Efflux of lipid from fibroblasts to apolipoproteins: dependence on elevated levels of cellular unesterified cholesterol

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Abstract Earlier work from this laboratory showed that enrichment of cells with free cholesterol enhanced the efflux of phospholipid to lipoprotein acceptors, suggesting that cellular phospholipid may contribute to high density lipoprotein (HDL) structure and the removal of sterol from cells. To test this hypothesis, we examined the efflux of [^{3}H]cholesterol (FC) and [^{32}P]phospholipid (PL) from control and cholesterol-enriched fibroblasts to delipidated apolipoproteins. The percentages of [^{3}H]cholesterol and [^{32}P]phospholipid released from control cells to human apolipoprotein A-I were 2.2 ± 0.5%/24 h and 0.8 ± 0.1%/24 h, respectively. When the cellular cholesterol content was doubled, efflux of both lipids increased substantially ([^{3}H]FC efflux = 14.6 ± 3.6%/24 h and [^{32}P]PL efflux = 4.1 ± 0.3%/24 h). Phosphatidylcholine accounted for 70% of the radiolabeled phospholipid released from cholesterol-enriched cells. The cholesterol to phospholipid molar ratio of the lipid released from cholesterol-enriched cells was approximately 1. This ratio remained constant throughout an incubation time of 3 to 48 h, suggesting that there was a coordinate release of both lipids. The concentrations of apoA-I, A-II, A-IV, E, and Cs that promoted half-maximal efflux of phospholipid from cholesterol-enriched fibroblasts were 53, 30, 68, 137, and 594 nM, respectively. With apoA-I and A-IV, these values for half-maximal efflux of phospholipid were identical to the concentrations that resulted in half-maximal efflux of cholesterol. Agarose gel electrophoresis of medium containing apoA-I that had been incubated with cholesterol-enriched fibroblasts revealed a particle with α to pre-β mobility. We conclude that the cholesterol content of cellular membranes is an important determinant in the ability of apolipoproteins to promote lipid removal from cells. We speculate that apolipoproteins access cholesterol-phosphatidylcholine domains within the plasma membrane of cholesterol-enriched cells, whereupon HDL is generated in the extracellular compartment. The release of cellular lipid to apolipoproteins may serve as a protective mechanism against the potentially damaging effects of excess membrane cholesterol. **—Bielicki, J. K., W. J. Johnson, R. B. Weinberg, J. M. Glick, and G. H. Rothblat.** Efflux of lipid from fibroblasts to apolipoproteins: dependence on elevated levels of cellular unesterified cholesterol. *J. Lipid Res.* 1992. **33:** 1699–1709.

Supplementary key words apolipoprotein A-I • phospholipid efflux • cholesterol efflux • cholesterol-enriched fibroblasts • reverse cholesterol transport

The high density lipoprotein (HDL) fraction of plasma consists of a heterogeneous mixture of particles that are enriched in protein and phospholipid (1). HDL is thought to initiate the reverse cholesterol transport process by promoting cholesterol efflux from extrahepatic cells (2).

Apolipoprotein A-I is the major protein constituent of plasma HDL, comprising 70% of the HDL apolipoprotein. A portion of the apoA-I secreted from cells has been shown to be in a lipid-deficient form (3–5), and about 3% of the total plasma apoA-I pool remains unassociated with lipoproteins (6). In contrast, apolipoprotein A-IV is found mostly free in plasma (7). The functions of apolipoproteins in the lipid-free state are unknown, but several studies indicate that they can promote cholesterol efflux from cells (8, 9).

Initial studies carried out by Stein and Stein (8) and Stein et al. (9) indicated that the removal of cellular cholesterol by delipidated apolipoproteins was accompanied by the release of phospholipid. We observed enhanced efflux of phospholipid to HDL when cells were enriched with cholesterol (10). In the present study, we characterized the efflux of cholesterol and phospholipid to various delipidated apolipoproteins. Our hypothesis was that cholesterol enrichment of fibroblasts would stimulate the efflux of phospholipid to apolipoproteins, and thereby promote the net release of cellular cholesterol. We found that apolipoproteins were inefficient at promoting choles-

Abbreviations: PL, phospholipid; FC, free (unesterified) cholesterol; HDL, high density lipoprotein; apo, apolipoprotein; PC, phosphatidylcholine; SM, sphingomyelin; lysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; FBS, fetal bovine serum; CS, calf serum; ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography.

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terol release from cells having normal levels of cholesterol. In contrast, substantial efflux was observed when fibroblasts were enriched with free cholesterol.

MATERIALS AND METHODS

Materials

Lipids, unesterified cholesterol (FC), cholesteryl methyl-ether, phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and lysophosphatidylcholine (lysoPC) were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA, essentially fatty acid-free) and fetal bovine serum (FBS) were also purchased from the same vendor. Reagent grade organic solvents were used, and were purchased from Fisher Scientific (Pittsburgh, PA). TLC plates were Anasil G (250 μm), and were obtained from Analabs (North Haven, CT). Trypsin, tissue culture media, calf serum, and electrophoresis grade agarose were purchased from Gibco (Grand Island, NY). Tissue culture flasks and plates were obtained from Falcon (Lincoln, NJ) and Corning Glass Works (Corning, NY), respectively. Gentamicin was from Tri Bio Labs (State College, PA). Radioisotopes, [^{32}P]orthophosphoric acid (carrier-free) and [^{3}H]cholesterol (58 Ci/mmol), were from New England Nuclear (Boston, MA). Dr. John Heider supplied Sandoz compound 58035.

Cell culture

The cell lines were maintained as described previously (10). Briefly, GM3468A normal human skin fibroblasts and WI38VA transformed lung fibroblasts were kept in minimum essential medium (MEM) supplemented with 10% FBS (v/v). J774 mouse macrophages and Fu5AH rat hepatoma cells were grown in RPMI containing 10% FBS (v/v) and MEM containing 5% calf serum (CS, v/v), respectively. All media were supplemented with 50 μg/ml of gentamicin. Cells grown in medium containing bicarbonate were kept at 37°C in an atmosphere of 95% air/5% CO_2. Cells in media buffered with 14 mM HEPES were used at 37°C in humidified air.

Lipoproteins, apolipoproteins and lipid dispersions

Lipoproteins were isolated from human plasma by sequential ultracentrifugation (11). Potassium bromide was used to adjust the density of plasma to yield the following fractions: VLDL (d < 1.006 g/ml), LDL (d 1.019–1.063 g/ml), HDL_3 (d 1.125–1.21 g/ml), and lipoprotein-deficient serum (LPDS, d > 1.21 g/ml). VLDL and LDL were dialyzed three times against 1 liter of phosphate-buffered saline (PBS) and then against MEM containing 14 mM HEPES (MEM-HEPES). The HDL_3 was chromatographed on heparin-Sepharose to remove particles containing apoE (12). The material that eluted from this column was concentrated using an Amicon ultrafiltration cell fitted with a P10 membrane. The concentrate was dialyzed against PBS that contained 50 units of penicillin G/ml and 50 μg of streptomycin sulfate/ml, and then against MEM-HEPES. The >1.21 g/ml fraction was treated with 20 units/ml of thrombin, and the fibrin-clot was pelleted by centrifugation. The supernatant from the clotted serum (LPDS) was stored at −20°C.

Purified apolipoproteins were obtained from the total human HDL fraction (d 1.063–1.21 g/ml) by ethanol–ether lipid extraction (13) followed by separation of apoA-I and A-II by anion exchange chromatography on a Q-Sepharose column in a fast protein liquid chromatography (FPLC) system (14). A mixture of total apoC was obtained from delipidated VLDL by gel filtration chromatography. Purified human apoE was a gift from Dr. Mahmood Hussain (The Medical College of Pennsylvania, Philadelphia, PA), and human apoA-IV was obtained as described by Weinberg and Jordan (15). Phospholipid liposomes and dispersions consisting of unesterified cholesterol and egg phosphatidylcholine (>2:1, mole:mole) were prepared as described previously (10).

Experimental conditions

Cells were grown to confluent monolayers in 22-mm culture wells. To label phospholipids, cells were rinsed with MEM-HEPES and then incubated for 2 days in bicarbonate-buffered MEM containing 10 mg protein/ml of LPDS and 5 μCi/ml of [^{32}P]phosphate. Half of the dishes were then treated for an additional 24 h with a cholesterol enrichment medium containing 100 μg cholesterol/ml of FC–PL dispersion (>2:1, mole:mole), 50 μg protein/ml of LDL, and 1 μg/ml of Sandoz compound 58035 to inhibit acyl-CoA:cholesterol acyltransferase (ACAT). This drug was added to medium in DMSO at a final concentration of 0.5% (v/v). To control for the egg phosphatidylcholine added to the enrichment medium, the remaining dishes of cells were incubated with a control medium containing 70–80 μg phospholipid/ml of phospholipid liposomes and 1 μg/ml of Sandoz 58035. Both the control and enrichment media contained 0.2% BSA and 5 μCi/ml of [^{32}P]phosphate. The cholesterol enrichment medium resulted in at least a twofold enrichment of cellular unesterified cholesterol. No cholesteryl ester was detected in cells because of the inclusion of Sandoz 58035.

When cells were labeled with [^{3}H]cholesterol and [^{32}P]phosphate, the enrichment medium was modified. The 2-day preincubation with LPDS and ^{32}P remained unaltered. Cells were enriched with cholesterol for 24 h as described above, but the control and enrichment media contained 1% FBS and 2 μCi/ml of [^{3}H]cholesterol. To prepare these control and enrichment media, [^{3}H]cholesterol was first added to 25% FBS and then this solution was sterilized by filtration (0.45 μm). Ethanol was

used to disperse the cholesterol into the medium; the final concentration of ethanol was 0.1% (v/v). The concentrated serum solution was diluted to 1% with MEM buffered with bicarbonate and the components of the control and cholesterol enrichment medium were added as described above.

Lipoproteins and apolipoproteins were diluted to final concentrations in MEM-HEPES and 0.5 ml was applied per dish. Bovine serum albumin (BSA) was added to the medium in the experiment reported in Table 1. In all other experiments, BSA was omitted from the medium because it was shown to have a minor stimulatory effect on phospholipid efflux (10). In most cases, the release of cellular lipid was measured after a 24-h incubation period.

Lipid analyses

At the end of each incubation, the media were removed from cell monolayers and centrifuged at 3300 *g* for 10 min. Butylated hydroxytoluene (10 μg/ml) was added to each sample to prevent the oxidation of phospholipids. Lipids were extracted from 80% of the supernatant of centrifuged media samples by the method of Bligh and Dyer (16). The chloroform phase from this partition was recovered, dried under a stream of nitrogen gas, and then redissolved in 0.5 ml of chloroform–methanol 2:1 (v/v). The radioactivity in one-fifth of this sample was quantitated by liquid scintillation counting (LSC).

Cellular lipids were recovered from monolayers by isopropanol (1 ml/dish) extraction (10), and butylated hydroxytoluene was added to a concentration of 5 μg/ml. The isopropanol extracts were dried under a stream of nitrogen gas and the lipid was further extracted from the dried residue by the method of Bligh and Dyer (16). The chloroform phase from this partition was evaporated under nitrogen. Lipids were redissolved in chloroform–methanol 2:1 (v/v). Aliquots were then taken for liquid scintillation counting and TLC.

Separation of phospholipid subclasses was achieved by a two-step, one-dimensional procedure (10), in which the first solvent consisted of chloroform–methanol–water 65:25:4 followed by a second development in chloroform–acetone–methanol–acetic acid–water 50:20:10:10:5. The distribution of radioactivity among each subclass was measured with a Radiomatic Imaging gas-flow ionization detector. The fractional distribution of either cellular or medium lipids was multiplied by the total radioactivity of phospholipids in cells or medium to determine the cpm in each phospholipid subclass.

Data analyses

The efflux data were expressed as a percentage of cellular lipid released from cells: (cpm in medium after 24 h/cpm in cellular lipids at t = 0) × 100. The results obtained for the efflux of each phospholipid subclass were expressed as the amount of radioactivity (cpm) in each of the individual subclasses as separated by TLC, divided by the cell protein content. For comparison, the data were also expressed as percentages of each subclass released from cells.

A GraphPAD Inplot software package (version 3.1, Graphpad Software Inc., CA) was used to analyze the efflux concentration-dependence curves. Data were fitted via a four-parameter logistics equation that generated the best-line through experimental data points and calculated the midpoint of these curves. The concentration of apolipoprotein that yielded half-maximal efflux was used as a criterion to determine which apolipoprotein was most effective at promoting phospholipid release from cells.

Agarose gel electrophoresis

To detect lipids associated with newly assembled lipoproteins, cholesterol-enriched fibroblasts were prepared as described above except that the labeling medium contained either 40 μCi/ml of [^{3}H]cholesterol or 50 μCi/ml of [^{32}P]phosphate.

Samples of media containing either apoA-I or HDL_3 that had been incubated with cells for 24 h were dialyzed three times at 4°C against a 500-fold excess of MEM-HEPES, using a 12,000 molecular weight cutoff membrane. Aliquots from each sample were removed before and after dialysis. Between 20 and 40% of radiolabeled lipids were lost during dialysis. This loss of lipid-radioactivity was probably due to adsorption of lipids to the dialysis chamber rather than degradation of phospholipids. This conclusion is substantiated by the observation that the recoveries of [^{3}H]cholesterol and [^{32}P]phospholipid differed by no more than 10%, indicating similar losses of both lipids. A 0.2-ml aliquot from each sample was electrophoresed for 3 h at 150 V (125 mA) according to the method of Noble (17). The dimensions of the gel were 200 × 120 × 6 mm (length × width × thickness). Individual lanes from a given gel were excised and 2.5-mm long segments were cut for quantification of radioactivity by LSC. Native LDL, VLDL, and HDL_3 were used as standards. These standards were run on the same gel as the medium from radiolabeled cells. The section of the gel containing the lipoprotein standards was stained for 1 h with Sudan Black and then destained with 50% ethanol.

Chemical analyses

Cholesterol mass was measured by gas–liquid chromatography, and cholesteryl methyl ether was used as the internal standard (18). Protein was measured by the method of Markwell (19). Phospholipid-phosphorus was determined by the method of Sokoloff and Rothblat (20). A single measurement of each analyte was taken from triplicate wells.

Statistics

Means and standard deviations were calculated. Unpaired *t*-tests were used, and $P < 0.05$ was the criterion for significance.

RESULTS

Efflux of phospholipid from cells to apolipoproteins

Initially, we tested whether apolipoprotein A-I (apoA-I) was able to promote phospholipid efflux from normal and cholesterol-enriched fibroblasts. We also compared this efflux to that obtained with HDL_3. With control cells, efflux to HDL_3 was about twice that obtained with BSA. When cells were enriched with cholesterol, phospholipid efflux to HDL_3 was enhanced by 50% (**Table 1**). These results are consistent with those that we reported previously (10). Delipidated apoA-I was not as effective as HDL_3 at promoting phospholipid efflux from control fibroblasts, yet when cells were enriched with cholesterol, efflux to apoA-I was enhanced by 62%. With only BSA in the medium, enrichment had no effect on phospholipid release.

To determine whether there existed a cell specificity for efflux to apoA-I, we examined the ability of apoA-I to promote phospholipid efflux from various cell lines. All of the different cell types used in this experiment were enriched with cholesterol at least threefold and then exposed to 25 μg/ml of apoA-I. In a 24-h period, the two fibroblast lines each released about 5% of their cellular phospholipid in the presence of apoA-I, which represented a fivefold increase in efflux compared to the basal release of phospholipid to protein-free medium (**Table 2**). ApoA-I promoted about a twofold increase in phospholipid efflux from J774 macrophages and Fu5AH rat hepatoma cells.

Based on the above results, we used normal human skin fibroblasts for subsequent characterizations of phospholipid efflux. The results in **Table 3** indicate that apoA-I, A-II, and a combination of C isoforms, when added to cells at 100 μg/ml, were able to stimulate variable degrees of phospholipid release from control cells. When cells were enriched with cholesterol, efflux to apoA-II was increased by 25%, while a 54% increase was observed with apoA-I and Cs. The apoCs promoted the greatest efflux of phospholipid compared to the other apolipoproteins.

TABLE 1. Efflux of phospholipid from control and cholesterol-enriched fibroblasts to HDL_3 and apoA-I

Cell Type[a]	Acceptor[b]	Efflux[c]
		%/24 h
Control	BSA	4.5 ± 0.6
	HDL_3	8.2 ± 1.1
	apoA-I	5.9 ± 0.8
FC-enriched	BSA	3.5 ± 0.4
	HDL_3	12.4 ± 0.8[d]
	apoA-I	9.5 ± 0.6[d]

[a]The cholesterol contents of control and cholesterol-enriched fibroblasts were 23.0 ± 1.9 and 62.0 ± 4.3 μg/mg cell protein, respectively.
[b]BSA was present in all media. Concentrations were as follows: BSA, 2 mg/ml; HDL_3, 500 μg protein/ml; and apoA-I, 500 μg protein/ml.
[c]Values are means ± SD, n = 3.
[d]$P < 0.01$, compared to efflux from control cells to the same acceptor.

TABLE 2. Efflux of [^{32}P]phospholipid from cholesterol-enriched cells to protein-free medium and apoA-I

Cell Type[a]	Efflux of Phospholipid	
	MEM-HEPES	ApoA-I[b]
	%/24 h	
GM3468A skin fibroblasts	1.1 ± 0.2	5.4 ± 0.5
WI38VA lung fibroblasts	0.9 ± 0.1	5.0 ± 0.4
Fu5AH rat hepatoma	0.5 ± 0.1	0.8 ± 0.1
J774 Mouse macrophages	1.0 ± 0.1	2.3 ± 0.1

[a]The free cholesterol to phospholipid molar ratio of each cholesterol-enriched cell type (from top to bottom) was 0.6 ± 0.03, 0.54 ± 0.02, 0.42 ± 0.01, and 0.69 ± 0.01.
[b]The concentration of apoA-I was 25 μg/ml.

Efflux of phospholipid subclasses to apoA-I

Phospholipids in cells and media were separated into subclasses by TLC (**Fig. 1**). With control fibroblasts, PC accounted for about 50% of the radioactive phospholipid released to apoA-I after 24 h. The remaining 50% of the label was associated with lysophosphatidylcholine, sphingomyelin, and phosphatidylethanolamine. These results were similar to those obtained with HDL_3 (10). Cholesterol enrichment enhanced the release of PC, which accounted for 70% of the radiolabeled phospholipid released from the cells, as well as release of SM, although to a lesser degree (Fig. 1). Cholesterol enrichment had no effect on the efflux of phosphatidylethanolamine (PE). Very similar patterns of efflux were observed with all of the other apolipoproteins used in this study (data not shown). Thus, cholesterol enrichment of cells primarily stimulated PC efflux.

Dependence of phospholipid efflux on apolipoprotein concentration

Initial comparisons were made between apoA-I and apoCs using cholesterol-enriched fibroblasts, and the data from a representative experiment are shown in the top panel of **Fig. 2.** Maximal efflux was obtained at approximately 100 and 1500 nM of apoA-I and Cs, respectively. It was also apparent from these results that the apoCs promoted greater maximal phospholipid release than apoA-I (maximal efflux to A-I = 4%/24 h vs. maximal efflux to Cs = 7%/24 h). These results are consistent with the differences in efflux between apoA-I and Cs presented in Table 3. A similar difference in maximal release was

TABLE 3. Efflux of phospholipid from control and cholesterol-enriched fibroblasts to different apolipoproteins

Cell Type[b]	Efflux[a]			
	MEM-HEPES	A-I[c]	A-II[c]	Cs[c]
	%/24 h			
Control	1.8 ± 0.2	2.6 ± 0.4	4.0 ± 0.4	4.1 ± 0.3
FC-enriched	1.0 ± 0.2[d]	4.0 ± 0.4[d]	5.0 ± 0.4[d]	6.3 ± 0.6[d]

[a]Values are means ± SD, n = 3.
[b]The cholesterol contents of control and cholesterol-enriched fibroblasts were 19.2 ± 3.0 and 46.7 ± 8.5 μg/mg cell protein, respectively.
[c]Concentration of apolipoprotein was 100 μg protein/ml.
[d]$P < 0.05$, compared to control.

also observed between apoA-II and apoE. ApoA-II was able to promote efflux over the lower concentration range, although at the higher concentrations apoE had a greater capacity for promoting the release of cellular phospholipid.

To compare the relative efficiency with which a given apolipoprotein promoted phospholipid efflux, the above experimental data were analyzed with a curve-fitting program (Materials and Methods) that generated the best-fit line through the experimental data points and calculated the concentration of apolipoprotein that stimulated half-maximal efflux of phospholipid from cells. This parameter was used as a measure of the effectiveness of apolipoproteins at promoting phospholipid release from cells. The concentrations of apoA-I and Cs that promoted half-maximal efflux were 58 and 734 nM, respectively (experiment shown in top panel of Fig. 2). These results indicated that on a molar basis apoA-I was able to stimulate the removal of phospholipid from cholesterol-enriched cells at much lower concentrations than apoCs.

Table 4 lists the concentrations of each apolipoprotein that promoted half-maximal efflux of phospholipid from cholesterol-enriched fibroblasts. For comparisons, the results were expressed in terms of molar and mass units. On a molar basis, A-I, A-II, and A-IV were equivalent. ApoE was less effective, and 10-fold more of the apoCs was required to promote as much efflux as apoA-I. When the results were normalized to the apolipoprotein mass used in efflux studies, apoA-I and A-II were still the most effective although expressing the results in this manner reduced the apparent differences between apoCs and the other apolipoproteins.

To determine whether there may be a relationship between cholesterol and phospholipid release from cells, we examined the efflux of both lipids to various concentrations of apoA-I and A-IV (**Fig. 3**). With each apolipoprotein, half-maximal efflux of cholesterol and phospholipid were obtained at essentially the same concentration (86 nM for apoA-I, and 68 nM for apoA-IV). These results suggest the possibility of a close link between the efflux of cholesterol and the efflux of phospholipid from cholesterol-enriched fibroblasts to delipidated apolipoproteins.

Kinetics of efflux of cholesterol and phospholipid from cells to apoA-I

The time courses for cholesterol and phospholipid efflux from control and cholesterol-enriched cells to apoA-I are shown in **Fig. 4.** Lipid release from control cells reached a maximum in 6 h or less, and then showed no further increase in the next 42 h. With cholesterol-enriched cells, efflux of both cholesterol and phospholipid reached a maximum in 24 h. It is apparent that cholesterol enrichment led to a substantial stimulation (> fivefold) in the 24 h release of cholesterol.

In a separate study, the efflux of cholesterol from enriched cells to apoA-I was compared to the efflux obtained

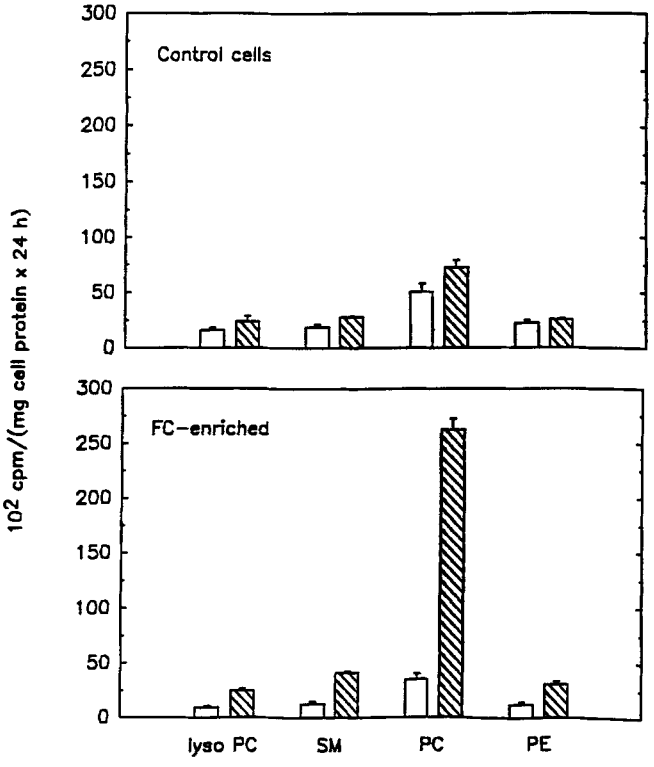

Fig. 1. Efflux of phospholipid subclasses from control and cholesterol-enriched fibroblasts to apoA-I. Normal human skin fibroblasts were labeled with [^{32}P]phosphate and enriched with cholesterol as described in Materials and Methods. The cholesterol content of cells was 18.6 ± 0.8 and 41.0 ± 6.2 μg free cholesterol/mg cell protein for control and cholesterol-enriched cells, respectively. ApoA-I was added to cells in MEM-HEPES at a concentration of 25 μg/ml. After a 24-h incubation, the medium was removed and the lipids were extracted and then separated by TLC. The results were expressed as the amount of radioactivity recovered in each phospholipid subclass, normalized to cellular protein. Open bars denote efflux to protein-free MEM-HEPES and the hatched bars show efflux to medium containing apoA-I. The percentage release of lysoPC, SM, PC, and PE to apoA-I was 3.97 ± 1.11, 5.08 ± 0.83, 1.54 ± 0.24, and 1.10 ± 0.05% for control cells, and 2.72 ± 0.49, 6.35 ± 0.69, 3.71 ± 0.23, and 1.49 ± 0.13% for cholesterol-enriched cells. Values are means ± SD, n = 3.

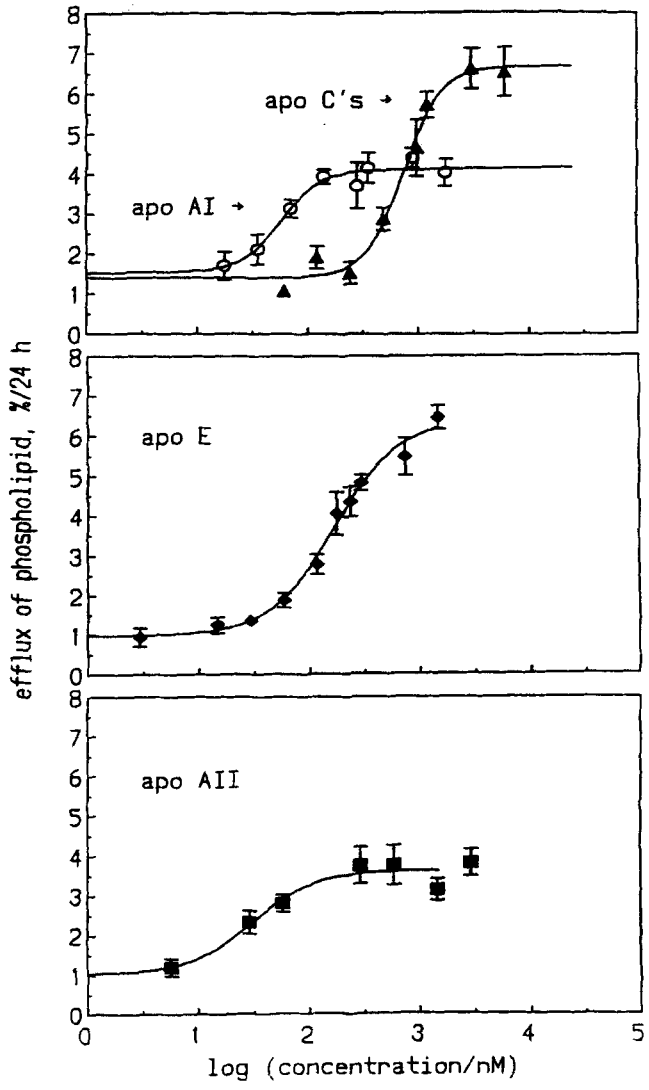

Fig. 2. Dependence of phospholipid efflux from cholesterol-enriched fibroblasts on apolipoprotein concentration. Normal human skin fibroblasts were labeled with [^{32}P]phosphate and enriched with cholesterol as described in Materials and Methods. The cholesterol content of enriched cells ranged from 46.7 to 50.0 μg free cholesterol/mg cell protein. Increasing amounts of each apolipoprotein were added to cells in MEM-HEPES. Efflux was expressed as the percentage of radiolabeled phospholipid released from cells in 24 h. Values are means ± SD, n = 3. Lines through the data points were generated with a GraphPAD Inplot software package (version 3.1, Graphpad Software Inc., CA) using a four-parameter logistic equation that was a logarithmic transformation of the Hill equation.

with HDL_3. Very little release of lipid was observed in the absence of an acceptor (**Fig. 5**). When apoA-I was added to the medium, there was a time-dependent increase in the release of cholesterol and phospholipid from cells, and this release was similar to that observed in the bottom panel of Fig. 4. HDL_3 also promoted phospholipid efflux, and the extent of this release was about the same as with apoA-I. HDL_3 served as a much better acceptor of cellular cholesterol, compared to delipidated apoA-I. The ability of HDL_3 to promote cholesterol efflux from cells is attributable to its relatively phospholipid-rich surface, which serves to solubilize cholesterol (2). ApoA-I was added to the cells as a delipidated apolipoprotein. It has been suggested that free apolipoproteins first promote the efflux of cellular phospholipid forming a phospholipid/protein complex which then serves as a cholesterol acceptor (8). Thus, it might be predicted that cholesterol release would lag behind phospholipid efflux, and that the molar ratio of cholesterol to phospholipid in the A-I medium would increase over time. From the kinetic data in Fig. 5, and from specific activity measurements of cellular lipids, we were able to estimate the mass of cholesterol and phospholipid released from cells. The results in **Fig. 6** show that the cholesterol to phospholipid molar ratio of lipid released from cells to apoA-I remained constant throughout the experiment, indicating that both lipids were released coordinately (within the 3-h resolution of the initial measurements). Although a similar pattern was observed for the efflux to HDL_3, this does not reflect the contribution of lipid influx to estimates of net mass transfer. Therefore, the similarities in efflux patterns do not necessarily indicate a common mechanism by which these two acceptors promote efflux.

Identification of newly formed lipoprotein particles

To determine whether the lipid accumulating in the medium of cells exposed to apoA-I was associated with a discrete particle, we used agarose-gel electrophoresis. To allow detection of newly formed lipoprotein particles in the efflux medium, we increased the specific activity of [^{32}P]phosphate in the labeling medium 10-fold. After the incubation of cells with either HDL_3 or apoA-I, the medium was removed and dialyzed against MEM-HEPES. As shown in the upper panel of **Fig. 7,** no lipoprotein particles were observed in protein-free medium that had been

TABLE 4. *Concentration of apolipoprotein yielding half-maximal efflux of phospholipid from cholesterol-enriched fibroblasts*

	Concentration	
Apolipoprotein	nM	μg/ml
A-I (7)[a]	53 ± 23[b]	1.50 ± 0.65[b]
A-II	31	0.54
	30	0.52
A-IV	68	3.10
E	167	5.70
	109	3.72
Cs (4)[a]	594 ± 259[b,c]	4.68 ± 2.04[b,d]

[a]Number of experiments.
[b]Means ± SD.
[c]$P < 0.0003$, compared to apoA-I.
[d]$P < 0.0035$, compared to apoA-I.

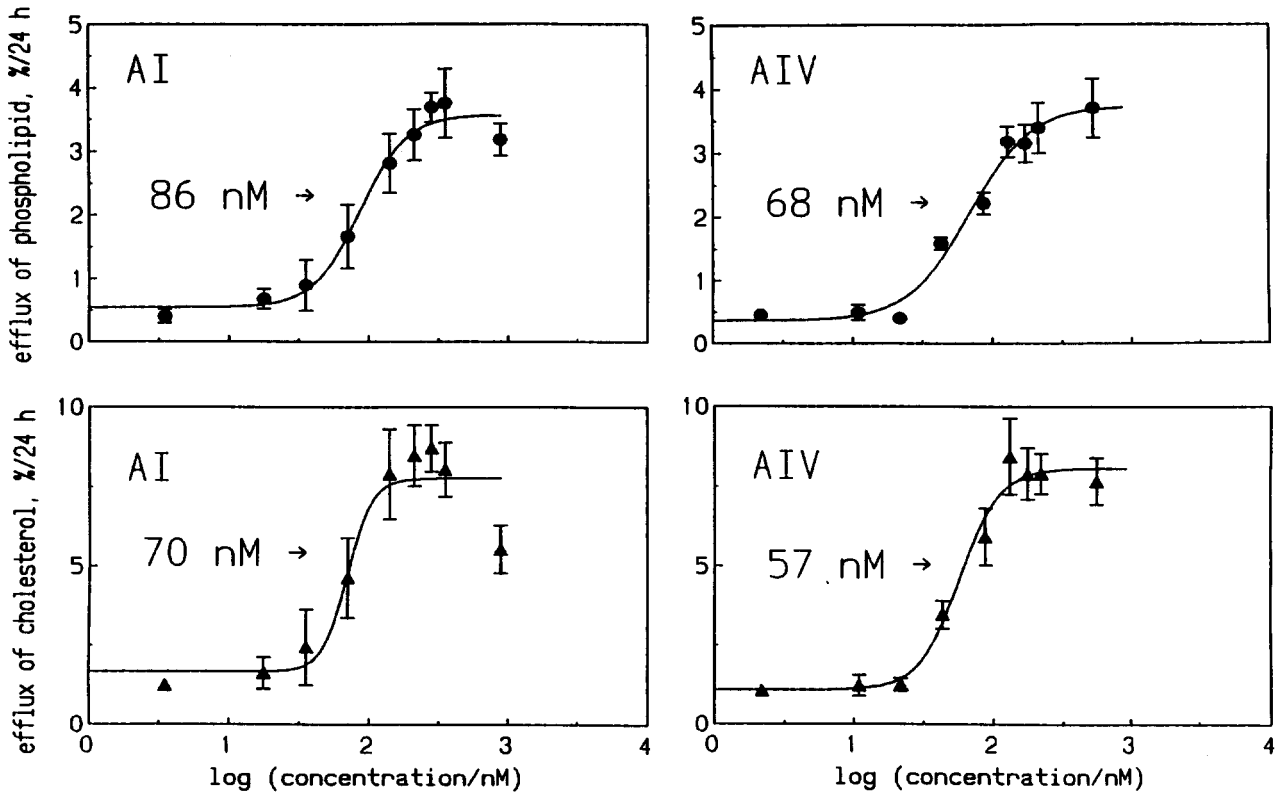

Fig. 3. Dependence of cholesterol and phospholipid efflux from cholesterol-enriched fibroblasts on apoA-I and A-IV concentration. Normal human skin fibroblasts were labeled with [^{3}H]cholesterol and [^{32}P]phosphate, and enriched with cholesterol as described in Materials and Methods. The cholesterol content of enriched cells was 41.6 ± 7.1 μg free cholesterol/mg cell protein. Increasing amounts of apoA-I and A-IV were added to cells in MEM-HEPES. Efflux was expressed as the percentage of radiolabeled lipid released from cells in 24 h. Values are means ± SD, n = 3.

incubated with cholesterol-enriched cells. The nature of the material producing the peak of radioactivity at about 120 mm is unknown, although it was observed in all samples of medium that were incubated with [^{32}P]phosphate-labeled cells. This material may represent excess free [^{32}P]phosphate or high molecular weight phosphorylated compounds that are retained after dialysis. The efflux of phospholipid to apoA-I resulted in the formation of a distinct peak of radioactivity that migrated between VLDL and HDL_3. To provide additional evidence that the peak of ^{32}P observed in the apoA-I efflux medium was actually a lipoprotein particle, we repeated this experiment using cells prelabeled with [^{3}H]cholesterol (**Fig. 8**). The radiolabeled cholesterol released to apoA-I exhibited α-mobility, suggesting the formation of a lipoprotein particle. In other experiments, [^{3}H]cholesterol and [^{32}P]phospholipids comigrated to the α-position on gels, and this material could be extracted from the agarose gel with organic solvents. The extracted lipids migrated with authentic cholesterol and phospholipid standards when analyzed by TLC (data not shown).

DISCUSSION

Results reported here and elsewhere (21) support the hypothesis that lipoprotein formation may occur by the transfer of cellular lipid to lipid-free apolipoproteins. Whether this is a physiologically significant pathway for HDL assembly has yet to be determined. We did not observe substantial efflux of lipid from cells that had normal levels of cholesterol. Earlier studies by Stein et al. (8, 9) and Jackson et al. (22) documented that apolipoproteins promoted lipid removal from ascites and smooth muscle cells, but the medium was not analyzed for the presence of newly assembled lipoproteins.

Recent evidence suggested that efflux of lipid from cholesterol-loaded cells to apolipoproteins resulted in the extracellular assembly of HDL. Hara and Yokohama (21) demonstrated that "free" apolipoproteins promoted lipid removal from mouse peritoneal macrophages that had been induced to form foam cells by preincubation with acetylated-LDL. The pre-β particles they described had a relatively high unesterified cholesterol content, and thus

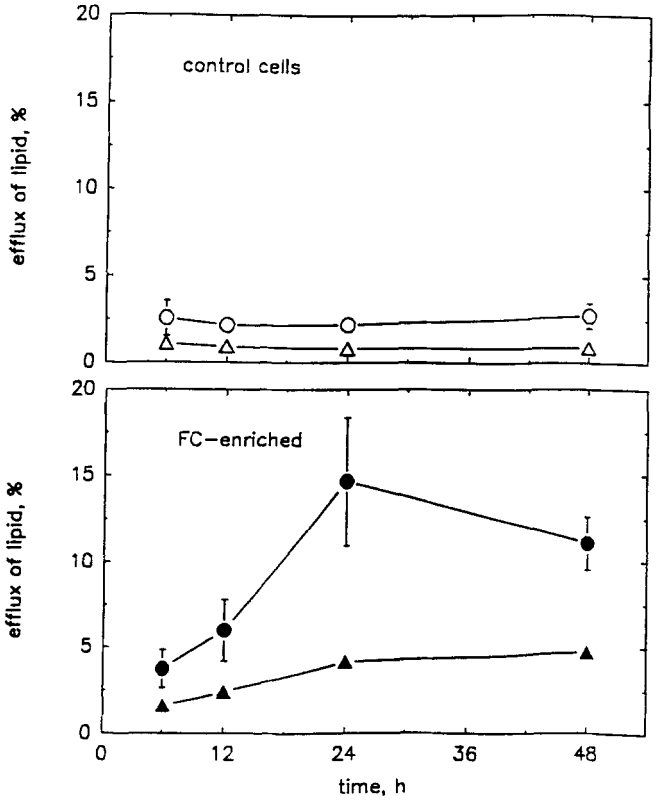

Fig. 4. Time courses of efflux of cholesterol and phospholipid from control and cholesterol-enriched fibroblasts to apoA-I. Normal human skin fibroblasts were labeled with [^{3}H]cholesterol and [^{32}P]phosphate, and enriched with cholesterol as described in Materials and Methods. The cholesterol content of cells was 26.9 ± 0.7 and 50.0 ± 1.1 μg free cholesterol/mg cell protein for control and cholesterol-enriched cells, respectively. ApoA-I was diluted to a final concentration of 10 μg/ml with MEM-HEPES. Efflux values are expressed as the percentage of radiolabeled lipids released from cells. Data for [^{3}H]cholesterol efflux are denoted by circles and [^{32}P]phospholipid efflux by triangles. Values are means ± SD, n = 3. Error bars were smaller than symbols in most cases.

the composition of this particle was different from the pre-β1 particle described by Castro and Fielding (23), which appeared to be an unusually efficient acceptor of cellular cholesterol. Our data are not entirely consistent with a model in which phospholipids are first released from cells to form nascent, pre-β HDL particles, which are then able to promote cholesterol removal from cells. Instead, our results suggest that both cholesterol and phospholipid may be released from cells coordinately. The concentration-dependence curves for efflux of cholesterol and phospholipid were also similar. These observations would be consistent with a model in which the release of free cholesterol and phospholipid was mechanistically linked. This link may be the result of an initial rapid release of phospholipid followed closely by the efflux of cholesterol, or the simultaneous removal of both cholesterol and phospholipid from the cell membrane. Detailed kinetic studies analyzing efflux at time points earlier than 3 h will be necessary to distinguish between these two possibilities.

Hara and Yokohama (21) reported that the nascent HDL particle derived from macrophages had pre-β mobility in agarose gels, while we observed a lipoprotein particle that had either α mobility or near α mobility from the medium of fibroblasts. Differences between cell types and methods of cholesterol enrichment may influence the

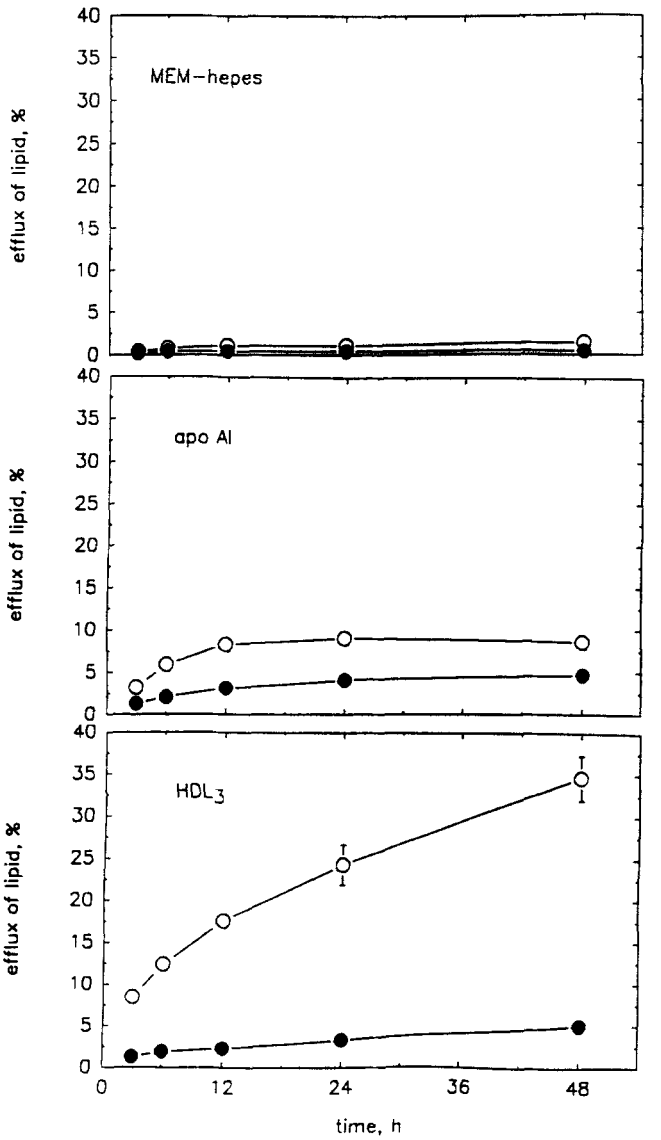

Fig. 5. Time courses of efflux of cholesterol and phospholipid from cholesterol-enriched fibroblasts to apoA-I and HDL_3. Normal human skin fibroblasts were labeled with [^{3}H]cholesterol and [^{32}P]phosphate, and enriched with cholesterol as described in Materials and Methods. The cholesterol content of enriched cells was 34.8 ± 0.5 μg free cholesterol/mg cell protein. ApoA-I and HDL_3 stock solutions were diluted with MEM-HEPES to final concentrations of 10 and 100 μg protein/ml, respectively. Efflux was expressed as the percentage of radiolabeled lipids released from cells. Data for [^{3}H]cholesterol efflux are denoted by open circles, and the closed circles symbolize [^{32}P]phospholipid efflux. Values are means ± SD, n = 3.

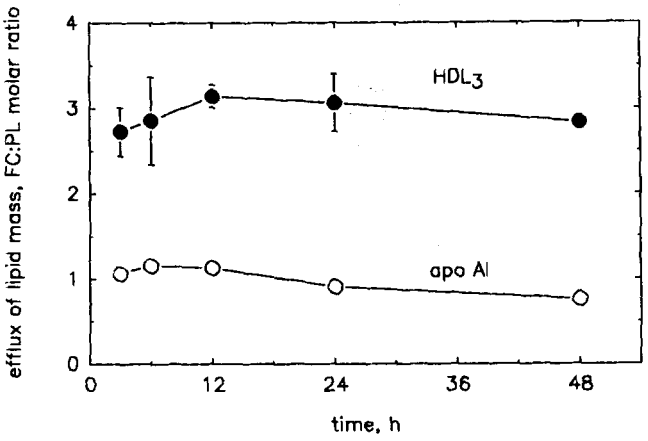

Fig. 6. Molar ratio of cell-derived cholesterol and phospholipid released to HDL_3 and apoA-I. From the specific activities of cellular [^{3}H]cholesterol (2696 cpm/nmol unesterified cholesterol) and [^{32}P]phospholipid (4033 cpm/nmol phospholipid) the efflux of lipid mass was calculated using the data presented in Fig. 5. The open circles denote the cholesterol:phospholipid molar ratio of the lipids released to apoA-I, and the closed circles correspond to the data obtained for HDL_3. For HDL_3, these data ignore the contribution of lipid influx to net release of lipid mass. Values are means ± SD, n = 3.

amount and types of lipids released from cells. Differences in the lipid composition of these particles may affect lipoprotein charge and movement in agarose. Based on estimates of lipid mass release (Fig. 6), we would predict that the particle isolated from fibroblasts is enriched in free cholesterol. However, we have not yet been able to isolate this material in a sufficient quantity to determine its chemical composition. Therefore, a direct comparison with the particle described by Hara and Yokohama (21) is impossible.

The enhanced efflux of lipid from cholesterol-enriched fibroblasts to lipid-free apolipoproteins may represent a pathway by which large free cholesterol- and phospholipid-rich HDL particles are generated in the interstitium. In dogs, interstitial lipoproteins have been shown to contain apoE and apoA-IV (24, 25). The concentration of apoA-IV in plasma is roughly 15 mg/dl, and a majority of this apolipoprotein is in a "lipid-free" form (7). We observed that the concentration of apolipoprotein that promoted maximal efflux of phospholipid from cells ranged from 3 to 12 μg/ml, depending on which apolipoprotein was used. Whether the concentration of apoA-IV reaches these levels in interstitial fluid is unknown, but it seems likely that the apoA-IV that is filtered across the endothelium would be available for promoting lipid removal from cholesterol-enriched cells.

The mechanism by which apolipoproteins promote lipid release from cells is unknown. Two general mechanisms can be proposed; one in which the apolipoproteins interact with specific cell surface proteins, and another in which the apolipoproteins associate with lipid domains within the plasma membrane. Our data cannot resolve these two models. Savion and Gamliel (26) showed that free apolipoproteins will bind to the surface of endothelial cells, and the K_d values for the binding of apoA-I and apoA-IV were 20 and 14 nM, respectively. Incubation of these cells with acetylated-LDL resulted in a fourfold increase in the binding of apolipoproteins to the plasma membrane. The concentration range for this specific

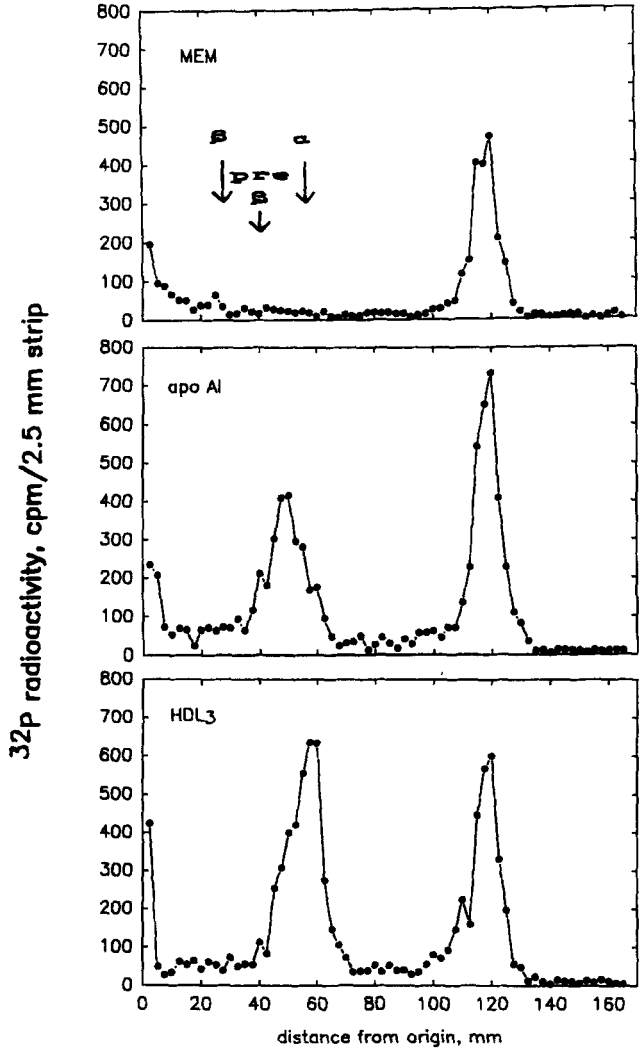

Fig. 7. Demonstration of lipoprotein particle resulting from efflux of [^{32}P]phospholipid from cholesterol-enriched cells to apoA-I. Normal human skin fibroblasts were labeled with 50 μCi/ml of [^{32}P]phosphate and enriched with cholesterol as described in Materials and Methods. ApoA-I and HDL_3 stock solutions were diluted with MEM-HEPES to final concentrations of 10 and 100 μg protein/ml, respectively. After a 24-h efflux period, the media were removed from cells and dialyzed against MEM-HEPES to remove free-[^{32}P]phosphate. Two-tenths of a milliliter from each sample was electrophoresed as described in Materials and Methods. Lanes corresponding to a given acceptor were excised from the gel and cut into 2.5-mm long strips for liquid scintillation counting. The migration distances of lipoprotein standards were 35, 42, and 55 mm for LDL (β), VLDL (pre-β), and HDL_3 (α), respectively.

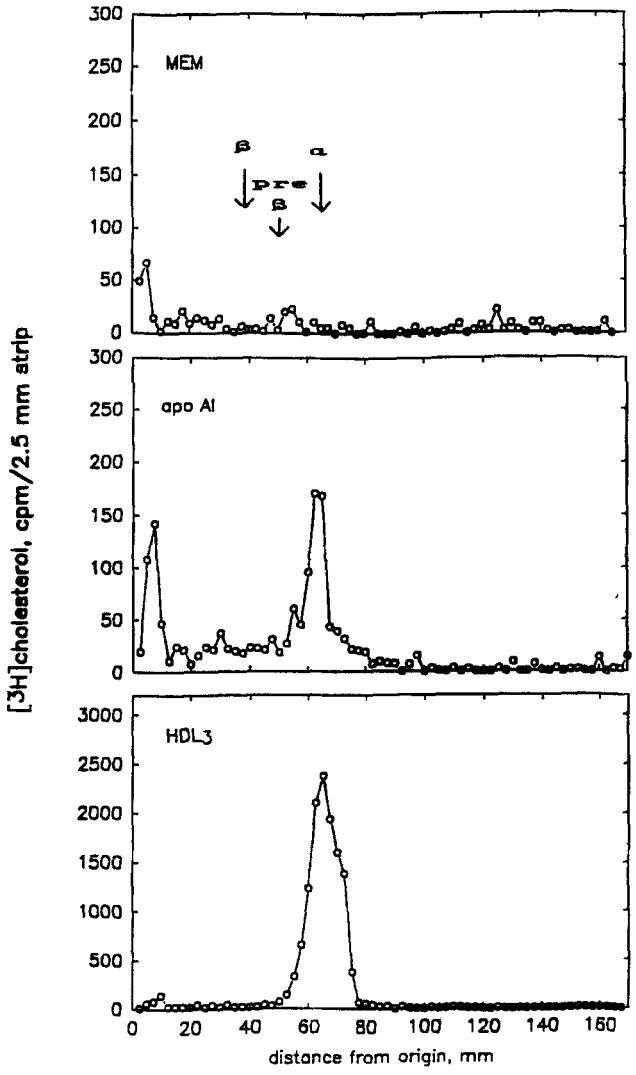

Fig. 8. Association of cellular [^{3}H]cholesterol with newly assembled lipoprotein resulting from efflux of lipid to apoA-I. Normal human skin fibroblasts were labeled with 40 μCi/ml of [^{3}H]cholesterol and enriched with cholesterol as described in Materials and Methods. The efflux conditions and the electrophoresis were as described in Fig. 7. Lanes were cut into 2.5-mm long strips and radioactivity was quantitated by liquid scintillation counting. The migration distances for lipoprotein standards were 40, 48, and 64 mm for LDL (β), VLDL (pre-β), and HDL_3 (α), respectively.

binding is similar to the values we obtained for the stimulation of both cholesterol and phospholipid efflux using cholesterol-enriched fibroblasts. Although these observations would be consistent with an apolipoprotein receptor mechanism, they do not exclude the possibility that the apolipoproteins are stimulating lipid efflux by interacting with membrane lipid domains. Cholesterol enrichment increases the free cholesterol content of the plasma membrane, and this enrichment may generate packing defects within the phospholipid bilayer that could enable apolipoproteins to bind reversibly to the plasma membrane. When the apolipoproteins dissociate, they may remove segments of the membrane containing unesterified cholesterol and phospholipid.

The predominant phospholipid subclass released from cells after cholesterol enrichment was phosphatidylcholine, and the cholesterol to phospholipid molar ratio of lipid released from cholesterol-enriched cells was estimated to be approximately 1. This ratio remained constant throughout a period of time ranging from 3 to 48 h, indicating that there was a parallel release of both lipids from cells. For these reasons, we propose that apolipoproteins can preferentially access cholesterol-phosphatidylcholine domains (27) within the plasma membrane of cholesterol-enriched fibroblasts resulting in the formation of newly assembled lipoprotein particles.

The preparation of apoCs used in this study was a mixture of C-I, II, and III and thus the activities of the individual species cannot be assessed. Apolipoprotein C-III was unable to promote lipid release from cholesteryl ester-loaded macrophages (21). The presence of this apolipoprotein in the C mixture may decrease the ability of C-I and C-II to promote phospholipid efflux from cells. The C apolipoproteins range in molecular weight from 6550 to 8837. Apolipoproteins A-I, A-II, and A-IV vary in mass from 17,000 to 46,000 daltons, and were equally effective at promoting efflux from cells. These apolipoproteins may meet a minimum size requirement (or contain a minimum number of amphipathic alpha helices) for promoting efflux from cells which the Cs do not.

We conclude that apolipoproteins promote lipid removal from cells. This process was largely dependent on the cellular free cholesterol content, wherein cholesterol enrichment of cells was necessary before substantial efflux of cholesterol and phospholipid was observed. We speculate that apolipoproteins remove lipid from cholesterol-phosphatidylcholine domains of the cellular membrane of cholesterol-enriched fibroblasts, whereupon HDL is generated in the extracellular compartment. Efflux of lipid from cholesterol-enriched cells to apolipoproteins may represent an additional protective mechanism against the potentially damaging effects of excess membrane cholesterol.

Special thanks to Dr. Michael C. Phillips for encouragement and advice. This work was supported by Program Project Grant HL-22633 and Training Grant HL-07443 from the National Heart, Lung, and Blood Institute of the National Institutes of Health.

Manuscript received 1 May 1992 and in revised form 25 June 1992.

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