LDL Inhibits the Mediation of Cholesterol Efflux From Macrophage Foam Cells by ApoA-I–Containing Lipoproteins
A Putative Mechanism for Foam Cell Formation

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Although the accumulation of cholesterol in macrophages appears to be an initial step in atherogenesis, low-density lipoprotein (LDL), a major risk factor for atherosclerosis, does not promote cholesterol accumulation in macrophages in its native form. On the other hand, apolipoprotein (apo) A-I–containing lipoprotein removes cholesterol from cholesterol-loaded macrophages (foam cells) and prevents cholesterol from accumulating in the cells. We examined the effect of LDL on cholesterol removal by two species of apoA-I–containing lipoproteins, one containing only apoA-I (LpA-I) and the other containing apoA-I and apoA-II (LpA-I/A-II). When foam cells were incubated with LpA-I or LpA-I/A-II, cellular cholesterol mass was reduced. In contrast, when LDL was added, the cholesterol-reducing capacities of these lipoproteins were dose-dependently inhibited by LDL. In the presence of LDL, LpA-I and LpA-I/A-II removed free cholesterol preferentially from LDL rather than from the plasma membrane of foam cells. In addition, a fair amount of cellular cholesterol was directly moved to LDL rather than to LpA-I or LpA-I/A-II. The cellular cholesterol that moved to LDL was completely compensated for by the cholesterol influx from LDL to foam cells. Thus, net cholesterol efflux (a combination of influx and efflux) from foam cells was inhibited by LDL. These results, taken together, indicate that LDL may accelerate foam cell formation by inhibiting cholesterol removal from the cells and that elevated levels of plasma LDL may become a risk factor for atherosclerosis by inhibiting the function of LpA-I and LpA-I/A-II at the cellular level. (Arterioscler Thromb. 1993;13:1307-1316.)

KEY WORDS • reverse cholesterol transport • lipoprotein A-I • lipoprotein A-I/A-II • HDL • LDL • foam cells • atherosclerosis • lecithin:cholesterol acyltransferase

The accumulation of cholesteryl esters (CEs) in macrophages (foam cells) is characteristic of the early stage of atherosclerotic lesions, and this accumulated cholesterol is primarily derived from low-density lipoproteins (LDLs). However, because LDL in its native form cannot convert macrophages into foam cells, it remains unclear why LDL is atherogenic at the cellular level. Goldstein et al proposed a possible mechanism for LDL-derived cholesterol influx into macrophages: The LDL receptor is either absent or is poorly expressed in macrophages, but macrophages do have scavenger receptors to take up and degrade chemically modified or cell-modified LDL. Through this scavenger-receptor pathway, macrophages take up large amounts of cholesterol, which are converted to foam cells.

On the other hand, the cholesterol removal system from macrophage foam cells also functions at cellular levels. High-density lipoprotein (HDL) is thought to play a major role on this cholesterol removal system. Under physiological conditions, HDL coexists with LDL and possibly with modified LDL. Therefore, if this removal system functions efficiently, the accumulation of cholesterol would be less likely to occur. In other words, cholesterol accumulation in macrophages might occur as a result of a disturbance in the balance of the cholesterol influx-efflux system. To date, less attention has been directed to the effect of LDL on the HDL-mediated cholesterol removal system. We explored the notion that elevated levels of LDL may block this removal system and accelerate cholesterol accumulation in macrophages. For lipoprotein samples, we used two species of apolipoprotein (apo) A-I–containing lipoproteins isolated by immunoaffinity: (1) lipoprotein containing apoA-I but no apoA-II (LpA-I) and lipoprotein containing apoA-I and apoA-II (LpA-I/A-II), and (2) a subtraction of conventional HDL isolated by ultracentrifugation (HDL₃).

Methods

Materials

All chemicals used were obtained from commercial sources. [1,2-³H]cholesterol ([³H]FC, 47.5 Ci/mmol) and [4,14C]cholesterol ([¹⁴C]FC, 53.1 mCi/mmol) were purchased from Du Pont–New England Nuclear, Boston, Mass. Plasma for LpA-I, LpA-I/A-II, HDL₃, and LDL was obtained from five independent groups of healthy adult normolipidemic volunteers from staff and students.
in the Kumamoto University School of Medicine (each group included five men and five women 18 to 25 years of age; all had fasted overnight). Five independent pooled plasma samples were used for the following studies.

**Lipoproteins**

LpA-I and LpA-I/A-II were isolated from each fresh pooled plasma sample by a combination of anti-apoA-I and anti-apoA-II immunosorbent columns, as previously described. Briefly, fresh plasma was applied on an anti-apoA-I column, and after washing extensively with 0.01 mol/L tris(hydroxymethyl)aminomethane (Tris), 0.5 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4 (buffer A), the column was eluted with 0.1 mol/L acetic acid and 1 mmol/L EDTA, pH 3.0. Each effluent was immediately adjusted to pH 7.4 with 1.0 mol/L Tris solution and dialyzed against 0.15 mol/L NaCl and 1 mmol/L EDTA, pH 7.4 (buffer B). Finally, the sample was concentrated in buffer B using a ultrafiltration cell (Amicon Corp, Danvers, Mass) equipped with a PM-10 membrane and was then applied on an anti-apoA-II column. The column was washed with buffer A to obtain LpA-I. The bound fraction was eluted from the column to obtain LpA-I/A-II. Both LpA-I and LpA-I/A-II were dialyzed and concentrated. In this procedure, >90% of lipids and apolipoproteins applied were recovered in the unbound and bound fractions. A combined sample (A-ILp) in which LpA-I and LpA-I/A-II were mixed at an equiprotein ratio was obtained. LDL (d, 1.019 to 1.063) and HDL (d, 1.125 to 1.21) were isolated from a portion of the fresh pooled plasma sample by sequential ultracentrifugation at 150,000g for 22 hours at 4°C and dialyzed against buffer B. These lipoproteins were used immediately after isolation in the following experiments. Acetyl LDL and [3H]FC-labeled acetyl LDL were prepared as described. Briefly, absolute ethanol (100 μL) containing 10 μCi of [3H]FC or 0.1 μCi of [3C]FC was incorporated into LDL or LpA-I or LpA-I/A-II, as previously described. Briefly, absolute ethanol (100 μL) containing 10 μCi of [3H]FC or 0.1 μCi of [3C]FC was placed in culture wells, and the ethanol was dried off by nitrogen flush. Then, each lipoprotein (500 μg protein) in 500 μL phosphate-buffered saline (PBS) was added to the well, and the [3H]FC or [3C]FC was equilibrated with FC in each lipoprotein by incubation at 4°C for 16 hours. The concentrations of lipoproteins are given in terms of the protein content of the lipoproteins.

**Rat Macrophage Monolayers**

All cell culture experiments were done at 37°C in humidified air containing 5% CO2. Peritoneal macrophages were harvested in PBS from nonstimulated male Wistar rats (170 to 200 g), centrifuged at 800g for 3 minutes, and suspended at 2 to 3 × 10⁶ cells per milliliter in Dulbecco's modified Eagle's medium containing 3% bovine serum albumin (BSA), 100 U/mL penicillin, and 100 μg/mL streptomycin (medium A). To each 22-mm plastic dish was added 1.0 mL of the cell suspension, and the preparation was incubated for 4 hours. Cell monolayers formed were washed three times with 1 mL PBS and used for cholesterol efflux experiments.

**Cholesterol Efflux From Macrophage Foam Cells**

Adhered macrophages were converted to foam cells by a 16-hour incubation with 100 μg/mL of unlabeled or [3H]FC-labeled acetyl LDL in medium A, as previously described. After a 6-hour equilibration, these foam cells were washed twice with 1.0 mL PBS containing 0.2% BSA and with PBS and subjected to efflux assays by incubating with 1.0 mL medium A containing 400 μg LpA-I, LpA-I/A-II, A-ILp (LpA-I+LpA-I/A-II), HDL3, and the combination of these lipoproteins and LDL (0 to 800 μg). LDL (0 to 800 μg/mL) was also incubated with foam cells. For control conditions, parallel incubations were performed without lipoproteins. At 24 hours after the onset of efflux experiments, the culture medium was removed to analyze the total radioactivity and esterification rate; the wells were then washed twice with 1.0 mL PBS containing 0.2% BSA and four times with PBS, and the cellular lipids were then extracted. Unless otherwise specified, the data derived from these efflux assays were the mean of quintuplicate runs in five separate experiments.

**Lipid Extraction**

FC and CEs were extracted directly from macrophage monolayers. Briefly, to each well was added 0.6 mL hexane/isopropanol (3:2), and the cells were incubated for 30 minutes at room temperature. The organic solvent was saved, and the cells were extracted again with 0.6 mL of the same solvent. The combined extracts were dried under nitrogen and dissolved in 120 μL isopropanol. Aliquots were used for both radioactivity and the mass estimations described below. After lipid extraction, cells in each well were washed with 1.0 mL PBS and dissolved in 0.5 mL of 0.1 mol/L NaOH for 10 minutes at 37°C. The supernatant was saved, and the wells were again treated with 0.3 mL of 0.1 mol/L NaOH. The combined supernatant was used to determine cell protein concentrations.

**Cellular Radioactivity**

Aliquots (20 μL) of lipid extracts were spotted in duplicate on a thin-layer chromatography (TLC) plate (Merck) and developed in n-hexane/diethyl ether/acetic acid/methanol (85:20:1:1). Spots corresponding to FC and CEs were cut out from the plate, and the radioactivities were determined.

**Mass Determination**

The cellular mass of FC and CEs was quantified by a modification of the enzymatic/fluorometric method, as described previously. Briefly, a remaining portion (20 μL) of the lipid extract was added to 0.4 mL of the enzyme mixture, and the preparation was incubated at 37°C for 1 hour (for FC) or for 2 hours (for total cholesterol), followed by the addition of 0.8 mL of 0.5 mol/L NaOH to halt the reaction. Fluorescence intensity was measured with excitation at 310 nm and emission at 407 nm. Values were quantified by comparison with standard curves obtained using cholesterol and cholesteryl oleate for FC and total cholesterol, respectively. The CE value was calculated by subtracting FC from total cholesterol. Standard curves were constructed for each set of experiments.
FC Movement Between ApoA-I–Containing Lipoproteins and LDL

Movement of FC between apoA-I–containing lipoproteins and LDL was studied using [3H]FC in LDL, LpA-I, or LpA-I/A-II. The mixture of labeled LDL and unlabeled LpA-I or LpA-I/A-II (protein ratio, 1:0) was incubated for 24 hours. Aliquots of the mixture were taken out just after mixing, at 5, 10, and 30 minutes, and at 1, 3, 6, and 24 hours. Radioactivity of [3H]FC or [3H]CE in LDL and LpA-I or LpA-I/A-II was determined as described below. Experiments using labeled LpA-I or LpA-I/A-II and unlabeled LDL were also performed under similar conditions.

FC Movement From Foam Cells to ApoA-I–Containing Lipoproteins or LDL

Movement of FC from foam cells to apoA-I–containing lipoproteins or LDL was performed using [3H]FC-labeled foam cells. Radiolabeled foam cells were incubated for 24 hours in medium A containing 400 µg/mL of LpA-I or LpA-I/A-II and varying concentrations of LDL. Aliquots of the medium were taken out at 5 and 30 minutes and 3, 5, 12, 18, and 24 hours for the time-course study. In the case of the esterification study, 3H-labeled foam cells were incubated for 24 hours in medium A containing 400 µg/mL of LpA-I and 400 µg/mL of [14C]FC-labeled LDL. Radioactivity of [3H]FC or [14C]FC and [3H]CE or [14C]CE in LDL and LpA-I or LpA-I/A-II was determined as described below.

Radioactivity Determination

To determine the radioactivity associated with LpA-I and LpA-I/A-II, LDL was precipitated from culture medium using phosphotungstate-MgCl2.24-25 Radioactivity associated with LDL was determined by the difference between the radioactivity before and after precipitation. The lipids in culture medium were extracted with methanol/chloroform (2:1 [vol/vol]). The extracts were dried under nitrogen flush and dissolved in 60 µL isopropanol. Aliquots (20 µL) of lipid extracts were spotted in duplicate on a TLC plate, and radioactivities of FC and CE were determined as described above.

Protein and Lipid Analysis

Lipid concentrations of lipoprotein samples and culture medium were analyzed using an autoanalyzer and enzymatic methods16-18 or by selective precipitation of LDL using phosphotungstate-MgCl2.24-25 The protein content of lipoproteins was determined by the method of Lowry et al.25

Electrophoretic Analysis

Agarose gel electrophoresis was performed using a Pol-E Film system for lipoprotein (Ciba Corning, Medfield, Mass).

Statistical Evaluation

The Wilcoxon signed rank test and paired t test were used to evaluate the data.

Results

Effect of LDL on Macrophages and Foam Cells

When macrophages were incubated with 400 µg/mL LDL for 24 hours, cellular FC and CE mass were slightly increased (Table 1). This indicates that macrophages possess the LDL receptor but that the activity was not enough for foam cell formation. In contrast, when cholesterol-loaded macrophages (macrophage foam cells) were incubated with the same amount of LDL, the cellular cholesterol mass did not change (Table 1). In both cases, after incubation the mobility of LDL on agarose gel electrophoresis did not change when compared with the LDL before incubation, and lipid composition was also unchanged (data not shown). Therefore, these results suggest three possibilities: (1) LDL has no effect on the cholesterol in foam cells. (2) The influx and efflux of FC molecules occurred at equal mass. (3) Although LDL was taken up via the LDL receptor, LDL could remove the same amount of cellular cholesterol, and as a result, the cellular cholesterol mass did not change. For clarification, we performed experiments using radiolabeled foam cells. When these cells were subjected to a 24-hour incubation with an increasing amount of LDL, a significant amount of radioactivity was recovered in the incubation medium (Fig 1A). Fifty percent to 70% of cellular [3H]FC was reduced, yet the cellular [3H]CE level did not change (Fig 1A). On the other hand, the cellular cholesterol mass, both of FC and CE, did not change (Fig 1B). Because net removal of intracellular FC leads to CE reduction,4,11,12,16 the unchanged cellular CE mass and cellular [3H]CE level suggested that intracellular FC removal and CE influx via the LDL receptor did not occur. The LDL receptor was suppressed in foam cells, and efflux of cellular FC was completely compensated for by the influx from LDL into the plasma membrane of foam cells (a one-for-one exchange reaction between plasma membrane FC and FC in LDL). As a result, LDL had no effect on the cellular cholesterol mass in foam cells.

Table 1. Effect of LDL on Cellular Cholesterol Mass of Macrophages or Macrophage–Foam Cells

<table>
<thead>
<tr>
<th></th>
<th>FC (nmol/mg protein)</th>
<th>CE (nmol/mg protein)</th>
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<tbody>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LDL</td>
<td>57±7</td>
<td>0±0</td>
</tr>
<tr>
<td>+LDL</td>
<td>73±11</td>
<td>35±11</td>
</tr>
<tr>
<td>Macrophage foam cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-LDL</td>
<td>130±18</td>
<td>125±7</td>
</tr>
<tr>
<td>+LDL</td>
<td>133±10</td>
<td>128±16</td>
</tr>
</tbody>
</table>


| FC, free cholesterol; CE, cholesteryl ester; LDL, low-density lipoprotein. Values are mean±SEM of quintuplicate experiments. Macrophages were converted to foam cells by a 16-hour incubation with 100 µg/mL acetyl LDL. These foam cells and unloaded macrophage monolayers were incubated in medium A containing no lipoprotein (−LDL) or 400 µg/mL LDL (+LDL) for 24 hours at 37°C, respectively. After incubation, the cells were washed twice with 1.0 mL phosphate-buffered saline containing 0.2% bovine serum albumin and four times with phosphate-buffered saline; cellular lipids were then extracted. The cellular mass of FC and CE was determined by an enzymatic/fluorometric method and expressed as nanomoles per milligram cell protein as described in “Methods.”

Effect of LDL on Cholesterol Efflux From Foam Cells Mediated by ApoA-I–Containing Lipoproteins and HDL3

When foam cells were subjected to a 24-hour incubation with 400 µg LpA-I, LpA-I/A-II, A-ILp, and HDL3,
FIG 1. Line graphs show the effect of low-density lipoprotein (LDL) on cholesterol efflux from macrophage foam cells. CE indicates cholesteryl ester; FC, free cholesterol. Macrophages were incubated for 16 hours at 37°C in 1.0 mL medium A containing 100 μg protein of [3H]FC-labeled acetyl LDL. After a 6-hour equilibration, cells were incubated for 24 hours with the indicated concentrations of LDL (0 to 800 μg/mL). On termination of each incubation, the radioactivity released into the medium was determined (A). Cells were washed twice with 1.0 mL phosphate-buffered saline containing 0.2% bovine serum albumin and four times with phosphate-buffered saline, and cellular lipids were extracted to determine radioactivity (A) and mass (B). [3H]FC and [3H]CE were separated by thin-layer chromatography, and the radioactivities were determined. The mass of cellular FC and CE was quantified by the enzymatic/fluorometric method, as described in “Methods.” The data derived from these efflux assays were the mean ± SEM of triplicate runs in three separate experiments. Mean values of radioactivity of cellular [3H]FC and [3H]CE for 100% were 269 and 142 x 10^6 dpm/mg cell protein, and those of FC mass and CE mass were 130 and 125 nmol/mg cell protein, respectively.

cellular [3H]FC and [3H]CE decreased by 50% to 60% (Fig 2A and 2B; LDL, 0 μg). A significant difference was not observed in total radioactivities excreted into the incubation medium, thereby indicating that the efflux of cellular cholesterol was constant in all cases (Fig 3; LDL, 0 μg). At mass levels, these lipoproteins similarly decreased the cellular CE mass, but only LpA-I and A-ILp significantly decreased the cellular FC mass. LpA-I/A-II and HDL₃ had no apparent effect on the cellular FC mass (Fig 2C and 2D). The intracellular FC removal leads to CE reduction; hence, our data indicate that LpA-I/A-II and HDL₃ removed only the FC mass generated by CE hydrolysis. In other words, the influx of FC from LpA-I/A-II and HDL₃ was smaller than the cellular FC efflux by the FC mass generated by CE hydrolysis. In case of LpA-I and A-ILp, the influx from these lipoproteins to foam cells was much less than that of LpA-I/A-II and HDL₃.

When varying amounts of LDL were added to the incubation medium containing 400 μg/mL LpA-I, LpA-I/A-II, A-ILp, or HDL₃, total radioactivities recovered in incubation media did not change significantly (Fig 3). Reduction of cellular [3H]FC was also not affected in any case (Fig 2A). In contrast, the reduction of cellular [3H]CE by LpA-I, LpA-I/A-II, A-ILp, and HDL₃ was dose-dependently weakened by the addition of LDL (Fig 2B). At mass levels, cellular FC mass-reducing capacities of LpA-I and A-ILp were similarly weakened by the addition of LDL (Fig 2C). The cellular CE mass-reducing capacity of LpA-I, LpA-I/A-II, A-ILp, and HDL₃ was weakened by the addition of LDL in a dose-dependent manner (Fig 2D). Significant cellular CE reduction by these lipoproteins and cellular FC reduction by LpA-I and A-ILp were not observed when we added more than 400 μg/mL LDL. These data, taken together, suggest that FC influx from the mixture of these lipoproteins and LDL into foam cells was increased when compared with apoA-I-containing lipoproteins or HDL₃ only.

**Effect of LDL on Esterification of Cholesterol Excreted From Foam Cells**

In a previous report, we showed that both LpA-I and LpA-I/A-II possess esterification activity of FC by lecithin:cholesterol acyltransferase (LCAT) and that the cholesterol-reducing capacity of LpA-I but not LpA-I/A-II is closely linked to this esterification activity. According to Fielding and Fielding, the inhibition of LCAT results in an increase in the FC influx from lipoproteins to cells but has no effect on the corresponding FC efflux from the plasma membrane. This seems to be similar to the results given in the previous section. Therefore, we examined the effect of LDL on the esterification activity in LpA-I. LpA-I, LDL, and the combination of LpA-I and LDL were incubated in PBS without foam cells at 37°C for 24 hours. As shown in Table 2, when LpA-I only was incubated, the CE mass increased, and the FC mass decreased. These data confirm that the esterification of FC by LCAT does occur in LpA-I. In the case of LDL only, neither the FC
nor the CE mass changed. When the combination of LpA-I and LDL was incubated, the CE mass in the incubation medium increased, and the FC mass decreased. The increased CE mass and the decreased FC mass were similar to findings in the case of LpA-I only. These results, taken together, indicate that LDL had no effect on the esterification activity in LpA-I. We also studied the effect of LDL on the esterification of FC excreted from foam cells. When radiolabeled foam cells were incubated with LpA-I and LDL, esterification of FC excreted from foam cells was remarkably reduced by the addition of LDL (Fig 4). This means that LCAT in

![Diagram](https://example.com/diagram.png)

**Fig 2.** Line graphs show the effect of low-density lipoprotein (LDL) on cholesterol efflux from foam cells stimulated by apolipoprotein A-I (ApoA-I)–containing lipoproteins and high-density lipoprotein3 (HDL3). FC indicates free cholesterol; CE, cholesteryl ester; Lp, lipoprotein; and A-IIp, LpA-I+LpA-I/A-II. Macrophage monolayers were converted to foam cells by a 16-hour incubation with 100 μg/mL of [3H]FC-labeled acetyl LDL. After equilibration, foam cells were incubated for 24 hours with a combination of 400 μg/mL LpA-I, LpA-I/A-II, A-IIp, or HDL3 and varying concentrations of LDL. Control experiments were done without including lipoproteins. After incubation, the cells were washed twice with phosphate-buffered saline containing 0.2% bovine serum albumin and four times with phosphate-buffered saline; then cellular lipids were extracted. Cellular [3H]FC (A) and [3H]CE (B) were determined by thin-layer chromatography. The mass of cellular FC (C) and CE (D) was quantified as described in "Methods." The data derived from these efflux assays were the mean±SEM of quintuplicate runs in five separate experiments. Mean values of FC and CE for 100% are 128 and 125 nmol/mg cell protein, respectively.

![Diagram](https://example.com/diagram.png)

**Fig 3.** Line graph shows the effect of low-density lipoprotein (LDL) on [3H]cholesterol efflux from foam cells mediated by lipoprotein (Lp) A-I, LpA-I/A-II, LpA-I+LpA-I/A-II (A-IIp), or high-density lipoprotein3 (HDL3). Macrophages were preincubated with [3H]free cholesterol–labeled acetyl LDL. After equilibration, macrophage foam cells were incubated for 24 hours with 400 μg/mL LpA-I, LpA-I/A-II, A-IIp, or HDL3 and varying concentrations of LDL. At the end of incubation, culture medium was removed, and the radioactivities were determined. The data are the mean±SEM of quintuplicate runs in five separate experiments.
TABLE 2. Changes of Cholesterol Mass in Lipoprotein A-I, LDL, and the Mixture of Lipoprotein A-I and LDL After Incubation

<table>
<thead>
<tr>
<th></th>
<th>Change of FC (nmol)</th>
<th>Change of CE (nmol)*</th>
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<tbody>
<tr>
<td>LpA-I</td>
<td>-78±15</td>
<td>74±12</td>
</tr>
<tr>
<td>LDL</td>
<td>0±5</td>
<td>0±11</td>
</tr>
<tr>
<td>LpA-I+LDL</td>
<td>-86±17</td>
<td>78±9</td>
</tr>
</tbody>
</table>

LDL, low-density lipoprotein; FC, free cholesterol; CE, cholesterol ester; LpA-I, lipoprotein A-I. Values are mean±SEM (n=5).

Lipoproteins (1 mg protein) were incubated in 1.0 mL phosphate-buffered saline at 37°C for 24 hours. For LpA-I+LDL, a mixture of LpA-I (1 mg) and LDL (1 mg) was incubated in 1.0 mL phosphate-buffered saline at 37°C for 24 hours. After incubation, FC and CE concentrations of the samples were analyzed. Data are expressed as the difference before and after incubation.

*Moles of CE were calculated from the molecular weight of cholesteryl linoleate.

LpA-I preferentially esterifies the FC derived from LDL. To confirm this, 3H-labeled foam cells were incubated with the combination of LpA-I (400 μg/mL) and [14C]FC-labeled LDL (400 μg/mL), and the origin of produced CE was determined according to the specific radioactivity of [3H]FC and [14C]FC. As expected, LCAT in LpA-I preferentially esterified FC from LDL (CE mass originated from the ratio of CE mass to CE mass from foam cells [20/1]; data not shown). We then examined FC transfer between LpA-I or LpA-I/A-II and LDL and between foam cells and lipoproteins.

Movement of FC Between LpA-I or LpA-I/A-II and LDL

When [3H]FC-labeled LDL was incubated with LpA-I, [3H]FC rapidly moved to LpA-I, and equilibration between LDL and LpA-I was completed within 60 minutes (Fig 5A). Esterification of [3H]FC was observed after 10 minutes of incubation. Specific radioactivity (the ratio of [3H]FC to CE mass) was significantly higher in LpA-I than in LDL after 30 minutes of incubation; hence, FC efflux from LDL was apparently greater than FC influx from LpA-I (Table 1). When labeled LpA-I was incubated with LDL, movement of [3H]FC to LDL was similar to that from labeled LDL to LpA-I (Fig 5B).

Specific radioactivity was significantly higher in LpA-I than in LDL, thereby suggesting that the FC efflux from LpA-I was less than the FC influx from LDL (Table 3). These results lead to the notion that there is a bidirectional FC movement between LDL and LpA-I, that there is a net FC movement from LDL to LpA-I, and that esterification of FC from LDL does occur. Similar results were obtained in the case of LpA-I/A-II (data not shown). To confirm these phenomena at mass levels, we determined the lipid compositions of LpA-I and LpA-I/A-II before and after incubation with LDL. The mixture of LpA-I or LpA-I/A-II and LDL was incubated in PBS for 24 hours at 37°C (1 mL of PBS containing 2 mg of each lipoprotein). LpA-I and LpA-I/A-II were also incubated without LDL in PBS. After incubation, LDL was precipitated, and the lipid composition of LpA-I and LpA-I/A-II was determined. The protein mass of LpA-I and LpA-I/A-II did not change by incubation with LDL. Data were compared with the lipid composition of these lipoproteins incubated at 4°C. As shown in Table 4, in LpA-I only was FC decreased and CE increased by the action of LCAT. However, after incubation with LDL, there were no significant changes of total lipid mass and lipid composition in LpA-I. As shown in Table 2, after incubation with LDL the total FC decreased and CE increased in the mixture of LpA-I and LDL. Thus, our observations are interpreted to mean that there is a net FC movement from LDL to LpA-I, that the displaced FC is esterified by LCAT in LpA-I, and that the CE formed is transferred to LDL, possibly by the action of cholesteryl ester transfer protein in LpA-I. In LpA-I/A-II only, as shown in Table 4, FC decreased and CE increased slightly by the action of LCAT. However, differing from LpA-I, total lipid mass increased significantly after the incubation with LDL. With respect to lipid composition, the FC content increased, but the CE content did not change at the mass level. This suggests that the LCAT activity in LpA-I/A-II is too low to esterify all of the FC moved from LDL and that there is a net FC movement from LDL to LpA-I/A-II.

Movement of FC From Foam Cells to LpA-I or LDL

As shown in Fig 6A, when radiolabeled foam cells were incubated with LpA-I and varying concentrations of LDL, radioactivity associated with LpA-I decreased as a function of the concentration of LDL. When labeled foam cells were incubated with LpA-I only, movement of cellular [3H]FC to LpA-I was much slower than that between lipoproteins (Fig 6B; compare with Fig 5 and Table 3). The equilibration was completed.
Fig 5. Line graphs show the movement of [3H]free cholesterol between lipoprotein (Lp) A-I and low-density lipoprotein (LDL). [3H]Free cholesterol-labeled LDL (A) and LpA-I (B) were incubated with unlabeled LpA-I and LDL at a protein ratio of 1.0. At several time points of incubation, to determine the radioactivity associated with LpA-I, LDL was precipitated from the culture medium using phosphotungstate-MgCl₂. Radioactivity associated with LDL was determined by the difference between the radioactivity before and after precipitation. The data are the mean±SEM of triplicate runs in five experiments.

Discussion

In our previous report, we found that the interaction of LpA-I or LpA-I/A-II with macrophage foam cells induced a mass reduction in cholesterol from these cells, but related mechanisms differed. The cholesterol mass-reducing capacity of LpA-I but not of LpA-I/A-II was closely linked to the esterification activity by LCAT associated with LpA-I. On complete inactivation of LCAT, the cholesterol-reducing capacity of LpA-I was lost, yet that of LpA-I/A-II was not affected. In addition, we also described the relation between affinity-purified HDL (LpA-I and LpA-I/A-II) and HDL isolated by ultracentrifugation (HDL₂ and HDL₃) more than 70% of HDL₂ particles were LpA-I, and >70% of HDL₃ particles were LpA-I/A-II. The lack of cholesterol-reducing capacity of HDL₂ was attributed to the loss of LCAT during ultracentrifugation. The inhibition of LCAT associated with

after 18 hours of incubation. Esterification of cellular [3H]FC was not observed before 5 hours of incubation because of the lower specific radioactivity (the ratio of [3H]FC to FC mass) in LpA-I. For the time-course study, labeled foam cells were incubated with 400 μg/mL LpA-I in the presence of the same concentration of LDL. The total radioactivity excreted into the medium did not change when LDL was added; however, distribution of the radioactivity markedly changed. After incubation with LpA-I and LDL, radioactivity recovered in the LDL increased as a function of the amount of LDL (Fig 6B). Specific radioactivity of [3H]FC in LpA-I was significantly higher than that in LDL at 30 minutes of incubation. However, the specific radioactivity in LDL then became significantly higher than that in LpA-I (Table 5). These results indicate that before 30 minutes of incubation, cellular cholesterol is preferentially moved to LpA-I and that a large amount of cellular cholesterol may be directly moved to LDL. In the case of LpA-I/A-II as shown in Table 5, a phenomenon similar to that seen with LpA-I was observed. However, specific radioactivities in LpA-I/A-II were always higher than those in LDL throughout the incubation period.

Table 3. Specific Radioactivity of Free Cholesterol in Lipoprotein A-I and LDL After Incubation of Labeled LDL or Labeled Lipoprotein A-I With Each Unlabeled Counterpart

| Specific radioactivity of FC after elapsed incubation time (dpm/nmol) |
|---|---|---|---|---|---|---|---|
| Minutes | 0 | 5 | 10 | 30 | 1 | 3 | 6 | 24 |
| Labeled LDL with LpA-I | | | | | | | | |
| LDL | 5589 | 4857 | 4923 | 4591 | 4506 | 4426 | 4350 | 4245 |
| LpA-I | 0 | 3887 | 4331 | 5248 | 5421 | 6066 | 6315 | 6530 |
| Labeled LpA-I with LDL | | | | | | | | |
| LDL | 0 | 5006 | 6299 | 7363 | 7878 | 7805 | 7710 | 7393 |
| LpA-I | 62875 | 32973 | 26678 | 15520 | 12647 | 12592 | 13735 | 13830 |

LDL, low-density lipoprotein; FC, free cholesterol; LpA-I, lipoprotein A-I. Values are the mean of three different experiments. Variations were less than 10%.

[3H]FC-labeled LDL or LpA-I was incubated with unlabeled LpA-I or LDL at 1.0 protein ratio in phosphate-buffered saline, respectively. At several time points of incubation, aliquots of the mixture were taken, and LDL was precipitated from the culture medium, as described in "Methods." Radioactivity associated with LDL was calculated by the difference between the radioactivity before and after precipitation.
Table 4. Lipid Composition of Lipoprotein A-I and Lipoprotein A-I/A-II Before and After Incubation With or Without LDL

<table>
<thead>
<tr>
<th></th>
<th>Percent of total lipid</th>
<th>FC/PL</th>
<th>Total lipid mass*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>CE</td>
<td>TG</td>
</tr>
<tr>
<td>LpA-I 4°C</td>
<td>9.7±0.7</td>
<td>26.8±1.7</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>LpA-I 37°C</td>
<td>5.9±0.5</td>
<td>31.3±1.3</td>
<td>3.9±0.6</td>
</tr>
<tr>
<td>LpA-I with LDL 37°C</td>
<td>9.2±0.7</td>
<td>27.9±1.5</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>LpA-I/A-II 4°C</td>
<td>7.0±0.5</td>
<td>29.0±2.0</td>
<td>5.0±1.1</td>
</tr>
<tr>
<td>LpA-I/A-II 37°C</td>
<td>5.0±0.4</td>
<td>30.7±2.2</td>
<td>5.9±1.5</td>
</tr>
<tr>
<td>LpA-I/A-II with LDL 37°C</td>
<td>8.4±0.5</td>
<td>27.3±1.7</td>
<td>4.4±1.3</td>
</tr>
</tbody>
</table>

FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid; Lp, lipoprotein; LDL, low-density lipoprotein. Values are mean±SD (n=3).

LpA-I or LpA-I/A-II (2 mg protein) was incubated with or without 2 mg LDL in 1.0 mL phosphate-buffered saline at 4°C or 37°C for 24 hours. After incubation, LDL was precipitated using phosphotungstate-MgCl₂. Lipid compositions of supernatants (LpA-I or LpA-I/A-II) were analyzed. Temperatures indicate the following: 4°C, before incubation; 37°C, after incubation.

*Lipid mass was expressed relative to the value before incubation.

LpA-I/A-II had no effect on the cholesterol-reducing capacity of LpA-I/A-II. This may explain why HDL₃ is effective in cellular cholesterol reduction. Such being the case, in the following discussion we highlight only LpA-I and LpA-I/A-II.

We found that LDL inhibits the cholesterol-reducing capacity of LpA-I and LpA-I/A-II in a dose-dependent manner. Two different mechanisms seem to be involved in this effect of LDL: (1) In the presence of LDL, LpA-I and LpA-I/A-II remove FC preferentially from LDL rather than from the plasma membrane of foam cells. (2) LDL changes the distribution of cholesterol excreted from foam cells. A fair amount of cholesterol is directly moved to LDL rather than LpA-I or LpA-I/A-II. Cellular cholesterol moved to LDL is completely compensated for by the cholesterol influx from LDL to foam cells. Thus, net cholesterol efflux (a combination of influx and efflux) from foam cells is inhibited by LDL.

To shed light on the inhibitory effect of LDL on the cholesterol-reducing capacity on LpA-I, we focused on

![Graph A. Distribution of Radioactivity](#)

![Graph B. Time course of distribution](#)

**Fig 6.** Line graphs show the effect of low-density lipoprotein (LDL) concentration and incubation time on the distribution of foam cell–derived radioactive cholesterol between lipoprotein (Lp) A-I and LDL. A, Radiolabeled foam cells were incubated for 24 hours in medium A containing 400 μg/mL LpA-I and varying concentrations of LDL. B, For the time-course study, radiolabeled foam cells were incubated with LpA-I only, LDL only, and the combination of LpA-I and LDL (400 μg/mL each) for 24 hours. Radioactivity associated with LpA-I or LDL and the distribution of radioactivity between LpA-I and LDL were determined at each time point as described in "Methods." The data are the mean±SEM of triplicate runs in three experiments.
TABLE 5. Specific Radioactivity of Free Cholesterol in Lipoprotein A-I or Lipoprotein A-I/A-II and LDL After Incubation With Labeled Foam Cells

<table>
<thead>
<tr>
<th>Minutes</th>
<th>Specific radioactivity of FC after elapsed incubation time (dpm/nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>LpA-I</td>
<td>2.42</td>
</tr>
<tr>
<td>LDL</td>
<td>2.23</td>
</tr>
<tr>
<td>LpA-I/A-II</td>
<td>4.73</td>
</tr>
<tr>
<td>LDL</td>
<td>3.08</td>
</tr>
</tbody>
</table>

LDL, low-density lipoprotein; FC, free cholesterol; Lp, lipoprotein. Values are the mean of three different experiments. Variations were less than 10%.

Macrophages were converted to foam cells by a 16-hour loading with 100 μg/ml of [3H]labeled acetylated LDL. After a 6-hour equilibration, cells were incubated with 400 μg/ml LpA-I or LpA-I/A-II in 1.0 ml medium A containing 400 μg/ml LDL. At several time points of incubation, aliquots of the mixture were taken, and LDL was precipitated from the mixture as described in "Methods." Radioactivity associated with LDL was calculated by the difference between the radioactivity before and after precipitation.

The results obtained in the present study might weaken the concept that a specific interaction of apoA-I with the plasma membrane is a key step in net cholesterol removal from cells.30,31 As shown in Fig 2, cellular FC and CE reduction by apoA-I-containing lipoproteins was significantly weakened by the addition of LDL. LDL did not inhibit the binding of apoA-I-containing lipoproteins to the plasma membrane of foam cells (authors’ unpublished observation). Although the interaction of apoA-I with the plasma membrane may be important, our present data strongly suggest that without a net FC mass efflux from the plasma membrane, CE mass reduction does not occur.

Recent studies of foam cell formation have focused on the recognition of modified LDL by scavenger receptors. On the basis of a substantial body of evidence, cholesterol that has accumulated in foam cells is assumed to be derived from oxidized LDL.32,33 However, there is no available evidence that elevated levels of LDL, a major risk factor for atherosclerosis, accelerate the synthesis of oxidized LDL. We propose that elevated levels of plasma LDL can accelerate foam cell formation by blocking net...
cholesterol removal from macrophage foam cells. In our unpublished data, the mean plasma apoB levels in normolipidemic Japanese adults was approximately 70 mg/dL. This means that the protein ratio of LpA-I or LpA-I/A-II to LDL and A-Ip to LDL is approximately 1.0 and 2.0, respectively, in normolipidemic plasma. In plasma, both LpA-I and LpA-I/A-II are present; therefore, approximately 50% of the cholesterol-reducing capacity of apoA-I-containing lipoproteins in plasma might be blocked by LDL.

Several studies show that the ratio of apoA-I-containing lipoproteins to LDL is higher in interstitial fluid than in plasma. If so, then the cholesterol removal system may function in the interstitial space. However, in hypercholesterolemic subjects, this system may be blocked by LDL-containing lipoproteins to LDL is higher in interstitial fluid than in plasma. If so, then the cholesterol removal system may function in the interstitial space. However, in hypercholesterolemic subjects, this system may be blocked by LDL.

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References
30. Shottte JP, Oran JF, Bierman EL, Oran JF. High density lipoprotein cell receptors promotes translocation of cholesterol from intracellular membranes to the cell surface. J Biol Chem. 1987;262:12904-12907.
LDL inhibits the mediation of cholesterol efflux from macrophage foam cells by apoA-I-containing lipoproteins. A putative mechanism for foam cell formation.

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