Role of Apolipoproteins in Cholesterol Efflux from Macrophages to Lipid Microemulsion: Proposal of a Putative Model for the Pre-β High-Density Lipoprotein Pathway†

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ABSTRACT: Lipid microemulsion of phospholipid and triglyceride with the size of low-density lipoprotein was capable of removing cholesterol from cholesterol-loaded mouse peritoneal macrophages, resulting in reduction of intracellularly accumulated cholesteryl ester. Apolipoproteins (apo) A-I, A-II, C-III, and E bound to the surface of the microemulsion did not modulate the interaction of the microemulsion with the cells in terms of the cholesterol efflux. The cholesterol removal by the microemulsion was enhanced by some 30% only when apoA-I, -A-II, and -E were present in excess to provide their free forms in the medium, but apoC-III did not show such an effect even by its excess amount. The kinetics including the results with apoC-III were consistent with a model that the apparent enhancement was due to generation of pre-β high-density lipoprotein (HDL)-like particles upon the interaction of free apolipoproteins with macrophages [Hara, H., & Yokoyama, S. (1991) *J. Biol. Chem. 266*, 3080–3086]. However, preβ-HDL-like particle was not detected after 6- and 24-h incubation in the medium where cholesterol efflux to the emulsion was maximally enhanced by the apolipoproteins, and cholesterol and phospholipids removed from the cells were all found with the microemulsions. It was also shown separately that the lipids in preβ-HDL-like particles generated by apoA-I and macrophages were rapidly, within the order of minutes, transferred to the apolipoprotein-covered microemulsions when they were incubated together. Thus, the data were consistent with a model that the free form of certain apolipoproteins, such as apoA-I, -A-II, and -E but not apoC-III, generates preβ-HDL-like particles with cellular lipids in situ and these particles act as mediators for cholesterol transfer from the cells to other lipoproteins.

High-density lipoproteins (HDL)[1] or reconstituted HDL-like particles are known to be potent cholesterol acceptors when cholesterol-loaded cells are exposed to these lipoproteins in the medium in vitro (Werb & Cohn, 1971; Stein & Stein, 1973; Brown et al., 1980; Daniels et al., 1981; Rothblat & Phillips, 1982). This is one of the supportive evidences for a hypothetical role of HDL in prevention or regression of atherosclerosis and considered to be the first step of so-called reverse cholesterol transport. Nonspecific free cholesterol exchange between the cell surface and HDL or HDL-like particle is considered as a main mechanism of this reaction (Rothblat & Phillips, 1982; Johnson et al., 1986, 1988; Karlin et al., 1987). However, the fact that cholesteryl ester accumulated in macrophages is almost depleted by exposing the cells to HDL solution repeatedly (Brown et al., 1980) may hint an additional and more "active" mechanism for cholesterol removal by HDL from macrophages. Involvement of specific protein–protein interaction has been proposed (Oram et al., 1983; Slotte et al., 1987; Aviram et al., 1988; Mendez et al., 1991) but remains somewhat controversial (Karlin et al., 1987; Tabas & Tall, 1988; Mendel & Kunitake, 1988). Excretion of cholesterol with apolipoprotein (apo) E may explain part of such a process in cholesterol-accumulated macrophages, but the reduction of the mass of cholesteryl ester by this mechanism is minimal (Basu et al., 1982). On the other hand, it was recently reported that cellular cholesterol appears in the "preβ-HDL" fraction in the very initial stage of the removal and then migrates to the other HDL fractions when fibroblasts are incubated with human plasma (Castro & Fielding, 1988). This approach may suggest the presence of a mediator for cholesterol transfer from the cells to lipoproteins.

We have shown that certain apolipoproteins in their free form in solution interact with cholesterol-loaded macrophages, generate HDL-like particles, and as a result reduce intracellularly accumulated cholesteryl ester (Hara & Yokoyama, 1991). The K_m values for these reactions are of the same order as those of K_d for the interaction of these apolipoproteins with phospholipid surface and more importantly as low as putative free apolipoprotein concentrations in plasma or in peripheral lymph. The HDL-like particles generated by this reaction were composed of free cholesterol, phospholipids, and apolipoproteins, and migrated as "preβ-HDL" in electrophoresis. The V_{max} values were 3–4-fold lower than that for cholesterol removal by HDL. Thus, this reaction is "one-way" removal of the cellular lipids and, therefore, may have physiological importance as the first step of cellular cholesterol removal in the peripheral tissues where the cell are exposed to the interstitial fluid rather than blood plasma. This may explain why cellular cholesterol appears in "preβ-HDL" prior to the other plasma lipoprotein fractions.

In this paper, we report further investigation about roles of apolipoproteins in cellular cholesterol efflux to the lipoproteins in the medium. As a model lipoprotein, we have used the lipid microemulsions of phosphatidylcholine and triolein with the

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[1] Abbreviations: HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); TLC, thin-layer chromatography.

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size of low-density lipoprotein (LDL) (Tajima et al., 1983). The advantages of the use of this particle are the following: (1) binding of various apolipoproteins to this particle was intensively studied, and therefore partitioning of free and bound forms of the proteins can be calculated for the various conditions using the binding parameters (Tajima et al., 1983; Yokoyama et al., 1985); (2) the gross structure of the particles is not altered by binding of apolipoproteins to the surface so that the function of apolipoproteins can be investigated without considering the change in particle structure (Tajima et al., 1983; Yokoyama et al., 1985). A putative role of "preβ-HDL"-like products from the interaction of free apolipoproteins with the cells is discussed in relation to the mechanism of cellular cholesterol removal by plasma lipoproteins.

Experimental Procedures

Lipoproteins, Apolipoproteins, and Lipid Microemulsions. Apolipoproteins (apo) A-I and A-II were isolated from the human plasma HDL fraction as described previously (Tajima et al., 1983). ApoE and apoC-III were isolated from the human plasma very-low-density lipoprotein fraction at the National Cardiovascular Center, Osaka, Japan, as described previously (Tajima et al., 1983; Yokoyama et al., 1985). Isolated apolipoproteins were lyophilized and stored at −70 °C in argon gas, and their aqueous solutions were prepared as previously described in a respective manner for each apolipoprotein (Tajima et al., 1983; Yokoyama et al., 1982, 1985). Total HDL was obtained from fresh human plasma as a fraction of density 1.09–1.21 g/mL. Lipid microemulsion with an average diameter of 26 nm was prepared from egg phosphatidylcholine (Avanti, Pelham, AZ) and triolein (Sigma, St. Louis, MO) with a starting weight ratio of 1:1 as previously described (Tajima et al., 1983). After sonication of the lyophilized lipids in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, the emulsion was isolated by ultracentrifugation at 99 000 rpm in a Beckman TL100 ultracentrifuge and by subsequent gel permeation chromatography in a Sepharose CL4B column. The fractions of the top of the inclusion peak of lipids were collected and concentrated by ultracentrifugation as the final preparation, where the average weight ratio of phosphatidylcholine to triolein was 0.85. The microemulsion solution was kept under argon gas at 4 °C and used within 1 week.

Loading Macrophages with Cholesteryl Ester. Low-density lipoprotein (LDL) was labeled with [1,2-^{3}H]cholesteryl oleate (45.4 Ci/mmol, purchased from Amersham, Canada) according to the method described elsewhere (Nishikawa et al., 1985; Hara & Yokoyama, 1991) and then acetylated (Basu et al., 1976; Hara & Yokoyama, 1991). Mouse peritoneal macrophages were prepared as described elsewhere from ICR mice (male or female, 20–30 g) (Hara & Yokoyama, 1991). The cells were collected by peritoneal lavage and dispensed into 3.5-cm plastic dishes [2×10^6 cells in 1 mL of RPMI 1640 medium (Flow Laboratories) per dish], and adhesive cells to the dish were obtained as the macrophage preparation (Hara & Yokoyama, 1991). The cells were incubated with the labeled acetylated LDL (50 μg as protein) in 1 mL of RPMI 1640 medium containing 2 mg/mL bovine serum albumin with or without 2 μCi/mL [*methyl*-^{3}H]choline chloride (15 Ci/mmol, Amersham) for 24 h and then incubated without lipoprotein for 24 h again (Hara & Yokoyama, 1991).

Cholesterol Removal from the Cells. The cholesterol-loaded cells were incubated with the lipid microemulsions, apolipoproteins, or both or with HDL in 1 mL of RPMI 1640 medium containing 2 mg/mL bovine serum albumin at 37 °C. The culture medium was removed after a certain period of incubation, centrifuged at 10 000 rpm for 2 min in a Beckman Microfuge to remove the cell components, and analyzed (Hara & Yokoyama, 1991). Total radioactivity of the medium was counted in a Beckman LS6000TA scintillation counter using liquid scintillator (Aqueous Counting Scintillant, code 196290). For some of the experiments, lipids were extracted from the medium by the method of Bligh and Dyer (1959), analyzed by thin-layer chromatography (TLC) to differentiate radioactivities in phospholipids and free and esterified cholesterol (Hara & Yokoyama, 1991). The medium was also analyzed by density gradient ultracentrifugation for the density 1.02–1.25 g/mL with sucrose as described previously for counting radioactivity or analysis by TLC of each density fraction (Hara & Yokoyama, 1991). The cells were washed, and the lipids were extracted with hexane/2-propanol (3:2 v/v) for the analysis by TLC. Specific radioactivity of cholesterol was determined for some experiments by measuring the amount of cholesterol by gas–liquid chromatography (Hara & Yokoyama, 1991). Choline-containing phospholipid and triglyceride in the culture medium were measured using an enzymatic assay kit for the respective lipid obtained from Wako Chemicals, Richmond, VA.

Transfer of Lipids. The culture medium obtained after incubation of the cholesterol-loaded macrophages (labeled with both cholesterol and choline) with 7.14×10^{-7} M apoA-I was incubated with the lipid microemulsion at the final concentration of 7.14×10^{-7} M apoA-I and 2.56×10^{-4} M phosphatidylcholine at 37 °C. After 30-min and 4-h incubation, the mixture was analyzed by density gradient ultracentrifugation. The radioactivity of cholesterol, phospholipids, and lysophospholipids was counted after the lipids were extracted and separated by TLC for each density fraction.

Binding Study of Apolipoproteins to the Lipid Microemulsion. ApoA-I and -A-II were incubated with the lipid microemulsion in the culture medium (RPMI 1640 containing 2 mg/mL bovine serum albumin and other necessary reagents for the cell culture, such as penicillin and streptomycin). After incubation at 37 °C for 30 min, 200 μL of the mixture was centrifuged in a Beckman TL100 ultracentrifuge using a TLA100 rotor for 1 h at 4 °C. Apolipoprotein concentration was measured for the top and bottom 100-μL fractions of each tube by a single radial immunodiffusion technique using an agarose gel plate containing specific antiserum for each apolipoprotein, purchased from Daiichi Pure Chemical Ind., Tokyo. Free protein background in the top fraction was calculated using the protein concentration in each bottom and a partitioning factor of the free apoA-I between the top and the bottom obtained from the results with the control solution without lipid ultracentrifuged at the same time (Tajima et al., 1983; Yokoyama et al., 1985). Lipid-bound protein was determined as previously described by subtracting the calculated free protein from the total protein in the top fraction (Tajima et al., 1983; Yokoyama et al., 1985). Binding parameters were calculated by least-square regression analysis of the linearized plot of the data (Tajima et al., 1983; Yokoyama et al., 1985).

Results

Cholesterol-loaded macrophages were incubated with the lipid microemulsion, 2.56×10^{-4} M (200 μg/mL) phosphatidylcholine, without apolipoproteins. During the course of incubation up to 30 h, free cholesterol in the medium continuously increased, and cellular cholesteryl ester reciprocally decreased (Figure 1). Approximately 25% of total cellular cholesterol was excreted into the medium at 30 h of incubation, which may account for some 5 μg of cholesterol efflux/dish based on the estimated specific radioactivity. The concen-

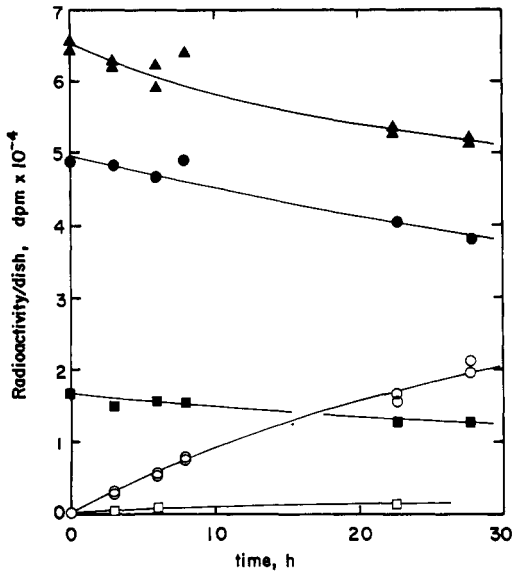

FIGURE 1: Efflux of cellular cholesterol by phosphatidylcholine/triolein microemulsions. Macrophages loaded with [^{3}H]cholesterol were exposed to the medium containing the lipid microemulsion with an average diameter of 26 nm, 2.56 × 10^{-4} M as phosphatidylcholine. Radioactivities in free cholesterol in the medium and in free and esterified cholesterol in the cells were determined. (○) Free cholesterol in the medium with the microemulsion; (□) free cholesterol in the medium without the emulsion; (▲) total cholesterol in the cells; (■ and ●) free and esterified cholesterol in the cells. Cell protein per dish was 120 ± 7 μg, and the specific radioactivity of cholesterol was estimated as approximately 4000 dpm/μg for total cellular cholesterol.

Table I: Binding Parameters of Apolipoproteins for Phosphatidylcholine/Triolein Microemulsions with a Diameter of 26 nm[a]

	apoA-I	apoA-II	apoC-III	apoE
K_d (×10^7 M)	1.6	2.5	5.3	11.8
B_{max} (×10^3 (mol of protein/mol of PC))	2.39	9.35	12.15	2.68

[a] These values represent the mean values published in previous papers (Tajima et al., 1983; Yokoyama et al., 1985) and have been used for calculation of partitioning of apolipoproteins between emulsion-bound and free forms in the culture medium.

tration of choline-containing phospholipid and triglyceride remained constant throughout the incubation time, showing that there is no significant fusion or uptake of the lipid emulsion by the cells relative to the amount present in the medium.

Binding of apolipoproteins to the microemulsion was intensively characterized in previous publications (Tajima et al., 1983; Yokoyama et al., 1985). The values of K_d and the maximum binding level (B_{max}) for equilibrium binding were accurately measured in the neutral sodium phosphate buffer containing 0.15 M NaCl in those studies (Table I) (Tajima et al., 1983; Yokoyama et al., 1985). In order to confirm that these parameters are valid in the culture medium, binding parameters of apoA-I and apoA-II to the lipid microemulsion were estimated in the culture medium used in this series of experiment. For apoA-I, K_d was (5.4 ± 6.4) × 10^{-7} M, and the maximum binding level (B_{max}) was (3.2 ± 0.3) × 10^{-3} mol of protein/mol of phospholipid, and for apoA-II K_d was (3.7 ± 1.6) × 10^{-7} M, and B_{max} was (7.3 ± 1.9) × 10^{-3} mol/mol. These values were in very good agreement with those listed in Table I considering the limit of accuracy of the method of single radial immunodiffusion used for this measurement. Therefore, the values listed in Table I were used for calculation of free and lipid-bound apolipoproteins in the culture medium.

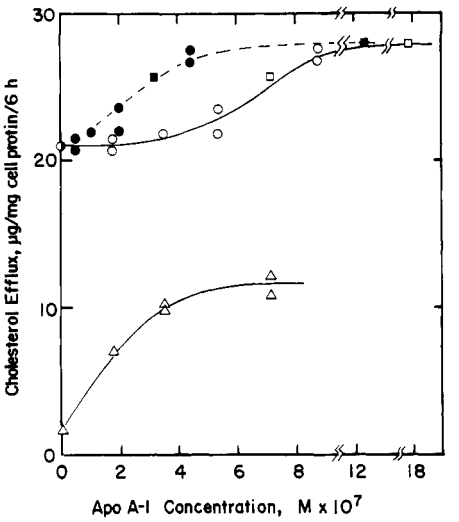

FIGURE 2: Effect of apoA-I concentration on cholesterol efflux to the lipid microemulsion from macrophages. The cholesterol-loaded cells were incubated with the lipid microemulsion, 2.56 × 10^{-4} M as phosphatidylcholine, in the presence of various amounts of apoA-I for 6 h at 37 °C, and cholesterol in the medium was measured using the radioactivity and specific radioactivity of cholesterol measured for a few representative points. The cholesterol efflux was measured in the absence of the lipid microemulsion at the same time as a control. (○ and □) Free cholesterol efflux as a function of total apoA-I concentration in the medium in the different series of experiments in the same condition; (● and ■) the same data plotted as a function of free apoA-I concentration in the mixture calculated using the binding parameters listed in Table I; (△) free cholesterol efflux by apoA-I without the lipid microemulsion. Cell protein per dish was 76 ± 6 μg.

Kinetics of cholesterol removal by the lipid microemulsion were examined in the absence of apolipoprotein and in the presence of apoA-I. The lipid microemulsion was preincubated with apoA-I, as 5.13 × 10^{-4} M phosphatidylcholine and 7.14 × 10^{-7} M apoA-I for 1 h at room temperature in the culture medium, where apoA-I binds to the emulsion to cover approximately 47% of the emulsion surface estimated according to the binding parameters in Table I, and then added to the cell culture. The microemulsion without apolipoproteins was also used in the same manner. The phosphatidylcholine concentration in the culture medium was 0 and (0.26–5.2) × 10^{-4} M where the non-lipid-bound apoA-I concentration was estimated from 1.4 × 10^{-8} to 1.4 × 10^{-7} M when it is present in this particular experimental condition. Radioactivity in the medium was determined after 6-h incubation at 37 °C. The kinetic profiles were almost identical regardless of the presence or absence of apoA-I, showing that apoA-I did not modulate the interaction of the microemulsion at all with respect to the cholesterol efflux. Apparent K_m and V_{max} values of the reaction calculated from the double-reciprocal plot of the data were (2.47 ± 0.31) × 10^{-5} M and 12 100 ± 620 dpm/6 h for the emulsion without apoA-I and (2.21 ± 0.28) × 10^{-5} M and 12 600 ± 490 dpm/6 h with apoA-I, being determined as the intercepts of the x and y axes using least-squares linear regression, respectively. The order of K_m in terms of the molarity of the microemulsion particles is on the order of 10^{-8}–10^{-9} M assuming 2600 phospholipids/particle. On the other hand, the K_m for cholesterol removal by HDL from macrophages is on the order of 10^{-7} M HDL particle molarity (data not shown), in good agreement with those estimated by using the published data for macrophages (Brown et al., 1980), fibroblasts (Daniels et al., 1981), and rat hepatoma cells (Karlin et al., 1987).

In the presence of a constant amount of the lipid microemulsion, the effect of various apolipoproteins on the rate of

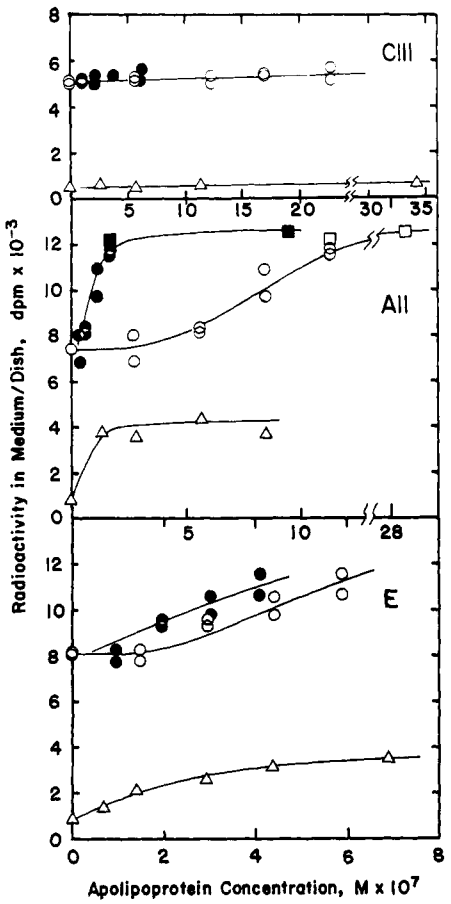

FIGURE 3: Effect of apoC-III, -A-II, and -E concentration on cholesterol efflux to the microemulsion from macrophages. Experimental procedures are the same as Figure 3. Symbols are the same; (○ and □) free cholesterol efflux as a function of total apolipoprotein concentration in the medium in the different series of experiments in the same condition; (● and ■) the same data plotted as a function of free apolipoprotein concentration in the medium calculated using the binding parameters listed in Table I; (△) free cholesterol efflux by free apolipoproteins without lipid microemulsion. Cell proteins per dish were 95 ± 12 μg for apoC-III experiments and 77 ± 9 μg for apoA-II and apoE.

cholesterol efflux was investigated. The concentratuion of the emulsion used was 2.56×10^{-4} M as phospholipids where the rate of the efflux is about reaching the V_{max}. Figure 2 shows the results with apoA-I (open symbols). Until the concentration of apoA-I reaches a certain point, the rate of the efflux was virtually constant. Then the rate increases as the apoA-I concentration increases and reaches the plateau where the rate is some 33% higher than that with the plain emulsion. The same data were then plotted against the calculated concentration of free apoA-I in the medium by using the binding parameters listed in Table I (closed symbols in Figure 3).[2] This curve is superimposable to the kinetics of cholesterol efflux induced by free apoA-I where preβ-HDL-like particles are generated in the medium [triangles in Figure 2; refer to Hara and Yokoyama (1991)], when the base line by the presence of the emulsion is subtracted.

The same type of analysis was carried out for the experiments with apoA-II, -C-III, and -E (Figure 3). The increase of the rate by apoA-II and -E appeared similar to that by

[2] The concentration of free apolipoprotein (P_f) is calculated using the equation $P_f = P_t - \{K_d + B_0 + P_t - [(K_d + B_0 + P_t)^2 - 4B_0P_t]^{1/2}\}(1/2)$, where P_t is the total protein concentration, K_d is a dissociation constant, and B_0 is the maximum binding level of the protein in the incubation mixture calculated as the product of B_{max} in Table I and phospholipid concentration.

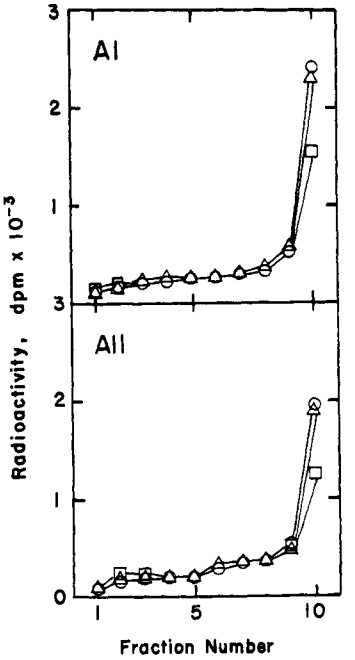

FIGURE 4: Density gradient ultracentrifugation analyses of the medium for distribution of cholesterol. The cholesterol-loaded macrophages (44 ± 3 μg of cell protein/dish) were incubated with the lipid microemulsion, 2.56×10^{-4} M phospholipid, in the preence of apoA-I [(□) 1.79×10^{-7} M; (△) 7.14×10^{-7} M; (○) 1.79×10^{-6} M] or apoA-II [(□) 2.81×10^{-7} M; (△) 1.24×10^{-6} M; (○) 2.81×10^{-6} M] for 6 h. At the highest concentration, 70% of apoA-I and 33% of apoA-II are calculated to be in the free form. The medium, 1 mL, was mixed with 0.5 mL of sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, and overlayered on the double layers of 65% sucrose (d = 1.31 g/mL), 0.5 mL, and 34% sucrose (d = 1.15 g/mL), 1.0 mL, in a quick-seal tube for a Beckman TLA 100.3 rotor. After centrifugation at 99 000 rpm in a Beckman TL100 ultracentrifuge for 1.5 h at 4 °C, the sample was collected from the bottom for 10 fractions. Densities: fraction 1 = 1.24 g/mL; 5 = 1.10 g/mL; 10 = 1.03 g/mL.

apoA-I (open symbols in each panel). The data are plotted against free apolipoproteins present in the mixture calculated from the binding constants in Table I (closed symbols in each panel), and the curves are superimposable to those with free apolipoprotein (triangles), respectively. There was no increase of the rate observed with apoC-III, which did not have a function of generating the HDL-like particle by interacting with the cell surface (Hara & Yokoyama, 1991). Thus, the increase of the rate of cholesterol efflux into the medium is likely to be due to the presence of free apolipoproteins in the mixture. The maximum rate reached was about 3 times higher than that with free apolipoproteins and as high as that with HDL.

By the direct interaction of free apolipoproteins in the solution with macrophages, preβ-HDL-like particles are generated with the cellular lipids (Hara & Yokoyama, 1991). The culture medium was examined for the presence of these particles by density gradient ultracentrifugation when the cholesterol efflux was presumably enhanced by the presence of free apolipoprotein in the medium. Figure 4 shows the distribution of cholesterol in the medium when various amount of apolipoproteins were present in the medium with the microemulsion after 6-h incubation with the cells. Even with great excess of apolipoproteins, when 70% of apoA-I and 33% of apoA-II were free form, all cholesterol radioactivity was detected with the microemulsion in the top fractions. The results were the same after 24-h incubation with the cells.

In order to observe directly the interaction of the microemulsion and the preβ-HDL-like particles generated by the

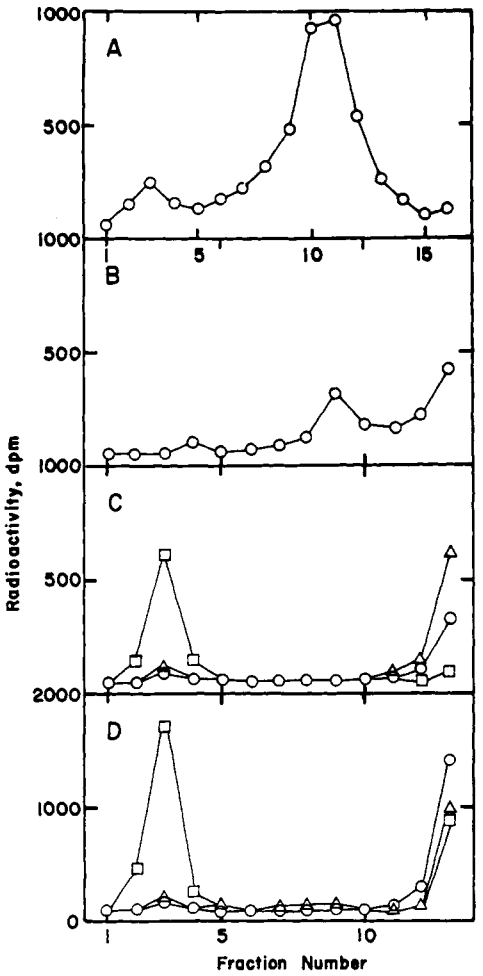

FIGURE 5: Transfer of lipids from HDL-like particles generated by apoA-I and macrophage to the lipid microemulsions. The macrophages preloaded with [^{3}H]cholesterol and prelabeled with [^{3}H]choline were incubated with 7.14×10^{-7} M apoA-I for 6 h to generate HDL-like particles in the medium. The medium containing such particles labeled with [^{3}H]cholesterol and [^{3}H]choline-containing phospholipids was removed and then mixed with lipid microemulsion preincubated with apoA-I and incubated at 37 °C for 30 min and 4 h. The final concentration in this mixture of phosphatidylcholine was 2.56×10^{-4} M and apoA-I 7.14×10^{-7} M. The mixture was analyzed by density gradient ultracentrifugation between 1.02 and 1.25 g/mL as described in the legend for Figure 4. (A) A control experiment: The culture medium containing the HDL-like particle before incubation with the lipid emulsion, 1.5 mL, was analyzed by density gradient centrifugation and fractionated into 16 fractions. Radioactivity of each fraction was directly counted without separation by TLC. (B) Radioactivity of cholesterol in density fractions after the medium was incubated with the emulsion for 30 min. The sample was fractionated into 13 fractions. For each density fraction, lipids were extracted and analyzed by TLC for separation of cholesterol from phospholipids. (C) The sample after 4-h incubation. Radioactivities in cholesterol (○), phosphatidylcholine + sphingomyelin (△), and lysolecithin (□) were determined after the lipids were extracted and separated by TLC. (D) The analysis of culture medium of macrophages after incubation of the cell with both the lipid microemulsion, 2.56×10^{-4} M phosphatidylcholine, and 7.14×10^{-7} M apoA-I for 6 h. The medium was directly analyzed by density gradient ultracentrifugation. Lipids were extracted from each density fraction and then analyzed by TLC. Symbols are the same as for panel C.

reaction of apolipoprotein with the cells, the culture medium of incubation of the cells with free apoA-I for 6 h was removed and then incubated with the microemulsion at 37 °C (Figure 5). After 30-min incubation, most of the cholesterol radioactivity was already transferred to the emulsion, and almost all cholesterol and phospholipids (phosphatidylcholine + sphingomyelin) were transferred to the emulsion fraction after 4-h incubation. Thus, the lipids in preβ-HDL-like particles were shown to be rapidly transferred to model lipoprotein particles.

Discussion

Lipid microemulsion composed of phosphatidylcholine and triolein with the diameter of LDL was used as an acceptor of cellular cholesterol in order to study the role of apolipoproteins in the reaction. As we mentioned in the introduction, the advantage of the emulsions over the use of phospholipid unilamellar vesicles is that the emulsions are more stable with regard to structure when apolipoproteins bind to their surface (Tajima et al., 1983; Yokoyama et al., 1985). The functions of apolipoprotein can be investigated without considering the factor of changing the gross structure of lipoprotein particles. It is also important that binding of apolipoproteins (A-I, A-II, C-II, C-III, and E) has been intensively characterized for this emulsion (Tajima et al., 1983; Yokoyama et al., 1985) and for the emulsions containing other lipids as well (Okabe et al., 1988). The necessary binding parameters are all available to calculate partitioning of the proteins between free and bound forms in a given condition (Tajima et al., 1984; Funahashi et al., 1989; Milner et al., 1991).

The microemulsion was shown to be a potent acceptor of cellular cholesterol when it was exposed to the macrophages loaded with cholesterol. The apparent K_m value of this reaction was as low as 10^{-8}–10^{-9} M in terms of the particle concentration, suggesting the lipid particle with phosphatidylcholine membrane surface has a high affinity to interact with macrophage cell membrane in terms of the free cholesterol efflux. This result agrees with the previous finding that cholesterol is exchanged between the cell surface and lipoprotein by a passive diffusion mechanism being dependent upon cholesterol content in each lipid surface and other factors (Lange et al., 1980; Rothblat & Phillips, 1982; Yeagle, 1985; Yeagle & Young, 1986). Since the microemulsion used in this study has no cholesterol, the reaction resulted in net efflux of cholesterol from the cell (Rothblat & Phillips, 1982) and a subsequent shift of equilibrium between free and esterified cholesterol in the cells (Brown et al., 1980). Even after 30-h incubation, cholesterol/phospholipid in the emulsion was as little as 1:40 in weight ratio (1:20 in molar ratio), which is far below the equilibrium with plasma membrane. Therefore, back-flow of cholesterol from the medium to the cell was negligible in this condition. The effect of apoE secreted by macrophages (Basu et al., 1982) may become the factor to be considered for this apparent high affinity. However, the concentration of apoE in the medium is estimated lower than 10^{-8} M (Hara & Yokoyama, 1991) so that the majority of the emulsion particles are free from apoE in this experimental condition (Yokoyama, 1990). Thus, the gross effect of apoE on the affinity of the emulsions for macrophages is unlikely to be observed in such a condition. The value of V_{max} was as much as $^1/_2$–$^2/_3$ of the V_{max} with HDL. None of apoA-I, -A-II, -C-III, and -E influenced the rate of cellular cholesterol removal by the emulsion when most of those proteins were present bound to the surface of the emulsion. Values of the apparent K_m and V_{max} obtained for the lipid particles with some 47% of its available surface covered by apoA-I were identical to those without any apolipoproteins. Thus, it is unlikely that any specific interaction is induced between the lipid particles and the cell surface by the presence of these apolipoproteins on the lipid particle surface, in terms of cholesterol efflux reaction from the cell.

However, the rate of efflux was enhanced by some 30% as increasing apoA-I, -A-II, and -E to such a concentration that provides the free apolipoproteins in the mixture, and it reached

the rate equivalent to that of V_{max} for HDL. The data of cellular cholesterol efflux were analyzed as a function of free apolipoproteins present in the mixtures calculated using the binding parameters previously determined for each apolipoprotein (Tajima et al., 1983; Yokoyama et al., 1985). The kinetics became almost superimposable to those for the direct interaction of these apolipoproteins in free form with macrophages in which preβ-HDL-like lipoproteins were generated in situ with cellular lipids. On the other hand, no increase of the cholesterol efflux rate was observed with apoC-III even in its excessive amount. This result agrees with the fact that apoC-III does not generate preβ-HDL-like particles upon interaction with macrophages. Thus, it is rational to postulate that free apoA-I, -A-II, and -E in the mixture are responsible for enhancing the cellular cholesterol efflux.

Although these effects seemed related to generation of preβ-HDL-like lipoproteins, these particles were not detected in the medium of several hours incubation, and all cholesterol originating in the cell was recovered with the emulsion fraction even when the efflux was maximally enhanced by apoA-I and -A-II. This result suggests that these lipids may not stay with these particles long enough to be detected in the presence of the lipid microemulsions. In fact, the radioactive cholesterol and phospholipids in the particle were all transferred to the emulsion within a short period of time when the preβ-HDL-like particle generated by apoA-I and macrophage was incubated with the lipid microemulsion. The half-life of the particle was estimated to be less than 10 min. Thus, preβ-HDL-like particles newly generated by free apolipoproteins with the cellular lipids last only for a short time and may act as mediators for the transfer of cellular lipids to lipid microemulsion covered with apolipoproteins.

Castro and Fielding have shown that cellular cholesterol appears in the preβ-HDL fraction in the very initial phase of the efflux from the cells and then is transferred to other lipoproteins when fibroblasts are incubated with the whole human plasma (Castro & Fielding, 1988). It is reasonable to assume that generation of preβ-HDL may also be possible in the system of fibroblast and plasma because the K_m value of the free apoA-I concentration for generating preβ-HDL-like particles is as low as $^1/_{400}$th of the plasma apoA-I concentration and may be consistent with the free apoA-I concentration in plasma or peripheral lymph (Hara & Yokoyama, 1991). This may be the reason why cellular cholesterol appeared in the preβ-HDL fraction first: it is not a cholesterol acceptor but is generated from cellular lipid and apolipoproteins. Thus, the data presented in the current paper are consistent with the data shown in their experiments. In order to provide further evidence, detection of such a mediator particle in the medium with the emulsion and apolipoproteins is required. Since the life of the mediator seems very short and its amount in the steady state seems very small, pulse–chase experiments with radiolabeled lipids may only demonstrate the presence of such a particle (Castro & Fielding, 1988). However, it was necessary to increase the specific radioactivity of cellular cholesterol to 10^8 dpm/μg (Castro & Fielding, 1988), which is higher than the level used in the current study by the factor of 10^4. We had difficulty to raise it to such a high level because we used radiolabeled cholesteryl ester rather than radiolabeled cholesterol to label the cells in order to avoid an artifact of having a plastic culture dish labeled (Hara & Yokoyama, 1991).

The mechanism of the transfer of lipids from preβ-HDL-like particles to apolipoprotein/lipid emulsion particles is not known. It could be explained by a collision and/or fusion process resulting in simple exchange of lipids because the lipid pool size of preβ-HDL is almost negligible compared to the emulsion. The chemical composition of preβ-HDL-like particles generated by macrophages and apoA-I or -A-II was somewhat unusual even though we may admit sizable errors in its estimation; relatively large amounts of cholesterol are found compared to that of phospholipids (Hara & Yokoyama, 1991). These particles seem less stable than plasma lipoproteins because they readily bind to the surface of polyallomer ultracentrifuge tubes or Teflon tubes in high salt concentration so that we had to use sucrose to isolate the particles (Hara & Yokoyama, 1991). Therefore, the fusion of the particles to the surface of the lipid emulsion may be considered as one of the possible mechanisms for the transfer of the lipids. The apparent high K_m value for the cholesterol efflux reaction to HDL relatively to that with the lipid microemulsion may represent integrated kinetics of the lipoprotein particles and free apolipoproteins in HDL preparations.

Generation of preβ-HDL-like particles is a process of pure net lipid efflux from cells whereas cholesterol flux between lipoprotein and the cell surface is bidirectional nearly in equilibrium (Rothblat & Phillips, 1982; Johnson et al., 1986, 1988; Karlin et al., 1987). In order to establish the role of generation of this particle in net cellular lipid removal by certain lipoprotein subclasses, the use of a microemulsion containing cholesterol in equilibrium with cell surface would provide us with more specific information in the future experiments.

Consequently, we may conclude that (1) apolipoproteins do not seem to modulate the lipoprotein/cellular interaction directly in terms of cellular lipid efflux; (2) free apolipoproteins generate preβ-HDL-like lipoproteins which mediate the efflux of cellular lipids to other lipoproteins, and (3) this proposed mechanism is consistent with the role of preβ-HDL in the initial phase of cellular cholesterol efflux. Although this is a part of the mechanism of cellular lipid efflux, more han half of the efflux still seems to be due to simple exchange of lipids between the cell membrane and lipoprotein surface because the lipid microemulsion itself was shown to be a very potent cellular cholesterol acceptor in our experiments.

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Association and Metabolism of Exogenously-Derived Lysophosphatidylcholine by Cultured Mammalian Cells: Kinetics and Mechanisms

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ABSTRACT: The association and metabolism of exogenously-derived lysophosphatidylcholine (lysoPC) with cultured mammalian cells from a variety of sources was studied, and a mechanism was defined by computer modeling for Vero cells. Cell monolayers were incubated with radiolabeled lysoPC, and the kinetics of disappearance from the medium, association with the cells, and metabolism by the cells of lysoPC were monitored both in the absence and in the presence of fetal bovine serum. Exogenously-supplied lysoPC first associated with cell membranes, followed by an almost complete conversion to phosphatidylcholine (PC). The kinetics of partitioning and metabolism were identical regardless of whether the exogenously-supplied lysoPC was labeled with [*methyl*-^{3}H]choline or with [1-^{14}C]palmitate. A two-step mechanism, consisting of a reversible partitioning of exogenous lysoPC into the cell membrane followed by enzymatic reacylation to PC, was found to adequately describe the observed kinetics in the presence of 0 or 0.5% fetal bovine serum. The effect of temperature on the individual rate constants and on the overall process was examined. An Arrhenius plot indicated an acute temperature sensitivity between 15 and 23 °C, consistent with a dependence on the lipid phase of the membrane and a regional phase transition temperature characteristic of mammalian cells. The acute temperature sensitivity was almost entirely due to the temperature dependence of reacylation. A multistep mechanism was established by combining the kinetic constants determined under conditions of low exogenous protein with the binding constant between lysoPC and serum protein. This mechanism accurately predicts the rates of uptake of exogenously-derived lysoPC with cultured cells in the presence of serum concentrations between 0 and 10%. A survey of a variety of cultured cells indicated that the kinetics of association and metabolism of exogenously-derived lysoPC is cell-type specific.

Phosphatidylcholine (PC) is the major phospholipid component of cellular membranes. It is an important substrate for hormone-activated phospholipases. Moreover, lysophosphatidylcholine (lysoPC) has been shown to be a stereospecific chemoattractant for mouse lymphoblastic cells (Hoffman et al., 1982) and for human monocytes (Quinn et al., 1988), and it has been postulated that lysoPC may be generated at sites of inflammation, tissue injury, and repair (Hoffman et al., 1982; Quinn et al., 1988). In addition, one causative agent of schistosomiasis, the parasite *Schistosoma mansoni*, uses lysoPC to lyse adherent human red blood cells

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