Efflux of intracellular versus plasma membrane cholesterol in HepG2 cells: different availability and regulation by apolipoprotein A-I

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Abstract We have compared the efflux of cholesterol from different cellular pools of human hepatoma cells HepG2 using intact cells or isolated membrane fractions. To label different pools, cells were incubated with either unesterified [^{14}C]cholesterol that had been incorporated into high density lipoproteins ([^{14}C]FC-HDL), low density lipoproteins ([^{14}C]FC-LDL), or phosphatidylcholine liposomes ([^{14}C]FC-PC), or with [^{14}C]acetate. Cell fractionation revealed that labeling of cells with [^{14}C]FC-PC resulted in the incorporation of [^{14}C]cholesterol almost exclusively into the plasma membrane (PM), while incubation with [^{14}C]FC-HDL resulted in the majority of [^{14}C]cholesterol incorporation into the PM, but with a smaller component associated with lysosomes. Labeling with [^{14}C]FC-LDL or [^{14}C]acetate led to an accumulation of [^{14}C]cholesterol predominantly in lysosomes or the endoplasmic reticulum (ER), respectively. When the kinetics of [^{14}C]cholesterol efflux was analyzed after pulse-labeling of different cellular pools, half-times of cholesterol efflux from lysosomes and ER were significantly longer than that from PM. In another set of experiments, when both labeling and efflux times varied, efflux of [^{14}C]cholesterol from the PM to human serum after 1.5 h pulse and chase incubations was double that from lysosomes and 8-fold that from ER. Extension of the incubation times from 1.5 to 3 h diminished the difference in cholesterol efflux from different membranes. Further incubation to 6 h almost abolished the different responses. Cell-free preparations of membranes, obtained from cells labeled with [^{14}C]cholesterol, showed no differences in cholesterol efflux. No differences in the distribution of [^{14}C]cholesterol released into serum among lipoprotein subfractions was observed. Pretreatment of the serum with Fab fragments of polyclonal rabbit anti-human apolipoprotein A-I antibodies reduced its ability to promote efflux of cholesterol from the ER by 77%, but had no effect on cholesterol efflux from the PM. Fab fragments of non-immune IgG had no effect on the efflux of both ER and PM cholesterol. We conclude that the availability of cellular cholesterol for efflux from HepG2 cells is strongly influenced by its subcellular location, and is regulated by apolipoprotein A-I.—**Sviridov, D., and N. Fidge.** Efflux of intracellular versus plasma membrane cholesterol in HepG2 cells: different availability and regulation by apolipoprotein A-I. *J. Lipid Res.* 1995. **36:** 1887–1896.

Supplementary key words cholesterol efflux • intracellular trafficking • HDL • reverse cholesterol transport

A growing body of data supports the concept that cellular cholesterol is not uniformly distributed among the various cell membranes, and that cholesterol associated with different membranes has different metabolic fates. It is generally accepted that at least two distinct cholesterol pools exist in a cell: the plasma membrane and the intracellular cholesterol pools (1–3). The intracellular cholesterol pool has been further subdivided on the basis of the subcellular location of cholesterol, e.g., lysosome (2, 4), endosome (4), and the endoplasmic reticulum pools (5). Further delineation of hepatic cholesterol pools is suggested on the basis of the metabolic properties of cholesterol within these pools, e.g., newly synthesized cholesterol pool (5), bile acid (6) and lipoprotein (7) precursor cholesterol pools, or on the basis of cholesterol exchange kinetics, into "fast" and "slow" pools (8, 9). Little is known about the communication between these anatomical, metabolic, and kinetic cholesterol pools, or about intracellular cholesterol trafficking prior to efflux.

Efflux of cholesterol from cells is mediated by HDL. HDL picks up cholesterol diffusing from the outer surface of plasma membrane (PM) into the surrounding aqueous phase, a nonspecific process which may or may not be accompanied by the net flux of cholesterol (10). There is uncertainty, however, regarding the regulation of cholesterol efflux. Oram et al. (3), Hokland et al. (11), and Slotte, Oram, and Bierman (12) suggested that the transfer of cholesterol from intracellular pool(s) into PM

Abbreviations: apoA-I, apolipoprotein A-I; DTT, dithiothreitol; ER, endoplasmic reticulum; FC, free (unesterified) cholesterol; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HDL, high density lipoprotein; LDL, low density lipoprotein; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PM, plasma membrane(s); PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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prior to its desorption is rate-limiting and that this process is regulated by the interaction of HDL with its receptor. Rothblat et al. (8) and Mahlberg and Rothblat (9), however, suggested that cholesterol trafficking inside the cell is not rate-limiting and cholesterol efflux depends entirely on the rate of cholesterol exchange between cells and acceptor, i.e., on the packing of cholesterol in the plasma membrane and the properties of an acceptor. Both hypotheses assume the existence of different cholesterol pools, although they differ with regard to the definition of a "pool."

Human hepatoma cells HepG2 produce an interesting model for investigating cholesterol trafficking and the influence of cholesterol acceptors on the pathway of cholesterol efflux. Like extrahepatic cells, HepG2 cells also shed cellular cholesterol independently of lipoprotein assembly and secretion (13). In contrast to fibroblasts, however, a number of more distinguishable cholesterol pools seem to be present in liver cells: hence our use of HepG2 cells to study the availability of cholesterol from different cellular pools for the efflux into human serum. In this study we found a significant difference in the efflux of cholesterol from intracellular membranes versus plasma membrane in HepG2 cells. In addition, we established that blocking of serum apoA-I with Fab fragments of polyclonal antibodies reduces efflux of intracellular, but not PM cholesterol.

MATERIALS AND METHODS

Cells

Human hepatoma cells HepG2 were grown in a CO_2-incubator (5% CO_2, 95% air) in 75-cm^2 flasks or 6-well (35 mm diameter) tissue clusters (Costar, Cambridge, MA) coated with collagen (14). Cultures were maintained in Dulbecco's modified minimum essential medium containing 10% FCS, 20 mM HEPES, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3.7 mg/ml sodium bicarbonate (all reagents from Flow-ICN). Cells were 90–100% confluent at the beginning of the experiments.

Human serum and lipoproteins

Human blood was collected from healthy volunteers, immediately cooled on ice and allowed to stand for 2–3 h on ice for clot formation. After clot retraction, blood was centrifuged for 30 min at 2000 *g* to remove blood cells and the fibrin clot. The serum was removed and stored frozen at -80°C. Preliminary experiments revealed no differences between plasma and serum in the abundance of lipoprotein subfractions on nondenaturing two-dimensional electrophoresis (see below) or in their ability to promote cholesterol efflux.

LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated by sequential preparative ultracentrifugation (15) and purified by additional ultracentrifugation at the corresponding densities. Protein composition of the lipoproteins was checked by electrophoresis on 12% SDS-polyacrylamide gel. To label lipoproteins with [^{14}C]cholesterol, 2 mg of each lipoprotein was incubated for 4 h at 4°C with 0.5 MBq of [4-^{14}C]cholesterol (NEN, North Ryde, NSW; sp act 2.1 GBq/mmol) dissolved in ethanol, and 1% (w/v) bovine serum albumin (Sigma, St. Louis, MO, essentially fatty acid-free). Lipoproteins were then reisolated by centrifugation at the corresponding densities and dialyzed against PBS.

[^{14}C]cholesterol-phosphatidylcholine liposomes ([^{14}C]FC-PC) were prepared according to Arbogast et al. (16) with modifications. In brief, egg phosphatidylcholine (100 mg/ml in chloroform, Sigma) was dried under N_2 and in a final PC concentration of 1 mg/ml in PBS was sonicated and then centrifuged for 1 h at 436,000 *g* to remove multilamellar vesicles. [^{14}C]cholesterol was dried under N_2, PC liposomes were added to the final ratio 1:1 (mol/mol), and the mixture was sonicated in 1 ml PBS.

Antibody

Antisera against human apoA-I was produced in 5- to 6-month-old rabbits that received 100 μg of purified human apoA-I emulsified in 1 ml of PBS and Freund's complete adjuvant by subcutaneous injections. The animals were boosted 3 weeks after the first immunization and bled 2 weeks thereafter. Antiserum was then subjected to chromatography on proteinA-Sepharose (Pharmacia, North Ryde, NSW). Specific antibody was then eluted from an apoA-I-Sepharose 4B affinity column using 0.1 M glycine HCl, pH 2.8, and neutralized immediately with 1 M Tris buffer, pH 8.0. Specificity of antibody was checked by Western blotting (17) after electrophoretic separation of human serum on 12% SDS-polyacrylamide gels: only one band with the molecular weight of apoA-I was detected with anti-apoA-I antibodies. For the production of Fab fragments, affinity-purified antibody was subjected to papain digestion (18). Fab fragments were removed from undigested IgG and Fc fragments by passage through proteinA-Sepharose and their purity was assessed by 12% SDS-polyacrylamide gel electrophoresis. Non-immune IgG was isolated by affinity chromatography on proteinA-Sepharose of pre-immune rabbit plasma.

Cholesterol efflux

For labeling with [^{14}C]cholesterol, cells in 6-well clusters were washed twice with HBSS and incubated for the indicated periods of time in serum-free medium con-

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taining various amounts of [^{14}C]FC-PC, [^{14}C]FC-HDL, [^{14}C]FC-LDL, or [1-^{14}C]acetate (Amersham, North Ryde, NSW; sp act 2.15 GBq/mmol). After labeling, cells were washed six times with HBSS and further incubated for the indicated periods of time in medium without FCS and with 50% human serum (3% serum in the experiments with antibody). The medium was then collected and centrifuged for 15 min at 4°C at 30,000 *g* and used for further analysis. Cells were removed using a cell scraper, dispensed in 1 ml of distilled water, and dissolved by overnight incubation in 0.5 M NaOH.

Aliquots of cells and media were saponified and then incubated with 1.5 ml of extraction solution [isopropanol-n-heptane-H_2SO_4 40:10:0.03 (v/v/v)] for 1 h at room temperature; then 1 ml of distilled water and 1.5 ml of n-heptane were added and samples were incubated for another 1 h. The organic phase was dried, dissolved in chloroform, applied to TLC plates (Silica gel 60 A, Whatman, Singapore) that were developed in hexane-diethyl ether-methanol-acetic acid 40:15:5:1 (v/v/v/v). The band corresponding to free cholesterol was scraped into scintillation vials and counted. In the experiments where esterification of cholesterol was assessed, saponification was omitted and radioactivity in both free cholesterol and cholesteryl ester bands was analyzed; the amount of esterified cholesterol in cells after labeling with [^{14}C]acetate was determined as a difference in the amount of free cholesterol in samples with and without saponification. In some experiments cholesterol from the band was extracted into chloroform, dried, redissolved in the isopropanol, and further analyzed by reverse phase HPLC on a C-18 column (25 cm) using a solvent system of acetonitrile-isopropanol-water 44:54:2 (v/v/v) at 1 ml/min and room temperature. Radioactivity was measured using an online radiochromatographic detector (Canberra Packard, A-100).

The distribution of [^{14}C]cholesterol between lipoprotein fractions in the medium was determined by nondenaturing two-dimensional electrophoresis described by Castro and Fielding (19). ApoA-I-containing subfractions of lipoproteins were identified by Western blotting (17) and the distribution of [^{14}C]cholesterol among lipoprotein subfractions was analyzed using a Bioimager BAS-1000 (Fuji, Japan).

To study the effect of antibody on cholesterol efflux, medium without FCS and with 3% human serum (final apoA-I concentration 29 µg/ml) was incubated with indicated concentrations of Fab fragments of polyclonal rabbit anti-human apoA-I antibodies or Fab fragments of rabbit non-immune IgG for 4 h at 37°C. After incubation with antibodies this medium was used in the efflux experiments as described above. To analyze [^{14}C]cholesterol efflux from isolated membranes, aliquots of membrane preparations (see below) were incubated with 50% human serum in PBS for 3 h at 37°C; membranes were then sedimented by centrifugation at 100,000 *g* for 30 min at 4°C and the distribution of radioactivity in the pellet and the supernatant was analyzed using TLC as described above.

Subcellular fractionation of HepG2 cells

Cells (3 × 75-cm^2 flasks for each preparation) were labeled as described above. After labeling, cells were cooled to 4°C and washed 5 times with ice-cold HBSS. Cells were removed with a cell scraper, resuspended in 0.25 M sucrose in buffer A (5 mM Tris, 1 mM PMSF, 1 mM DTT, pH 7.0) (all reagents from Sigma) and homogenized by 10–20 strokes of a Teflon-glass homogenizer kept in ice. Nuclei, mitochondria, and undamaged cells were removed by centrifugation at 800 *g* for 10 min at 4°C, and crude membranes were sedimented by centrifugation at 200,000 *g* for 40 min at 4°C in a 70.1 Ti rotor. Pellets were resuspended in 2 ml of 65% sucrose in buffer A, homogenized, placed in the bottom of centrifuge tubes, overlaid with a discontinuous (six steps) sucrose gradient of 15–55% sucrose in buffer A, and centrifuged at 100,000 *g* for 3 h at 4°C in a SW41 Ti rotor. The gradient was then collected in six fractions, diluted with buffer A, and membranes were pelleted by centrifugation at 250,000 *g* for 1 h at 4°C. Pellets were resuspended in distilled water, aliquots were counted, and the rest was stored frozen at -80°C. The recovery of [^{14}C]cholesterol from the gradient was greater than 90%. Activity of 5′-nucleotidase, a marker of PM (20, 21), was assayed according to Gentry and Olsson (22). Activity of NADPH-cytochrome C reductase, a marker of ER membranes (20, 21), was assayed according to Sottocasa, Kuylenstiera, and Ernster (23). Acid phosphatase, a marker of lysosome membranes (20, 21), was assayed using a kit from Sigma according to the manufacturer's instructions.

The protein content of the cells, membranes, antibody, and lipoprotein preparations was determined according to Bradford (24).

Statistical analysis

In order to normalize the amount of [^{14}C]cholesterol to the number of cells, the amount of [^{14}C]cholesterol released from cells into the medium was expressed relative to cell protein content. Specific activity of the cellular total cholesterol was determined on washed cells after labeling incubation (time zero) and this value was used to calculate the amount of [^{14}C]cholesterol released into the medium. Kinetic curves were best fitted by a single exponential equation using "SimaStat-SigmaPlot" software. Rate constants for [^{14}C]cholesterol efflux (k_e) were calculated using these single exponential

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equations. A $t_{½}$ value in hours was calculated as follows: $t_{½} = \ln2/k_e$. In some experiments cholesterol efflux was expressed as a percent of total (radioactivity in cells plus medium) [^{14}C]cholesterol appearing in the medium. In the experiments with antibody, cholesterol efflux was expressed relative to the control (no antibody). The background values, i.e., amount of [^{14}C]cholesterol released from the cells to the serum-free medium, were subtracted. The proportion of [^{14}C]cholesterol in different subcellular fractions was calculated as the area under corresponding peaks and then expressed as a percent of the total area of all peaks.

Means ± standard errors of mean of triplicate or quadruplicate determinations (i.e., from three or four dishes) are presented. Statistical significance of differences was determined by Student's two-tailed *t*-test. All experiments were reproduced 2–3 times.

RESULTS

To label different cellular membranes with [^{14}C]cholesterol, cells were incubated with [^{14}C]cholesterol incorporated into HDL ([^{14}C]FC-HDL), LDL ([^{14}C]FC-LDL), or phosphatidylcholine liposomes ([^{14}C]FC-PC). In addition, cells were incubated with [^{14}C]acetate to label newly synthesized cholesterol pools. After labeling, cellular membranes were fractionated in a sucrose density gradient and identified according to the presence of marker enzymes. Distribution of the marker enzyme activity and [^{14}C]cholesterol in different subcellular fractions are presented at **Fig. 1.** Plasma membranes, characterized by the highest activity of 5′-nucleotidase, were recovered in the fraction with a density of 1.15 g/ml (Fig. 1A) and were clearly separated from lysosomes (i.e., the fraction containing the highest acid phosphatase activity) that were recovered at a density 1.25 g/ml (Fig. 1C). The NADPH:cytochrome C reductase activity, characterizing the endoplasmic reticulum, was recovered at a density intermediate between PM and lysosomes (d 1.20 g/ml) (Fig. 1B). Although the fraction corresponding to the ER overlapped PM and lysosome fractions, the procedure achieved a significant enrichment of different membrane fractions, similar to that reported by Magargal, Dickinson, and Slakey (20).

After incubating cells with [^{14}C]FC-PC, the major portion (87%) of the label was associated with the fraction corresponding to PM (Fig. 1A). The remainder (13%) of [^{14}C]cholesterol was present in the lysosome fraction. Labeling of cells with [^{14}C]FC-HDL resulted in the incorporation of 65% [^{14}C]cholesterol into PM and 35% into lysosomes. Seventy five percent of newly synthesized cholesterol labeled with [^{14}C]acetate was isolated in ER membranes with no other major peaks (Fig.

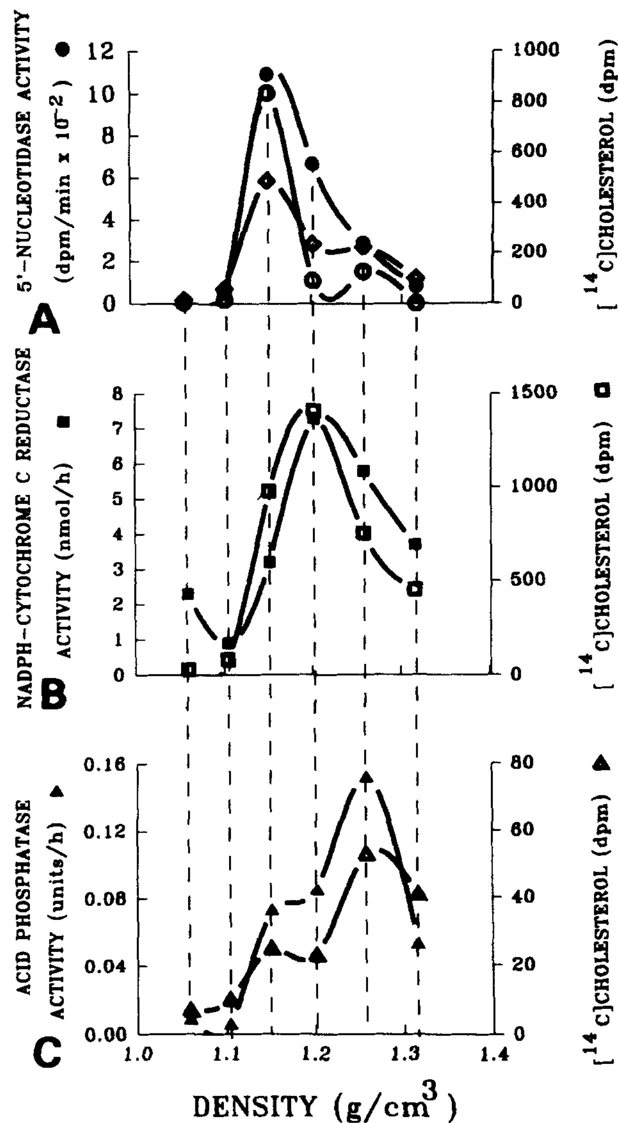

Fig. 1. Distribution of [^{14}C]cholesterol and marker enzymes among cellular membranes after sucrose density gradient fractionation of HepG2 cells labeled with [^{14}C]FC-PC (A), [^{14}C]FC-HDL (A), [^{14}C]acetate (B) or [^{14}C]FC-LDL (C). HepG2 cells were incubated for 3 h with [^{14}C]FC-PC (A, ○), [^{14}C]FC-HDL (A, ◇) (final protein concentration 45 μg/ml), [^{14}C]FC-LDL, (C)(final protein concentration 50 μg/ml)(final activity of all three 2.5 kBq/ml) or [^{14}C]acetate (B)(final activity 50 kBq/ml). After incubation, cells were washed, removed with a cell scraper, and fractionated by sucrose density gradient ultracentrifugation as described in Materials and Methods. Isolated membrane fractions were diluted with PBS, sedimented by additional centrifugation for 40 min at 250,000 *g* at 4°C, and resuspended in distilled water. Activity of 5′-nucleotidase (A), NADPH-cytochrome C reductase (B) and acid phosphatase (C) were determined as described in Materials and Methods. Aliquots of each fraction were saponified, lipids were extracted, cholesterol was isolated by TLC as described in Materials and Methods, and the amount of [^{14}C]cholesterol in each fraction was determined.

1B). Incubation of cells with [^{14}C]FC-LDL led to the incorporation of 69% of [^{14}C]cholesterol into the lysosomes and 31% into PM fraction (Fig. 1C). The amount of cholesterol that was esterified after labeling

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TABLE 1. Esterification of cholesterol associated with different cellular pools

Labeled with	Location	[14C]cholesteryl Esters
		% of total [14C]cholesterol
[14C]FC-PC	PM	1.9 ± 0.06
[14C]FC-HDL	PM and lysosomes	1.6 ± 0.01
[14C]FC-LDL	Lysosomes	3.0 ± 0.03[a]
[14C]acetate	ER	2.9 ± 0.14[a]

HepG2 cells were incubated for 3 h with [14C]FC-HDL (final protein concentration 45 μg/ml), [14C]FC-LDL (final protein concentration 50 μg/ml), [14C]FC-PC (final activity of all three 2.5 kBq/ml), or [14C]acetate (final activity 50 kBq/ml). After incubation cells were washed, removed with the cell scraper, lipids were extracted and separated by TLC as described in Materials and Methods. Amount of [14C]cholesteryl esters after labeling of cells with [14C]acetate was determined as a difference in the amount of [14C]cholesterol before and after saponification. Means ± standard error of the mean of quadruplicate determinations are presented.

[a]$P < 0.001$ (vs. [14C]FC-PC).

of different cellular membranes is shown in **Table 1.** Esterification of [14C]cholesterol associated with PM was 1.5- to 2-times less than that associated with intracellular compartments, lysosomes, and the endoplasmic reticulum. The total cholesterol content (mass) of the cells was not altered by the labeling procedures (not shown). When cells were labeled as described above for different times (1.5–6 h), the amount of [14C]cholesterol in cells increased linearly for all four labeling procedures (not shown).

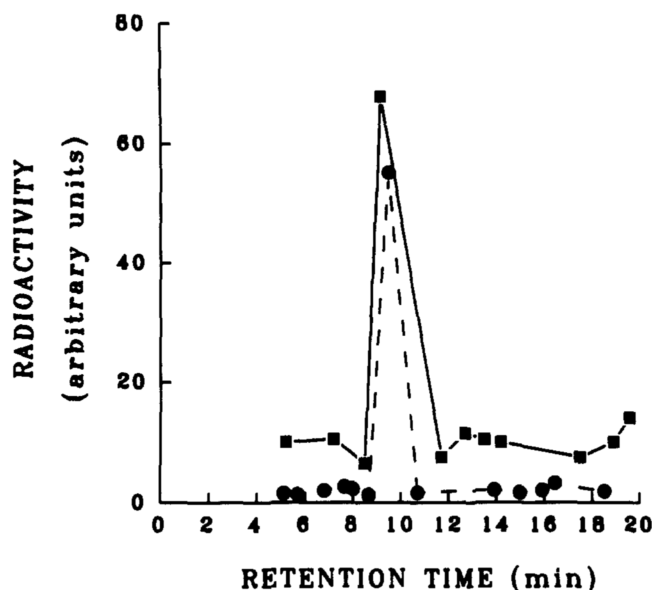

Fig. 2. HPLC analysis of [14C]cholesterol released from cells labeled with [14C]acetate. Labeling of HepG2 cells with [14C]acetate and efflux incubation (3 h each) were performed as described in Materials and Methods. After efflux incubation, medium was removed and centrifuged for 15 min at 30,000 *g*. Lipids were extracted and cholesterol was isolated by TLC as described in Materials and Methods. The cholesterol band was then extracted by chloroform (solid line) and together with commercial sample of [14C]cholesterol (dashed line) was analyzed by reverse phase HPLC with the radiochromatographic detector as described in Materials and Methods.

Cholesterol efflux was evaluated as a transfer of [14C]cholesterol from prelabeled cells to human serum. However, because labeling of cells with [14C]acetate results in incorporation of the label in all cellular compounds, newly synthesized cholesterol released from the cells could be contaminated with labeled cholesterol precursors or products of cholesterol oxidation that are able to move from ER to PM (25) and that cannot be separated from cholesterol by TLC. To investigate this possibility we analyzed the medium by reverse phase HPLC, which enabled us to separate different sterols. Seventy percent of newly synthesized sterols released into medium was represented by cholesterol, compared to 87% in the commercial sample of [14C]cholesterol (**Fig. 2**). No other sterols were detected as a peak, and remaining radioactivity was represented by background "noise" (Fig. 2).

When labeled cells were incubated for 3 h in serum-free medium, the amount of the [14C]cholesterol released was 11.7%, 7.7%, 14.2%, 13.3% for, respectively, PM, PM and lysosomes, ER, and lysosomes when compared to that in the presence of 50% serum (difference between values was not statistically significant). Ninety percent of the [14C]cholesterol released in the absence of serum was recovered in the lipoproteins secreted by HepG2 cells and their detailed analysis is reported elsewhere (13).

The time-course of cholesterol efflux after labeling different cellular cholesterol pools is presented in **Fig. 3** and kinetic parameters of the efflux are presented in

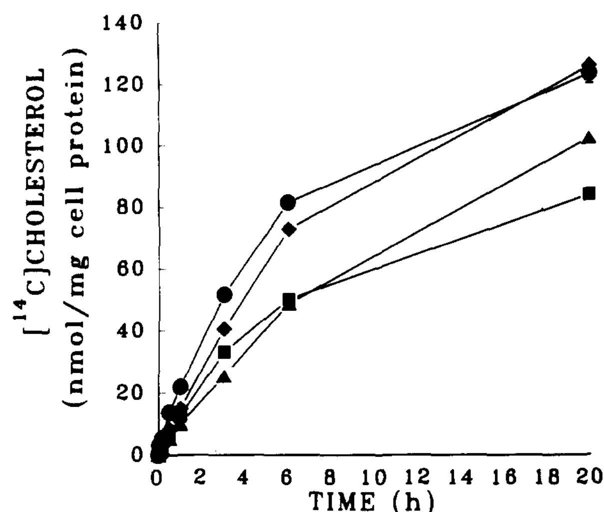

Fig. 3. Time-course of [14C]cholesterol efflux from different cholesterol pools of HepG2 cells. HepG2 cells were labeled for 3 h as described in Fig. 1. After labeling cells were washed and further incubated for the indicated periods of time with the medium containing 50% human serum. After the second incubation, medium was removed and centrifuged for 15 min at 30,000 *g*. Lipids were extracted and saponified and cholesterol was isolated by TLC as described in Materials and Methods. Means ± standard error of the mean of triplicate determinations are presented. (●) PM; (◆) PM and lysosomes; (▲) lysosomes; (■) ER.

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TABLE 2. Kinetic parameters of cholesterol efflux from different cellular pools

Labeled with	Location	k_e	$t_{½}$
		h^{-1}	h
[14C]FC-PC	PM	0.175 ± 0.009	3.96
[14C]FC-HDL	PM and lysosomes	0.120 ± 0.004[a]	5.78
[14C]FC-LDL	Lysosomes	0.075 ± 0.003[a]	9.24
[14C]acetate	ER	0.145 ± 0.006[b]	4.95

Parameters were calculated as described in Materials and Methods from the data presented at Fig. 3.
[a]P < 0.01; [b]P < 0.02 (vs. [14C]FC-PC).

Table 2. When PM cholesterol was labeled with [14C]FC-PC, the curve best fitted a single exponential equation with kinetic parameters similar to those observed by others (10) for exchange with a single kinetic pool. When lysosome or ER membranes were labeled with [14C]FC-LDL and [14C]acetate, respectively, the kinetic curves were still best fitted by a single exponential equation, but the efflux was significantly slower when compared to the efflux from PM (Fig. 3, Table 2). When both PM and lysosomes were labeled by [14C]FC-HDL, the efflux values were intermediate between values for PM and lysosomes separately (Fig. 3, Table 2). This is compatible with the assumption that another process, presumably an exchange between PM and intracellular cholesterol, becomes rate-limiting and slows down the efflux.

The cholesterol that initially associated with a particular subcellular fraction will eventually be redistributed among other cellular pools (26). The duration of both the labeling procedure and the efflux incubation can potentially affect the redistribution of [14C]cholesterol. Accordingly, different times for both the labeling and subsequent efflux were tested in a set of experiments designed to assess the availability for efflux of cholesterol present in different pools. When different cellular cholesterol pools were labeled for 1.5 h, and cells were washed and then incubated for another 1.5 h with human serum, a pronounced difference in the [14C]cholesterol efflux from different pools was observed (**Fig. 4, left**). About 50% of the [14C]cholesterol incorporated into the PM of cells was then released into the serum. Only 30% of the [14C]cholesterol was released from cells when both PM and lysosomes were labeled with [14C]FC-HDL. Even less [14C]cholesterol was released from lysosomes and ER: respectively, 22% and 6% of the initial [14C]cholesterol. Although less marked, the differences in the efflux from various pools were still seen when the time of labeling and efflux were both increased to 3 h: 63% of [14C]cholesterol was released from the PM during this time, while 49%, 37%, and 42% were released from PM and lysosomes, lysosomes alone, and ER, respectively (Fig. 4, middle). Extension in time of the labeling and efflux to 6 h each further reduced the differential efflux of [14C]cholesterol into the medium with 53%, 48%, 45%, and 40% of the [14C]cholesterol released from PM, PM and lysosomes, lysosomes alone, and ER, respectively (Fig. 4, right). The only fraction that continued to show a significant difference regarding cholesterol efflux with longer incubation was the ER.

To assess whether the intact cell structure is required for maintaining the difference in cholesterol availability in different pools, similar experiments were performed using isolated membranes instead of cultured cells. HepG2 cells were labeled with [14C]FC-HDL, [14C]FC-LDL, [14C]FC-PC, or [14C]acetate for 3 h and then homogenized and their subcellular organelles were fractionated. Plasma membranes were isolated from the cells labeled with [14C]FC-HDL and [14C]FC-PC, lysosomes from the cells labeled with [14C]FC-LDL, and ER membranes from cells labeled with [14C]acetate. When these various membrane fractions were separately incubated with human serum for 3 h, about 50% of the total [14C]cholesterol was released (**Fig. 5**). No statistically significant difference was found in the efficiency of [14C]cholesterol efflux from different membranes.

To determine whether the [14C]cholesterol released from different cellular pools is picked up by different lipoprotein subfractions, we used nondenaturing two-dimensional electrophoresis to separate lipoproteins. No difference was found in the relative distribution of

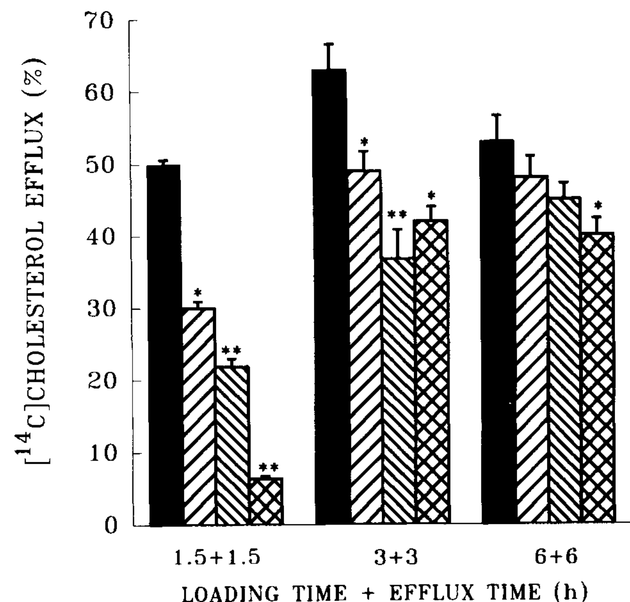

Fig. 4. Cholesterol efflux from different cholesterol pools of HepG2 cells. Experiments were performed as described in Fig. 3, except that the labeling time varied as indicated. Results are expressed as percent of total (cells plus medium) [14C]cholesterol appearing in the medium. Means ± standard error of the mean of quadruplicate determinations are presented. PM (■); PM and lysosomes (▨); lysosomes (▧); ER (▩); *P < 0.01 (vs. PM); **P < 0.05 (vs. PM and lysosomes).

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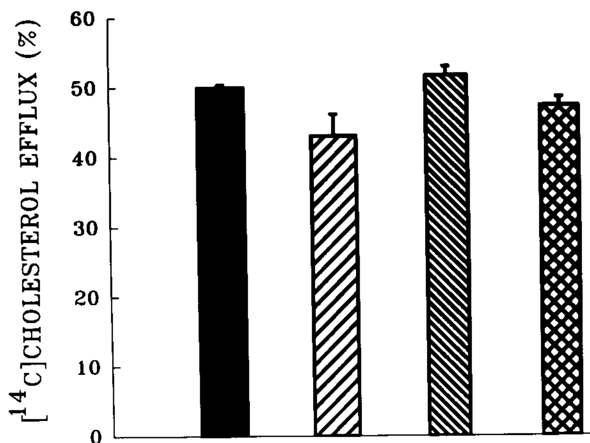

Fig. 5. Cholesterol efflux from isolated membrane fractions. HepG2 cells were labeled for 3 h as described in Fig. 1. Cells were then washed, removed, and fractionated as described in Materials and Methods. Aliquots of membranes were resuspended in PBS and incubated with 50% human serum for 3 h at 37°C. Membranes were then sedimented by centrifugation at 100,000 *g* for 30 min at 4°C, lipids from both the medium and the pellet were saponified and extracted, and cholesterol was isolated by TLC as described in Materials and Methods. Results are expressed as percent of total (membranes plus medium) [^{14}C]cholesterol appearing in the medium. Means ± standard error of the mean of quadruplicate determinations are presented. PM ([^{14}C]FC-PC) (■); PM([^{14}C]FC-HDL) (▨); lysosomes (▧); ER (▩).

[^{14}C]cholesterol among LDL, preβ-HDLs, and αHDL fractions (not shown), nor was there a difference in the esterification of [^{14}C]cholesterol released into the medium from cells labeled in different pools: 8.2 ± 2.2% (mean ± standard error of mean of quadruplicate determinations) of [^{14}C]cholesterol in the medium was esterified after 1.5 h incubation for each of four samples.

To investigate the possible role of apolipoprotein A-I in the efflux of intracellular and plasma membrane cholesterol, human serum (final concentration 3%) was pretreated with increasing concentrations of Fab fragments of rabbit anti-human apoA-I antibodies. When this serum was used as an acceptor and cells were labeled predominantly in the ER fraction (labeling with [^{14}C]acetate), the ability of serum to promote cholesterol efflux from ER was decreased in a dose-dependent manner up to a maximum of 77%. (**Fig. 6A**). The amount of [^{14}C]cholesterol remaining in the cells increased reciprocally (not shown). Treatment of serum with Fab fragments of nonimmune rabbit IgG had no effect on its ability to promote cholesterol efflux (Fig. 6A). In contrast, when the PM of HepG2 cells was labeled with [^{14}C]FC-PC, pretreatment of serum with Fab fragments of either anti-apoA-I or nonimmune IgG had no effect on the ability of the serum to promote cholesterol efflux from the PM (Fig. 6B).

DISCUSSION

Transfer of cellular cholesterol to a cholesterol acceptor in the plasma is considered a key step in reverse cholesterol transport. The final stage in this process, transfer of cholesterol from the plasma membrane to HDL, is a passive diffusion of cholesterol through the

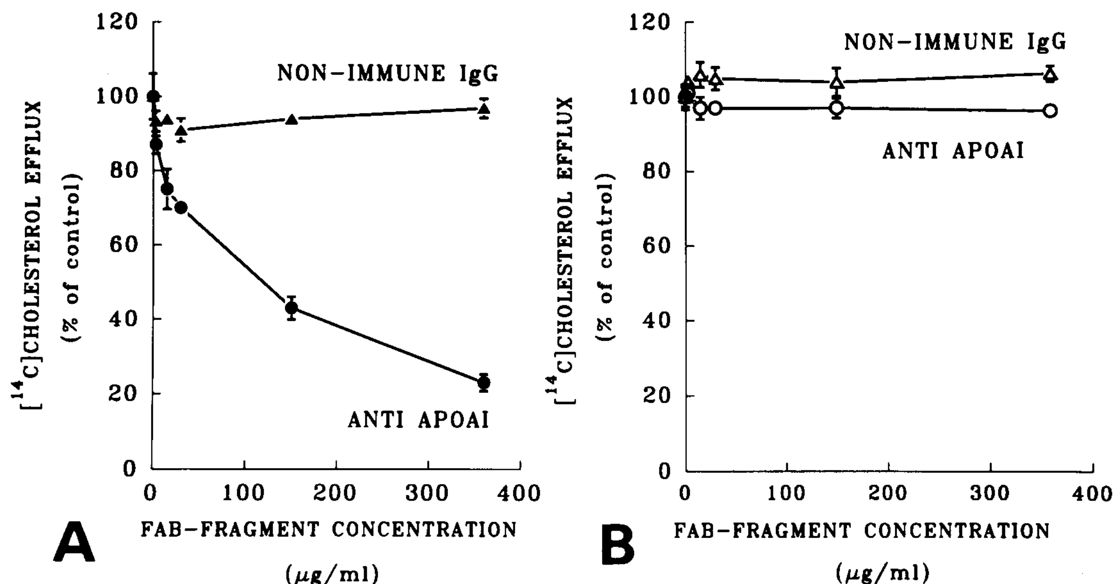

Fig. 6. Dose-dependence of the effect of Fab fragments of anti-apoA-I antibody or non-immune IgG on the efflux of cholesterol from ER (A) or PM (B). ER (A) or PM (B) of HepG2 cells were labeled by incubation with [^{14}C]acetate or [^{14}C]FC-PC, respectively, as described in Fig. 1. Human serum (final concentration 3%) was incubated with indicated concentrations of Fab fragments of rabbit anti-human apoA-I or rabbit non-immune IgG for 4 h at 37°C. After labeling cells were washed and incubated with the pretreated serum for 3 h. Medium was then removed, centrifuged for 15 min at 30,000 *g*, and cholesterol was isolated by TLC as described in Materials and Methods. Results are expressed relative to

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aqueous phase to the acceptor in the plasma (for review see refs. 8, 10). However, the results of numerous studies suggest that, besides the availability and physicochemical characteristics of the acceptor, other cell regulatory factors operate in order to prevent uncontrolled fluctuations of the cholesterol composition of the membranes. Two major hypotheses have been proposed to explain the cellular regulation of cholesterol efflux. Oram and Bierman (3, 11, 12) suggested that the rate-limiting step in the process is the transfer of cholesterol from the intracellular compartments to PM, a step that is regulated by interaction between HDL and its putative receptor. Rothblat and Mahlberg (8, 9) suggested that the rate of cholesterol efflux is mainly influenced by the rate of diffusion of cholesterol from the plasma membrane into the aqueous phase, a passive process that is not directly regulated by receptors. In the present work we explored the problem of the rate-limiting step of cholesterol efflux, and its regulation, by directly comparing efflux from the different cellular membranes and by studying the role of apoA-I in this process using apoA-I specific antibodies. If the availability of cholesterol for efflux from various subcellular fractions differs, the explanation presumably is because distinct pathways of intracellular cholesterol trafficking influence eventual efflux, and thus potentially regulate the process of cholesterol release.

To study cholesterol efflux from different subcellular pools we used human serum as an acceptor of cholesterol. It has been demonstrated previously that lipoprotein subfractions other than the major αHDL fraction may participate in cholesterol efflux (27, 28) and the use of serum provides the cells with all the potential circulating cholesterol acceptors. This will reduce the risk of introducing errors that may occur as a result of a selective dependency of the efflux from any one membrane fraction on a particular type of acceptor particle.

Differential labeling of cellular membranes was achieved by presenting [^{14}C]cholesterol in different forms that targeted it to the different cellular compartments. While the procedure resulted in the preferential labeling of a certain cholesterol pool, a small proportion of [^{14}C]cholesterol was also found in other pools. It is unclear to what extent this reflects rapid movement of [^{14}C]cholesterol from one pool into another, or is the result of the limitations in the separation of the membranes during cell fractionating. The data presented in Fig. 1, therefore, would represent at least some degree of underestimation of the enrichment of certain pools with [^{14}C]cholesterol.

The degree of specific subcellular localization of label that was achieved using different [^{14}C]cholesterol donors and [^{14}C]acetate was sufficient to enable a comparative measurement of efflux to be undertaken. This was apparent due to a striking difference in the availability of [^{14}C]cholesterol from different membranes for efflux that was observed in the presence of human serum. Kinetic analysis showed that half-time of cholesterol efflux from lysosomes and ER is, respectively, twice and 25% longer when compared with the efflux from PM. The difference was even more pronounced in the short-term experiments: two and eight times more [^{14}C]cholesterol was released from the PM than from the lysosomes and ER, respectively, after short pulse and chase incubation. This may even be an underestimation, because a proportion of labeled cholesterol initially incorporated into lysosomes or ER has already redistributed to the PM by the beginning of the chase incubation. It is interesting to note that the difference between $t_{1/2}$s of the efflux from PM and ER is smaller than the difference between the amounts of released [^{14}C]cholesterol, which is consistent with the hypothesis that part of newly synthesized cholesterol may form another pool with very long $t_{1/2}$. Overall, however, the result is consistent with the view that the rate-limiting step of cholesterol efflux involves the transfer of cholesterol from intracellular compartments to PM, and that cholesterol in the various compartments (i.e., lysosomes and ER) is not universally available for transfer. Different availability of cholesterol in different compartments for efflux was demonstrated only in the intact cells, but not in isolated membranes, which implies that the cause of the delay is intracellular cholesterol trafficking, and is not the result imposed by the labeling technique.

The rate of cholesterol trafficking from intracellular compartment to PM and back varies from one cell type to another, but is generally very fast (for review see refs. 26, 29). Thus, $t_{1/2}$ of the movement of cholesterol from lysosomes to PM in Chinese hamster ovary cells was estimated as 42 min (30), and $t_{1/2}$ of the movement of cholesterol from ER to PM in human fibroblasts was about 18 min (25), which is less than the usual $t_{1/2}$ of cholesterol efflux (10). Cholesterol delivered from the intracellular compartments to PM is not necessarily released from cells, it can be rapidly redirected back to the intracellular compartments for esterification (1, 31). It was also established that intracellular cholesterol trafficking is a specific and regulated process (26, 32). This opens an interesting possibility that cholesterol from intracellular compartments is not delivered uniformly to different domains in the PM, i.e., intracellular cholesterol may be delivered predominantly to the specific domain that is less available for efflux (e.g., "cholesterol rich domain" (8)) and most of this could be redirected back to ER for esterification. If labeling of PM delivers [^{14}C]cholesterol uniformly to all domains, difference in the efflux of cholesterol from intracellular pools and PM can be explained not by intracellular cholesterol traffick-

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ing but by the trafficking of cholesterol between different domains in PM.

According to Slotte, Oram, and Bierman (12) and Mendez, Oram, and Bierman (33) the transfer of cholesterol from the intracellular compartments to the plasma membrane comprises the rate-limiting step of cholesterol efflux, a step that can be regulated by the interaction of HDL with its putative receptor. Several reports have been published demonstrating that blocking of apoA-I with monoclonal antibody inhibits cholesterol efflux (34–36). However, PM and intracellular cholesterol are not distinguished in these reports. We found that blocking of apoA-I with Fab fragments of polyclonal anti-apoA-I antibodies reduces efflux of intracellular but not plasma membrane cholesterol. This finding again confirmed that the transfer of cholesterol from intracellular compartments to PM is the rate-limiting step of efflux and is stimulated by apoA-I. As efflux of cholesterol from PM was not affected, it was unlikely that the antibody altered the lipid binding properties of apoA-I or affected the metabolism of cholesterol associated with apoA-I-containing particles. The most probable explanation is that the antibody blocked interaction of apoA-I with a putative receptor that regulates the transfer of cholesterol from the intracellular compartments to the PM prior to efflux. We have previously demonstrated that polyclonal anti-apoA-I antibody inhibits HDL binding to cells (37). In this context our data support the hypothesis of Oram and Bierman (3, 11, 12) that intracellular trafficking is the rate-limiting step and the most important regulator of cholesterol efflux. It cannot be excluded, however, that blocking of HDL by antibody may have different effects on the efflux of cholesterol from the different pools because intracellular cholesterol is delivered selectively to the cholesterol-poor (i.e., "HDL-dependent" (8)) domain of PM prior to efflux, while labeling of PM labels cholesterol selectively in the cholesterol-rich (i.e., "HDL-independent" (8)) domain. We, however, are not aware of any reports demonstrating selective delivery of intracellular cholesterol to cholesterol-poor domains of PM, and it would also contradict the data showing that PM cholesterol is more readily available for efflux.

It should be pointed out that in our experiments we did not quantify the net flux of cholesterol between cells and serum lipoproteins; rather, we specifically measured release of radiolabeled cholesterol from different pools in HepG2 cells. The bidirectional flux of cholesterol between cells and an acceptor determines the net cholesterol flux when there is one type of lipoprotein particle in the incubation medium, but interpretation of bidirectional flux is complex in the presence of whole serum because the cellular cholesterol content is affected by all lipoproteins present in the serum (38). Thus, results of our study will not predict how the cholesterol content of a specific pool will change (e.g., efflux of cholesterol from intracellular compartments may be compensated for by cholesterol biosynthesis, LDL uptake etc). We used the tracer technique to compare movement to serum of cholesterol from various intracellular membranes with that from the PM. That we observed differences in efflux of [^{14}C]cholesterol from various membranes demonstrates that intracellular pathways are the likely rate-limiting and regulated steps of cholesterol efflux.

In conclusion, we have demonstrated that the availability of cholesterol for efflux from different subcellular pools varies considerably and is at least partially controlled by the interaction of apoA-I with the cell.

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