Lecithin:Cholesterol Acyltransferase Reaction on Cellular Lipid Released by Free Apolipoprotein-Mediated Efflux†

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ABSTRACT: Lecithin:cholesterol acyltransferase (LCAT) reaction was studied in free apolipoprotein-mediated cellular lipid efflux from mouse peritoneal macrophages and human skin fibroblasts. When the cells were incubated with lipid-free human apolipoproteins (apo) A-I or A-II, pre-β high density lipoprotein (HDL) particles were generated by removing cellular cholesterol and phospholipid. Cholesterol was esterified by LCAT in such particles generated with human apoA-I, but not in those with apoA-II. The reactivity of the apoA-I-pre-β-HDL particles with LCAT was in the same order as that in human plasma HDL and in phosphatidylcholine/cholesterol unilamellar vesicles activated by apoA-I when compared on the rate of percent cholesterol esterification. However, cholesterol efflux mediated by apoA-I was not enhanced by active cholesterol esterification in the medium from either type of cells. Thus, it is unlikely the LCAT reaction on newly generated pre-β-HDL directly causes further cellular cholesterol efflux. In control experiments, LCAT esterified cholesterol on human plasma HDL in the cell medium regardless of its origin, either HDL or cells. Cholesterol esterification on HDL was unable to enhance cellular cholesterol efflux significantly but reduced the influx of cholesterol from HDL to cell, resulting in the increase of net efflux of cellular cholesterol, in agreement with the results previously demonstrated.

Cellular cholesterol efflux is the first step of the cholesterol transport from peripheral tissues to the liver by the plasma lipoprotein system, but the mechanism of this reaction is not fully understood. Free cholesterol in the cell is exchangeable with its extracellular pools such as plasma lipoproteins' surface by a physicochemical mechanism, presumably diffusion through aqueous phase (Rothblat & Phillips, 1982; Johnson et al., 1986, 1988; Karlin et al., 1987). The net efflux by this mechanism may therefore be achieved by gradient of cholesterol content between cellular and lipoprotein surfaces (Johnson et al., 1986, 1988; Karlin et al., 1987). On the other hand, specific interaction site of the plasma membrane with high density lipoproteins (HDL)[1] may play a role in the net cholesterol efflux to HDL by mobilizing intracellular cholesterol to the cellular surface (Slotte et al., 1987; Aviram et al., 1989; McKnight et al., 1992).

We have demonstrated that many lipid-free apolipoproteins with amphiphilic α-helical segments (apoA-I, A-II, A-IV, E, and insect apoIII) mediate net efflux of cellular cholesterol and phospholipids (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Li et al., 1993). This reaction generates pre-β-HDL-like particles composed of cellular free cholesterol and phospholipid and results in the reduction of intracellularly accumulated cholesteryl ester. The V_{max} of the reaction is about one-third of the HDL-mediated apparent cholesterol efflux, which is almost equivalent to the net cholesterol efflux achieved by HDL (Johnson et al., 1986), and the concentration of the apolipoproteins required for the reaction is very low, such as $^1/_{500}-^1/_{1500}$ of plasma apoA-I concentration for the K_m. Therefore, it is conceivable that lipid-free apolipoproteins in the interstitial fluid directly carry out such a reaction, or that apolipoproteins dissociate from HDL to interact with the cell surface. The same type of reaction was also demonstrated by other groups with different cell lines in different conditions (Bielicki et al., 1992; Mendez et al., 1994; Forte et al., 1993). Cellular lipid efflux by this mechanism is consistent with the appearance of cellular cholesterol in pre-β-HDL fraction in the earliest phase of the incubation of plasma with cells in culture (Castro & Fielding, 1988; Miida et al., 1992; Huang et al., 1994).

Esterification of cholesterol on HDL by lecithin:cholesterol acyltransferase (LCAT) is thought to play a significant role in cellular cholesterol efflux by maintaining a cholesterol concentration gradient between the cell and HDL surfaces to enhance the efflux (Glomset, 1968), based on the observation that reducing free cholesterol in plasma by LCAT reaction lead to the decrease of cholesterol in erythrocytes (Murphy, 1962). However, it has never been observed that extracellular cholesterol esterification directly stimulates the efflux of cholesterol from the cells in culture to lipoproteins in such a manner as proposed (Stein et al., 1978; Ray et al., 1980; Fielding & Fielding, 1981; Kilsdonk et al., 1993). Some authors reported that cholesterol esterification reduces free cholesterol influx from HDL to the cells in culture resulting in the net decrease of cellular cholesterol in hepatocytes (Ray et al., 1980) and in fibroblasts (Fielding & Fielding, 1981), while the others described that there was no effect of cholesterol esterification on the net removal of cholesterol by HDL from human endothelial cells (Kilsdonk et al., 1993). Thus, it has not yet been completely established what the direct role of LCAT in cellular cholesterol efflux.

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[1] Abbreviations: HDL, high density lipoproteins; apo, apolipoprotein; LCAT; lecithin:cholesterol acyltransferase; LDL low density lipoproteins.

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Therefore, it is necessary to study the influence of the LCAT reaction on free apolipoprotein-mediated cellular lipid efflux. It is to be asked whether or not LCAT is capable of esterifying cholesterol on the pre-β-HDL particles generated by free apolipoproteins−cellular interaction and whether such cholesterol esterification would result in a significant effect on cellular cholesterol efflux by this mechanism.

In this paper, we demonstrate cholesterol esterification by LCAT in the pre-β-HDL generated by apolipoprotein−cell interaction. However, such extracellular cholesterol esterification by LCAT did not cause enhancement of the net efflux of the cellular cholesterol mediated by lipid-free apoA-I. In contrast, it was shown that the LCAT reaction in HDL did not directly cause significant stimulation of the efflux of cellular cholesterol from macrophages or fibroblasts but resulted in the increase of net cholesterol removal by HDL from cells by suppressing the influx of cholesterol from HDL to cells.

EXPERIMENTAL PROCEDURES

Reagents. Egg phosphatidylcholine was purchased from Avanti Polar Lipids (Pelham, AL). RPMI culture medium was from Flow Laboratory (Missisouga, ON). Other chemicals and reagents used were of the highest quality among those commercially available.

LCAT, Lipoproteins and Apolipoproteins. LCAT was isolated from human or pig plasma as described elsewhere (Ryan et al., 1992; Czarnecka & Yokoyama, 1993). Pig LCAT was highly isolated to 16 000-fold (Czarnecka & Yokoyama, 1993). The partially isolated enzymes from human plasma (2600-fold) (Ryan et al., 1992), being not contaminated by apolipoproteins, was also used in some experiments. The enzyme preparations were concentrated by ultrafiltration to the appropriate concentration when required for the experiments (Ryan et al., 1992). Human HDL was obtained as a fraction with density 1.063−1.21 g/mL of fresh human plasma. ApoA-I and A-II were isolated from HDL by delipidation and an ion-exchange chromatography with DEAE-cellulose (Whatman DE-52) as previously described (Hara & Yokoyama, 1991; Hara et al., 1992). In order to inactivate LCAT, freshly prepared human plasma or HDL fraction was incubated in 10 mM *N*-ethylmaleimide at room temperature for 3 h, followed by thorough dialysis. In addition, HDL was labeled with [1,2-^{3}H]cholesteryl oleate (Amersham) by incubating the plasma with phosphatidylcholine vesicles containing the radiolabeled lipid according to the method previously described in order to observe the uptake of HDL by macrophages (Francis et al., 1991).

Measurement of Cholesterol Esterification. The activity of LCAT was measured as cholesterol esterification on lipoprotein particles. Fresh human HDL was labeled with [7-^{3}H]cholesterol (New England Nuclear) by incubating the isolated lipoprotein with radiolabeled cholesterol in a complex with bovine serum albumin in the presence of 10 mM *N*-ethylmaleimide at 37 °C for 3 h. The labeled HDL was reisolated by ultracentrifugation, dialyzed, and used for the substrate. As alternative substrate, egg phosphatidylcholine small unilamellar vesicles were prepared with 20 mol % cholesterol containing the radiolabeled cholesterol as described elsewhere (Yokoyama et al., 1980). The vesicles were preincubated with human apoA-I in the weight ratio of the protein to phospholipid of 1/4 for 30 min at room temperature before the LCAT reaction started (Yokoyama et al., 1980). These substrates were incubated with the enzyme at 37 °C for 2 h. The reaction was terminated, lipid was extracted with chloroform/methanol (2:1 by volume), and the radioactivity of free and esterified cholesterol was determined after separating those by thin layer chromatography (with a solvent hexane/diethyl ether/acetic acid, 80:20:1) (Komaba et al., 1992; Yokoyama et al., 1980). The LCAT activity was expressed in various experiments as a percentage of that in pooled fresh plasma which had cholesterol esterification activity of 56.4 nmol/mL/h for its own lipoproteins.

Cellular Cholesterol Efflux. Mouse peritoneal macrophages were obtained by peritoneal lavage as described previously (Hara & Yokoyama, 1991). The macrophages, of the density of 30−40 μg of cell protein/dish in the standard preparation procedure (unless otherwise specified), were loaded with radiolabeled cholesterol by incubating with acetylated low density lipoprotein containing [1,2-^{3}H]cholesteryl oleate (Amersham) as the final specific radioactivity of total cholesteryl ester 16 160 dpm/mmol, prepared according to the method reported elsewhere (Hara & Yokoyama, 1991), in 1 mL of RPMI medium containing 2 mg of bovine serum albumin for 24 h. Total cellular cholesterol after loading was 200−300 μg/mg of cell protein. When cellular choline/phospholipid was to be labeled, [*methyl*-^{3}H]-choline (New England Nuclear) was added to the medium for this incubation (Hara & Yokoyama, 1991; Li et al., 1993). Human skin fibroblast, Detroit 551, in a confluent stage, as 40−60 μg of cellular protein/dish, was loaded and labeled with the tritiated cholesterol by incubating the cells in the same medium with unmodified LDL incorporated with [1,2-^{3}H]cholesteryl oleate as described above. Total cellular cholesterol after loading was 70−100 μg/mg of cell protein. The cells were then washed and incubated another 24 h without lipoproteins. To label the cells with radioactive cholesterol, [1,2-^{3}H]cholesterol, to reach higher specific radioactivity, the fibroblasts were incubated with the labeled cholesterol mixed with albumin (2%) for 24 h and were washed. The cells were then harvested by the trypsin treatment and then placed in new plastic culture dish and cultured again for 24 h.

The cholesterol loaded and phospholipid-labeled macrophages were incubated with the 1-mL medium containing apoA-I or apoA-II, 10 μg, for 24 h. In order to detect the density peak of free cholesterol and choline phospholipid, the medium was removed and analyzed by sucrose density gradient ultracentrifugation as described previously (Hara & Yokoyama, 1991). The aliquot of each medium after incubation of the cholesterol-loaded but not choline-labeled cells with apoA-I and apoA-II, 10 μg/1 mL, respectively, were incubated with LCAT at 37 °C for 2 h. The lipids were extracted with chloroform/methanol and analyzed by thin layer chromatography, and radioactivity of free and esterified cholesterol was counted.

The cholesterol-labeled macrophages or fibroblasts were incubated in the 1-mL medium containing apoA-I with and without LCAT unless otherwise specified. The radioactivity of free and esterified cholesterol in the medium was counted at appropriate incubation times. The radioactivity in the medium was directly considered as the net cholesterol efflux from the cells because there was no cholesterol in the medium at the initial stage of the incubation. The cells were also incubated with the LCAT-inactivated normal plasma HDL in the presence and absence of LCAT, and the

appearance of radiolabeled cellular cholesterol in the medium was measured as radioactivity in free and esterified cholesterol. The same experiments were carried out with the LCAT-inactivated whole human plasma. In these latter cases, the radioactivity in the medium was considered as the "efflux" of cholesterol originating in the cells, and the possible "influx" from lipoprotein to the cells was not directly measured.

HDL was labeled with [^{14}C]cholesterol (New England Nuclear) according to the same procedure as described above for [^{3}H]cholesterol and incubated with [^{3}H]cholesterol-labeled fibroblasts in the presence and absence of LCAT in the medium. Both efflux and influx of cholesterol was measured during the incubation by counting each isotope in esterified and unesterified cholesterol in the medium and cells. The amount of cholesterol in each flux was calculated using the specific radioactivity of each isotope in the initial condition based on the measurement of free cholesterol in HDL and fibroblasts, respectively (Li et al., 1993).

Unless otherwise specified, each data point was duplicated in every experiment within an error of 10%, and the data shown represented the average.

RESULTS

After incubation of the cholesterol-and-choline-labeled macrophages with apoA-I and A-II for 24 h, efflux of free cholesterol and choline phospholipid was observed in the medium, without detecting cholesteryl ester. Density gradient analysis of the medium after the incubation demonstrated that the peaks of free cholesterol and phosphatidylcholine coincided at around 1.1 g/mL both with apoA-I and with apoA-II, being consistent with our previous finding that pre-β-HDL-like lipoprotein was generated by the reaction (Figure 1) (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Li et al., 1993). Thus, lipoprotein particles generated with cellular lipids and apolipoproteins and resembling pre-β-HDL contained both substrates of LCAT, phosphatidylcholine and cholesterol. The molar ratio of cholesterol to phosphatidylcholine was estimated to be 3.4 and 4.0 for the apoA-I and apoA-II particles, respectively, from the radioactivity of each lipid in the medium and specific radioactivity of each lipid in the cells (Hara & Yokoyama, 1991). The molar ratio of cholesterol to total choline phospholipid including sphingomyelin was around 2.4 and 2.7 for apoA-I and apoA-II particles, respectively (Hara & Yokoyama, 1991). In comparison to the chemical composition of various pre-β-HDL in the previous publications (Table 1), the composition of the particles thus generated seems to be consistent with those isolated from the lymph of cholesterol-fed dogs rather than those from human plasma, considering large assay errors to be allowed especially for the particles generated in the culture.

Figure 2 shows cholesterol esterification on these particles by human LCAT. Cholesterol esterification was demonstrated only with the medium containing the pre-β-HDL generated with apoA-I, and the esterification was almost negligible with the particles formed by apoA-II. The rate of cholesterol esterification with these particles was compared to the rates observed with HDL and apoA-I-activated unilamellar vesicles. Because the rate is proportional to the substrate concentration within the range used (up to 700 μM choline phospholipid and 200 μM free cholesterol as HDL) (Czarnecka & Yokoyama, 1993), the comparison was made

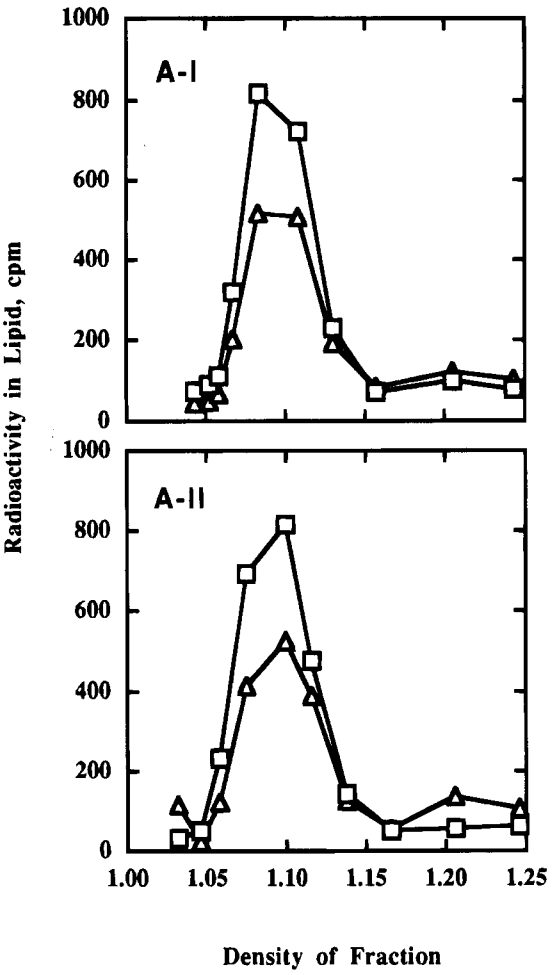

FIGURE 1: Demonstration of HDL-like particles by density gradient analysis of the lipids in the medium of cholesterol-loaded macrophages after incubation with apolipoproteins. The cells had been labeled for cholesterol and choline phospholipid and then incubated with apoA-I (top panel) and apoA-II (bottom panel), 10 μg/mL respectively, for 24 h as describe previously (Hara & Yokoyama, 1991). One mililiter of the medium was applied for sucrose density gradient ultracentrifugation in a Beckman TL 100 table top ultracentrifuge, and lipids in each fraction were extracted and analyzed by thin layer chromatography (Hara & Yokoyama, 1991). Radioactivity of free cholesterol (squares) and phosphatidylcholine (triangles) was counted and demonstrated in this figure. The peak of the control medium (bovine serum albumin) was about 5% of those demonstrated in this figure. The average cellular protein per dish was 217 $\pm$ 21 μg for this experiments.

on the basis of the percentage esterification of cholesterol (Table 2). The rate of percent cholesterol esterification on the pre-β-HDL particles formed with apoA-I was comparable to the rate observed with the both substrates for the LCAT assay, though the entire kinetic study is required for more detail.

Cholesterol-labeled macrophages were then incubated with apoA-I in the presence and absence of human LCAT. Since there is no lipid in the medium in the initial condition, cellular cholesterol efflux measured as radioactivity in the medium directly indicates overall net cellular cholesterol efflux in these experiments. The LCAT activity in the medium was adjusted for 5% and 10% of the control plasma. Without LCAT, all the cholesterol originating in the cells and found in the medium was free cholesterol. When the culture medium contained LCAT, cholesteryl ester was continuously generated in the medium as the incubation was carried out up to 24 h (Figure 3). The activity of LCAT in the medium was maintained reasonably constant during the period of the

Table 1: Chemical Composition of Pre-β-HDL (in Weight %)

	protein	phospholipid	cholesterol		triglyceride
			free	esterified	
human plasma[a]	91.3	6.6	0.3	2.5	
dog lymph (control)[b]	37.1	41.9	8.1	11.8	1.9
(cholesterol fed)[b]	35.7	40.0	15.3	9.0	
dog lymph (cholesterol fed)[c]	30.8	46.2	17.0	6.6	
A-I/macrophage[d]	48.1	30.0	21.9	0	0
A-II/macrophage[d]	68.1	14.8	17.0	0	0

[a] Average values of three cases published by Kunitake et al. (1985). [b] Adopted from the table by Sloop et al. (1987). [c] Read-out values from the figure published by Dory et al. (1983). [d] The particles used in this study being adopted from the data by Hara and Yokoyama (1991), with the values of phospholipid as the sum of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine.

Table 2: Percent Esterification of Cholesterol by LCAT

substrate	free cholesterol (μM)	phosphatidylcholine (μM)	LCAT concentration (percent of plasma)[a]	esterification (percent/2 h)
HDL[b]	144	479	37.9	3.7 ± 0.6
vesicle[c]	111	561	31.3	1.8 ± 0.3
medium/A-I[d]	2.15	0.63	12.0	4.3
medium/A-I[d]	2.15	0.63	6.0	2.9
medium/A-II[e]	2.78	0.70	14.4	0.1

[a] The pooled fresh plasma used for this standardization had cholesterol esterification activity as a rate of 56.4 nmol/mL/h for its own lipoproteins. [b] Human HDL labeled with radioactive free cholesterol was used for substrate in the total incubation volume 190 μL. The data is mean ± SD for the three incubations. [c] ApoA-I-activated unilamellar vesicles of phosphatidylcholine and cholesterol in a total incubation volume of 230 μL. The data are the mean ± SD for the three incubations. [d] Macrophage culture medium after incubation with apoA-I, 10 μg/mL, was incubated with LCAT. The 500 μL medium was incubated in the total volume of 600 μL. The data are the average of the two incubations. [e] Macrophage culture medium after incubation with apoA-II, 10 μg/mL, was incubated with LCAT. The 400 μL medium was incubated in a total volume of 500 μL. The data are the average of the two incubations.

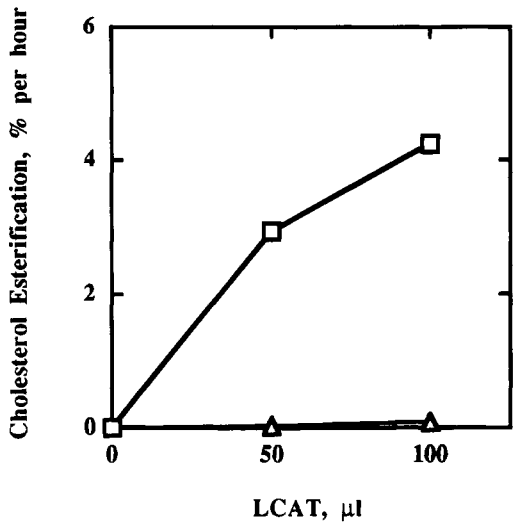

FIGURE 2: Cholesterol esterification on the HDL-like particles generated by macrophage lipids and apolipoproteins. The cholesterol-loaded and -labeled macrophage culture medium after 24 h incubation with apoA-I and apoA-II, 10 μg/mL, respectively, was incubated with LCAT for 2 h. The LCAT preparation used contained 72% activity of the control plasma. The apoA-I medium (500 μL) containing 1.29 nmol of free cholesterol, 0.38 nmol of phosphatidylcholine, and 0.15 nmol of sphingomyelin was incubated in the final volume of 600 μL (squares), and 400 μL of the apoA-II medium, containing 1.39 nmol of free cholesterol, 0.35 nmol of phosphatidylcholine, and 0.17 nmol of sphingomyelin, was incubated in the final volume of 500 μL (triangles). The average cellular proteins per dish was 94.5 ± 8.2 μg for this experiment.

incubation (Figure 3). The ratio of cholesteryl ester to the total efflux cholesterol in the medium continuously increased up to the maximum 16% with the high dose of LCAT. However, even with this continuous and substantial cholesterol esterification by LCAT in the medium, no significant increase was observed in the overall net cholesterol efflux from cholesterol loaded macrophages into the medium (sequential method for matched pairs) (Figure 3).

On the basis of these data, further extent of cholesterol esterification was attempted using highly isolated pig LCAT to observe its effect on the apoA-I-mediated cellular cholesterol efflux from the macrophages and the fibroblasts (Figure 4). The ratio of cholesteryl ester was almost 50% of the total cellular cholesterol found in the medium after 24 h in this condition in both cases. Total net cellular cholesterol efflux from macrophages was even somewhat lower by such high rate of cholesterol esterification (Figure 4, top) and that from fibroblasts showed no change (Figure 4, bottom). Thus, cholesterol esterification on the pre-β-HDL generated with apoA-I did not cause any additional net cellular cholesterol efflux from either cell.

To compare with the finding above, cholesterol flux between cell and HDL isolated from plasma was observed in the presence and absence of LCAT. Cholesterol was labeled with ^3H in human fibroblasts and with ^{14}C in HDL, respectively. During the incubation of the labeled HDL and fibroblasts in the presence and absence of LCAT, each labeled cholesterol was traced in the medium. Figure 5 shows esterification of both cellular and HDL-originating cholesterol in the medium indicating that cholesterol from these two pools are almost equally used by LCAT, though HDL-originating cholesterol may slightly be more esterified. Thus, LCAT does not preferentially use cholesterol molecules from either sources to generate cholesteryl ester on HDL. Figure 6 demonstrates influx and efflux of cholesterol in the presence and absence of LCAT during the incubation described in Figure 5. The efflux represents appearance in the medium of cholesterol that is originating in cellular pool at each incubation time, and the influx is indicated as disappearance from the medium of cholesterol that is originating in HDL. Calculation was based on the specific radioactivity of each isotope (^3H for cellular cholesterol and ^{14}C for HDL free cholesterol). There was no apparent effect of the presence of LCAT on the efflux of cellular cholesterol, while influx of HDL cholesterol into the cell was suppressed

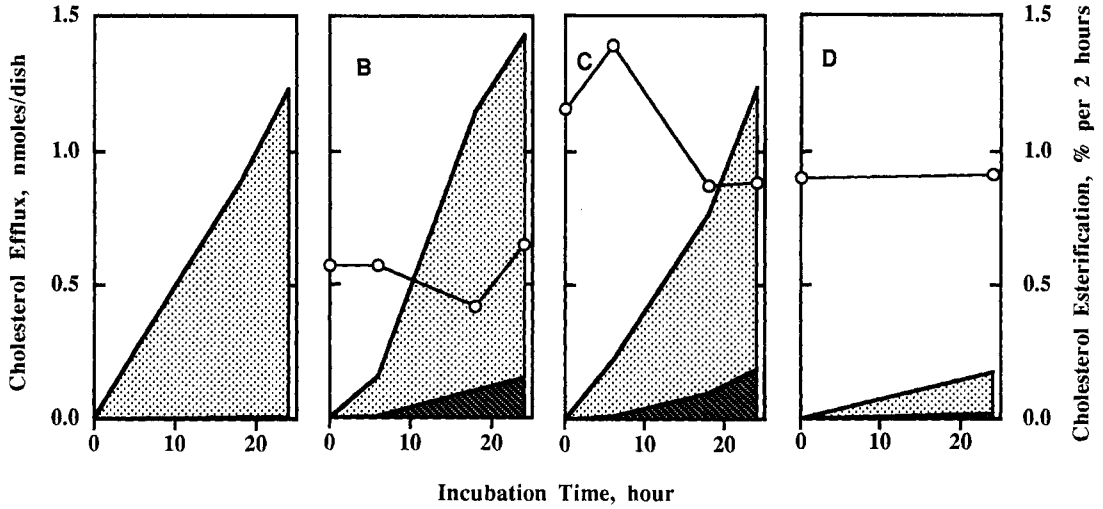

FIGURE 3: Cholesterol efflux from macrophage to the apoA-I-containing medium in the presence of LCAT. The cells loaded and labeled with cholesterol, average cellular protein 45.4 ± 5.5 μg, were incubated with the medium, 500 μL, containing apoA-I and/or LCAT. (Panel A) 10 μg/mL apoA-I without LCAT; (panel B) apoA-I plus LCAT, 5% of plasma activity in the medium; (panel C) apoA-I plus LCAT, 10% of plasma activity in the medium; (panel D) LCAT, 10% of plasma activity, without apoA-I. Free and esterified cholesterol in the medium were determined from radioactivity of each lipid in the medium and cellular specific radioactivity of total cholesterol (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Li et al., 1993) at 6, 18, and 24 h of incubation. A 95 μL aliquot of the medium at each incubation time was assayed for the LCAT activity using the substrate of apoA-I-activated vesicles in the same condition as described in Table 1. Data represent total cholesterol in the medium, divided into free cholesterol (upper shadowed area) and esterified cholesterol (lower hatched area). Open circles indicate the LCAT activity in the medium at each incubation time expressed as percent cholesterol esterification. No LCAT activity was detected in the medium without LCAT (experiment of panel A). The results of cholesterol efflux with neither apoA-I nor LCAT were similar to the results in panel D. Difference between the efflux in panels A and B, and A and C, were statistically insignificant on the basis of the sequential method for matched pairs.

by the LCAT reaction. This results also indicate that [^{14}C]-cholesterol influx may account for at most 12% of cellular cholesterol after 6 h of incubation. Such estimation may explain slightly higher apparent esterification of HDL-cholesterol in Figure 5.

Figures 7 and 8 further demonstrated lack of enhancement of cellular cholesterol efflux to HDL by LCAT reaction, from macrophages and fibroblasts, respectively. Even in such an extreme condition as 85% of cholesterol originating from cellular pool was esterified in the medium, no significant increase was observed in overall cellular cholesterol appearance in the medium (Figure 7). The lack of significant effect of LCAT on the cholesterol efflux from fibroblasts was also observed with various concentration of HDL (20−100 μg of protein) in the linear time course up to 20 h including the very initial phase up to 2.5 h (data not shown). In addition, the whole human plasma (1−4% in the medium, LCAT-inactivated) was incubated with either macrophages or fibroblasts in order to observe the overall effect of the presence of other plasma components such as other lipoproteins (LDL and very low density lipoproteins) and lipid transfer proteins. There was no enhancement by the LCAT reaction of cellular cholesterol efflux to the medium.

Throughout the experiments with macrophages, the cholesterol esterification by LCAT consistently seemed to decrease rather than increase cellular cholesterol efflux. To identify the cause of this, some factors were examined. Neither the presence of high concentration of albumin in the medium (5%) nor the additional lysolecithin (10% of phosphatidylcholine) had significant effect on the effect of LCAT reaction. The uptake of HDL-cholesteryl ester by macrophages was not affected by LCAT reaction. Therefore, the apparent lower cholesterol efflux from macrophage in the presence of LCAT reaction may not be attributed to the effect of lysolecithin generated by LCAT. It was not caused either by the cellular uptake of HDL due to modification of HDL particle by cholesterol esterification as measured the uptake of HDL-cholesteryl ester. Thus, the reason for this remains unclear.

DISCUSSION

HDL-like particles were generated with cellular phospholipids and free cholesterol in the medium when apoA-I and apoA-II were extracellularly added to the cholesterol-loaded macrophages, confirming our previous reports (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Li et al., 1993). These particles were previously characterized as pre-β-HDL-like lipoproteins with respect to their density, electrophoretic mobility, and their chemical compositions (Hara & Yokoyama, 1991). Cholesterol esterification by LCAT was demonstrated in such particles generated by apoA-I. The rate of percent cholesterol esterification in the medium was in the same order as the rate with human native HDL and as the rate of the reaction with unilamellar vesicles with egg phosphatidylcholine containing 20 mol % cholesterol when maximally activated by apoA-I. This is the demonstration of the LCAT reaction with its both substrates directly and exclusively derived from cellular lipid pool. Cholesterol esterification was almost negligible when similar HDL-like particles were generated with apoA-II. Thus, the lipoprotein particles generated by apoA-I−macrophage interaction also fulfill the properties of HDL as a substrate of LCAT. Lack of reactivity of the apoA-II particles is also consistent with the previous report for deactivation of the LCAT reaction by apoA-II on HDL (Chung et al., 1979). High content of sphingomyelin in the particles (Hara & Yokoyama, 1991) may cause lower rate of cholesterol esterification (Subbaiah & Liu, 1993), which cannot be confirmed unless using the control particles with less sphingomyelin.

These results seem consistent with the data reported by other groups showing cholesterol esterification by LCAT on

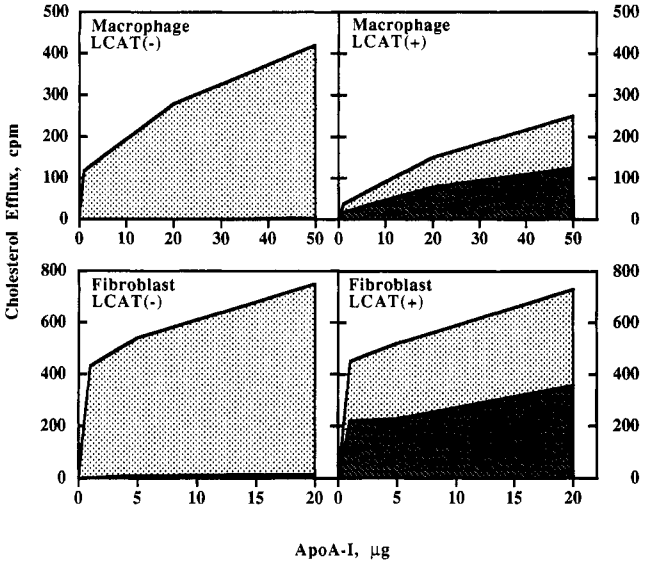

FIGURE 4: Effect of cholesterol esterification in the medium on the efflux of cholesterol from macrophages and fibroblasts mediated by various amount of lipid-free human apoA-I. (Top two panels) Mouse peritoneal macrophages was loaded with radiolabeled cholesterol (radioactivity in cellular free cholesterol was 3526 ± 887 cpm/dish, and esterified cholesterol was 29.1 ± 0.7% of total cellular cholesterol in radioactivity) and then incubated with the indicated amount of human apoA-I for 24 h with and without LCAT isolated from pig plasma (45% of the activity of control plasma as a final concentration in the medium). The lipid was extracted from the medium and analyzed by thin layer chromatography. Data represent total cholesterol in the medium, divided into free cholesterol (upper shadowed area) and esterified cholesterol (lower hatched area). Total cholesterol efflux was lower in the presence of LCAT by $p < 0.05$ (sequential method for matched pairs). (Bottom two panels) Human skin fibroblasts was loaded with radiolabeled cholesterol via LDL containing with radiolabeled cholesteryl ester (radioactivity in cellular free cholesterol was 10 553 ± 2376 cpm/dish and esterified cholesterol was 41.7 ± 3.2% of total cellular cholesterol in radioactivity) and then incubated with the indicated amount of human apoA-I for 24 h with and without pig LCAT (60% of the activity of control plasma as a final concentration in the medium). The lipid was extracted from the medium and analyzed by thin layer chromatography. Data represent total cholesterol, divided into free cholesterol (upper shadowed area) and esterified cholesterol (lower hatched area).

the lipoproteins in peripheral lymph (Dory et al., 1983, 1986) or with pre-β-HDL in plasma (Neary et al., 1991; Miida et al., 1992). Thus, the results were consistent with the hypothesis that pre-β-HDL can be generated *in situ* by the interaction of lipid-free apolipoproteins with the surface of peripheral cells (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992; Li et al., 1993).

Such cholesterol esterification by LCAT, however, failed to demonstrate significant enhancement of the net cellular cholesterol efflux by the mechanism of free apolipoprotein–cell interaction. Whereas the free cholesterol/phospholipid molar ratio in plasma HDL is much lower than 1, pre-β-HDL generated by apoA-I–cell interaction has that ratio higher than 1, and thus there is virtually no gradient of cholesterol content between the cellular surface and such lipoproteins (Table 1) (Hara & Yokoyama, 1991). This may mean that this particle poorly accepts cholesterol from cell once it is generated (Johnson et al., 1986, 1988; Phillips et al., 1987). It is also possible that high content of sphingomyelin in such particles (Hara & Yokoyama, 1991) may result in poorer cholesterol efflux (Gold & Phillips, 1990). Cholesterol esterification results in decrease of free cholesterol in such lipoproteins but an equimolar amount of

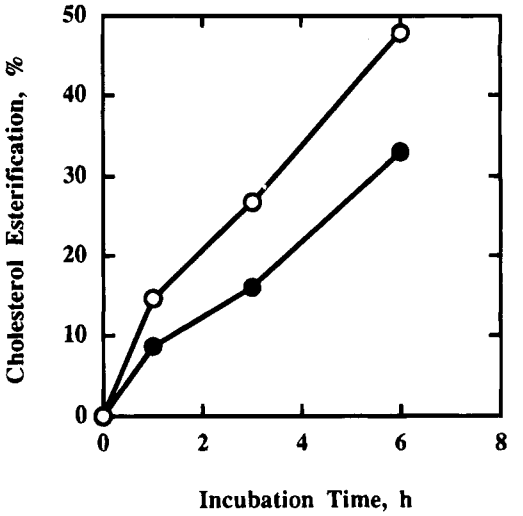

FIGURE 5: Esterification of cholesterol originating from the HDL free-cholesterol pool and the cell membrane free-cholesterol pool, in the medium containing HDL and LCAT. HDL-free cholesterol (0.66 μg/dish) was labeled with ^{14}C, and cellular free cholesterol was labeled with ^{3}H (1.6 μg/dish). Esterification of cholesterol was measured in the presence of LCAT (50% of plasma activity) for HDL (open circles) and cellular (closed circles) cholesterol. Values were calculated on the basis of the initial specific radioactivity of each free cholesterol pool. The rate with HDL-originating cholesterol is statistically higher than that with cellular cholesterol by $p < 0.05$ (sequential method for matched pairs).

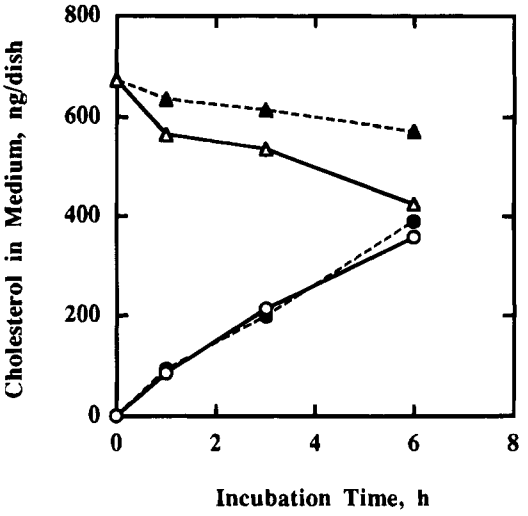

FIGURE 6: Efflux of cellular cholesterol to HDL and influx of HDL cholesterol into the cells in the presence and absence of LCAT. Experimental conditions are described in the legend for Figure 5. Triangles indicate cholesterol originating in HDL free cholesterol showing its influx to the cells as it decreases in the medium, and circles indicate efflux of cholesterol originating from the cellular free cholesterol pool, with LCAT (closed symbols with broken lines) and without LCAT (open symbols with solid lines). Difference of the influx between the presence and absence of LCAT was statistically significant by sequential method for matched pairs ($p < 0.01$).

glycerophospholipid also decreases as lysophospholipid products are removed from the lipoprotein by albumin (Hara & Yokoyama, 1991; Czarnecka & Yokoyama, 1993). Therefore, the LCAT reaction may not decrease cholesterol/phospholipid but rather increase it in the particles, unless additional phospholipid is provided from other sources such as other lipoproteins. Indeed, cholesterol efflux was not enhanced even after 50% of cholesterol was esterified. Adding other lipoproteins as a whole plasma was unable to show significant effect of providing additional source of

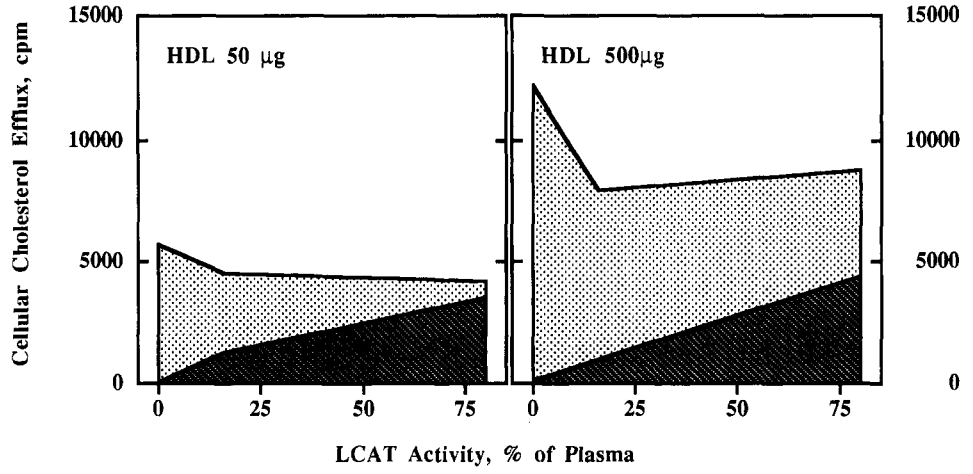

FIGURE 7: Effect of cholesterol esterification on cellular cholesterol efflux from macrophages to HDL. Cholesterol-loaded mouse peritoneal macrophages (radioactivity of cellular free cholesterol was 4546 ± 228 cpm/dish and esterified cholesterol was 26.6% ± 1.2% of total cellular cholesterol in radioactivity) were incubated with HDL (50 and 500 μg of protein) in the presence of various amount of LCAT (up to 80% of the activity of control plasma as a final concentration in the medium). Data represent total cholesterol in the medium, divided into free cholesterol (upper shadowed area) and esterified cholesterol (lower hatched area).

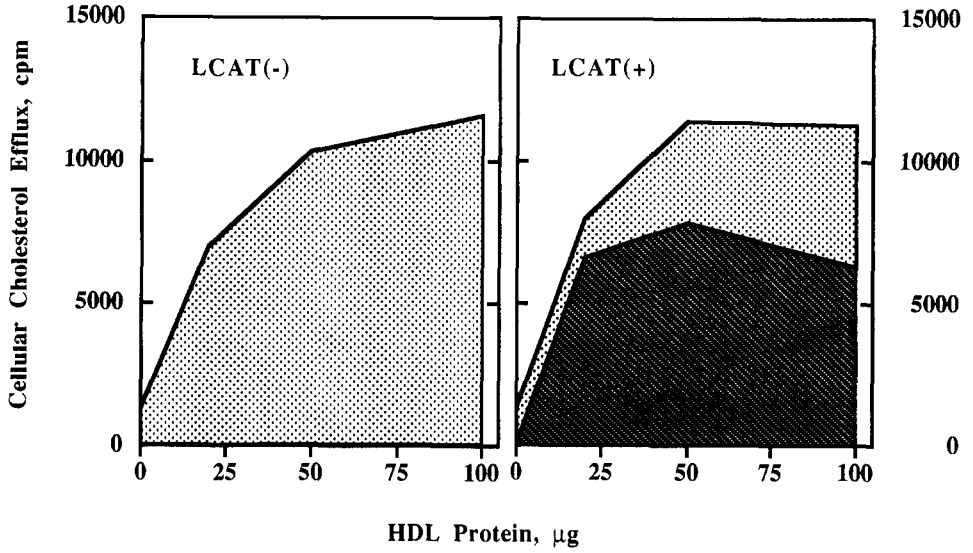

FIGURE 8: Effect of cholesterol esterification on cellular cholesterol efflux from fibroblasts to HDL. The cells were labeled with [^{3}H]-cholesterol (radioactivity in cellular cholesterol was 37 860 ± 697 cpm/dish and esterified cholesterol was 4.9 ± 0.6% of total cellular cholesterol in radioactivity) and incubated with various amount of HDL in the presence and absence of LCAT (75% of plasma activity). Data represent total cholesterol in the medium, divided into free cholesterol (upper shadowed area) and esterified cholesterol (lower hatched area).

lipids under the limited experimental condition. Thus, cholesterol esterification does not act as a direct driving force for further efflux of cellular cholesterol to the newly generated pre-β-HDL.

Thus, LCAT reaction has been shown not to be an additional direct driving force for the cellular cholesterol removal by lipid-free apolipoprotein. Further studies should be conducted to observe the effect of cholesterol esterification in the presence of additional factors such as removal of free and esterified cholesterol from the particles generated or supply of phospholipid as a complementary substrate. Experimental design for such a study, however, may not be simple because of instability of the pre-β-HDL generated in the presence of other lipoproteins (Hara & Yokoyama, 1992), because of the nonspecific cholesterol efflux to any other lipid surface in the medium such as phospholipid vesicles, microemulsions, and other lipoproteins (Hara & Yokoyama, 1992) and of the requirement of the transfer activities for certain lipids.

The LCAT reaction also failed to demonstrate direct enhancement of cellular cholesterol efflux to HDL either from macrophage or fibroblasts. Indeed, in many of the previous works (Stein et al., 1978; Ray et al., 1980; Fielding & Fielding, 1981; Kilsdonk et al., 1993), the effect of LCAT has never been demonstrated for significant direct enhancement of cellular cholesterol efflux. The net effect of LCAT was only by the decrease of influx of cholesterol from HDL to the cells (Ray et al., 1980; Fielding & Fielding, 1981) in complete agreement with our present report.

The decrease of free cholesterol in HDL would generate the flow of cellular cholesterol toward HDL due to the greater gradient of cholesterol content between HDL and cellular surface (Glomset, 1968). However, many papers agree that the time course of cholesterol efflux to HDL is slow from various cells in culture such as fibroblasts, macrophages, and vascular smooth muscle cells, with a half-time of several hours or longer (Daniels et al., 1981; Karlin et al., 1987; Johnson et al., 1988; Mahlberg & Rothblat, 1992; Yancey

& St. Clair, 1992). The LCAT reaction may not enhance this rate if the rate-limiting step is rather off-rate of the efflux.

On the other hand, classical work by Murphy (1962) demonstrated direct effect of cholesterol esterification on the net cholesterol efflux from erythrocytes. This was in fact clearly confirmed by our own preliminary data that cellular cholesterol efflux from human erythrocytes increased exactly as much as the esterified cholesterol on HDL in the condition used (Czarnecka and Yokoyama, unpublished data). The time course of cholesterol exchange between HDL and erythrocytes in this condition has a half-time of less than 1 h and is more or less compatible to that for cholesterol exchange between vesicles or lipoproteins (i.e., HDL and LDL) (Nishikawa et al., 1988). Interestingly, cholesterol esterification on HDL also directly causes net move of cholesterol from LDL to HDL as much as the esterified cholesterol on HDL by LCAT (Ko et al., 1994). Thus, LCAT seems to have direct effect on cholesterol transfer if the rate of its bilateral exchange is much faster than the rate of esterification.

The efflux rate of cholesterol from certain types of cells is reportedly fast and may strongly depend on the acceptor concentration, such as hepatoma cell lines and erythrocytes (Karlin et al., 1987; Gold & Phillips, 1990; Steck et al., 1988). Thus, it is possible that LCAT may directly enhances the efflux cholesterol to HDL not only from erythrocytes but also from certain types of cells in culture in some appropriate condition. It may explain why low LCAT activity in plasma causes problem only in some particular organs including erythrocytes, renal tubule, and potentially cornea.

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