Effect of High Density Lipoproteins Modified with Dilinoleoylphosphatidylcholine Vesicles on [³H]Cholesterol Release from Macrophages

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Summary High density lipoprotein 3 ($\text{HDL}_3=1.12 < d < 1.21$) was modified with dilinoleoylphosphatidylcholine, and the effects of these modified HDL_3 on the removal of [³H]cholesterol from rat peritoneal macrophages were studied. On incubation of HDL_3 with polyenephosphatidylcholine (PPC) vesicles, phospholipid-rich high density lipoproteins ($1.18 < d < 1.24$) were formed. The apolipoprotein AI (apo AI) and AII (apo AII) contents of these modified HDL_3 were about 3 to 40% of those of native HDL_3 . Removal of [³H]cholesterol from macrophages was investigated using rat peritoneal macrophages prelabeled with [³H]cholesterol emulsion or with [³H]cholesterol oleate-labeled acetylated low density lipoproteins ($1.019 < d < 1.063$). The modified HDL_3 s facilitated the removal of [³H]cholesterol from macrophages. The extent of removal of [³H]cholesterol from macrophages was higher with modified HDL_3 than with native HDL_3 , and among modified HDL_3 subfractions, fractions IV and V, whose ratio of apo AI to phospholipid (AI/PL) were 0.44 and 0.58, respectively, were more efficient [³H]cholesterol acceptors than native HDL_3 (AI/PL=1.46). These results indicate that the effectiveness of HDL_3 in removing [³H]cholesterol from macrophages was enhanced by modification with PPC.

Key Words: dilinoleoylphosphatidylcholine, high density lipoprotein, macrophages, apo AI, cholesterol release

Intracellular cholesterol ester contents are regulated by incorporation of cholesterol ester associated with low density lipoproteins (LDL, $1.019 < d < 1.063$),

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synthesis of cholesterol in the cells, and its efflux from the cells [1–4]. High density lipoproteins (HDL, $1.063 < d < 1.24$) are thought to remove cholesterol from cells [4], and their capacity to do this is regulated by their protein and lipid compositions [5]. Epidemiological studies have shown that the plasma level of HDL is negatively correlated with the incidence of atherosclerotic cardiovascular disease [6]. Thus, treatment that increases in the HDL level may prevent cardiovascular disease. HDL can be modified by incubation with polyenephosphatidylcholine (PPC), which results in a change in the fluidity of the lipoprotein [7].

In this work, the effects of various phospholipid vesicles and apolipoprotein AI, CI, and CIII on the release of [^3H]cholesterol from macrophages were studied. In addition, modified HDL₃ were prepared by incubation of HDL₃ with polyenephosphatidylcholine vesicles, and the effectiveness of these modified HDL₃ in the removal of [^3H]cholesterol from rat peritoneal macrophages was compared with that of native HDL. Furthermore, the effect of modified HDL on the release of [^3H]cholesterol from rat peritoneal macrophages that had been incubated with [^3H]cholesterol oleate-labeled acetylated LDL was determined.

MATERIALS AND METHODS

Materials. [^3H]Cholesterol (specific activity = $142 \mu\text{Ci/mol}$) and [^3H]cholesterol oleate (SA, $120 \mu\text{Ci/mol}$) were purchased from New England Nuclear, Boston. Dipalmitoylphosphatidylcholine was obtained from Sigma Co., Ltd., St. Louis. Polyenephosphatidylcholine was provided by Natterman (West Germany); it contains lecithin, more than 80% of which was dilinoleoyl-sn-glycerophosphocholine from soybeans.

Preparation of lipoproteins. Total high density lipoproteins (HDL, $d = 1.063$ – 1.210 g/ml) were isolated from the plasma of normal fasting individuals by ultracentrifugation in a solution of KBr. Samples were centrifuged in a type 42 rotor operated at 48,000 rpm for 22 h. Lipoproteins were dialyzed against standard buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.9% NaCl. Total HDL were further fractionated into HDL₂ and HDL₃ by zonal ultracentrifugation in a gradient of NaBr ($d = 1.00$ – 1.40 g/ml) as described by Patsch *et al.* [8]. Zonal ultracentrifugation was performed at 41,000 rpm and 10°C for 22 h in a Beckman Ti-14 zonal rotor. The density of lipoproteins was defined by the density at that point in the gradient, where each lipoprotein was eluted under the above conditions of zonal ultracentrifugation.

Preparation of dipalmitoylphosphatidylcholine (DPPC) vesicles and polyenephosphatidylcholine (PPC) vesicles. DPPC or PPC vesicles (20 mg) in chloroform were evaporated under N_2 gas, and 5 ml of standard buffer was added. The mixture was sonicated for 15 min in an ultrasonic apparatus (Tomi, Tokyo).

Preparation of apolipoproteins AI (apo AI), CI (apo CI), and CIII (apo CIII). Apo AI was purified from HDL by gel filtration by the method of Jackson and Gotto [9]. Apo CI and apo CIII were prepared by the method of Brown *et al.* [10].

Preparation of [^3H]cholesterol-laden macrophages. Resident rat peritoneal macrophages were harvested from male Wistar-King strain rats in phosphate-buffered saline as described by Brown *et al.* [11]. Usually the peritoneal fluids from 4 rats were combined and centrifuged at $600 \times g$ for 10 min. The cells were then suspended in Dulbecco-modified Eagle (DME) medium containing 2% bovine serum albumin (Sigma, Fraction V) and were dispensed into 4 dishes (50×50 mm) and incubated in a humidified CO_2 (5%)-incubator at 37°C . After incubation for 1 h, nonadherent cells were removed by washing the monolayers twice with DME medium. [^3H]Cholesterol (100 μCi), as a microcrystalline suspension in 0.9% NaCl prepared by the method of Stein and Stein [12], was added, and incubation was carried out for 24 h. After incubation, the cells were washed five times with DME, suspended in DME containing 2% bovine serum albumin and dispensed into dishes (25×25 mm) at 2×10^6 cells/dish.

Preparation of modified HDL. The HDL_3 fraction (40 mg phospholipid) obtained by zonal ultracentrifugation was incubated with PPC vesicles (40 mg) containing 20% deoxycholate in 50 ml of standard buffer consisting of 0.9% NaCl and 10 mM Tris-HCl (pH 7.4), for 24 h at 37°C , and then dialyzed against standard buffer 3 times for 24 h. This sample was used as modified HDL_3 in the experiments. For subfractionation of modified HDL_3 , the mixture obtained as described above was subjected to zonal ultracentrifugal analysis in a gradient of NaBr ($d=1,000$ – $1,300$ g/ml) and the collected fractions were divided into five groups. Each group was concentrated with an Amicon and dialyzed against the standard buffer.

Preparation of [^3H]cholesterol oleate-labeled acetylated LDL and determination of hydrolyzed [^3H]cholesterol. LDL was labeled with [^3H]cholesterol oleate by the method of Krieger *et al.* [13], and then acetylated with acetic anhydride by the method of Brown *et al.* [11]. Hydrolyzed [^3H]cholesterol from [^3H]cholesterol oleate was analyzed as follows: After the incubation of [^3H]cholesterol oleate-labeled acetylated LDL with macrophages, Folch's lipid extraction mixture [14] was added to the medium or cells. These extracted lipids were subjected to thin layer chromatography (Silica Gel 60 F_{254} , Merck, Darmstadt) using a solvent system of petroleum ether/diethylether/acetic acid, 90/10/1 by volume.

Incubation of [^3H]cholesterol-laden macrophages with phospholipid vesicles of HDL_3 or modified HDL_3 . [^3H]Cholesterol-laden macrophages (2×10^6 /dish) were incubated in 2 ml of DME medium containing 2% bovine serum albumin and indicated amounts of apolipoproteins, phospholipid vesicles, HDL_3 or modified HDL_3 . After incubation for 6 h in CO_2 incubator, the radioactivity in the medium was counted with a scintillation counter.

Other assay methods. Protein was determined by the method of Lowry *et al.* [15] using bovine serum albumin as standard. Phospholipid phosphorus was analyzed by the method of Bartlett [16]. Total cholesterol was determined with cholesterol ester hydrolase, cholesterol oxidase, and peroxidase [17]. Free cholesterol was determined with cholesterol oxidase and peroxidase [17]. Esterified cholesterol was determined by subtracting free cholesterol from total cholesterol. Triglyceride

was determined with lipoprotein lipase, glycerol dehydrogenase, and diaphorase [18]. Apo AI and apo AII concentrations were determined by the single radial immunodiffusion method with kits provided by Eiken Co., Tokyo.

RESULTS

Effects of apolipoproteins AI, CI, and CIII on the release of [³H]cholesterol from macrophages

Addition of apo CI, CIII, or AI to the medium did not significantly increase the release of [³H]cholesterol from macrophages compared with that in the absence of these apolipoproteins, as shown in Fig. 1A. PPC vesicles, which have a transition temperature less than that of DPPC vesicles (42°C), had higher activity for releasing [³H]cholesterol than did DPPC vesicles. Addition of the apolipoprotein CI, CII, or AI in the presence of DPPC vesicles (Fig. 1B) or PPC vesicles (Fig. 1C) increased the release over that with DPPC or PPC vesicles alone, the effects being more than additive (Figs. 1B and 1C).

Zonal ultracentrifugal analysis after incubation of HDL₃ and PPC vesicles

Incubation of HDL₃ with PPC vesicles resulted in the appearance of new lipoprotein complex fractions at densities of 1.14 to 1.28. These were collected in five fractions, as shown in Fig. 2, and the lipid compositions and apolipoprotein

Fig. 1. Effects of apolipoproteins on the release of [³H]cholesterol from macrophages in the presence or absence of DPPC or PPC vesicles. A, [³H]cholesterol-laden macrophages (2×10^6 cells) were incubated with apo AI (20 μg), CI (20 μg) or CIII (20 μg) in 1.0 ml of DME medium containing 2% bovine serum albumin. At the indicated times, the cells and medium were harvested and centrifuged and radioactivity in the supernatant was measured. B and C, same as A, but with addition of DPPC vesicles (200 μg) and PPC vesicles (200 μg), respectively. The values for percent release are means of triplicate. Standard deviation was less than 5%.

Fig. 2. Zonal ultracentrifugation of HDL₃ and PPC liposomes incubated with HDL₃. A, HDL₃ (40 mg PL) was subjected to zonal ultracentrifugation. B, HDL₃ (40 mg PL) was incubated with 40 mg phospholipid of PPC vesicle suspension containing 12 mg of deoxycholic acid in 50 ml of medium containing 0.9% NaCl, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA for 24 h. Then the incubation media were subjected to zonal ultracentrifugal analysis in a linear gradient of $d=1.000$ to $d=1.4$.

Table 1. Compositions of modified HDL₃s.

	Fraction No.					HDL ₃
	I	II	III	IV	V	
Density (g/ml)	1.14	1.18	1.22	1.26	1.28	1.23
Protein (%)	12	16	21	33	42	52
Esterified cholesterol	14	18	10	4.0	2.3	14.6
Cholesterol	5.1	3.7	3.7	1.8	1.2	3.6
Triglyceride	8.3	10.2	8.1	4.7	2.3	5.7
Phospholipid	61	51	58	56	52	24
Apo AI (μ g/mg phospholipid)	35	250	253	445	580	1,461
Apo AII	9	58	91	148	200	487

All determinations were performed in triplicate. Standard deviation was less than 6%.

concentrations of each fraction were measured, with the results shown in Table 1. The protein contents of the fractions were lower, and their phospholipid contents higher, than those of native VDL₃. The amount of protein decreased, but the cholesterol ester, cholesterol, and triglyceride contents increased, with decreasing

density of the fraction. The contents of apolipoproteins AI and AII were very low: even in the highest density fraction, the contents of AI and AII were about 30% of those in native HDL₃, while in the lowest density fraction, the apo AI and AII contents were about 3% of those of native HDL₃.

[³H]Cholesterol releasing activity of modified HDL₃

The [³H]cholesterol-releasing activities of various HDL and PPC vesicles were studied. As shown in Fig. 3, the amount of [³H]cholesterol released from macrophages increased with increasing phospholipid content of each type of complex. The amount released increased up to 200 μ g of added phospholipid and then reached a plateau. At more than 300 μ g of phospholipid, the modified HDL₃ was more active than native HDL₃. PPC vesicles showed less releasing activity than HDL₃. These results indicate that the ability of HDL₃ to effect removal of [³H]-cholesterol from macrophages was increased by modification of the lipoprotein with PPC vesicles. Next, the effects of subfractions of modified HDL₃ were examined. As shown in Table 2, fractions IV and V showed higher activities than fractions I, II, and III. Compared with the native HDL₃, fraction I was less active, but fractions IV and V were much more active based on an equal phospholipid content (400 μ g) of each complex.

Release of [³H]cholesterol from macrophages incubated with [³H]cholesterol oleate-labeled acetylated LDL

Denatured LDL is incorporated into macrophages as reported by Brown *et al.*

Fig. 3. Effects of HDL₃, modified HDL₃, and PPC vesicles on release of [³H]cholesterol from macrophages loaded with [³H]cholesterol. Macrophages from rat peritoneal cavity were loaded with [³H]cholesterol previously according to the method described in "Materials and Methods." The macrophages (2×10^5 cells) were dispensed into dishes (25×25 mm) containing 1 ml of 2% bovine serum albumin and DME medium, and indicated amounts of PPC vesicles, HDL₃, or modified HDL₃ in terms of phospholipid were added. Incubation was performed for 4 h, and the radioactivity in the medium was counted.

Table 2. [^3H]Cholesterol release from [^3H]cholesterol-laden macrophages in the presence of various HDLs.

Fraction	[^3H]Cholesterol release into medium ($\times 10^5$ dpm/400 μg phospholipid)
Native HDL ₃	1.98 $\pm$ 0.21
Modified HDL (Total)	2.62 $\pm$ 0.31
PPC	1.44 $\pm$ 0.18
Modified HDL ₃	
Fraction I	1.62 $\pm$ 0.16
II	2.02 $\pm$ 0.12
III	2.22 $\pm$ 0.22
IV	2.88 $\pm$ 0.20
V	2.64 $\pm$ 0.15

Rat peritoneal macrophages (2×10^5 cells) prelabeled with [^3H]cholesterol (2×10^6 dpm) were dispensed in dishes (25×25 mm) containing 2 ml of DME medium and 2% bovine serum albumin, and 400 μg phospholipid of indicated lipoprotein complexes or PPC vesicles were added. After incubation for 4 h, the radioactivity in the medium was counted. Values are mean $\pm$ SD of triplicate.

[11]. Therefore, we next examined the removal of [^3H]cholesterol from macrophages that had taken up [^3H]cholesterol oleate-labeled acetylated LDL. In the preparation of acetylated LDL, 98% of the [^3H]cholesterol was esterified, but 64% of that in macrophages was esterified, indicating that the incorporated LDL was degraded by acid cholesterol esterase in the macrophages. After incubation of labeled macrophages with modified HDL₃, 83% of the radioactivity of free [^3H]cholesterol was found in the medium, indicating that on hydrolysis of acetylated LDL-[^3H]cholesterol oleate in macrophages, [^3H]cholesterol was released into the medium.

Release of [^3H]cholesterol by the macrophages from [^3H]cholesterol oleate in acetylated LDL was enhanced by the addition of HDL₃ (Fig. 4). When the same amounts of phospholipid (400 μg) of HDL₃ and modified HDL₃ were added, the [^3H]cholesterol release was greater in the presence of modified HDL than in that of native HDL₃, as shown in Fig. 4.

DISCUSSION

Jackson *et al.* [5] reported that apo AI, AII, or apo CI, II, III alone was ineffective for removing cellular [^3H]cholesterol from Landschütz ascites cells, but that the cholesterol removal was enhanced when phosphatidylcholine vesicles were added with these apoproteins. Our results on the effects of apo AI, CI, and CIII using rat peritoneal macrophages were essentially the same as theirs. As for the role of lipid fluidity in [^3H]cholesterol removal, it is reported that modified HDL₃ with unsaturated fatty acid-rich diet have no different releasing activity from that with saturated fatty acid-rich diet, indicating that perturbation in the unsaturation of the HDL phospholipids does not affect the removal of cholesterol from cells [19]. In our *in vitro* study, PPC vesicles were more efficient than DPPC vesicles

Fig. 4. Effects of HDL₃ and modified HDL₃ on release of [³H]cholesterol from macrophages loaded with [³H]cholesterol oleate-labeled acetylated LDL. Macrophages from the peritoneal cavity of rats were incubated with [³H]cholesterol oleate-labeled-acetylated LDL for 24 h at 37°C in a CO₂ incubator. The cells were washed and 2×10^6 macrophages were incubated in dishes containing 2 ml of DME medium in the presence of 400 µg phospholipid of HDL₃ or modified HDL₃ for the indicated times. The medium was centrifuged at $1,000 \times g$ for 5 min, and the radioactivity in the supernatant was counted.

for the release of [³H]cholesterol. Jackson *et al.* [5] showed that linoleoyl phosphatidylcholine-apo AI, AIII, or CIII complexes had higher activity than palmitoyl phosphatidylcholine-apolipoprotein complexes.

We found that modified HDL rich in phospholipid had higher activity for the release of [³H]cholesterol from macrophages than did native HDL₃. As shown in Table 2, fractions IV and V were the most active per phospholipid content among the subfractions of modified HDL₃ and native HDL₃. The ratio of apo AI/phospholipid in fraction IV is only about 30% of native HDL₃ (Table 1): the value of apo AI/phospholipids in native HDL₃ were three times greater than that required for maximal activity for promoting the release of [³H]cholesterol from macrophages. Thus, upon incubation of HDL₃ with PPC vesicles, excess apolipoprotein would be associated with the phospholipid vesicles, possibly augmenting the [³H]cholesterol-releasing activity of the particles. Precise mechanism should be further investigated with emphasis on the physical properties of lipid particle surface modified with other lipids or apolipoproteins.

When [³H]cholesterol oleate-labeled acetylated LDL were incubated with macrophages, free [³H]cholesterol was released from the macrophages. This finding can be explained by the scavenger pathway proposed by Ho *et al.* [3]; namely, [³H]cholesterol oleate in acetylated LDL is incorporated into macrophages by phagocytosis and hydrolyzed in lysosomes and the resulting free cholesterol is then transferred to the cell membrane and released. This pathway was enhanced by the addition of HDL₃ (Fig. 4); and when the same amount of modified HDL₃ as native HDL₃, based on phospholipid content, was added to the incubation medium,

the [^3H]cholesterol releasing activity of the former was higher than that of the native HDL₃.

Lecithin cholesterol acyltransferase (LCAT) in plasma converts nascent HDL to esterified cholesterol-rich particles, and is believed to facilitate removal of cholesterol from cells [20]. The process of removal of [^3H]cholesterol from the cells consists of three steps: the release from the cells, association with HDL and esterification to make HDL more efficient as an acceptor [21]. In our assay system, LCAT activity was negligible; but the release of [^3H]cholesterol occurred, indicating that the fluidity of the acceptor phospholipid and apolipoproteins were important in the first two steps. These conclusions are consistent with the report of Zierenberg *et al.* [7] that cholesterol transfer is not dependent on LCAT activity and that the surface properties of HDL are the limiting factor for cholesterol uptake and not the activity of LCAT [7].

In 1960, Byers and Friedman reported that infusion of soybean phosphatides into the rabbits accelerated the regression of atheroma [22]. To relate our results to their observations, studies are required on whether modified HDL can promote the removal of net cholesterol from cells, and facilitate the transport of the released cholesterol into the liver.

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