Cellular Cholesterol Efflux

ROLE OF CELL MEMBRANE KINETIC POOLS AND INTERACTION WITH APOLIPOPROTEINS AI, AII, AND Cs

Efflux of $[^{14}C]$cholesterol from various cells was monitored in the presence of discoidal complexes of egg phosphatidylcholine and purified apolipoproteins, containing either apoAI, AII, or Cs. Particles containing apoAI were more efficient acceptors than those containing apoAII or Cs when the donor cells were J774 macrophages. No differences were observed when the same acceptor preparations were exposed to Fu5AH rat hepatoma or rabbit aortic smooth muscle cells. The differential efficiency of apolipoproteins in stimulating cholesterol removal from J774 cells was maintained in a plasma membrane-enriched fraction isolated from the same cells. Nonlinear regression analysis of kinetic data obtained from J774 cells exposed to apoAI demonstrated that the cholesterol efflux was best fitted to a curve describing the release from two kinetic compartments. Approximately 10% of cholesterol was transferred from a rapidly exchangeable pool with a $t_{1/2}$ ranging between 1.5 and 3 h, and the remaining fraction was released from a slower pool with a $t_{1/2}$ of about 20 h. Modulation of cholesterol efflux from J774 cells by either varying the concentration or the apolipoprotein composition of the acceptors influenced the size of the pools and the $t_{1/2}$ of the slow pool. Kinetics of cholesterol efflux from membranes isolated from J774 cells also best fit a two-compartment model and modification of the apolipoprotein composition of the acceptor induced a pattern of changes in pool size and half-time similar to that described for whole cells. In the three cell lines studied, we consistently resolved a slow pool with a half-time ranging between 15 and 20 h. In smooth muscle cells only the slow pool was evident, whereas in Fu5AH a very large fast pool was also resolved. In contrast to J774 cells, apolipoprotein composition of the acceptor did not influence the pools in these two cell lines. These results led us to propose a new model regarding the influence of multiple kinetic pools of cholesterol on the regulation of cholesterol desorption from the cell membrane.

Reverse cholesterol transport is a process by which excess cholesterol is transported from peripheral cells to the liver for excretion. The first step of this pathway involves the transfer of cholesterol molecules from the plasma membrane of the donor cell to the acceptor lipoprotein particle. HDL, or subfractions of HDL, are believed to be the physiological acceptors. The most generally accepted mechanism for this process involves the movement of cholesterol molecules through an aqueous phase (for a review, see Refs. 2, 3). A variety of studies have demonstrated that this transfer is influenced by the physical and compositional characteristics of the acceptor particle, such as the size (4, 5), the lipid composition, particularly the cholesterol to phospholipid mass ratio (6, 7), and in some cases, the apolipoprotein composition (8, 9). This latter aspect has recently become the focus of considerable interest. Early investigations have demonstrated that the addition of apolipoproteins to phospholipid liposomes enhances the efficiency of the phospholipid in stimulating cholesterol release. Part of this increased efficiency was attributed to the ability of apolipoproteins to convert liposomes to smaller apolipoprotein-phospholipid structures and, hence, increasing the relative surface area of the particles (4). However, additional investigations have demonstrated that factors other than increased surface area must be invoked to explain the increased cholesterol release provoked by some apolipoproteins. For instance, when acceptor particles are reconstituted with phosphatidylcholine and purified apolipoproteins so that the complexes are similar in lipid to protein ratio and in size, it is observed that acceptors containing apoAI are consistently more efficient in removing cellular cholesterol than particles containing apoCs (4). However, this difference between apolipoproteins is exhibited only with some cell types, since the particles have identical effects when incubated with other cell types (4). We have also recently demonstrated that, in J774 macrophages, discoidal complexes of egg PC and apoAI are more active in removing cholesterol from both the lysosomal and membrane pools when compared to similar acceptors reconstituted with either apoAI or apoCs (10). In addition to the studies from this laboratory, investigations on the Ob 1771 mouse adipose cell line have indicated that incubation of this cell with acceptor particles containing apoAI or apoAIV results in the depletion of cellular cholesterol, whereas exposure of these cells to particles containing either apoAI or apoE produces no such depletion (9, 11).

The studies cited above suggest that apolipoproteins can influence cellular cholesterol efflux by mechanisms that are

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The abbreviations used are: HDL, high density lipoproteins; BSA, bovine serum albumin; egg PC, egg phosphatidylcholine; SMC, rabbit aortic smooth muscle cells; $t_{1/2}$, half-time of efflux; $k$, rate constant of transfer from a pool expressed as the fraction of initial content transferred/unit of time (dependent variable); $v$, natural log base; $t$, or specific time point (independent variable); $C_e$, cell cholesterol content at time $t$ (independent variable); $C_0$, or Cs, initial cholesterol content in pool 1 and 2, respectively (dependent variables).
not solely related to physicochemical parameters, and the purpose of the present study is to obtain additional data to further elucidate the mechanism(s) underlying this physiologically important phenomenon.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unless otherwise indicated, chemicals and tissue culture materials were as described previously (10, 12, 13). Polyethylene glycol was purchased from Sigma and Dextran T500 from Pharmacia LKB Biotechnology Inc. All reagents employed for membrane enzyme assays were from Sigma. Procedures used for apolipoprotein purification and preparation of egg phosphatidylcholine-apolipoprotein complexes, used as cholesterol acceptors, were identical to those reported in previous investigations (4, 10).

**Cell Culture and Labeling of Cellular Cholesterol—**Unless otherwise specified, MEM and RPMI were buffered with 24 mM bicarbonate, and cultures were maintained at 37°C in an atmosphere of 5% CO2. Smooth muscle cells (SMC) were isolated from New Zealand rabbit aorta and cultured in MEM containing 10% fetal bovine serum, as described by Minar et al. (14, 15). For the experiments, SMC were plated in 35-mm dishes at an approximate density of 2 × 105 cells/ml, using 2 ml/dish. Fu5AH rat hepatoma cells and J774 murine macrophages were maintained in MEM, 5% calf serum and RPMI-10% fetal bovine serum, respectively. Four × 105 Fu5AH cells/ml and 7.5 × 105 J774 cells/ml were plated on 35-mm dishes, using 2 ml/dish. Two days after plating, cells were rinsed and cellular cholesterol was labeled during a 2-day incubation with [3H]cholesterol (0.5-0.6 μCi/ml) in medium containing 25% serum, maintaining 2.5% serum remaining.

**Preparation of J744 Plasma Membranes—**J774 macrophages were plated on 100-mm dishes at a density of 9 × 105 cells/ml, using 10 ml/dish and labeled with [3H]cholesterol as described above. Plasma membranes were isolated by following the procedure of Brunette and Till (16), using an aqueous two-phase polymer separation system. The solvents used for isolation of plasma membranes (designated as "top" and "bottom" phases) were prepared by combining polyethylene glycol and dextran T500, as described in (16). Briefly, cells from 16 dishes were scraped with a rubber policeman and centrifuged for 10 min at 1000 rpm. The pellet was resuspended in 20 ml of 1 mM ZnCl2 and from this suspension, membranes were harvested in a cell disruption bomb apparatus (Parr Instrument Co., Moline, IL) for 10 min at 250 psi (17). Cell breakage and nuclei integrity were monitored by microscopic examination. The cell lysate was centrifuged at 4°C for 30 min at 3,000 rpm. The pellet was suspended in 10 ml of top phase, vortexed, and combined to 10 ml of bottom phase. The mixture was centrifuged at 4°C for 20 min at 10,000 rpm (Beckman, L7 ultracentrifuge). The membranes formed a white fluffy interphase which was collected and washed twice with water by centrifugation at 3,000 rpm for 30 min. The pellet was resuspended in 4.5 ml of RPMI buffered with 12.5 mM HEPES. The purification and recovery in the final preparation were monitored by measuring in the homogenate and the membrane fractions the following parameters: alkaline phosphatase activity as a plasma membrane marker (18), protein (19), phospholipid (20), and cholesterol (21) contents. These assays indicated that membrane preparations were enriched approximately 2-3-fold in plasma membrane marker enzyme activity. Forty-two percent of the alkaline phosphatase, 20 ± 3% of the protein, 15 ± 2% of the cholesterol, and 18 ± 3% of the phospholipids present in the total homogenate were recovered in the membrane fraction (values are mean ± S.D., n = 3).

**Kinetics of Cholesterol Efflux—**On the day of the experiment, confluent cell monolayers were rinsed three times with medium containing 1% BSA and incubated for 2 h at 37°C with 1% BSA to allow equilibration of the [3H]cholesterol in the membranes. Unless specified, [3H]cholesterol efflux was initiated by adding 2.5 ml of medium containing 1% BSA and the indicated acceptor. Aliquots of media were carefully removed every hour for the first 4 h of efflux and every 1 or 2 h thereafter up to the end of the experiment, usually 12 h. The samples were collected in conical tubes chilled on ice, and centrifuged for 5 min in a Beckman microfuge (setting 8). Aliquots of supernatant were counted for 3H label by liquid scintillation. The experiments were ended by harvesting the remaining medium and the cell monolayers in cold phosphate-buffered saline. Determination of cholesterol efflux from membrane preparations was performed following the general procedure of Bellini et al. (22). Briefly, incubations were conducted in 10-ml glass vials at 37°C in an orbital shaking water bath. Efflux was initiated by adding the membranes (final concentration: 100 μg of protein/ml) to the medium containing the indicated acceptor. The volume was adjusted to 8 ml with RPMI buffered with HEPES at a final concentration of 1% BSA. At each time point, 2 aliquots of 200 μl were taken, centrifuged at 4°C in a 42.2T1 rotor (Beckman) at 30,000 rpm for 15 min (Beckman L8 ultracentrifuge). 100 μl of supernatant was then counted by liquid scintillation.

**Analytical Procedures—**Procedures used for cell lipid extraction and for protein and lipid analysis were as described before (10, 12). Efflux data collected at various time points were analyzed by using a nonlinear regression program (Graphpad Inplot, version 3.1, Graphpad Software, San Diego, CA), based on the method of Marquardt (23), in which goodness-of-fit is quantified by the least-squares method. Equations resolving an exponential decay of cellular label cholesterol were selected. The best fit between first or second order reaction was determined by examining both the graphs of the curves and the residual plots and by performing a statistical F test (24). Eventual problems of local minima were tested by repeating the analysis several times with different starting values for the variables.

**RESULTS**

In our first series of experiments, we compared the ability of particles containing either apoAI, AII, or Cs to promote cholesterol efflux from rabbit aortic SMC, Fu5AH rat hepatoma cells, and J774 murine macrophages. As shown in Fig. 1, we observed that, in the presence of equivalent concentrations of the same preparations of acceptors, the fractional efflux measured after 12 h varied among cell types, with efflux being slowest in SMC and fastest with the Fu5AH cells. We also observe that in SMC and Fu5AH cells, all the apolipoprotein complexes containing either apoAI, AII, or Cs were equally efficient in promoting cholesterol efflux. In contrast, with J774 macrophages, apoAI particles were better acceptors than those containing apoAI or Cs. These results, consistent with previously published data (4, 10, 25), indicate the existence of fundamental differences between cells which directly influence the ability of a cell to release its cholesterol.

A possible mechanism by which apoAI particles may function as a better cholesterol acceptor with the J774 cells involves the stimulation by apoAI of some intracellular metabolic pathway leading to the selective removal of intracellular pools of cholesterol. To test this hypothesis we compared cholesterol efflux from J774 cell monolayers and isolated plasma membranes prepared from the same cells. As shown in Fig. 2, the difference between acceptors observed in J774 monolayers was maintained with isolated plasma membranes, particles containing ApoCs, apoAI, and apoAI, respectively, ranking from the least to the most efficient acceptor. These results indicate that in J774 cells the modulation of cholesterol efflux is not linked to intracellular events, but rather is primarily related to the interaction between the apolipoprotein moiety of the acceptor and the plasma membrane.

![Fig. 1. Cholesterol efflux from various cell types as induced by particles containing apoAI, AII, or Cs. SMC, Fu5AH, and J774 cells, labeled with [3H]cholesterol, were incubated in RPMI containing 1% BSA and particles containing either apoAI (S), apoAI (C), or apoCs (X) at a concentration of 800 μg of phospholipids/ml. Cholesterol (FC) efflux, determined as described under "Experimental Procedures," is expressed as the percent of cholesterol released in the media after 12 h. Values are mean ± S.D., n = 3.](image-url)
Kinetic studies were conducted to further characterize the mechanism of cell cholesterol removal by extracellular acceptors. A large number of data points were generated over a length of time sufficient to accurately resolve, by computer analysis, the mathematical model reflecting this process. Typical kinetic data obtained with complexes containing apoAI are shown for SMC (Fig. 3), Fu5AH (Fig. 4), and J774 macrophages (Fig. 5). For each cell type, experimental values were plotted against two curves, one fitting a first order reaction (i.e. equation with a single exponential term: \( C_t = C_0 e^{-kt} \)) and another fitting a second order reaction (i.e. equation with the sum of two exponential terms: \( C_t = (C_0 e^{-kt1}) + (C_0 e^{-kt2}) \)). This later equation assumes efflux from two parallel domains which would mix at a very slow rate relative to their rate of desorption. The goodness of the fits was evaluated by both graphical examination and statistical methods (24).

In the case of SMC (Fig. 3), the curve fitting a first order reaction gives the best fit; the visual analysis of the graphs as well as residual plots and \( F \) test (\( p = 0.06 \)) indicate that the addition of a second exponential term does not significantly improve the fit. In contrast, the kinetic data obtained with Fu5AH or J774 cells as cholesterol donors (Figs. 4 and 5, respectively) are in both cases, better resolved by an equation including two exponential terms. This conclusion is supported by the following points: 1) visual examination of the graphs suggests a second order plot, 2) the residual plots from the first order equation (inset, panel A) show a random distribution of the experimental values around the curve, and 3) statistical \( F \) test indicates that the two fits are significantly different, with \( p < 0.00001 \). Together, these results demonstrate that the kinetics of cholesterol removal, as induced by apoAI complexes, are different between the cell types tested. In SMC, all the cholesterol transferred to the acceptor originates from a single exchangeable pool and undergoes unidirectional flux at a rate \( k_h \). In contrast, cholesterol transferred between either J774 or Fu5AH cells and the acceptors distributes between two distinct kinetic compartments having different rates of transfer.

A two-pool kinetic system describing the movement of cholesterol between cells and exogenous acceptors could be the result of two different physiological models: 1) a cellular pool model in which cholesterol is removed from two distinct kinetic compartments and does not undergo any significant back influx; or 2) a bidirectional flux model in which cellular cholesterol forms a single kinetic pool and cholesterol present in the medium forms the second kinetic pool. The kinetics for the cellular pool model would be fit by an equation with two exponential terms, similar to that used in the nonlinear regression analysis presented in Figs. 3–5, and this fit would persist under conditions in which there was no cholesterol influx. In the bidirectional flux model, net cholesterol movement is a result of bidirectional flux, occurring at different rates designated \( k_i \) for influx and \( k_e \) for efflux, and is resolved by the equation: \( C_t = C_0(e^{-kt1}) + B \), in which \( B \) is an equilibrium value (26). This model applies when cholesterol is present in the acceptor, such as in HDL, or when the concentration of acceptor is limiting and influx is significant (26). When experimental efflux data were computer analyzed using the equations fitting each of these models, both gave satisfactory fits and could not be discriminated with certainty. Because these two models have very different physiological implications, it was important to establish which one accurately reflects cholesterol efflux. The two models could be resolved by studying the kinetics of efflux under conditions in which cholesterol influx was negligible. If there was no influx, the bidirectional flux model should yield data that were best fit by an equation using a single exponential term, whereas the cellular two pool model should continue to best fit a two exponential equation. To address this question, kinetics of cholesterol movement between J774 cells and apoAI particles were studied under conditions in which the possibility of influx of cholesterol from the medium back to the cells was prevented. This was achieved by replacing the efflux medium containing apoAI complexes with fresh acceptors every hour over 12 h. The kinetics obtained are shown in Fig. 6, in which the experimental values are plotted against the curves predicting a single (panel A) and a two-compartment model (panel B). Residual plots (insets, Fig. 6) and statistical \( F \) test indicate that the two-exponential function fit the data best (\( p < 0.00001 \)). In another series of experiments, the volume of medium containing apoAI particles was increased from 2 to 6 ml. If cholesterol influx was a significant factor, addition of different amounts of acceptor would be expected to alter the kinetics. We observed that the efflux of cholesterol from J774 cells was similar under all conditions and was still better resolved by a two-pool model (data not shown). Thus, our results indicate that the second order kinetics of cholesterol transfer between J774 cells and acceptors containing apoAI is not affected when influx is suppressed. This finding strongly favors the model predicting that in the present experimental system, the cholesterol removed from the cells originates from two distinct cellular pools and does not undergo any appreciable back influx. In addition, the data presented in Table I, which demonstrate that similar changes of cellular cholesterol content were obtained when calculated using either isotopic or mass determination, indicate that factors such as \( de novo \) cholesterol synthesis are not complicating the kinetic analysis.

Compartmental analysis not only determines the best fit but also provides an estimated value of the parameters characterizing each of the kinetic pools, i.e. the half-time of transfer (\( t_{1/2} \)), derived from the rates \( k_1 \) and \( k_2 \), and the size of the pools (C1 and C2) expressed in percent. The parameters of each of the kinetic compartments identified in J774 cell monolayers incubated in the presence of particles containing apoAI are presented in Table II. Since the final extent of efflux as well as the parameter values may significantly vary between experiments, data are presented for four independent experiments. This analysis consistently resolves a rapidly transferred pool with a \( t_{1/2} \) of efflux ranging from 1.4 to 2.8 h.

**Fig. 2.** Cholesterol efflux from J774 macrophage monolayers and isolated membrane preparations in the presence of particles containing apoAI, AII, or Cs. J774 macrophages labeled with \(^{3}H\)cholesterol and membranes isolated from the same cells were incubated with particles containing apoAI (\( \square \)), AII (\( \bigcirc \)), or Cs (\( \blacksquare \)) at a concentration of 400 pg of phospholipid/ml. Cholesterol (FC) efflux, determined as described under "Experimental Procedures," is expressed as the percent of cholesterol released in the media after 8 h. Values are mean ± S.D., n = 3 for J774 monolayers, and n = 2 for J774 membranes.
and a slow pool with a t_{1/2} value ranging between 17 and 24 h. Between 8 and 13% of the amount of cholesterol removed from J774 cells by apoAI particles originates from the fast pool, the remaining coming from the slow pool.

By using the same experimental and analytical approaches, the effect of the concentration of acceptors on the pools was studied and is presented in Table III. When J774 cells are exposed to increasing concentrations of acceptors containing apoAI (from 200 to 1000 μg of phospholipids/ml), the stimulation of efflux (from 29 to 45% after 12 h) is associated with a reduction in the t_{1/2} for the slow pool (from 30 to 16.9 h) and a significantly increased contribution of cholesterol from the fast pool (from 6.4 to 9.6%), with a concomitant reduction of the slow pool. The t_{1/2} for the fast pool is stable. A similar pattern is observed when the extent of efflux is modulated by the apolipoprotein composition of the acceptor particle. As illustrated in Table IV, an increase in the efficiency of the acceptor, in the order apoCs < apoAII < apoAI, is associated with: 1) the expansion of the fast pool and a concomitant reduction in the size of the slow pool and 2) the reduction of the half-time of desorption of the slow pool.

Table V shows the parameters obtained for cholesterol transfer from plasma membranes isolated from J774 cells incubated with acceptors containing various apolipoproteins. Although the t_{1/2} of the slow pool is faster in plasma membrane than in whole cells, a very similar pattern is observed with both whole cells and plasma membrane preparations regarding the changes in half-time and pool size as modulated by different acceptors.

Kinetic parameters for cholesterol efflux from SMC and Fu5AH are shown in Table VI. In SMC, cholesterol is removed from a single domain with a t_{1/2} of 20 h. In contrast, in Fu5AH about 50% of cholesterol is removed from the fast pool with a t_{1/2} of 1.8 h, the remaining being transferred from the slower domain with a t_{1/2} of 15 h. In both cell systems, apolipoprotein composition of the acceptor did not influence the kinetics of cholesterol efflux.

**DISCUSSION**

A widely accepted model for the transfer of cholesterol from cells is that of passive diffusion, in which cholesterol desorbs from the plasma membrane and is taken up by the acceptor.
FIG. 4. Kinetics and nonlinear regression analysis of cholesterol removal from Fu5AH rat hepatoma cells in the presence of particles containing apoAI. Kinetics of [3H]cholesterol efflux were measured and analyzed under the same conditions as those described for Fig. 3.

In the presence of excess acceptor particles, this desorption appears to constitute the rate-limiting step of cell cholesterol efflux, since it is a rather slow process when compared to the rate of translocation of cholesterol from intracellular compartments to the plasma membrane (27, 28). The rate at which this critical process occurs has been reported to vary largely, depending on the cell system used and the physicochemical characteristics of the acceptor particles (4, 25). The present investigation was conducted in an attempt to further elucidate the mechanism involved in the regulation of cellular cholesterol transfer from various cell types to acceptors containing the major apolipoproteins of HDL, namely apoAI, AII, and Cs.

This work represents a continuation of a recently published study in which we observed that in J774 macrophages particles containing apoAI were better acceptors of both lysosomal and plasma membrane cholesterol, as compared to particles containing apoAII or Cs (10). These observations, together with those of DeLamatre et al. (4), suggested that in some cells cholesterol desorption from the membrane was specifically affected by apolipoproteins. This was supported by the observation that the differential efficiency between apolipoproteins occurred at all concentrations, including the highest, at which efflux is maximum and becomes independent of acceptor concentration (4, 10, 29). These studies were conducted by using discoidal complexes of egg PC and purified apolipoproteins as cholesterol acceptors (30). Particles of the same size and lipid composition provide suitable conditions for specifically exploring the influence of apolipoproteins on cholesterol transfer between cells and acceptor particles (4, 7, 10).

In the present studies, we compared the ability of these various acceptors to remove cholesterol from J774 macrophages, Fu5AH hepatoma cells, and aortic smooth muscle cells (Fig. 1). Particles containing apoAI were more efficient cholesterol acceptors than those containing apoAII or Cs when the donor cells were J774 macrophages. However, no such differences were observed when the same acceptor preparations were exposed to Fu5AH hepatoma or SMC. These results are consistent with earlier studies by DeLamatre et al. (4), showing higher cholesterol efflux in the presence of particles containing apoAI when the donor cells were human.
fibroblasts, L cell mouse fibroblasts, and J774 murine macrophages and no differences between particles for cholesterol removal from Hep-G2 human hepatoma or Fu5AH rat hepatoma cells. Other investigators have also suggested that the apolipoprotein composition of the acceptor particle influences the ability of the acceptor to stimulate cholesterol efflux (8, 9, 31). The intriguing differences between cell types observed in our laboratory support the hypothesis that the variation between apolipoproteins is primarily a cell-related phenomena rather than a simple reflection of the physical and compositional properties of the acceptor lipoproteins.

One model which has been proposed to explain the greater efficiency of particles containing apoAI in removing cholesterol is that involving the interaction of the apolipoprotein moiety of the acceptor with the putative HDL receptor (9, 11). However, our previous studies have demonstrated that cross-linking the apolipoproteins on the particle, a procedure that blocks specific binding to the HDL receptor (32), did not affect the ability of the acceptor to stimulate efflux from J774 cells (10). Another possible explanation for the apolipoprotein specificity observed in some cell types invokes the selective removal of intracellular pools of cholesterol having a rate of translocation to the plasma membrane slower than the rate of desorption to the exogenous acceptors (33). This hypothesis, however, has been ruled out by the observation that the differential efficiency of the various apolipoprotein complexes in stimulating cholesterol removal from J774 cells does not require cell integrity and is maintained in a plasma membrane-enriched fraction isolated from the same cells (Fig. 2). These results demonstrate that the effects of apolipoproteins are primarily linked to processes occurring at the level of the plasma membrane.

Nonlinear regression analysis of kinetic data obtained from J774 cells exposed to apoAI complexes indicates that cholesterol efflux is consistently best fitted to a line describing the release from two kinetic compartments (Figs. 4 and 5). We also have verified that these pools are not a reflection of bidirectional flux of cholesterol between cell donor and acceptor, but rather are located within the cells (Fig. 6). Although the numerical values describing the two pools may differ among experiments, the data for cholesterol efflux from J774, as induced by apoAI particles, consistently differentiate a
Fig. 6. Kinetics and nonlinear regression analysis of cholesterol efflux from J774 macrophages in the absence of eventual influx. J774 macrophages, labeled with [4C]cholesterol, were incubated with apoAI (400 μg of phospholipids/ml). Every hour, efflux medium was removed and replaced by fresh acceptors. Aliquots of media collected over the 12-h period and cells harvested at the end of the experiment were analyzed for label content. Experimental data, presented as mean ± S.D., n = 4, and expressed as the fraction of cholesterol remaining in the cells at the indicated time, are plotted against a curve generated by nonlinear regression computer analysis and fitting either a single (panel A) or a two-compartment (panel B) model. Residual plots for each of the two fits are shown in the insets.

Table I
Comparison of J774 cells cholesterol content as determined by mass and isotope

J774 macrophages labeled with [4C]cholesterol were incubated with BSA alone or BSA plus acceptors containing apoAI (800 μg of phospholipids/ml). Cell cholesterol content was determined both by GLC and isotope counts at times 0 and 12 h. Mass and cpm measured at time 0 are taken as 1.00 and data at 12 h are normalized to these values. Initial cell content was 12.7 ± 0.9 μg of cholesterol/dish. No cholesteryl ester was detected at any time. (Values are mean ± S.D., n = 3).

| Time (h) | Medium  | Mass Fraction of cholesterol remaining in cells | Isotope Fraction of cholesterol remaining in cells |
|---------|---------|-----------------------------------------------|-----------------------------------------------|
| 0       | BSA     | 1                                              | 1                                             |
| 12      | BSA     | 0.88 ± 0.06                                   | 0.85 ± 0.00                                   |
| 12      | apoAI/PC| 0.49 ± 0.03                                   | 0.48 ± 0.01                                   |

RAPIDLY TRANSFERRED compartment of cholesterol with a t1/2 value between 1.5 and 3 h and a second pool with a much slower desorption, with a t1/2 of about 20 h (Table II). Modulation of the extent of cholesterol efflux from J774 cells by either varying the concentration (Table III) or the apolipoprotein composition of the acceptors (Table IV) appears to directly influence the characteristics of the kinetic pools. An increased efflux is associated with two major changes: 1) an enlarged fast pool, and hence a reduced slow pool, and 2) a substantial reduction in the t1/2 of efflux from the slow pool. The t1/2 of the fast pool is relatively independent of the nature and concentration of the acceptor. The two kinetic pools revealed in whole J774 cells appear to be located within the plasma membrane since the kinetics of cholesterol efflux from membrane preparations isolated from J774 cells also best fit a two compartment model. In addition, modification of the apolipoprotein composition of the acceptor induces a pattern of changes in pool size and half-time in the membranes similar to that described in whole cells (Table V).

Multiple kinetic pools of cholesterol have been observed by
other investigators using a variety of experimental systems, including mycoplasma (34), tissue culture cells (35-37), or artificial membranes (5). The experimental conditions used in the present studies allow an accurate resolution of kinetic compartments having a t₁/₂ of transfer ranging between 1 and 25 h. Therefore, kinetic pools transferred with a t₁/₂ value out of these limits would not be detected. However, the existence of such pools has been suggested. For instance, a very rapidly exchangeable pool with a t₁/₂ in the range of 1-30 min could be resolved in both artificial membranes and tissue culture cells when using a fluorescent cholesterol analog, dehydroergosterol (37, 38). This cholesterol domain may be related to the small fraction of fibroblast cholesterol which has been shown to be selectively and very rapidly transferred to apoAI-containing HDL particles having a pre-β1 electrophoretic mobility (39).

### Table II

**Parameters of cholesterol kinetic pools in J774 macrophages in the presence of acceptors containing apoAI**

Kinetic studies of cholesterol efflux were conducted as described under “Experimental Procedures” using J774 macrophages labeled with ³⁵C]cholesterol and particles containing apoAI (400 µg of phospholipids/ml). Efflux values after 12 h are mean ± S.D., n = 3. For each experiment, time points were determined in triplicate and were computer analyzed using a nonlinear regression program. Estimated parameters were calculated for an equation describing the sum of two exponential functions (see text). p values were derived from F tests, performed to compare the goodness of the models fitting either a first or second order function.

| Experiment t₁/₂ | Pool 1 | Pool 2 | % efflux in 12 h | p value (F test) |
|-----------------|--------|--------|----------------|-----------------|
| Size            | t₁/₂   | h      | %              | h               |
| %               | h      | %      |
| 1               | 8.7    | 1.4    | 91.4           | 23.6            |
| 2               | 10.8   | 2.0    | 89.2           | 19.8            |
| 3               | 12.7   | 2.8    | 87.3           | 27.0            |
| 4               | 8.4    | 1.4    | 91.7           | 22.9            |

### Table III

**Effect of concentration of apoAI acceptors on cholesterol kinetic pools in J774 macrophages**

Kinetic studies of cholesterol efflux were conducted in J774 macrophages labeled with ³⁵C]cholesterol in the presence of the indicated concentration of particle containing apoAI as described under “Experimental Procedures.” Efflux values after 12 h are mean ± S.D., n = 3. For each condition, time points were determined in triplicate and were computer analyzed using a nonlinear regression program. Estimated parameters were calculated for an equation describing the sum of two exponential functions (see text). The fits resolving each set of data were all significantly different from each other, as indicated by F test with p values less than 0.00001.

| Conc. of acceptors | Pool 1 | Pool 2 | % efflux in 12 h | p value (F test) |
|-------------------|--------|--------|----------------|----------------|
| µg PL/ml           | t₁/₂   | h      | %              | h               |
| µg PL/ml           | %      | h      | %              | h               |
| 200                | 6.4    | 1.5    | 93.6           | 28.9 ± 0.1      |
| 400                | 8.4    | 1.4    | 91.7           | 22.8 ± 1.8      |
| 1000               | 9.6    | 1.2    | 90.4           | 45.3 ± 1.8      |

### Table IV

**Effect of apolipoprotein composition of the acceptors on cholesterol kinetic pools in J774 plasma membranes**

Kinetic studies of cholesterol efflux were conducted as described under “Experimental Procedures” using plasma membranes isolated from J774 macrophages labeled with ³⁵C]cholesterol in the presence of the indicated acceptors at a concentration of 800 µg phospholipids/ml. Efflux after 12 h are mean ± S.D., n = 3. For each condition, time points were determined in triplicate and were computer analyzed using a nonlinear regression program. Estimated parameters were calculated for an equation fitting the sum of two exponential functions (see text). The fits resolving each set of data were all significantly different from each other, as determined by F test with p values less than 0.00001.

| Acceptor         | Pool 1 | Pool 2 | % efflux in 12 h |
|------------------|--------|--------|----------------|
| Size             | t₁/₂   | h      | %              |
| %               | h      | %      |
| apoCs           | 6.1    | 1.6    | 94.0           | 24.3 ± 0.8      |
| apoAII          | 8.0    | 1.6    | 92.1           | 19.2 ± 0.6      |
| apoAI           | 12.0   | 1.8    | 88.2           | 13.6 ± 1.0      |

### Table V

**Effect of apolipoprotein composition of the acceptors on cholesterol kinetic pools in J774 plasma membranes**

Kinetic studies of cholesterol efflux were conducted as described under “Experimental Procedures” using plasma membranes isolated from J774 macrophages labeled with ³⁵C]cholesterol in the presence of the indicated acceptors at a concentration of 800 µg phospholipids/ml. Efflux after 12 h are mean ± S.D., n = 3. For each condition, time points were determined in triplicate and were computer analyzed using a nonlinear regression program. Estimated parameters were calculated for an equation describing the sum of two exponential functions (see text). The fits resolving each set of data were all significantly different from each other, as determined by F test with p values less than 0.00001.

| Acceptor         | Pool 1 | Pool 2 | % efflux in 8.5 h |
|-----------------|--------|--------|----------------|
| Size            | t₁/₂   | h      | %              |
| %               | h      | %      |
| apoC           | 8.2    | 0.8    | 91.7           | 12.2 ± 0.3      |
| apoAI          | 10.7   | 0.9    | 89.0           | 8.5 ± 0.6       |
| apoAI          | 10.6   | 0.8    | 89.2           | 7.9 ± 0.3       |

### Table VI

**Cholesterol kinetic pools in rabbit aortic SMC and Fu5AH rat hepatoma cells**

Kinetic studies of cholesterol efflux were conducted as described under “Experimental Procedures” using SMC or Fu5AH cells labeled with ³⁵C]cholesterol in the presence of acceptors containing either apoAI, AII, or Cs at a concentration of 400 µg phospholipids/ml. For each condition, time points were determined in triplicate. Since they were not significantly different, results obtained for each cell type in the presence of the various types of acceptors were combined. Efflux at the end of the experiments are mean values ± S.D., n = 9. Estimated parameters were calculated for the equations fitting the data best, i.e. a single exponential for SMC and the sum of two exponential functions for Fu5AH cells (see text).

| Acceptor     | Pool 1 | Pool 2 | % Efflux in 23 h |
|--------------|--------|--------|----------------|
| Size         | t₁/₂   | h      | %              |
| %            | h      | %      |
| SMC          | 99.0   | 20.0   | 53.8 ± 0.7     |
| ApoAI, AII, or Cs | 52.1  | 1.8    | 47.5 ± 1.8     |

| Acceptor     | Pool 1 | Pool 2 | % Efflux in 11 h |
|--------------|--------|--------|----------------|
| Size         | t₁/₂   | h      | %              |
| %            | h      | %      |
| Fu5AH        | 70.7   | 1.5    | 71.0 ± 2.5     |
The question of the location of these domains has not been resolved and it has been suggested that they may be a reflection of the asymmetric distribution of cholesterol either between the inner and outer membrane leaflets (34) or between lateral domains within the same leaflet (37). Although our present studies cannot resolve this issue, it should be pointed out that two kinetic pools are still observed in our experiments which used open sheets of membranes (16). This observation would favor a model involving two pools located within the same leaflet.

We have previously demonstrated that the minimum \( t_{1/2} \) for cholesterol efflux differs considerably among cell types, ranging from 2 h to more than 24 h (25), and these differences were maintained when using membrane preparations (22). The present study suggests that these dissimilarities in efflux may be a reflection of differences in the size of the kinetic pools of cholesterol within these cells (Tables IV and VI). In the three cell lines studied, we consistently resolved a slow pool with a half-time ranging between 15 and 20 h. In the rabbit aortic SMC only the slow pool was evident, and the lack of fast pool would explain the slower release of cholesterol from these cells. The very rapid efflux consistently noted with the Fu5AH rat hepatoma cells appears to be a reflection of a kinetic distribution of cell cholesterol (26). It was appropriate, therefore, to assume that cellular cholesterol formed a single kinetic pool. The present analysis using apolipoprotein-egg PC complexes as acceptors indicates that Fu5AH cholesterol may reside in two kinetic pools. Although this observation represents an improvement of our understanding of cholesterol dynamics in cells, it does not change our previous conclusions regarding mechanisms of sterol efflux and effects of HDL structure on this process (26, 40). The J774 macrophage cell line, used most extensively in the present investigation, demonstrates characteristics intermediate between the hepatoma and the smooth muscle cell lines. These results led us to propose that the critical rate of desorption of cholesterol from the membrane to the acceptor is regulated by the distribution of cholesterol within membrane pools.

In contrast to the J774 cells, neither the SMC nor the Fu5AH cells exhibited a change in the kinetic parameters describing efflux when exposed to acceptors containing different apolipoproteins. The ability of apolipoproteins to modulate these kinetic domains is therefore rather specific for the cell type, which suggests differences in the nature of these membrane domains. The mechanism by which the apolipoproteins can modulate the characteristics of membrane pools of cholesterol remains to be established. Two general models can be suggested, one involving the direct interaction of the entire acceptor particle with the plasma membrane, or a mechanism in which free apolipoproteins dissociate from the acceptor and are incorporated into the membrane. This latter possibility is particularly attractive in view of a recent report showing that incorporation of a sufficient amount of apoAI into egg PC bilayer vesicles enhances the rate of cholesterol transfer (41). If the association of free apoAI with a local region of the plasma membrane occurred, it could modulate the rate of desorption from distinct kinetic pools of cholesterol. Recent reports (42, 43) have also shown that lipid-free apolipoproteins can stimulate the release of phospholipids from cells. We could speculate that such release could induce local modifications of cholesterol packing in the membrane, changing the rate of desorption of cholesterol into the aqueous phase.

Our observations are consistent with the knowledge recently acquired on cholesterol packing in biological membranes. There is now evidence of cholesterol-rich and cholesterol-poor domains within the same leaflet of the plasma membrane and a number of important biological functions have already been proposed for these structural domains (for review, see Ref. 37). The distribution of membrane cholesterol between distinct kinetic pools has been suggested for a number of cell systems (36, 37, 44) and was shown to be sensitive to a variety of factors, including fatty acid-binding protein (45), temperature, drugs affecting cholesterol metabolism (37), membrane cholesterol, and protein content (44). We now suggest that the large variations in the rates of cholesterol transfer observed among cell types can be explained by fundamental differences in the distribution of membrane cholesterol between rapidly and slowly exchangeable pools. In some cell types, apolipoproteins have the ability to regulate the rate-limiting step of cholesterol efflux by differentially affecting the size and rate of desorption from the cholesterol domains. We believe that future investigations should focus on the structural characterization of the cholesterol domains among cell types and on the elucidation of their interaction with apolipoproteins. Greater knowledge at the level of microdomains of cholesterol within cell membrane may reconcile the discrepancies and controversy presently existing between laboratories, in regard to the regulatory mechanisms of cell cholesterol removal.

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