media in cell labeling, cell equilibration, and cellular efflux stages. Typically, $1\text{ }\mu\text{Ci}$ [^3H]cholesterol per mL in ethanol was injected into the media. Where indicated, cholesterol flux studies were also performed with mouse L-cell fibroblasts. Mouse L cells were handled exactly as described for Fu5AH cells, except that they were plated in media containing 10% FBS.

Cholesterol Enrichment of Cells—For cellular mass experiments, UC-enriched Fu5AH hepatoma cells were generated by incubating cells that were $\approx 80\%$ confluent with 1 mL MEM supplemented with 5% FBS and UC/PL dispersions ($250\text{ }\mu\text{g}$ cholesterol per milliliter of dispersion) for 48 hours²⁸ in the presence of $1\text{ }\mu\text{g}$ Sandoz 58035 per milliliter of

medium.²⁸ To generate cells that were both UC and EC enriched, Sandoz compound 58035 was omitted²⁸ during both cholesterol enrichment of the cells and the subsequent incubations with cholesterol acceptors.

Cell Equilibration—Eighteen hours before the start of the efflux experiment, medium containing 1% BSA and 1 μg Sandoz 58035 per milliliter of medium was exchanged for the radiolabeling or cholesterol-enrichment medium and left on cells to ensure the removal of non-cell-associated UC. Three washes were performed at the start of the experiment followed by the addition of 1 mL of the appropriate test medium (MEM/HEPES/1% BSA/1 μg Sandoz 58035) unsupplemented (control) or supplemented with serum, VLDL, LDL, or HDL (or control media) with or without LUVs. Particles either individually or in combination were preincubated overnight at 37°C to allow remodeling to occur and were subsequently added to medium before addition onto cells. The cells were maintained at 37°C throughout plating and incubation and typically had specific activities of 0.03 to 0.05 $\mu\text{Ci}/\mu\text{g}$ UC.

Sampling—For UC efflux experiments, at the indicated time points, 75- μL aliquote of the extracellular medium were removed over 24 hours and placed into a Millipore multiscreen assay system to filter dislodged cells and membrane fragments.¹⁶ Fifty microliters of the eluant was placed in scintillation vials and counted according to standard procedures. For bidirectional flux and mass determinations (at the indicated time points) and at the end of efflux experiments, media were removed and cells were rinsed three times with 3 mL PBS containing 1 mmol CaCl_2 and 0.5 mmol MgSO_4 per L before lipid and protein extraction and quantitation. Briefly, cellular lipids were obtained after incubation with 2 mL isopropanol at room temperature overnight. The solvent was evaporated under N_2 , the lipids were solubilized in toluene, and an aliquot was taken for liquid scintillation counting. To determine cellular sterol mass, cholesteryl methyl ether was added as an internal standard to each well during isopropanol extraction and sterol content was quantified by the method of Ishikawa et al.²⁹ as modified by Klansek et al.²⁷ Total cholesterol (equal to unesterified and esterified forms) and UC concentrations were determined by gas-liquid chromatography. UC concentrations were determined directly and EC was calculated by the difference. After evaporation of the isopropanol, cellular protein was extracted from the wells by addition of 2 mL of a 1% SDS solution in 0.1 mol/L NaOH. The protein content was measured by the method of Lowry et al.³⁰ as modified by Markwell et al.²⁵

Separation of Particles After an 8-Hour Incubation on Cells—Medium from triplicate wells containing LUVs alone ($[^3\text{H}]\text{CHE}$ labeled), HDL alone, or the combination were removed from cells, immediately cooled in ice water, and then filtered. Aliquots of 100 μL medium were fractionated using 1 mL mini BioGel A15 m columns by centrifugation at 4°C.³¹ Medium applied onto the columns was subjected to an initial spin of 2000 rpm for 2 minutes in a Beckman benchtop centrifuge (GPR centrifuge) so that the LUVs and HDL were separated within 10 minutes of removal from the cells. Thereafter, 100 μL of cold Tris-buffered saline was added to each column, and repeated cycles of centrifugation and addition of buffer were performed for a total of three buffer washes. Preliminary studies indicated that this procedure of three buffer application and centrifugation cycles did not elute HDL

from the columns, and indeed no detectable radioactivity was found in the eluant obtained from media after an 8-hour exposure of cells to HDL alone. An aliquot of the eluant was taken for counting in a Beckman LS 3801 liquid scintillation counter equipped with a $^3\text{H}/^{14}\text{C}$ dual-label program. The recovery of ^3H CHE label in the eluant was used to calculate the fraction of ^{14}C UC cellular label associated with the LUVs. The fraction of cellular ^{14}C UC label associated with HDL was calculated by the difference.

Viability of Cells—The extent of adenine release as described by Reid et al.³² was used to assess membrane integrity. Radiolabeled adenine was incorporated into cellular pools by adding 0.5 μCi ^3H adenine in MEM supplemented with 1% BSA and 1 μg compound 58035 per mL to Fu5AH cells 24 hours before efflux experiments (adenine release was monitored in plates run in parallel to cholesterol efflux experiments). Twelve hours later, the cells were washed with MEM/1% BSA to allow for label equilibration. The cells were subsequently washed three times with media as described above before efflux media containing the various acceptor particles were applied. The initial amount of cellular ^3H adenine was quantified by extracting cells with 2 mL of a 1% SDS solution in 0.1 mol NaOH per L.

Kinetic Analysis and Statistical Treatment

Data Analysis—The fractional release of cellular UC determined experimentally was analyzed as described in detail for these systems by Davidson et al.¹⁶ Briefly, the kinetic analysis assumed a closed system in which UC exists in one of two kinetic pools, either the cellular UC pool or the acceptor UC pool. The equilibration of UC between these pools was fit to the single-exponential equation: $Y = H_1 e^{-gt} + H_2$, which has been shown to fit the data as well as a double-exponential equation by comparison of correlation coefficients. Y represents the fraction of radiolabeled UC remaining in the cell at a given time, t is the incubation time in hours, H_1 is a preexponential term that reflects the fraction of cellular UC that becomes lost to the medium during approach of steady state, g is the sum of the rate constants for efflux (k_e) and influx (k_i), and H_2 is a constant that represents the fraction of labeled cell UC that remains associated with the cells at equilibrium due to a constant retrograde flux of UC from the extracellular acceptor back into the cells. H_1 , g , and H_2 are variables that can be fit to the experimental data by a computer software package (GraphPad Software Inc). The $t_{1/2}$ for efflux in hours was calculated as $t_{1/2} = \ln 2 / k_e$, where $k_e = g \times H_1$.

Statistical Analyses—Experiments were performed in triplicate cell wells and where indicated, rate constants were derived for each replication. Moreover, experiments were done at least in duplicate. The data were analyzed using ANOVA. When the ANOVA indicated differences among groups, pairwise comparisons between groups were performed using the Student-Neumann-Keuls q test. Values of $P < .05$ were considered statistically significant. Unless otherwise stated, data shown represent the mean $\pm$ SD obtained from triplicate wells.

Results

Lipoprotein/LUV Mixtures as Acceptors

Two strategies were employed to maximize the ability to detect remodeling and shuttling. First, to maximize remodeling, LUVs, serum, and lipoproteins, separately or in the indicated combinations, were preincubated for 18 hours before addition to the cells to allow interactions between particles to occur. Second, to maximize shuttling, relatively low concentrations of serum or lipoproteins were used, a strategy that also mimicked the low lipid levels seen in interstitial fluid.¹⁵ Fig 1A shows the loss of [³H]UC from cultured cells to these mixtures. Both LUVs and serum promoted efflux, though the effects were small, consistent with prior studies.^{33,34} The sum of efflux to these preparations over the 24-hour period was also small (shown as “additive”). In contrast, the combination of LUVs with serum was synergistic, producing a loss of labeled UC from the cells during the 24-hour time course that was far greater than expected on a strictly additive basis. Substantial cholesterol efflux was seen when LUVs (1 mg PL per milliliter) were added to a wide range of serum (1 % to 10%/mL), with the greatest synergy apparent at the lower end of this range.

Examination of separate lipoprotein classes revealed that LUVs added to either VLDL or LDL failed to produce any synergistic loss of cellular cholesterol over a range of lipoprotein concentrations (data not shown). Strong synergy, however, was seen between LUVs and HDL (Fig 1B). LUVs and HDL each promoted cellular cholesterol efflux, though the effects were small, consistent with prior studies,^{16,35} whereas the combination produced near-complete loss of labeled UC from the cells during the 24-hour incubation. The calculated sum of efflux to these preparations over the 24-hour period was also small (shown as “additive”). As previously shown, the rates and extent of UC efflux exhibit a linear dependence on acceptor concentration over a range of 10 to 200 $\mu\text{g/mL}$ HDL PL.^{16,35} Thus, under the conditions chosen, an additive effect in the absence of any interactions between particles is expected. Using adenine release as a measure of cell integrity, there was no detectable cellular toxicity (<18% of total cellular adenine label was released over 24 hours to MEM controls and <15% to MEM supplemented with LUVs plus HDL) (cf Reference³⁵). Enhanced cholesterol efflux was seen when LUVs (1 mg PL per milliliter) were added to a wide range of HDL concentrations (5 to 200 μg HDL PL per milliliter), with the greatest synergy apparent at the lower end of this range (data not shown). Substantial synergy was also seen between LUVs and the $d>1.21\text{-g/mL}$ fraction of plasma, which contains VHDL, and between LUVs and a small, discoidal POPC-apoA-I complex (data not shown). Synergy between LUVs and HDL was also observed with efflux of cholesterol from mouse L-cell fibroblasts (see Table 1).

To verify that these results with labeled cholesterol reflected movement of sterol mass, sterol-loaded Fu5AH cells were incubated with LUVs, HDL, or the combination in the absence of ACAT inhibitor. Fig 2 shows a substantial loss of cellular UC and EC stores to the combination of LUVs and HDL but not to either particle separately. As before, there was substantial synergy between LUVs and HDL that could not be explained by a simple additive effect. Mass data from a 24-hour incubation are shown, but similar effects were seen at 12 hours. Similar mass results were also obtained with cells that had not been loaded with

sterol (data not shown), although the synergy between LUVs and HDL to cause net mass depletion was more apparent in sterol-loaded cells.

Remodeling of LUVs and HDL During Coincubation

LUVs and HDL₃ were coincubated for 18 hours at a PL ratio of 10:1, and then reisolated by gel filtration chromatography (Fig 3A). Note that LUVs and HDL remain as distinct and well-separated particles even after an overnight coincubation. The purity of reisolated particles was verified by native PAGE and agarose gel electrophoresis of the material used for compositional and functional studies. Fig 3B through 3D represents electrophoretic scans obtained from native PAGE gels of the HDL coincubated with saline (Fig 3B), HDL coincubated with LUVs (Fig 3C), and the molecular-weight standards. No detectable change in the size distribution of HDL was observed before or after coincubation with saline or LUVs. To determine whether higher ratios of LUV to HDL PL could alter HDL size, particles were incubated at a 40:1 ratio, followed by native PAGE, which similarly showed no change in HDL size (data not shown).

Table 2 lists the alterations in HDL composition after incubation with LUVs at a 10:1 PL ratio. As expected,¹⁸ the HDL became depleted in UC and enriched in PL. Although no detectable loss of HDL protein mass could be detected by chemical assay of the reisolated HDL, a small amount of protein, amounting to $\approx 8\%$ of the total mass found in HDL, was acquired by the repurified LUVs. SDS-polyacrylamide gel electrophoresis revealed that the LUVs adsorbed primarily apoA-I. The UC lost from the HDL was entirely accounted for in the LUV fraction. No cholesteryl ester moved between the particles.

Table 3 summarizes the diameters measured by native PAGE for HDL particles before and after coincubation with LUVs (10:1 PL ratio) and reisolation. Of particular note, HDL remodeled by LUVs showed little if any increase in size over native HDL or control HDL coincubated by saline, despite the significant increase in PL content (see Tables 2 and 3). HDL incubated with a different vesicle preparation, small unilamellar vesicles, does substantially increase in size.¹⁸ The theoretical estimates of particle diameters calculated from compositional data described in Table 2 are also listed. These estimates confirm that although remodeled HDL PL was enriched over twofold by coincubation with LUVs (see Table 2), there was at most only a 10% increase in the calculated particle diameter (Table 3). This difference is too small to detect accurately using native PAGE.²³ Electron microscopy performed on control and remodeled HDL particles revealed that remodeled particles are similar in size and shape to control particles (not shown).

Fig 4A shows cellular cholesterol efflux to control and remodeled HDL, which was added at equivalent particle numbers (ie, the same HDL EC concentration). The remodeled HDL was a significantly better acceptor of cellular cholesterol, both in terms of initial rate of sterol removal and in total capacity for labeled sterol after 24 hours. The $t_{1/2}$ for efflux to control HDL was 8.4 ± 0.6 hours and to remodeled HDL was 3.5 ± 0.5 hours. These enhancements could be attributed to the increased PL content of remodeled HDL: when added to cells at equivalent HDL PL concentrations,³⁶ the efflux rates were identical ($t_{1/2}$, 8.1 ± 0.5 hours and 8.1 ± 0.9 hours) between control and remodeled HDL (data not shown), consistent with the similar sizes of the particles.¹⁶

Fig 4B depicts cellular cholesterol efflux to remodeled LUVs and remodeled HDL separately and when added in combination. The calculated sum of efflux to remodeled particles is also shown. The remodeled LUVs exhibited no improvement over the original LUVs in their ability to promote cellular cholesterol efflux in the absence of small acceptors (cf Fig 1B). Despite the estimated loss of a tenth of the LUV PL to HDL (when LUVs and HDL are incubated at a ratio of 10:1), the rate and extent of UC efflux seen when remodeled LUVs are added onto cells at 1 mg PL per milliliter or when normalized for particle numbers were superimposable. Similarly, this slight change in LUV PL content during remodeling had no effect on UC efflux, even in the presense of HDL (data not shown). Thus, for comparison's sake, remodeled LUVs are presented in the recombination experiments at a concentration of 1 mg PL per milliliter. As noted above, remodeled HDL was a better cholesterol acceptor than the original HDL particle. However, there was still a large discrepancy ("residual synergy") between the sum of efflux to the remodeled particles individually compared with the efflux to the remodeled particles added in combination. Note that efflux to the original combination of LUVs and HDL (Fig 1B) and to the combination of remodeled LUVs and HDL (Fig 4B) was essentially identical, as expected.

The residual synergy, unexplained by remodeling, is also illustrated in Fig 4C. The time courses for the efflux of cellular cholesterol under three conditions are compared: (1) the calculated sum of efflux to the original LUVs and HDL added to cells separately ("original additive," taken from Fig 1B), (2) the calculated sum of efflux to the remodeled particles added separately ("remodeled additive," taken from Fig 4B), and (3) the observed efflux when LUVs and HDL are added in combination ("particle combination," taken from Fig 4B). The distance marked by arrow A (between original additive and particle combination) represents the total synergy, and the distance marked by arrow B (between remodeled additive and particle combination) represents residual synergy unexplained by particle remodeling. After 24 hours, this residual synergy was $\approx 60\%$ of the total synergy (height of arrow B divided by height of arrow A). Thus, under these conditions, remodeling accounted for $\approx 40\%$ of the total synergy between LUVs and native HDL in promoting cellular cholesterol efflux. Using a range of remodeled HDL concentrations, particle remodeling was consistently found to account for 20% to 50% of the total synergy observed when the two types of acceptor particles were incubated with cells (data not shown). These results indicate that 50% to 80% of the synergy between LUVs and HDL in causing cellular cholesterol efflux must arise from a process, such as shuttling, that requires ongoing interactions between LUVs and HDL.

Shuttling of UC from Cells to the LUV Sink

Several complementary approaches were used to demonstrate shuttling of cellular cholesterol from cells to the LUVs, ie, that LUVs act as a "sink" and that small acceptor particles act as the carriers for cellular cholesterol. One approach was to reisolate particles after an 8-hour incubation with [^{14}C]UC-labeled cells. HDL added alone acquired $16 \pm 1\%$ of cellular label, and LUVs added alone acquired $12 \pm 1\%$ of cellular label, consistent with Fig 1B. When LUVs and HDL were added in combination, $56 \pm 2\%$ of the [^{14}C]UC was found in the medium: $53 \pm 5\%$ of cellular [^{14}C]UC was associated with LUVs and only $3 \pm 5\%$ of cellular [^{14}C]UC was found in the HDL fraction. Thus, despite the increased efficiency and

capacity of remodeled HDL to accept cellular cholesterol (see Fig 4A), the continued presence of LUVs reduced the amount of cellular cholesterol accumulated by HDL. Also, despite the relative inability of LUVs to acquire cellular cholesterol in the absence of HDL, LUVs in the presence of HDL became the predominant reservoir of cholesterol in the extracellular medium. The simplest explanation is that HDL carried cholesterol from cells to the LUVs, and that HDL was continuously stripped of its cholesterol.

Another approach was to study the bidirectional flux of cholesterol between cells and HDL, in the absence or presence of LUVs. For these studies, higher concentrations of HDL than in the preceding studies were required to allow the detection of cholesterol influx from labeled lipoproteins into cells (LUV:HDL PL ratio of 5:1). Note that in the absence of LUVs, the equilibration of label in these studies is dependent on the amount of UC mass present in the media (initially in HDL) relative to that present in the cells. The addition of LUVs had a major effect on the influx of [^{14}C]UC from HDL back into the cells (compare solid and open circles, Fig 5). Over the 24-hour period, cellular acquisition of $\approx 25\%$ of HDL [^{14}C]UC in the absence of LUVs was reduced to $<6\%$ in their presence. This result indicates that LUVs caused the UC of HDL to become sequestered in a pool, presumably in the LUVs themselves, that is inaccessible to the cells. This conclusion is also supported by the observation that although the rates and extents of cellular cholesterol efflux were similar when cholesterol-free and cholesterol-containing LUVs (10:90 UC/PL mol/mol) were placed on cells (data not shown), there was no detectable influx of LUV UC label to cells. In contrast, LUVs had virtually no effect on the initial rate of cellular [^3H]UC efflux to the medium: during the first 3.5 hours, the $t_{1/2}$ for efflux to HDL alone was 3.0 ± 0.1 hours and to HDL and LUVs was 2.4 ± 0.1 hours. This result is consistent with the process of shuttling: cellular loss of cholesterol is mediated by HDL, regardless of the presence of LUVs. Thus, by blocking influx of HDL UC into cells while maintaining efflux of cellular UC to the medium, LUVs shifted the steady state distribution of [^3H]UC. After 24 hours, cells had lost $\approx 70\%$ of their [^3H]UC label to HDL but $>90\%$ to HDL plus LUVs.

The final approach to demonstrate shuttling was to use an artificial acceptor of cholesterol, cyclodextrins,^{35,37–40} that our preliminary studies showed to be unable to acquire PL from LUVs (data not shown). Hence, remodeling of the small acceptor does not occur when a mixture of cyclodextrin and LUVs is used to remove cellular cholesterol. Similar to the studies with HDL, a low concentration of 2-hydroxypropyl- β -cyclodextrin (1 mmol/L), shown previously not to promote significant efflux from cells, was chosen.³⁵ LUVs added to Fu5AH cells alone at a concentration of 1 mg PL per milliliter extracted only 1.5% of cellular [^3H]UC over a 2-hour time course, and 1 mmol/L cyclodextrin extracted only 2%. The combination, however, promoted extremely rapid loss of cellular cholesterol (40%) within 2 hours. These results are most compatible with the concept that, in the presence of a sink, small acceptors can act as shuttles for cellular cholesterol.

Discussion

The current study demonstrates substantial synergy between large and small acceptors of cholesterol in promoting cellular cholesterol efflux. Fig 6 illustrates the possible interactions when physically different acceptors are present simultaneously. In the simplest scenario,

barring any interactions between LUVs and small acceptors, it was anticipated that the extent of cholesterol release from cells should be predicted by the calculated sum of efflux to the two particles separately (the small acceptor shown is HDL). Instead, substantial synergy, greater than predicted by a strictly additive effect, was observed. With HDL, this synergy results from two complementary processes, namely, remodeling of the HDL by PL enrichment that converts it into a better cholesterol acceptor and shuttling of cholesterol from cells to LUVs by HDL.

The present findings resolve an apparent inconsistency between prior work using LUVs in vitro and in vivo. LUVs promote substantial cholesterol mobilization in vivo^{13,41} but are virtually inert in serum-free medium added to cells growing in culture.¹⁶ In the mid 1980s, Williams and colleagues¹¹ hypothesized that remodeling of HDL by liposomes and shuttling of cholesterol by HDL from cells to liposomes may be responsible for the large mobilization of tissue cholesterol into plasma after PL injection. Subsequent studies demonstrated that a remodeling of HDL by liposomes occurs in vitro¹⁸ and in vivo,^{13,18,42} although the ability of this remodeled HDL to promote cellular cholesterol efflux had not been examined. The current study substantially extends this prior work by directly elucidating the contributions to cellular cholesterol efflux of HDL remodeling and shuttling. The present results indicate that remodeling of HDL results in a more efficient acceptor of cellular cholesterol in vitro, but this increase in efficiency cannot account for the enhanced mobilization of cellular cholesterol observed when LUVs and HDL are combined. In fact, in the cell-culture system, most of the synergy between LUVs and HDL was the result of shuttling. The detection of shuttling will be most apparent with the combination of a very efficient small acceptor and a large acceptor incapable of promoting cellular UC mobilization on its own. In vivo, the contributions of remodeling and shuttling are not known, but it is likely that both occur. After injection of LUVs in vivo, HDL becomes PL enriched and UC depleted,^{13,18} consistent with the remodeling observed in vitro (Table 1). Also, virtually all of the cholesterol that is extracted from tissue stores following an injection of liposomes in vivo is carried by the liposomes themselves,^{13,18} yet such large particles penetrate poorly into the interstitial space. Thus, direct acquisition of this cholesterol by the liposomes in vivo is unlikely suggesting an essential role for shuttling of cholesterol by small acceptors.¹¹

The remodeling of HDL by LUVs results in particles that exhibit a greater efficiency and a larger capacity to extract cellular cholesterol. Ordinarily, HDL particles contain relatively few PL molecules (about 50),⁴³ and incoming UC molecules must compete with apolipoprotein molecules for interaction with these PLs.⁴⁴ PL enrichment by LUVs substantially increases the number of PL molecules per particle, which results in an enhancement in the initial rate of sterol removal and in the total capacity of remodeled particles. Despite the increased PL content of remodeled HDL, there was no detectable change in the particle diameter by PAGE or particle shape by electron microscopy. Hence there was no slowing in the kinetics of cholesterol removal compared with control HDL when matched for PL content (Fig 4A and “Results”). The apo A-I acquired by LUVs from HDL did not change the efficiency or capacity of remodeled LUVs to extract cellular cholesterol, consistent with prior observations.¹⁶

Shuttling of cellular cholesterol may be a general phenomenon allowing synergy between different acceptors: the combination of a large and a small acceptor overcomes inherent deficiencies in each particle. By themselves, small acceptors, such as HDL or cyclodextrins, are efficient acceptors of UC molecules from cells but have a low capacity. By themselves, large acceptors, such as LUVs, have a high capacity to solubilize cholesterol but are too large to be efficient acceptors. For at least four reasons, the small size of HDL enables it to efficiently extract cholesterol from cells ($t_{1/2}$, ≈ 3 to 7 hours) through its ability to approach the cell surface, where most UC transfer occurs.^{16,17,35} First, compared with large acceptor particles, HDL diffuses more rapidly and therefore more readily permeates the unstirred water layer that surrounds cells. Second, any glycocalyx barrier around cells would preferentially allow entry of HDL over larger particles. Third, these small particles can better access membrane regions, such as membrane folds or invaginations (eg, caveolae), that might be important in UC efflux.^{16,45,46} Fourth, small particles in vivo penetrate more efficiently into the interstitial space.⁸ In contrast, LUVs of ≈ 120 nm diameter, which contain $\approx 85\,000$ PL molecules, have an enormous capacity to solubilize cellular cholesterol, but because of their large size they are very inefficient at promoting cholesterol efflux from cell monolayers ($t_{1/2}$, ≈ 70 hours).¹⁶ LUVs exhibit much slower diffusion rates than HDL and take ≈ 2 hours to fully equilibrate across the unstirred water layer surrounding cells.¹⁶ LUVs are also inefficient at donating cholesterol to cultured cells, consistent with relatively slow off-rates for UC from the LUV bilayer ($t_{1/2} > 4$ hours).⁴⁷ The movement of cholesterol from HDL to larger particles, however, is very rapid ($t_{1/2}$, ≈ 3 minutes at 37°C).⁴⁸

Thus, when LUVs and HDL are combined on cells, HDL efficiently acquires cellular cholesterol and rapidly transfers it to LUVs. When present simultaneously, high efficiency and high capacity are achieved. During shuttling, the kinetic effect of LUVs is to act as a sink or trap for UC, blocking its reentry into the cells. Shuttling has virtually no effect on the initial rate of cellular UC efflux, which is mediated primarily by HDL, regardless of the presence of LUVs (Figs 4B and 5). During shuttling, the kinetic effect of the small acceptor is to act as a catalyst that accelerates the achievement of steady state between UC pools in cells and LUVs. For example, achievement of steady state between cells and the combination of LUVs with HDL resulted in the loss of $\approx 90\%$ of cellular [^3H]UC and took ≈ 24 hours (Fig 1B). By curve fitting the data of efflux to LUVs alone, achievement of the same steady state would have taken 100 hours or ≈ 4 days. The results with cyclodextrins show an even stronger effect.

Remodeling and shuttling may also occur in physiological settings among endogenous lipoproteins, in the absence of LUVs. Cellular UC acquired by pre- β -HDL eventually appears on the more abundant, α -migrating HDL species, with the concurrent loss of pre- β -HDL species.^{4-6,10} It is unclear whether this movement of cellular cholesterol from pre- β -HDL species to α -HDL is due solely to transformation (remodeling) of the pre- β particles into α -HDL or if a transfer of cholesterol (shuttling) also occurs. Definitive studies aimed at tracking the flow of UC have been difficult, owing to the low abundance of pre- β particles, which represent $< 4\%$ of the cholesterol mass in plasma, and to the rapid transformation into α -HDL by the action of lecithin:cholesterol acyltransferase in whole serum. It has been suggested that pre- β -HDL particles undergo a transformation as they acquire cellular lipids to become discoidal in nature⁷ and thus would likely exhibit slower off-rates for UC ($t_{1/2}$,

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Selected Abbreviations and Acronyms

apo	apolipoprotein CHE = [1,2- ³ H]cholesteryl hexadecyl ether
EC	esterified cholesterol
FBS	fetal bovine serum
LUV	large unilamellar phospholipid vesicle
MEM	minimum essential medium
MLV	multilamellar vesicle
PAGGE	Polyacrylamide gradient gel electrophoresis
PL	phospholipid
POPC	1-palmitoyl, 2-oleoyl phosphatidylcholine
RCT	reverse cholesterol transport
t_{1/2}	half-time for efflux
TBS	Tris-buffered saline
UC	unesterified cholesterol

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FIG 1.

Time courses of [³H]UC efflux from rat Fu5AH hepatoma cells to LUVs, serum, HDL, and combinations. Fu5AH cells trace-labeled with [³H]UC in 22-mm tissue-culture wells were incubated for 24 hours at 37°C in a humidified incubator (5% CO₂) with 1.0 mL of test medium (MEM/HEPES containing 1% BSA, 1.0 μg Sandoz compound 58035, and the indicated cholesterol acceptors). A, Time course for the release of UC label from cells exposed to POPC LUVs 1 mg PL per milliliter (▼), 1 % serum (■), and the combination of these two acceptors at these concentrations (▲). The calculated sum of cholesterol release to LUVs and to serum is shown by ◆, dashed line and is marked “additive.” B, Time course for the release of cellular [³H]UC by POPC LUVs 1 mg PL per milliliter POPC LUVs (▼), HDL₃ at 25 μg PL per milliliter (■), and the combination of these two acceptors at these concentrations (▲). The calculated sum of cholesterol release to LUVs and to HDL added separately is shown by ◆, dashed line and is marked “additive.” Data points represent the mean±SD of the value derived from three cell wells after correction for efflux to control media (MEM/HEPES containing 1% BSA). All curves were fit to a single-exponential decay equation as described in “Methods.”

FIG 2.

The depletion of cellular UC and EC mass in cholesterol-loaded Fu5AH cells exposed to LUVs, HDL, and the combination. A, Net loss of cellular UC mass expressed as micrograms UC per milligram cell protein. B, Net loss of cellular EC mass in micrograms EC per milligram cell protein after a 24-hour exposure to the indicated acceptors. Data shown represent the mean $\pm$ SD of the net loss of sterol measured from three cell wells after correcting for sterol mass present in cells exposed to control media (MEM/HEPES/1% BSA). UC levels in control cells after a 24-hour exposure to control medium were 22.5 ± 1.2 $\mu\text{g}/\text{mg}$ protein and EC levels were 53.4 ± 1.3 $\mu\text{g}/\text{mg}$ protein.

FIG 3.

Isolation and characterization of LUVs and HDL after coincubation. LUVs and HDL used to create remodeled particles were coincubated overnight at 37°C (ratio of LUV PL to HDL PL was 10:1) in the absence of cells. After coincubation, LUVs and HDL were separated on a Sepharose CL-4B column (90×1.5 cm) equilibrated with TBS and run at 0.75 mL/min at 25°C (A). Fractions corresponding to each repurified particle were pooled and concentrated before determination of particle diameter by native PAGE. Electrophoretic scans obtained from Phast Gel native PAGE of HDL coincubated with saline (B), HDL remodeled by LUVs (C), and molecular-weight standards (D) are shown. The peaks marked 1 through 4 of panel D represent the migration of the molecular-weight standards (1) thyroglobulin, (2) ferritin, (3) catalase, and (4) lactase dehydrogenase.

FIG 4.

Cholesterol efflux from cells to control and remodeled HDL particles. The cell efflux conditions were the same as described for Fig 1. A, Time courses for the release of labeled UC from cells exposed to HDL ($25 \mu\text{g PL per mL}$) that had been preincubated with saline (■ “original” HDL) and HDL remodeled (see “Methods” and Fig 3) by LUVs (○) placed on cells at the same particle numbers (ie, same EC concentrations) as the original HDL. B, Time courses for the release of cellular $[^3\text{H}]$ UC by remodeled LUVs at $1 \text{ mg PL per milliliter}$ (▽), remodeled HDL placed on cells at the same particle numbers present in original HDL at $25 \mu\text{g PL per milliliter}$ (○), and the combination of these remodeled particles at these concentrations (▲) versus time. The calculated sum of cholesterol release to remodeled LUVs and HDL is shown by —, line-dash-line and is marked “additive.” C, Relative contributions of remodeling and shuttling to cellular cholesterol efflux. The time courses for the release of cellular cholesterol to (1) the sum of release to original LUVs and

to HDL added separately (◆, dashed line, taken from Fig 1B), (2) the sum of release to remodeled LUVs and remodeled HDL added separately after reisolation (, line-dash-line, taken from Fig 4B), and (3) remodeled LUVs and remodeled HDL added in combination after reisolation (▲, taken from Fig 4B) are shown. The vertical arrow A (between “original additive” and “particle combination”) represents total synergy, and the distance marked by arrow B (between “remodeled additive” and “particle combination”) represents residual synergy. Data points represent the mean $\pm$ SD of [3 H]UC remaining in cells determined from three cell wells after correcting for efflux to control media. All curves were fit to a single-exponential decay equation as described in “Methods.”

FIG 5.

Bidirectional flux of [³H]UC from labeled cells to media containing [¹⁴C]UC-labeled HDL with or without LUVs. Fu5AH cells were prepared as described in Fig 1. [¹⁴C]UC-labeled HDL was generated as described in “Methods” and then coincubated with LUVs or saline before addition to cells. Data shown represent the fraction of [³H]UC remaining in cells and the fraction of [¹⁴C]UC that had accumulated in cells from media HDL at the indicated times. Solid symbols represent the efflux of [³H]UC (■) from cells to HDL₃ at 200 μg PL per milliliter and the influx of [¹⁴C]UC (●) from HDL₃. Open symbols represent the efflux of [³H]UC (□) and the influx of [¹⁴C]UC (○) in cells exposed to 200 μg PL per milliliter HDL₃ and 1 mg/mL LUV PL. Each data point represents the mean±SD obtained from three cell wells.

FIG 6.

Mechanisms for the synergy between LUVs and HDL in the mobilization of cellular cholesterol. A, Cholesterol efflux from a cell monolayer to LUVs and HDL in the absence of any interactions. B, Synergy between LUVs and HDL due to remodeling. HDL remodeled by LUVs becomes UC depleted and PL enriched, making it a more efficient acceptor for cellular cholesterol. C, Synergy between LUVs and HDL due to a continuous shuttling of UC from cells to LUVs by HDL, which acts as a carrier.

Table 1

Fraction of Cellular UC Label Remaining in Fu5AH Cells and Mouse L Cells After a 24-Hour Incubation With HDL and LUVs Separately or in Combination

Cell Type	HDL, 25 μ g PL/mL	LUVs, 1 mg PL/mL	Additive [*]	HDL and LUV Combination [†]
Rat Fu5AH hepatoma cells	0.87 $\pm$ 0.02	0.79 $\pm$ 0.02	0.66 $\pm$ 0.04	0.12 $\pm$ 0.13 [‡]
Mouse L-cell fibroblasts [*]	0.82 $\pm$ 0.05	0.66 $\pm$ 0.07	0.48 $\pm$ 0.09	0.20 $\pm$ 0.30 [‡]

Values are shown as mean $\pm$ SD of the value derived from three cell wells after correction for efflux to control media (MEM/HEPES/1 % BSA/1 μ g/mL Sandoz 58035).

^{*} Calculated as 1–[(efflux to HDL alone)+(efflux to LUVs alone)].

[†] Experimentally observed fraction of UC label remaining in cells after incubation with the combination of HDL and LUVs.

[‡] Significant difference between HDL and LUV combination ($P<.01$) and other treatments. Statistics between the other treatment groups are not indicated.

Table 2
Compositional Analyses of HDL Before and After Preincubation With LUVs or Saline

Particle	Ratio (wt/wt)				
	UC/EC	PROT/EC	PL/EC	UC/PL	PROT/PL
HDL ₃	0.147±0.014	5.79±0.64	2.41 ±0.24	0.061 ±0.005	2.42±0.26
HDL coincubated with saline	0.150±0.025	5.73±0.82	2.25±0.28	0.067±0.007	2.56±0.28
HDL remodeled by LUVs	0.001 ±0.001 *	5.76±0.47	5.20 + 1.38 *	0.001 ±0.001 *	1.16±0.25 *

PROT indicates protein. Values are shown as mean±SEM (average of two separate experiments with triplicate determinations). HDL remodeled by LUVs was generated after an overnight incubation of LUVs with the HDL₃ (10-fold excess LUV PL to HDL PL). HDL coincubated with saline was generated by incubating HDL₃ with an equivalent volume of saline.

* Significant difference between HDL remodeled by LUV and HDL coincubated with saline ($P<.01$); HDL₃ and HDL remodeled by saline were statistically indistinguishable from each other.

Table 3

HDL Particle Diameters Before and After Preincubation With LUVs or Saline

Particle	Particle Diameter Measured by Native PAGE, nm	Theoretical Estimation of Particle Size, nm [*]
Original HDL	9.1±0.6	8.5±0.4
HDL coincubated with saline	9.7±0.7	8.5±0.5
HDL remodeled by LUVs	9.8±0.4	9.4±0.6

Values are the average diameter±range of the indicated particles obtained after digitizing two gels.

^{*} Estimation of particle size from the partial specific volumes of the constituents according to the method of Shen et al.⁴³ Briefly, the HDL PL and protein contents in original and remodeled particles as described in Table 2 were used to estimate the particle diameters.