Regulation of Cellular Cholesterol Efflux by Lecithin:Cholesterol Acyltransferase Reaction through Nonspecific Lipid Exchange*

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Erythrocyte was found lacking in reactivity to lipid-free apolipoproteins to generate pre-β-high density lipoprotein (HDL) with the cellular lipid and, therefore, was used to study cellular cholesterol efflux to plasma lipoproteins exclusively by a nonspecific exchange mechanism. Over the range of hematocrit from 1-20% (cellular cholesterol pool of 2.5 μg to 50 μg per 250 μl), the fractional rate of cellular cholesterol efflux to lipoprotein was constant, and, therefore, absolute efflux rate was a linear function of the hematocrit of this range. In the absence of lecithin:cholesterol acyltransferase (LCAT), the cholesterol influx rate from lipoproteins was equal to the efflux rate from erythrocytes resulting in no net transfer of cholesterol, with either HDL or low density lipoprotein. In the presence of LCAT in the mixture of HDL and erythrocyte, cholesterol was esterified exclusively in HDL regardless of the origin. While the hematocrit was low and efflux of cellular cholesterol was slower than cholesterol esterification, the esterification of cell-originating cholesterol did not directly enhance the efflux. With high hematocrit that gives faster cholesterol efflux, the efflux was increased directly by the cholesterol esterification. On the other hand, the LCAT reaction significantly reduced HDL-cholesterol influx. The LCAT reaction thus induces substantial net cholesterol efflux from erythrocytes through a nonspecific cholesterol exchange mechanism.

Esterification of cholesterol in plasma by lecithin:cholesterol acyltransferase (LCAT) is believed to play a significant role in cellular cholesterol efflux. It maintains the gradient of cholesterol content between cellular and lipoprotein surfaces by reducing free cholesterol in lipoprotein, which is thought to generate continuous flow of cholesterol from cell to lipoprotein (Glomset, 1968). LCAT is known to act mainly on high density lipoprotein (HDL) (Akanuma and Glomset, 1968; Ko et al., 1994). However, many works using cultured cells failed to demonstrate direct enhancement of cellular cholesterol efflux by cholesterol esterification in HDL (Stein et al., 1978; Ray et al., 1980; Fielding and Fielding, 1981; Kilsdonk et al., 1993), while some of them showed the increase of net cellular cholesterol efflux by the LCAT reaction by reducing the influx of HDL cholesterol (Ray et al., 1980; Fielding and Fielding, 1981). Thus, a precise role of LCAT in the efflux of cellular cholesterol remains to be identified. This is related to the fact that a genetic defect of LCAT results in cholesterol accumulation only in a limited number of organs but not in general cholesterol deposit (Glomset et al., 1983).

Cellular cholesterol efflux is mediated by two distinct mechanisms. One is nonspecific exchange of cholesterol between the cell and lipoprotein surface (Hara and Yokoyama, 1992; Li et al., 1993), and the other is the reaction of lipid-free helical apolipoproteins with cellular surface to result in unidirectional removal of the cellular lipids and to generate new pre-β-HDL particles with the cellular lipid (Hara and Yokoyama, 1991, 1992; Li et al., 1993). The cellular lipid efflux to HDL is likely to involve both mechanisms as HDL-apoproteins dissociate from the lipoprotein to interact with high affinity association sites of the cells (Li et al., 1993, 1995). We demonstrated the efficient esterification of cholesterol by LCAT in the pre-β-HDL newly generated with the cellular phospholipid and cholesterol of macrophages and fibroblasts by lipid-free apolipoprotein (apo) A-I, but it did not enhance net cellular cholesterol efflux any further (Czarnecka and Yokoyama, 1995). Net cellular cholesterol efflux to HDL was increased by LCAT reaction only by decreasing the cholesterol influx from HDL to the cells while the efflux was not directly enhanced (Czarnecka and Yokoyama, 1995), in agreement with the results previously demonstrated by a few other authors (Ray et al., 1980; Fielding and Fielding, 1981).

In the work presented in this paper, we demonstrate that erythrocyte is lacking in the free apolipoprotein-mediated cellular lipid efflux. This means that cholesterol efflux from erythrocyte is by a nonspecific bidirectional movement exclusively. Therefore, the erythrocyte is a good model by which to study the factors including LCAT reaction that may affect cellular cholesterol efflux through a nonspecific cholesterol exchange mechanism. In addition, erythrocyte membrane in fact undergoes pathological change as one of a few symptoms of genetic LCAT deficiency (Glomset et al., 1983). Hence, we have chosen this cell to examine a role of LCAT reaction in cellular cholesterol efflux purely by nonspecific cholesterol exchange.
By using erythrocyte, the rate of cellular cholesterol efflux was easily modulated by changing the amount of the cells in the incubation mixture. When the cholesterol exchange rate was high, the LCAT reaction in HDL directly increased the efflux of cellular cholesterol toward HDL. When the exchange rate was low and the efflux rather than the esterification was a rate-limiting step, the increase of the net efflux was mainly by the decrease of the influx.

MATERIALS AND METHODS

LCAT, Lipoproteins, and Apolipoproteins—LCAT was isolated from pig plasma as described elsewhere to 16,000-fold (Czarnecka and Yokoyama, 1993). The preparation of the protein migration as a single band of molecular mass 67,000 Da by silver staining in electrophoresis in 0.5% agarose gel and by the analysis of apolipoprotein composition in polyacrylamide gel electrophoresis in the presence of SDS. LCAT was treated with 10 mM N-ethylmaleimide at room temperature for 2 h in order to inactivate endogenous LCAT. Lipid composition of the lipoprotein preparation was analyzed by enzymatic assay methods (Table I). Lipoprotein-cholesterol was labeled with either apoA-I or phosphatidylcholine. For measuring the influx of cholesterol from HDL to erythrocyte, at the same time, the [4-¹⁴C]cholesterol-labeled HDL was incubated with the [7-³H]cholesterol-labeled erythrocytes in the absence and presence of LCAT, and the radioactivity of the lipid in the medium was determined by the same procedure. In the same experiment using the [4-¹⁴C]cholesterol-labeled HDL and the [7-³H]cholesterol-labeled erythrocyte, esterification of cholesterol on HDL was measured for each radiolabel by thin layer chromatography in order to determine the origin of cholesterol esterified by LCAT. The influx of free cholesterol from HDL or LDL was alternatively measured by using the lipoproteins labeled with [7-³H]cholesterol and unlabelled erythrocytes.

RESULTS

Human erythrocyte was found lacking in the free apoA-I-mediated lipid efflux. Fig. 1 shows the efflux of cellular cholesterol and phosphatidylcholine from erythrocyte induced by human HDL and lipid-free apoA-I in the condition of 16% hematocrit. Unlike many other cells in culture, no lipid efflux was induced by lipid-free apoA-I from erythrocyte. Thus, cholesterol efflux from erythrocyte induced by HDL is mediated only by nonspecific cholesterol exchange between the surfaces of the cell and HDL, and this makes it possible to study cellular cholesterol efflux without influence of the free apolipoprotein-mediated lipid efflux. The same experiment with pig erythrocytes and reticulocytes also showed the specific lack of the free apoA-I-induced cholesterol efflux (data not shown).

When LCAT was present (0.6 µg), the incubation mixture of HDL and [³H]cholesterol-labeled erythrocytes, the enzyme esterified cholesterol whether cell-originating or HDL-originating (Fig. 2A). This particular experimental condition included 16% hematocrit (cellular cholesterol pool 40.4 µg), 50 µg of HDL-phospholipid, and 14.0 µg HDL-free cholesterol in 250 µl. Apparently percent esterification in HDL of cell-originating cholesterol was lower than that of HDL-originating cholesterol in the early phase of the radiolabeled cholesterol efflux, presumably due to the influx of HDL-free cholesterol to the erythrocytes and to the time lag caused by mixing of exogenous and endogenous cholesterol in HDL. At the 6-h incubation, percent esterification of cellular cholesterol in HDL was a function of the LCAT concentration in the medium (Fig. 2B). There was no cholesterol esterification by LCAT directly on erythrocytes in the incubation mixture of HDL and 0.1% BSA with and without LCAT at 37 °C in 250 µl, with continuous mixing. Hematocrit of the incubation mixture was chosen between 1 and 20% in order to provide various cellular cholesterol pool sizes. Activity of the LCAT in the mixture supernatant was 20% of that in the standard pooled human plasma, 10.8 nmol/mg/h (0.6 µg of LCAT/m) unless specified otherwise. The incubation was terminated by placing the mixture in ice, and then erythrocytes were removed by centrifu-
The effect of LCAT reaction on cellular cholesterol efflux was also observed in long time incubation in the mixture of 16% hematocrit, where the efflux and influx of radiolabeled cholesterol between HDL and erythrocyte were both linear, and the rates were nearly the same in the absence of LCAT (Fig. 3). The net efflux of cellular cholesterol was, therefore, almost negligible. Cholesterol esterification by LCAT (0.6 μg of LCAT in the mixture, 20% of the plasma LCAT activity in the medium) caused enhancement of cellular cholesterol efflux and reduction of HDL-cholesterol influx (Fig. 3). Thus, a substantial amount of net cholesterol efflux was generated by LCAT from erythrocytes to HDL. On the other hand, cholesterol was exchanged between erythrocytes and LDL (50 μg of phospholipid and 20.3 μg of free cholesterol) without causing net efflux. The rates were approximately the same as those with HDL in the absence of LCAT (Fig. 4). LCAT hardly esterified cholesterol in LDL causing no change of cholesterol flux (Fig. 4).

The effect of LCAT reaction on cellular cholesterol efflux was also observed in long incubation in the mixture of 16% hematocrit (cellular cholesterol pool 40.4 μg) and HDL-free cholesterol pool (1.7 μg). In the absence of LCAT, the cholesterol efflux was rapid and the radiolabeled cholesterol efflux reached nearly steady state equilibrium with back influx in a few hours (Fig. 5A). The influx of HDL cholesterol is reciprocal to the cellular cholesterol efflux reaching the steady state in the same time course (Fig. 5C). When LCAT was present (20% of the plasma activity), the cellular cholesterol efflux was prominently increased particularly in the late phase, and the increment was as much as the cholesterol esterified (Fig. 5B). The influx of HDL cholesterol was decreased by the LCAT reaction, again more markedly in the late phase (Fig. 5C). As a consequence, the net efflux of cellular cholesterol was negligible in the absence of LCAT and was induced substantially by the LCAT reaction (Fig. 6). These data are in contrast to the previous observation with the cells in culture that there was no increase of cellular cholesterol efflux but only a significant increase of cellular cholesterol influx by LCAT (Ray et al., 1980; Fielding and Fielding, 1981; Czarnecka and Yokoyama, 1995).

We hypothesized that rate of the cellular cholesterol efflux is a determining factor for the direct effect of LCAT on the efflux. To test this hypothesis, the efflux rate was manipulated by changing the hematocrit of the incubation mixtures having a constant amount of HDL (50 μg of phospholipid and 14.0 μg of free cholesterol), and the effect of the LCAT reaction on the efflux was examined in each condition. Fig. 7 represents the reactions with the hematocrit of 1%, 5%, and 15%, in the presence and absence of 0.6 μg of LCAT (20% of its activity in plasma). The rate of esterification of HDL-originating cholesterol was approximately 4.7 nmol/h in this experimental condition being estimated from the data in Fig. 2 (percent esterification) and Fig. 3 (influx of HDL-free cholesterol).

With the lowest hematocrit of 1%, cellular free cholesterol was about 2.5 μg, and this was equivalent to that of many similar experiments using the cells in culture. In this condition, efflux rate of radiolabeled cholesterol was in the same order as the rate from the cells in culture. As shown in the top panels of Fig. 7, the labeled cholesterol in the medium almost linearly increased up to 24 h indicating that the efflux did not reach the steady state equilibrium with the back influx within this incubation time. There was no increase of the cholesterol efflux by cholesterol esterification by LCAT (the top right panel of Fig. 7). The rate of the labeled cholesterol efflux increased as the hematocrit increased. The efflux was reaching the steady state equilibrium with the back influx within this incubation time.

any of these experimental conditions (data not shown).

In the initial 1 h of the incubation of the same mixture with 16% hematocrit, the efflux and influx of radiolabeled cholesterol between HDL and erythrocyte were both linear, and the rates were nearly the same in the absence of LCAT (Fig. 3). The net efflux of cellular cholesterol was, therefore, almost negligible. Cholesterol esterification by LCAT (0.6 μg of LCAT in the mixture, 20% of the plasma LCAT activity in the medium) caused enhancement of cellular cholesterol efflux and reduction of HDL-cholesterol influx (Fig. 3). Thus, a substantial amount of net cholesterol efflux was generated by LCAT from erythrocytes to HDL. On the other hand, cholesterol was exchanged between erythrocytes and LDL (50 μg of phospholipid and 20.3 μg of free cholesterol) without causing net efflux. The rates were approximately the same as those with HDL in the absence of LCAT (Fig. 4). LCAT hardly esterified cholesterol in LDL causing no change of cholesterol flux (Fig. 4).

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equilibrium with the back influx within the same time range (the middle and lower panels of Fig. 7). The cholesterol esterification in HDL induced significant increases of the efflux in this condition (the lower right panel). The increment of the efflux by LCAT reached the same extent as the amount of esterified cholesterol with the 15% hematocrit.

Further analysis was attempted for the data with various cellular cholesterol pools including those shown in Fig. 8. The rate of the cellular cholesterol efflux was estimated from the initial rate of the radiolabeled cholesterol efflux from the erythrocytes (calculated as a least square linear regression of the efflux data for the initial 2 h). The top panel demonstrates that the rate of the cholesterol efflux from erythrocyte is almost directly proportional to the cellular cholesterol pool size in the incubation mixture, showing that the fractional efflux rate of cellular cholesterol (percent of the cellular cholesterol pool) was constant over the range of the experimental condition. The lower panel shows that such increases of the rate of the cholesterol efflux (the x axis) resulted in higher increases of the cholesterol efflux by cholesterol esterification in HDL (the y axis). When the hematocrit is above 15%, the rate of the cholesterol efflux (6.2 nmol/h) would be as high as the esterification rate (4.7 nmol/h) (estimated from the data in Figs. 2 and 7). The increment of the efflux by LCAT seemed to reach the same extent as that of cholesterol esterification in such a condition.

**DISCUSSION**

Erythrocyte in peripheral blood, including reticulocyte, was shown to have no interaction with lipid-free helical apolipoproteins to generate pre-β-HDL with the cellular lipids. Therefore, cholesterol transfer between erythrocytes and plasma lipoproteins is exclusively by a nonspecific exchange mechanism. Cholesterol efflux rate from erythrocytes to HDL depended directly on the cellular cholesterol pool size. Without LCAT reaction, the efflux and influx rates between erythrocyte and HDL were the same, and no net flux was generated. Net cholesterol efflux was induced when LCAT esterified cholesterol in HDL. When the efflux (exchange) was slower than cholesterol esterification, a direct increase of the efflux by LCAT was not apparent, and the net cholesterol efflux was caused mainly by the reduction of cholesterol influx from HDL to the cells. When the cholesterol exchange was faster than the esterification, it directly caused the increase of cellular cholesterol efflux.

Specific lack of the reactivity to lipid-free apolipoprotein in erythrocyte has provided additional evidence that apolipoprotein-mediated cellular lipid efflux is an independent pathway of nonspecific cholesterol exchange. Erythrocytes seem to lose the interaction with helical apolipoproteins perhaps during the maturation in an earlier stage than reticulocyte, and, accordingly, it does not provide a direct lipid source for generation of new HDL in blood. By using erythrocytes, therefore, cellular cholesterol efflux can be studied without the influence of the apolipoprotein-cell interaction. It is also possible to make the cellular cholesterol pool in the experimental incubation mixture much larger than that of the cultured cells by more than an order of the magnitude. Accordingly, this system simulates a physiological condition in the bloodstream where HDL and erythrocyte interact directly in solution. Using cells in culture may not exactly mimic an in vivo microenvironment for the interaction of lipoprotein with the cells in tissues, in terms of the local density of cell membrane and its cholesterol pool size.

**Fig. 4.** The initial phase of the cholesterol efflux from erythrocytes to LDL. ([3H]Cholesterol-labeled erythrocyte (16% hematocrit) was incubated with unlabeled LDL 20.3 μg of free cholesterol (50 μg of phospholipid) at 37 °C in the presence and absence of LCAT as 20% of its plasma activity (0.6 μg). The same experiment was done except for using [3H]cholesterol-labeled LDL and unlabeled erythrocyte to observe the influx of LDL-free cholesterol. Each data point represents an average of triplicate experiments with an error bar for the standard error. A and B represent the efflux experiments in the absence and presence of LCAT, respectively. The shadowed area represents free cholesterol in the medium. Squares and diamonds in C represent the influx of LDL-free cholesterol in the absence and presence of LCAT, respectively.

**Fig. 5.** Entire time course of cholesterol flux between erythrocytes and HDL and the effect of the LCAT reaction. ([3H]Cholesterol-labeled erythrocytes were incubated with [14C]cholesterol-labeled HDL in the presence and absence of LCAT. Hematocrit was 16%. HDL-free cholesterol was 1.7 μg, and the LCAT activity in the medium was 20% of its activity (0.6 μg) in plasma 250 μl of the incubation mixture. A and B represent the efflux of cellular cholesterol measured by the increase of [3H]cholesterol in the medium in the absence and presence of LCAT, respectively. The shadowed area represents free cholesterol, and the hatched area represents esterified cholesterol in the medium. C represents the influx of HDL cholesterol to the cells measured by the decrease of [14C]cholesterol in the medium. The diamonds and squares represent presence and absence of LCAT in the mixture, respectively. Each data point represents an average of the triplicate experiments with a standard error.
exposed to lipoproteins in the adjacent interstitial fluid.

LCAT has been known to play a role in regulation of the net cholesterol efflux from erythrocytes. In his classical work, Murphy (1962) demonstrated the decrease of cholesterol in erythrocytes by incubating with plasma. It is also known that cholesterol accumulates in a limited number of organs in the patient with genetic or secondary LCAT deficiencies (Glomset et al., 1983; Simon and Scheig, 1970), and erythrocyte is one of the primary organs where such cholesterol accumulation takes place. These data implicate that lipids of erythrocyte membrane undergoes the direct influence of plasma lipoprotein metabolism as one of the major cholesterol reservoirs in blood (Mindham and Mayes, 1991).

The results of this study showed that HDL removes cholesterol from erythrocytes only in the presence of LCAT. When cholesterol is esterified in HDL, the influx of cholesterol from HDL to the cell is reduced as the free cholesterol in HDL decreases. However, the efflux is not increased directly by the LCAT reaction when its rate is slower than the esterification (Czarnecka and Yokoyama, 1995). The off-rate of the cellular cholesterol efflux is presumably a rate-limiting step in such a condition so that additional room for free cholesterol in HDL created by the cholesterol esterification cannot immediately be filled with cell cholesterol due to the slow efflux. Thus, a condition such as 1% hematocrit is equivalent to most of the in vitro experiments using the cells in culture, with respect to the cellular cholesterol pool size and the rate of cellular cholesterol efflux. The rate of cellular cholesterol efflux is presumably a rate-limiting step in such a condition so that additional room for free cholesterol in HDL created by the cholesterol esterification cannot immediately be filled with cell cholesterol due to the slow efflux. Thus, a condition such as 1% hematocrit is equivalent to most of the in vitro experiments using the cells in culture, with respect to the cellular cholesterol pool size and the rate of cellular cholesterol efflux.

The top panel (A) shows a linear increase of the cellular cholesterol efflux rate without cholesterol esterification as a function of cellular cholesterol pool size. The rate was calculated as an average of the slope in the initial part of the time course of the radiolabeled cellular cholesterol efflux (up to 2 h) of at least four individual experimental points using the data presented in Fig. 7. The bottom panel (B) shows the increment of the efflux by cholesterol esterification (squares) and relative cholesterol esterification (as ester/free cholesterol) (circles) as a function of the rate of the cellular cholesterol efflux calculated in the top panel. The efflux increment and relative esterification were calculated by using the data of the incubation of 6 and 24 h presented in Fig. 7. The error bars represent standard error.

Fig. 6. Net cholesterol efflux from erythrocytes to HDL. The data were calculated from the experiment shown in Fig. 5, as the difference between the efflux and influx at each experimental point. Diamonds represent the data with LCAT, and squares represent the data without LCAT.

Fig. 7. The efflux of cellular cholesterol to HDL from various hematocrits of erythrocytes. HDL (14 μg of free cholesterol and 50 μg of phospholipid) was incubated with [3H]cholesterol-labeled erythrocytes as 1, 5, and 15% hematocrit (Ht). Cellular choline-phospholipid was 3.4 μg, 17.5 μg, and 52.7 μg, and cellular cholesterol was 2.5 μg, 12.6 μg, and 37.9 μg, respectively, in the incubation mixture. Each data point represents an average of the duplicate experiments. The shaded area represents free cholesterol, and the hatched area represents esterified cholesterol.

Fig. 8. Analysis of the relationship between erythrocyte cholesterol pool size and the rate of cholesterol efflux from erythrocytes to HDL, and of that among the efflux rate, the increment of the efflux, and relative cholesterol esterification in HDL. The error bars represent standard error.
cellular cholesterol when the rate of cholesterol exchange between HDL and the cells is faster than cholesterol esterification.

Cholesterol accumulation varies over various organs in the patients with genetic or secondary defects of LCAT, resulting in a pathological change in a limited number of the organs (Glomset et al., 1983; Simon and Scheig, 1970). From the conclusion of this study, it can be speculated that the tissues lacking in a free apolipoprotein-mediated cellular lipid efflux pathway are more severely affected by LCAT deficiency because the LCAT-dependent nonspecific pathway is the only available mechanism to remove cholesterol from such tissues. Erythrocyte, and perhaps cornea as well, seems to be such a case since these cells may not synthesize the new protein required for the cells to interact with helical apolipoproteins. Other tissues are capable of shedding cholesterol via the apolipoprotein-mediated pathway even without the help of LCAT. It is also conceivable that the tissues having a slow rate of cholesterol exchange with HDL may be less affected by the deficiency of LCAT since the contribution to net cell cholesterol removal of the LCAT-mediated efflux is small.

This paper demonstrated that LCAT actively regulates cholesterol content of erythrocytes in the bloodstream. The question is whether this conclusion can be extrapolated to other tissues where the average rate of cholesterol between the cells and HDL is yet to be determined. Recently, Francis et al. (1995) reported that fibroblasts from homozygous Tangier disease patients (genetic HDL deficiency) showed specific and complete defect of the apolipoprotein-mediated cellular cholesterol efflux pathway. Therefore, cholesterol removal from the peripheral tissues is carried out only by the LCAT-dependent nonspecific mechanism in these patients. Interestingly, cholesterol esterification in the plasma of these patients is not drastically reduced in spite of their extremely low plasma HDL level (Clifton-Bligh et al., 1972; Ohtaki et al., 1983), and the accumulation of cholesterol is limited only in certain organs related to the reticuloendothelial system (Schaefer et al., 1980). Therefore, the LCAT-mediated net cellular cholesterol removal seems active and efficient through nonspecific cholesterol exchange in the tissues of many organs. Thus, together with the finding in the LCAT deficiency, we conclude that the two distinct mechanisms to induce net cellular cholesterol efflux back each other up in most of the tissues.

A role of HDL in net cellular cholesterol efflux thus seems to be dual. One is to carry helical apolipoproteins that dissociate from HDL and interact directly with cellular surface to remove the cellular lipid by generating new pre-β-HDL. LCAT does not play a significant role in this pathway (Czarnecka and Yokoyama, 1995). The other role of HDL is to provide the site for LCAT reaction to induce the net cholesterol efflux by reducing the influx of cholesterol from HDL to the cells and by increasing the efflux of cellular cholesterol through nonspecific cholesterol exchange.

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