Cell-Derived Unesterified Cholesterol Cycles Between Different HDLs and LDL for Its Effective Esterification in Plasma

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Pulse-chase incubations of human plasma with $[^3H]$cholesterol-laden skin fibroblasts or low density lipoproteins (LDL) and nonnaturating two-dimensional electrophoresis were used to study the transfer and esterification of cell-derived unesterified cholesterol (UC) in human plasma lipoproteins. Specific radioactivities ($[^3H]$UC per microgram of UC) were calculated, and net cholesterol mass transfer was quantified using a fluoroenzymatic assay to validate productive transfers of UC between high density lipoprotein (HDL) and LDL. Cell-derived UC was initially taken up by pre-$\beta$-HDL and subsequently transferred in the sequence pre-$\beta$-HDL$\rightarrow$pre-3-HDL$\rightarrow$3-HDL$\rightarrow$LDL. During the first 5 minutes of this process, only 5% of cellular cholesterol was esterified in pre-$\beta$-HDL and 3-HDL; the remainder reached LDL as UC. Cellular UC accumulating in LDL was then redistributed to various HDL particles via two pathways: 1) the partially LDL receptor-mediated uptake and secretion of UC by cells and 2) the direct transfer of UC to HDL, mostly to 3-HDL and a small amount to pre-$\beta$-HDL. UC was not transferred from LDL to HDL after inhibition of LCAT. The esterification of cellular $[^3H]$cholesterol in plasma was competitively inhibited by the addition of excess unlabelled LDL but not of excess HDL. However, both excess LDL and excess HDL prevented the esterification of cell-derived cholesterol in apolipoprotein B-free plasma. This demonstrated that LDL is the major source of UC to the LCAT reaction and that the transfer of UC from LDL to HDL is LCAT dependent. In conclusion, the effective esterification of cell-derived cholesterol in plasma involves a rapid transfer of UC via HDL particles to LDL from which it is distributed to pre-$\beta$-HDL and 3-HDL. Furthermore, we hypothesize that the transfer per se of cellular UC to LDL forms a cholesterol concentration gradient between cell membranes and HDL and thus a second, reverse cholesterol transport mechanism in addition to the esterification of cholesterol by LCAT. (Arteriosclerosis and Thrombosis 1993;13:445-458)

KEY WORDS • pre-$\beta$-HDL • 3-HDL • LDL • cell-derived cholesterol • reverse cholesterol transport • lipid transfer • lecithin: cholesterol acyltransferase

Several epidemiological studies have documented an inverse correlation between the plasma concentration of high density lipoprotein (HDL) cholesterol and the risk of coronary heart disease. The antiatherogenic role of HDL has mostly been attributed to its ability to take up excess cholesterol from cell membranes of nonhepatic cells and to deliver it to the liver (reviewed in References 2 and 3). It is generally held that the cholesterol-esterifying enzyme lecithin: cholesterol acyltransferase (LCAT) plays a pivotal role in this reverse cholesterol transport, since the esterification of cholesterol generates a concentration gradient and thereby a flux of cholesterol from the cell membranes into the plasma compartment (reviewed in References 2 and 3). Quantitatively, however, cell membranes appear to be only a minor donor of unesterified cholesterol (UC) to the LCAT reaction. Most UC has been shown to originate in apolipoprotein (apo) B-containing lipoproteins (LpB).4-6 LCAT esterifies UC from these two sources in two distinct HDL particles that differ by their mobility on agarose gel electrophoresis. Cell-derived UC is first taken up by small, discoidal, apo A-I-containing HDL with pre-$\beta$ mobility. From these pre-$\beta$-HDL, UC is rapidly transferred to larger pre-3-HDL and subsequently to pre-3-HDL to be esterified in the latter.7,8 Low density lipoprotein (LDL)-derived cholesterol is esterified in 3-HDL.9,10 Cholesterol esters (CEs) formed by LCAT in pre-$\beta$-HDL and 3-HDL are also further metabolized through independent pathways. CEs from 3-HDL are transferred to LDL, possibly by cholesterol ester transfer protein (CETP).10 By contrast, CEs generated in pre-$\beta$-HDL accumulate in 3-HDL without being transferred to LDL.10 Thus, UC derived from LDL and cells is metabolized in two independent compartments. However, in view of the putative key role of LCAT in reverse cholesterol transport, this raises two important questions: 1) What is the origin of UC in LDL? 2) How is effective reverse
transport of cellular cholesterol, which is only a minor substrate for LCAT, achieved?

We postulated that the UC in LDL originates from peripheral cells rather than from secreted very low density lipoproteins (VLDL) and then performed various pulse-chase experiments of plasma with fibroblasts or LDL, both labeled with $^3$H]cholesterol. Using nondenaturing gradient gel electrophoresis, we followed the transfer processes of cell and LDL-derived $^3$H]UC between pre-β-HDL, α-HDL, LDL, and cells. Our results suggest that cell-derived UC is transferred via HDL to LDL from where it is transferred to pre-β-HDL and α-HDL for esterification through three alternative pathways. Since LDL is the major source of substrate to the LCAT reaction, we conclude that the transfer of cellular UC to LDL and its ongoing redistribution from LDL to pre-β-HDL and α-HDL is important in intensifying the otherwise ineffective esterification of cell-derived cholesterol in the plasma compartment.

**Methods**

**Plasma Samples**

Blood was collected from three normolipidemic probands (Table 1) after overnight fasting and was cooled immediately in ice water. Streptokinase was used as the anticoagulant at a final concentration of 150 units/mL. Plasma was obtained by centrifugation at 4°C (200g, 30 minutes) and used immediately in the experiments described below.

**Preparation and Labeling of Lipoproteins**

LDL ($d=1.019–1.063$ g/mL) and HDL ($d=1.063–1.21$ g/mL) were isolated by standard preparative ultracentrifugation from fresh normal human plasma and dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. To inactivate LCAT, HDL was heat inactivated by incubation in a water bath at 56°C for 30 minutes when required. In some indicated experiments, plasma was depleted of LpB by heparin-Sepharose affinity chromatography. Briefly, 3 mL normal human plasma was applied to a heparin-Sepharose CL-6B column (Pharmacia-LKB, 1-cm diameter, 15-cm length) equilibrated with $5 \text{mM}$ tris(hydroxymethyl)-amino methane-hydrochloride (Tris-HCl) buffer (pH 7.4) containing 0.15 M NaCl. The unretained fraction, defined as apo B–free plasma, was collected and reconstituted to the original volume. The complete removal of LpB was ascertained by the turbidimetric quantification of apo B.

The lipoproteins retained by the column (LDL and VLDL) were eluted with the same buffer containing 3 M NaCl and dialyzed against a buffer containing $5 \text{mM}$ Tris-HCl and 0.15 M NaCl.

LDLs were radiolabeled by equilibration with a $[1,2-\text{H}]$cholesterol–albumin–agarose complex according to the method of Miida et al. Stability of the labeled LDL against oxidation was ascertained by agarose gel electrophoresis. The mean specific activity of $[\text{H}]$cholesterol in labeled LDL amounted to $3.4\pm 1.3\times 10^6$ cpm/μg protein or $7.9\pm 1.5\times 10^5$ cpm/μg UC (mean±SD).

**Cell Culture**

Normal skin fibroblasts as well as skin fibroblasts from a patient homozygous for familial hypercholesterolemia (FH fibroblasts) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum in 3.5-cm dishes as described by others. At the state of near confluence, cells of some dishes were loaded with 0.5 μCi $[1,2-\text{H}]$cholesterol (New England Nuclear, Boston, Mass.; 51.7 Ci/mmol) complexed with fetal calf serum and incubated for 72 hours at 37°C. The final specific radioactivity in the labeled cells amounted to $5.6\pm 1.9\times 10^6$ cpm/mg cell protein or $1.87\pm 0.51\times 10^6$ cpm/μg cell cholesterol (mean±SD).

**Pulse-Chase Experiments With Fibroblasts**

Fibroblasts loaded with radiolabeled cholesterol were washed six times with phosphate-buffered saline (PBS, pH 7.4). In pulse-chase experiments, 1 mL complete plasma or apo B–free plasma was first incubated with labeled fibroblasts (pulse). After incubation for either 1 or 5 minutes, the plasma was removed and used for chase incubations. Conditions and time intervals were varied as indicated in the "Results" section. Chase incubations were performed in the absence or presence of fibroblasts. In some instances, LpB's were removed from the plasma by precipitation with phosphotungstic acid/MgCl$_2$. In other cases, various amounts of HDL, LDL, or dithio-bis(2-nitrobenzoic acid) (DTNB, at a final concentration of 2 mM) were added to the complete or apo B–free plasma before starting the chase incubation.

After chase incubation, the plasma samples were either directly derivatized by the addition of chloroform/methanol (2:1, vol/vol) or their lipoproteins were separated by agarose gel electrophoresis or by two-dimensional electrophoresis (2DE; see below).

In some experiments the cellular content of radioactive UC and CE was also quantified. For this purpose, the cells were solubilized with 0.5N NaOH after each incubation, and cell-associated lipids were extracted with chloroform/methanol (2:1, vol/vol). UC and CE were separated by thin-layer chromatography (TLC), and their radioactivity was determined by liquid scintillation spectrometry.

**Experiments With Radioactively Labeled LDL**

To study the transfer of LDL-derived cholesterol, 100 μL radiolabeled LDL ($9.4\times 10^4$ cpm) was incubated with 1 mL normolipidemic plasma in either the presence or absence of the LCAT inhibitor DTNB. During
the incubation, the plasma was mixed by continuous shaking. Plasma aliquots were removed after different time intervals for the determination of radioactivity in either complete plasma or defined lipoproteins.

Nondenaturing Two-Dimensional Electrophoresis

The transfer of cell- or LDL-derived cholesterol between different plasma lipoproteins was determined by nondenaturing 2DE, for which agarose gel electrophoresis was followed by polyacrylamide gradient gel electrophoresis (PAGGE).2,3,10,15 In the first dimension, 20 μL plasma labeled with cell- or LDL-derived radioactive cholesterol was separated by electrophoresis in a 0.75% agarose gel and a 50 mM merbital buffer (pH 8.7) at 4°C. Two agarose gel strips containing the separated lipoproteins were then transferred to a 2–20% polyacrylamide gradient gel. The second-dimensional separation was performed at 40 mA for 4–5 hours at 4°C. Thus, after separation, every PAGGE gel contained two 2DE patterns of the lipoproteins from one individual. One half of the gel containing one set was first stored at 4°C. The second half of the gel was electrophoblotted onto a nitrocellulose membrane, on which apo A-I–containing lipoproteins were subsequently detected by using a sheep anti-human apo A-I antiserum (Boehringer Mannheim, Mannheim, FRG), a biotinylated rabbit anti-sheep immunoglobulin G antiserum, and streptavidin–horseradish peroxidase (Amersham). The immunoblot was then used as a template to localize the corresponding lipoproteins in the other half of the gel. These lipoproteins were cut out and their lipids extracted with chloroform/methanol (2:1, vol/vol) for 72 hours. In some experiments, the total radioactive cholesterol in various lipoprotein fractions was determined. In other experiments, UC and CE were first separated by TLC on silica gel plates to separately count their radioactivity.

To determine the recovery of radioactivity, the amounts of [3H]UC and [3H]CE from pre-β- HDL and α-HDL were compared with the radioactivity in the supernatant after precipitation with phosphotungstate/MgCl₂. The amount of [3H]UC and [3H]CE in LDL was similarly compared with the radioactivity in the infranatant after precipitation. The recoveries were 85 ±14% in pre-β-HDL plus α-HDL and 92±17% in LDL.

Determination of Cholesterol Net Mass Transfer

We determined the net mass transfer of cholesterol from fibroblasts to plasma, LpB, or HDL by comparing the decrease in UC seen in the presence of fibroblasts to the decrease seen in their absence. After washing four times with PBS, dishes with or without confluently growing fibroblasts were incubated with 2 mL of 5% plasma in PBS. The media were removed after 2 hours' incubation at 37°C. Aliquots of 0.5 mL were precipitated with phosphotungstate/MgCl₂. Lipids in either the total medium or in the precipitates (i.e., LpB) were extracted with chloroform/methanol for 10 hours.

Cholesterol mass in the extracts was quantified by the use of a modified fluorometric assay that has been reported previously.16 Briefly, the extracted lipids were dissolved with 100 μL isopropanol. Test reagent (0.4 mL) for the determination of UC (Boehringer Mannheim, 0.16 IU/mL cholesterol oxidase, 30 IU/mL horseradish peroxidase, 0.15 mg/mL p-hydroxyphenylacetate in 0.05 M sodium phosphate buffer, pH 7.0) was added to 20 μL of the dissolved lipids. After a 30-minute incubation at room temperature, 0.8 mL of 0.5 M NaOH was added and mixed. The relative fluorescence was measured using a phototfluorometer (Shimadzu, Tokyo, Japan) at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. The fluorescence intensities were calibrated using a dilution series of cholesterol standards ranging from 0.05 to 0.2 μg (Sigma).

In some indicated instances, incubations were performed in the presence of DTNB with LDL (50 μg protein per milliliter) or with 2 mL apo B–free plasma. The experiments were performed with plasma samples from three normolipidemic individuals and were repeated four times each. Transfer rates are presented as nanomoles unesterified cholesterol per milligram cell protein per 2 hours.

General Procedures

Total protein concentrations were measured according to the method of Lowry et al,17 using bovine serum albumin as the standard. Plasma concentrations of cholesterol, triglycerides, and HDL cholesterol were determined with an autoanalyzer (Boehringer/Hitachi, Mannheim, FRG). LDL cholesterol was calculated by the Friedewald formula.18 LCAT activity was determined by the esterification of [3H]cholesterol in apo A-I–containing proteoliposomes.19 The cholesterol esterification rate was determined using the fluoroenzymatic assay described above by quantifying the decrease of UC in plasma during a 60-minute incubation at 37°C.

Every experiment was performed at least four times using the plasma samples from the probands described...
above. In some instances, percent values are presented. They represent the concentration of UC or CE in one particle as a percentage of total UC or CE in all lipoproteins (i.e., pre-β-HDL+α-HDL+LDL) at any given time point.

**Results**

Transfer and Esterification of Cellular Cholesterol

Figure 1 presents an anti-apo A-I immunoblot of nondenaturing 2DE of human plasma. Besides the major HDL subtraction with α-mobility (i.e., α-HDL), several species of pre-β-migrating HDL were also identified, which we designated as pre-β1-HDL, pre-β2-HDL, and pre-β3-HDL, according to the original description by Fielding and coworkers.7-10,15

Similar anti-apo A-I immunoblots were used as templates to localize apo A-I–containing lipoproteins in plasma samples that had been pulsed with radiolabeled cellular cholesterol. Figure 2 summarizes the occurrence of radiolabeled total cholesterol in plasma and in various lipoprotein fractions during ongoing chase incubations. After a 1-minute pulse incubation, the percentages of radioactive cholesterol in pre-β1-HDL, pre-β2-HDL, pre-β3-HDL, α-HDL, and LDL

![Figure 2](image_url)
amounted to 30±7%, 7±3%, 8±3%, 49±3%, and 7±4%, respectively. During subsequent chase incubation with unlabeled cells, the radioactivity in pre-β-HDL decreased (preferentially in pre-β2-HDL), remained stable in α-HDL, and increased in LDL to 40% after 15 minutes of incubation. These observations were similar to those reported by Castro and Fielding7 and indicate that cell-derived cholesterol was first incorporated into pre-β-HDL and subsequently transferred to the various lipoproteins in the sequence pre-β2-HDL→pre-β3-HDL→α-HDL→LDL.

In subsequent studies, we analyzed the esterification of UC and the transfer of [3H]UC and [3H]CE in the various lipoproteins. To obtain amounts of labeled cholesterol in the lipoproteins sufficient to differentiate between [3H]UC and [3H]CE, the pulse incubations were extended to 5 minutes (Figures 3 and 4). After a 5-minute pulse incubation, about 55% of radioactive CE was recovered in pre-β2-HDL and 40% in α-HDL. During chase incubations, radiolabeled CE in pre-β2-HDL decreased to less than 20% after a 5-minute chase incubation, during which time radiolabeled CE increased to more than 70% in α-HDL. These observations are in agreement with those of Fielding and coworkers7,8,10 and indicate that cellular cholesterol is esterified in pre-β2-HDL, from which CEs are transferred to α-HDL. However, after comparing the absolute amounts of radioactive UC and CE in the various lipoproteins at various time points (Figure 3), it became apparent that transfer of [3H]UC to α-HDL and LDL was more rapid than its esterification in pre-β3-HDL. After a pulse incubation for 5 minutes, the absolute amounts of radiolabeled UC and CE in pre-β2-HDL were almost identical, but the absolute amounts of radioactive UC in α-HDL and LDL exceeded those of radioactive CE by a factor of 20. During the ongoing chase incubation, the absolute amounts of [3H]UC remained stable in pre-β2-HDL. They gradually decreased in α-HDL and LDL but were always much higher than those of [3H]CE (Figure 3). Expressed as a percentage, the concentration of [3H]UC reached a very low level in pre-β2-HDL and decreased in α-HDL from 60% to 40% within 20 minutes, during
which time it increased in LDL. Only small amounts of \[ \text{[H]} \text{CE} \] were found in LDL (Figure 3).

**Transfer of Radioactive Unesterified Cholesterol to LDL**

The decrease in the absolute amount of \[ \text{[H]} \text{UC} \] in HDL was accompanied by an initial increase and then little change in \[ \text{[H]} \text{UC} \] in LDL, suggesting that cell-derived UC was not taken up by LDL directly but was transferred to it via HDL. Another possible explanation is that HDL loses \[ \text{[H]} \text{UC} \] to the cells. To further investigate this, we repeated the chase incubations in the absence of cells. Transfer and esterification of cellular cholesterol occurred in a similar way as in the chase incubation with cells (Figure 3). However, after 20 minutes, 30% of \[ \text{[H]} \text{CE} \] was found in LDL. In the absence of cells, we also observed an increase of \[ \text{[H]} \text{UC} \] in LDL accompanied by a decrease of \[ \text{[H]} \text{UC} \] in \( \alpha \)-HDL (Figure 4). After 20 minutes, approximately 80% of \[ \text{[H]} \text{UC} \] was found in LDL. In normal plasma, 75% of UC is present in LDL. Therefore, to rule out any diffusional equilibration of labeled cholesterol and UC among the various lipoproteins as the source of \[ \text{[H]} \text{UC} \] in LDL, we extended the chase incubation. Figure 5 shows the accumulation of \[ \text{[H]} \text{UC} \] in LDL and HDL expressed as specific radioactivity (counts per minute of \[ \text{[H]} \text{UC} \] per microgram of UC). After 30 minutes, the specific radioactivity was 25% higher in LDL than in HDL (\( p<0.01 \), Student's \( t \) test), thus supporting our hypothesis that cell-derived \[ \text{[H]} \text{UC} \] is transferred from HDL to LDL independent of its equilibration with unlabeled UC.

To further test this hypothesis, we measured the net mass transfer of UC from cells to plasma, to \( \text{LpB} \), and LDL during incubation for 2 hours (Table 2). Both in the presence and absence of fibroblasts, the absolute amounts of UC decreased in plasma, HDL, and \( \text{LpB} \), presumably because of the esterification of cholesterol by LCAT. However, the decrease in UC was significantly less in the presence than in the absence of cells. After 2 hours, a net amount of 4.57±0.54 nmol UC per milligram cell protein was released into 100 \( \mu \)L plasma (2 mL of a 5% solution). Seventy-one percent of this UC was found in \( \text{LpB} \). The importance of \( \text{LpB} \) for the release of UC from cells was also shown by a reduction in efficiency of the net transfer of UC into the medium of almost 40% when apo B-free plasma was used (Table 2). The incubation of normal, LDL receptor-positive fibroblasts with LDL alone, as expected, resulted in a UC transfer from the medium to the cells. By contrast, FH fibroblasts released a small net amount of UC to LDL (Table 3).

Taken as a whole, these results indicate that the transfer of radiolabeled UC from cells to LDL via HDL...
FIGURE 5. Time course of changes in the specific radioactivity of \(^{3}H\)unesterified cholesterol (UC) in \(\alpha\)-HDL and LDL during chase incubation without cells. Pulse incubations with radiolabeled fibroblasts for 5 minutes were followed by chase incubations without cells for the indicated time intervals. \(\alpha\)-HDL and LDL were separated by one-dimensional agarose gel electrophoresis. Subsequent to lipid extraction, UC and CE were separated by thin-layer chromatography. Specific radioactivity is expressed as counts per minute of \(^{3}H\)UC per microgram UC in LDL and \(\alpha\)-HDL.

HDL, high density lipoprotein; LDL, low density lipoprotein.

not only reflected simple equilibration of labeled and unlabeled UC in these lipoproteins but also pointed to a net transfer of UC to LDL. Such net transfer of cellular UC to LDL presumably via HDL has not been reported before. Since in vivo the bulk of cholesterol in both HDL and LDL is esterified and since HDL is the major site of cholesterol esterification,\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) this rapid transfer raises the question of the metabolic fate of UC in LDL.

**Role of Fibroblasts in Transfer of Unesterified Cholesterol**

After both short and long pulses, the concentration of radiolabeled cholesterol in the plasma immediately decreased in the presence of unlabeled cells and then remained stable during the remainder of the incubation (Figures 2a and 3a). This suggests that during chase incubation, labeled cell-derived cholesterol was removed from the plasma by the cells, for example, via uptake of LDL. This assumption is supported by the observation that the amount of radioactive UC and CE in LDL steadily increased during the whole chase incubation when performed in the absence of cells (Figure 4) but reached a maximum after 5 minutes when performed in the presence of cells (Figure 3). Another important difference is that the amount of \(^{3}H\)UC in pre-\(\beta\)-HDL gradually decreased during the chase incubation in the absence of cells, whereas it remained constant or even increased in the presence of cells (Figures 3 and 4), suggesting that \(^{3}H\)UC taken up by the cells via LDL is redistributed to the plasma via pre-\(\beta\)-HDL.

To test the hypothesis that UC is retaken from LDL by the cells and redistributed, we modified the pulse-chase experiments. We compared the amounts of extracellular and intracellular radiolabeled cholesterol seen on incubation of whole or apo B–free plasma with normal (LDL receptor–positive) fibroblasts with the amounts of extracellular and intracellular radiolabeled cholesterol found on incubation of whole or apo B–free plasma with LDL receptor–negative fibroblasts from a patient with homozygous FH (FH fibroblasts; Figure 6). In whole plasma (Figure 6a), the amount of radiolabeled cholesterol decreased during incubation with normal and to a lesser extent with FH fibroblasts. A chase with normal cells for 20 minutes decreased the radioactivity in plasma by more than 30%; similar incubation with FH fibroblasts produced a reduction of less than 20%. The amount of radioactivity in apo B–free plasma was not reduced during incubation. Conversely, after incubation of whole plasma with normal fibroblasts for 20 minutes, a large amount of radiolabeled cholesterol was found in normal fibroblasts, 95±4% of which was not

| Table 2. Cholesterol Net Transport Rates in Plasma Incubated With Normal Fibroblasts |
|---------------------------------|-----------------|-----------------|-----------------|
| Plasma                          | HDL             | LpB             |
| (nmol)                          | (Percent)*      | (nmol)          | (Percent)*      |
| Plasma                          |                 |                 |                 |
| +4.57                           | 1.40            | +29             | +3.26           | +71              |
| (0.54)                          | (0.39)          | (6.3)           | (0.31)          | (6.4)            |
| Apo B–free plasma               |                 |                 |                 |
| +2.79                           | ...             | ...             | ...             | ...              |
| (0.31)                          | ...             | ...             | ...             | ...              |
| LDL (50 μg protein/mL)          |                 |                 |                 |
| ...                             | ...             | -2.25           | ...             | ...              |
| ...                             | ...             | (0.39)          | ...             | ...              |

Two milliliters of 5% plasma, 5% apolipoprotein (apo) B–free plasma, or low density lipoprotein (LDL, 50 μg protein/mL) was incubated with normal human fibroblasts or without cells at 37°C for 2 hours. Cholesterol net mass transfer was determined by a modified fluorometric assay. This allowed calculation of the difference between the reduction of unesterified cholesterol in high density lipoprotein (HDL) or LDL after 2 hours' incubation of plasma in the presence or absence of fibroblasts. Values are mean±(SD) of four experiments. LpB, apolipoprotein B-containing lipoproteins.

*Percentage of total unesterified cholesterol net mass transfer into the medium.
TABLE 3. Cholesterol Net Transport Rates in Plasma Incubated With Familial Hypercholesterolemia Fibroblasts

<table>
<thead>
<tr>
<th>Net transfer of unesterified cholesterol into</th>
<th>Plasma (nmol)</th>
<th>HDL (nmol)</th>
<th>LpB (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>+7.13 (0.54)</td>
<td>+2.40 (0.47)</td>
<td>+4.81 (0.62)</td>
</tr>
<tr>
<td>Apo B–free plasma</td>
<td>+2.87 (0.46)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>LDL (50 μg protein/mL)</td>
<td>...</td>
<td>+0.38 (0.23)</td>
<td>...</td>
</tr>
</tbody>
</table>

HDL, high density lipoprotein; LpB, apolipoprotein B–containing lipoproteins; apo, apolipoprotein; LDL, low density lipoprotein. Experimental conditions are the same as in Table 2 except that familial hypercholesterolemia fibroblasts were used. Values are mean±(SD) of four experiments.

*Percentage of total unesterified cholesterol net mass transfer into the medium.

In FH fibroblasts, by contrast, the amount of radiolabeled cholesterol was reduced by 50% and none was esterified. In the absence of LpB’s, only small amounts of radiolabeled CE and UC were detected intracellularly (Figure 6b). These results suggest that during chase incubations, [3H]UC is transferred from the plasma to the cells mainly via LpB (70–80%) and LDL receptor–mediated uptake (50%) but also to a considerable extent by a pathway independent of the LDL receptor.

The quantitative importance of LDL receptor–mediated removal of UC from the plasma by fibroblasts became obvious when we compared the net transfers of UC after 2 hours’ incubation with normal and FH fibroblasts (Table 3). In the presence of FH fibroblasts, net transfer of UC into plasma was increased by 56%, net transfer to LpB by 48%, and net transfer to HDL by 71%. As expected, incubation of FH fibroblasts with apo B–free plasma did not result in transfer rates different from normal cells; in contrast to normal cells, incubation of FH fibroblasts with apo B–free plasma also resulted in transfer rates different from normal cells.
...tion with LDL resulted in a net transfer of UC from FH cells into the plasma (Table 3).

Taken together, these results suggested that during chase incubations, fibroblasts take up both CE and UC via LDL. About 35% of UC \((7.13 - 4.57)/7.13\) nmol/mg per 2 hours) appears to be taken up by the LDL receptor pathway. Because the cells probably resecrete UC into the plasma, the cellular uptake of UC through LDL provides a pathway by which UC is redistributed from LDL to pre-\(\beta\)-HDL for esterification. This conclusion is supported by the finding of higher amounts of \([H]CE\) in pre-\(\beta\)-HDL when chase incubations were performed in the presence of cells (Figure 3) than in their absence (Figure 4).

**Transfer of Unesterified Cholesterol From LDL to HDL Subclasses**

To test whether UC from LDL is also directly redistributed to pre-\(\beta\)-HDL and/or \(\alpha\)-HDL for esterification, we performed pulse incubations of complete plasma with LDL that had been previously labeled with radioactive UC. On prolonged incubation with labeled LDL, the specific radioactivity of \([H]UC\) gradually increased in both \(\alpha\)-HDL and pre-\(\beta\)-HDL (Figure 7a). After a 0.5-minute incubation with labeled LDL, about 25% of \([H]UC\) was found in pre-\(\beta\)-HDL, with only a small amount in pre-\(\beta\)-HDL (Figures 7b and 7c). During incubation, the relative concentration of labeled

FIGURE 7. Bar graphs of distribution of low density lipoprotein (LDL)-derived \([H]unesterified cholesterol (UC) in various high density lipoprotein (HDL) particles. Native plasma was incubated with labeled LDL for the indicated time intervals. Each bar shows the mean value of five experiments. Panel a presents changes of the specific radioactivity (in counts per minute \([H]UC per microgram of UC); panel b presents changes in the absolute amounts; and panel c presents relative changes. Note the different orders of magnitude between the absolute amounts of \([H]UC in pre-\(\beta\)-HDL and \(\alpha\)-HDL in panels b and c.
UC decreased in pre-β-HDL but increased in α-HDL to nearly 100% after 15 minutes. This suggests that at least a proportion of LDL-derived UC is transferred to α-HDL by two mechanisms, one involving pre-β- and pre-β-β-HDL, and another independent of pre-β-HDL. Whereas the pre-β-HDL-independent pathway has previously been described by Miida et al., the pre-β-HDL-dependent pathway represents a novel finding.

Role of LDL as a Source of Cell-Derived Unesterified Cholesterol for Cholesterol Ester Production in Plasma

Both the rapid transfer of [3H]UC to LDL and its subsequent redistribution from LDL to pre-β-HDL and α-HDL suggest that LDL plays a crucial role in the esterification of cellular cholesterol in plasma. To test this hypothesis, pulse incubations of plasma with radio-labeled fibroblasts for 5 minutes were followed by cell-free chase incubations for up to 11 hours at 37°C in the presence or absence of the LCAT inhibitor DTNB (Figure 8). At each incubation time, radioactive UC and CE were determined in α-HDL and LDL. Without LCAT inhibition, the amount of [3H]UC decreased in HDL and increased in LDL to an equimolar extent during the first hour of incubation. During prolonged incubation, the amount of [3H]UC gradually declined in LDL but remained constant in HDL (Figure 8a). At the same time the amount of [3H]CE increased in HDL and...
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LDL (Figure 8b). After 11 hours the amount of [3H]CE present in HDL and LDL was equal to the amount of [3H]UC removed from LDL. After inhibition of LCAT by DTNB, [3H]UC was transferred from HDL to LDL during the first hour. However, the amount of [3H]UC in LDL was not reduced (Figure 8c). Thus, inhibition of LCAT not only prevented the esterification of [3H]UC but also inhibited its transfer from LDL to HDL. This supports the hypothesis that UC in LDL is a major source of CE production in HDL.

This conclusion is also supported by our finding that inhibition of LCAT by DTNB significantly decreased net UC mass transfer from fibroblasts into the plasma (Table 4). Although DTNB also decreased net transfer of UC to LDL and prevented the production of CE at the expense of UC in HDL, in the presence of DTNB HDL was depleted of UC (Table 4).

To further investigate the importance of LDL as a substrate donor for LCAT, we performed another series of experiments in which pulse incubations of whole or apo B–free plasma with radiolabeled fibroblasts were followed by chase incubations in the absence of cells but in the presence of excess, exogenously supplied, unlabeled LDL or heat-inactivated HDL. Under these conditions, unlabeled UC in the exogenous lipoproteins competes with labeled UC in the endogenous lipoproteins for both transfer and esterification. Only the addition of excess unlabeled LDL but not that of excess unlabeled HDL (up to 200 μg) significantly inhibited the esterification of cell-derived [3H]UC in plasma (not shown). Compared with complete plasma, esterification of cell-derived UC in apo B–free plasma was about 50% less effective. However, under this condition, the addition of both excess LDL and excess HDL effectively inhibited the esterification of cell-derived [3H]UC, although the inhibitory effect of HDL was less pronounced than that of LDL. Figure 9 shows that the inhibitory effects on [3H]UC esterification of excess unlabeled LDL in complete plasma and of both excess unlabeled LDL and HDL in apo B–free plasma were concentration dependent.

### Discussion

In this study, we demonstrated that LDL is both a major acceptor of cell-derived [3H]UC and a major donor of [3H]UC to the LCAT reaction. Pathways for the transfer of UC and CE are summarized in Figure 10. In agreement with Castro and Fielding, we found that cell-derived [3H]cholesterol is initially metabolized by pre-β-HDL (Figures 2–4). However, we observed that only a minor proportion of cell-derived [3H]UC (less than 5% in the first 5 minutes) was esterified in this lipoprotein while most remained unesterified and was rapidly transferred to α-HDL and LDL (Figures 3–5). Since the maximum of [3H]UC in α-HDL preceded that in LDL and since the amount of [3H]UC steadily decreased in α-HDL but increased in LDL, we assume that [3H]UC is transferred to LDL via α-HDL (Figures 3–5). From LDL, [3H]UC is then transferred to various HDL particles for esterification by cell-dependent and cell-independent pathways. The reasons for this assumption are as follows. First, during chase incubation with both LDL receptor–positive and LDL receptor–negative fibroblasts, the amount of [3H]cholesterol decreased in plasma and LDL but increased in the cells (Figure 6). Chase incubation of apo B–free plasma with cells of either type neither decreased [3H]cholesterol in plasma nor increased [3H]UC and [3H]CE in the cells, which suggests that cells take up [3H]UC via LDL. In the presence of HDL, the changes in intracellular and extracellular [3H]UC were reduced by 50% compared with normal cells; thus, cellular reuptake of [3H]UC must occur by both LDL receptor–dependent and –independent pathways (Figure 6). Since the amount of [3H]UC in pre-β-HDL gradually decreased during chase incubations in the absence of cells (Figure 4) but remained constant during chase incubations in the presence of cells (Figures 3 and 4), cellular reuptake of [3H]UC from LDL probably results in the resecretion of [3H]UC into the medium and thereby enhances its esterification in pre-β-HDL. Second, [3H]UC is also transferred to α-HDL by a cell-independent mechanism, which probably involves direct

### Table 4. Effects of Lecithin:Cholesterol Acyltransferase Inhibition by Dithio-bis(2-Nitrobenzoic Acid) on Cholesterol Net Transport Rates

<table>
<thead>
<tr>
<th>Condition</th>
<th>Net transfer of unesterified cholesterol into</th>
<th>Plasma (nmol)</th>
<th>HDL (nmol)</th>
<th>LpB (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma without DTNB (normal cells)</td>
<td>+4.57</td>
<td>1.40</td>
<td>+29</td>
<td>+3.26</td>
</tr>
<tr>
<td></td>
<td>(0.54)</td>
<td>(0.39)</td>
<td>(6.3)</td>
<td>(0.31)</td>
</tr>
<tr>
<td>Plasma with DTNB (normal cells)</td>
<td>+0.39</td>
<td>-2.56</td>
<td>...</td>
<td>+2.71</td>
</tr>
<tr>
<td></td>
<td>(0.31)</td>
<td>(1.62)</td>
<td>...</td>
<td>(1.53)</td>
</tr>
<tr>
<td>Plasma without DTNB (FH cells)</td>
<td>+7.13</td>
<td>+2.40</td>
<td>+35</td>
<td>+4.81</td>
</tr>
<tr>
<td></td>
<td>(0.54)</td>
<td>(0.47)</td>
<td>(4.7)</td>
<td>(0.62)</td>
</tr>
<tr>
<td>Plasma with DTNB (FH cells)</td>
<td>+2.57</td>
<td>+0.82</td>
<td>+31</td>
<td>+2.71</td>
</tr>
<tr>
<td></td>
<td>(0.34)</td>
<td>(0.23)</td>
<td>(6.3)</td>
<td>(0.47)</td>
</tr>
</tbody>
</table>

HDL, high density lipoprotein; LpB, apolipoprotein B–containing lipoproteins.

Two milliliters of 5% plasma was incubated with normal or familial hypercholesterolemia (FH) fibroblasts from a patient homozygous for familial hypercholesterolemia. Values are mean±(SD) of four experiments.

*Percentage of total unesterified cholesterol net mass transfer into the medium. FH cells denote fibroblasts from a patient homozygous for familial hypercholesterolemia.
and indirect transfer via pre-β-HDL, since 1) the bulk of [3H]UC (80%) is already seen in α-HDL after 30 seconds and 2) the remaining [3H]UC, which was located in pre-β-HDL, continuously decreased during incubation (Figure 7). It is very likely that direct transfer of UC from LDL to HDL depends on LCAT, since inhibition of this enzyme by DTNB inhibited not only the production of CE but also the removal of [3H]UC from LDL (Figure 8 and Table 4). In most experiments we studied the transfer of [3H]UC. Since UC rapidly diffuses between HDL and LDL (reviewed in References 22 and 23), any transfer of [3H]UC could be caused by nonproductive equilibration of labeled and unlabeled UC among the various lipoproteins. This possibility is easily ruled out with regard to the transient accumulation of cell-derived [3H]cholesterol in pre-β-HDL, since initially 30–40% of [3H]cholesterol occurred in these cholesterol-poor lipoproteins, although they account for less than 5% of plasma HDL (Figure 2). Seventy-five percent of plasma UC is present in LDL, which provides a large pool that is exchangeable with [3H]UC. However, we have good evidence that the accumulation of [3H]UC in LDL is also due to a directed transfer of UC to this
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Both the transfer of UC to LDL and the heterogeneity in its recycling to HDL are important for effective reverse cholesterol transport for two reasons: 1) The transfer of UC to LDL provides a reverse cholesterol transport mechanism that is independent of LCAT. 2) The redistribution of UC from LDL to various HDL intensifies the esterification of cell-derived cholesterol by LCAT.

It is generally held that the esterification of cell-derived cholesterol in plasma helps to maintain a cholesterol concentration gradient between cell membranes and the plasma compartment and the plasma compartment (reviewed in References 2 and 3). Therefore, a key role in reverse cholesterol transport has been attributed to LCAT. The transfer of UC to LDL via HDL observed by us would also form a cholesterol concentration gradient between cell membranes and HDL, since LDLs are rapidly eliminated by the liver through the LDL receptor pathway. Therefore, the transfer of UC to LDL may represent a second, reverse cholesterol transport mechanism that does not depend on LCAT activity. In our studies, such transfer took place in the presence of the LCAT inhibitor DTNB (Figure 8c and Table 4). Support for the existence of this alternative reverse cholesterol transport pathway in vivo comes from clinical studies of patients with familial LCAT deficiency. The LDL of patients with familial LCAT deficiency characteristically contains large amounts of UC, indicating that the transfer of UC to LDL also does take place in vivo when no functional LCAT is present. These patients accumulate large amounts of UC in various peripheral tissues, although the activity of the intracellular cholesterol-esterifying enzyme acyl coenzyme A: cholesterol acyltransferase in these patients is normal, indicating that the cells take up UC probably via LDL, since HDL is not present. The presence of an LCAT-independent, reverse cholesterol transport pathway would also provide a good explanation as to why the various genetic defects in LCAT do not put patients with familial LCAT deficiency or fish-eye disease at increased risk of coronary heart disease.

Since LCAT preferentially esterifies LDL-derived UC, the rapid transfer of cell-derived UC to LDL and its subsequent transfer to HDL appear to be the metabolic bases for its effective esterification in plasma. In vivo, this key role of LDL for regular cholesterol esterification in plasma is illustrated by the decreased LCAT activity in abetalipoproteinemia, which can be corrected by the addition to the plasma of LDL or VLDL but not of HDL or LCAT. Moreover, compared with the direct esterification of cell-derived UC in pre-β-HDL, the transfer of cell-derived UC to LDL and subsequently to α-HDL for esterification would also intensify the hepatic elimination of CE; Fielding and colleagues demonstrated that only the CE formed in α-HDL from LDL-derived UC is directed to LDL by CETP and can therefore be eliminated via hepatic LDL receptors. By contrast, the CE formed in pre-β-HDL accumulates in HDL, which apparently is a little reactive “sink” without being transferred to LDL.

For some time now, LpB’s have been known to act as the acceptor of CE transferred from HDL by CETP and thus to play a major role in reverse cholesterol transport (reviewed in Reference 3). Our studies demonstrate that most of the cell-derived UC is transferred to LDL.
via HDL without prior esterification. This novel pathway thus attributes another important function to LDL in reverse cholesterol transport, namely that of an intermediate acceptor of most cell-derived UC and the major donor of UC to the LCAT reaction.

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Cell-derived unesterified cholesterol cycles between different HDLs and LDL for its effective esterification in plasma.

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