Selective Inhibition of Free Apolipoprotein-Mediated Cellular Lipid Efflux by Probucol †

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Articles

Selective Inhibition of Free Apolipoprotein-Mediated Cellular Lipid Efflux by Probucol†

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ABSTRACT: We attempted to demonstrate selective modulation of lipid-free apolipoprotein-mediated cellular lipid efflux in order to test the hypothesis that it is an event independent of nonspecific physicochemical cholesterol exchange. Probucol, a unique cholesterol-lowering drug, was found to selectively suppress this pathway *in vitro* in mouse peritoneal macrophage. Probucol was given to the cells *via* the uptake of acetylated low-density lipoprotein (LDL) into which it had been incorporated. The uptake of lipoprotein-cholesteryl ester by the macrophage was the same whether the acetylated LDL was probucol-carrying or probucol-free, and probucol accumulated in the cell in parallel to cholesterol when carried by the lipoprotein. Incorporation of [^{35}S]methionine into cell protein was unaffected by probucol accumulated in the cells. The efflux of cellular cholesterol and phospholipid mediated by lipid-free human apolipoproteins (apo) A-I, A-II, and E was all completely inhibited by probucol. Reversible binding of free apoA-I to the cellular surface was also completely blocked by probucol in this condition. On the other hand, nonspecific cholesterol exchange between LDL and macrophage was unaffected by probucol. Thus, probucol selectively inhibited apolipoprotein-mediated cellular lipid efflux by blocking specific binding of free apolipoprotein to the cell without influencing nonspecific lipid exchange. In the absence of lecithin: cholesterol acyltransferase (LCAT) reaction, apparent cellular cholesterol efflux to high-density lipoprotein (HDL) was reduced by probucol by 40−70% while the rate of cholesterol influx from HDL to the cells was unaffected, resulting in cancellation of the net cellular cholesterol efflux to HDL. However, the increase of the net cholesterol efflux to HDL by LCAT was unaffected by probucol. Net cellular cholesterol efflux to HDL in the absence of LCAT, therefore, seems to depend on an apolipoprotein-mediated mechanism.

Cellular cholesterol efflux is one of the essential events of cell cholesterol metabolism since most of the cells in the peripheral tissues are unable to catabolize cholesterol. This has also been an important topic of studying the mechanism of intracellular cholesterol accumulation, in order to understand the process of the development of atherosclerotic vascular lesions. We have proposed that cellular lipid efflux is carried out by the two different mechanisms that are independent of each other; one is a nonspecific exchange of cholesterol and phospholipid between cell membrane and extracellular lipoproteins (Rothblat & Phillips, 1982; Johnson et al., 1988; Hara & Yokoyama, 1992; Li & Yokoyama, 1993) in which net efflux of cholesterol is induced by its esterification in high-density lipoprotein (HDL)[1] (Czarnecka & Yokoyama, 1995, 1996), and the other is direct interaction of apolipoproteins that have dissociated from HDL with the cell surface to generate new pre-β-HDL particles by removing cellular lipids (Hara &Yokoyama, 1991, 1992; Hara et al., 1992). The latter pathway depends on cellular factors such as a putative binding site for apolipoproteins on the cell surface (Li et al., 1995; Czarnecka & Yokoyama, 1996) and its subsequent linkage to regulation of cholesterol trafficking from the intracellular pool to a specific domain for the efflux by cellular signal transduction (Li & Yokoyama, 1995). The recent report demonstrated the selective defect of apolipoprotein-mediated lipid efflux in fibroblasts from patients with Tangier disease (Francis et al., 1995), providing an implication that generation of HDL by apolipoprotein−cell interaction serves as a major source of plasma HDL. Selective blockade by anti-apoA-I antibody Fab fragments of the cholesterol efflux from its intracellular pool of HepG2 cells to serum but not from the plasma membrane is also consistent with this view (Sviridov & Fidge, 1995).

Probucol is a unique lipid-lowering drug with many interesting effects. It lowers low-density lipoprotein (LDL)

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[1] Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; EDTA, ethylenediaminetetraacetic acid; apo; apolipoprotein; PBS, 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM EDTA; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; LCAT, lecithin:cholesterol acyltransferase; BSA, bovine serum albumin.

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apparently by a mechanism other than up-regulation of the LDL receptor (Yamamoto et al., 1986a). Interestingly, regression of cutaneous and tendinous xanthoma was observed in probucol-treated patients independently of its cholesterol-lowering effect (Davignon, 1986; Yamamoto et al., 1986a). In animal models, inhibition of the progression of vascular atherosclerotic change was demonstrated in strong relation to its powerful antioxidative potential against lipid peroxidation (Kita et al., 1987; Carew et al., 1987; Sasahara et al., 1994). In such a context, this drug triggered a number of research projects for prevention and regression of atherosclerosis by using other antioxidative compounds (Steinberg & Workshop Participants, 1992). However, probucol also significantly reduces plasma HDL, and it is aggravated by combination with some other drugs (Davignon et al., 1986; Yokoyama et al., 1988). These facts have made this drug very controversial. The recent large-scale clinical trial (PQRST) indeed failed to achieve a significant conclusion for the specific effect of probucol on regression of arteriosclerotic lesions in peripheral arteries (Walldius et al., 1994), and this may have been caused by the concomitant lowering of HDL by probucol (Johansson et al., 1995).

Although the mechanism of the action of probucol is virtually unknown, the hypothesis that its antioxidative effect prevents oxidative modification of LDL has been largely and widely accepted to explain the "antiatherogenic" effect of this drug (i.e., Parthasarathy et al., 1986). In relation to this hypothesis, an *in vitro* study showed that probucol prevents lipid accumulation in macrophages (Yamamoto et al., 1986a), but others reported it does not (Ku et al., 1990; Nagano et al., 1989). In addition, probucol reportedly suppressed macrophages to secrete interleukin 1 *in vitro* (Ku et al., 1988) and decreased the inflammatory process of the cells in vascular walls *in vivo* (Chang et al., 1995), but it may enhance chemotaxis of macrophages *in vitro* (Hara et al., 1991). For its HDL-lowering effect, some authors reported the increase of plasma cholesterol ester transfer protein (McPherson et al., 1991; Chiesa et al., 1993).

Thus, there are many controversial views over this unique drug. We undertook the study to observe the effect of probucol on the cells *in vitro* for further understanding of the cellular cholesterol efflux mechanism. Unlike the previous attempt of direct loading of probucol, a very hydrophobic compound, to the cells by using organic solvent (Yamamoto et al., 1986a; Zambon et al., 1992) or by conjugating with serum albumin (McLean et al., 1992), probucol was given to mouse peritoneal macrophages by the uptake of the probucol-carrying acetylated LDL through the scavenger receptor-mediated pathway. We believe that this is more relevant to mimic an *in vivo* manner of intracellular probucol accumulation since probucol is mostly carried by lipoprotein in plasma (Sudo et al., 1983) and the uptake of lipoproteins by macrophage is largely dependent on the scavenger receptor. The effect was examined for both of the two efflux pathways of cellular lipid: apolipoprotein-mediated and nonspecific exchange. The results revealed that probucol causes dramatic selective inhibition of the free apolipoprotein-mediated cellular lipid efflux without influencing the nonspecific efflux, which is interestingly somewhat analogous to the cells from Tangier disease (Francis et al., 1995). Thus, probucol was shown to be a useful tool to study this biologically regulated cellular cholesterol efflux pathway. Net cellular cholesterol efflux to HDL in the absence of LCAT was demonstrated to be dependent on the apolipoprotein−cell interaction.

EXPERIMENTAL PROCEDURES

Materials. Probucol, 4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol) with M_r = 516.84, was a gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo). RPMI medium 1640, penicillin−streptomycin, Tris, and fetal calf serum were purchased from Life Technologies Inc. Fatty acid-free bovine serum albumin (cell culture grade), chloramine T, 5,5′-dithiobis(2-nitrobenzoic acid), and triolein (>99%) were from Sigma Chemical, and sodium bromide and ethylenediaminetetraacetic acid (EDTA) were from BDH Inc. Egg phosphatidylcholine was purchased from Avanti. [1,2-^{3}H]-Cholesterol oleate (45 Ci/mmol), [*methyl*-^{3}H]choline chloride (15 Ci/mmol), and [4-^{14}C]cholesterol (53 mCi/mmol) were obtained from Amersham. ^{125}I carrier-free (17 Ci/mg in 10^{-5} M NaOH) was purchased from Du Pont Canada Inc. A kit for measuring the protein uptake of [^{35}S]-L-methionine (1255 Ci/mmol), Tran ^{35}S-label, and urea (ultra pure) were obtained from ICN Pharmaceuticals Inc.

Lipoproteins and Apolipoproteins. Lipoproteins were isolated from fresh human plasma by sequential ultracentrifugation in sodium bromide at a density of 1.006−1.063 g/mL for LDL and 1.125−1.21 g/mL for HDL_3. Lipoprotein-free plasma protein fraction was collected as a bottom fraction of density >1.21 g/mL. All plasma fractions were thoroughly dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM EDTA (PBS). Apolipoproteins (apo) A-I and A-II were isolated from the human HDL fraction using delipidation followed by anion-exchange column chromatography in 6 M urea as previously described (Yokoyama et al., 1982). Recombinant human apo E_3 was a generous gift from Mitsubishi Chemical Corp. (Yokohama). The apolipoproteins were dissolved in buffer before use in the experiments according to the methods previously described (Yokoyama et al., 1982, 1985; Tajima et al., 1983). The purity of lipoprotein was verified by electrophoresis on a 0.5% agarose gel. Apolipoprotein analysis of the lipoprotein fraction and verification of the purity of isolated apolipoproteins were done by electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS). A part of the HDL preparation was treated with *N*-ethylmaleimide (NEM) (5 mM) in order to inactivate the endogenous lecithin:cholesterol acyltransferase (LCAT) irreversibly (Czarnecka & Yokoyama, 1995).

Acetylated LDL Containing [^{3}H]Cholesteryl Oleate and Probucol. Incorporation of the radiolabeled cholesteryl ester and probucol into LDL was carried out according to the method previously described (Nishikawa et al., 1986). Lipid microemulsion (average diameter of 26 nm) was prepared by sonicating egg phosphatidylcholine (3 mg) with triolein (3 mg) or with triolein (2 mg) and probucol (1 mg) together with the radiolabeled cholesteryl oleate (300 μCi) in 20 mL of PBS under a nitrogen stream, and isolated by ultracentrifugation at 540 000*g* for 20 min (Tajima et al., 1983). LDL (15 mg as protein) was incubated with the microemulsion (3 mg of phospholipid) with or without probucol and lipoprotein-free plasma fraction (1.5 g of protein) at 37 °C for 48 h in the presence of dithionitrobenzoic acid (2 mM), aprotinin (20 units/mL), gentamycin (0.1 mg/mL), EDTA (0.5 mM), and NaN_3 (0.1% w/v) as described elsewhere

(Nishikawa et al., 1986). The mixture after the incubation was applied to a dextran sulfate−cellulose column (2.5 × 8 cm) equilibrated with PBS, and the bound LDL fraction was eluted with the same buffer except for 0.5 M NaCl. LDL was further purified by ultracentrifugal floatation at a density = 1.063 g/mL adjusted with NaBr at 257 000*g* for 24 h. Thus, the probucol-carrying LDL and probucol-free LDL (both containing the radiolabeled cholesteryl ester) were prepared in a parallel manner. Both LDLs were acetylated according to the methods described by Basu et al. (1982). LDL and HDL were directly labeled with [^{14}C]cholesterol by incubating lipoprotein by injecting the ethanol solution of the radiolabeled cholesterol into the lipoprotein solution as described by Czarnecka and Yokoyama (1993). After incubation at 37 °C for 2 h, the lipoproteins were reisolated by ultracentrifugal floatation.

Loading Mouse Peritoneal Macrophages with Acetylated LDLs. Peritoneal macrophages were obtained from BALB mice (20−35 g) by peritoneal lavage with cold RPMI medium 1640 containing 5 units/mL of penicillin and 5 μg/mL streptomycin (medium A) as described previously (Hara & Yokoyama, 1991). The probucol-free and probucol-carrying radiolabeled and acetylated LDL (50 μg of protein) in 1 mL of medium A containing bovine serum albumin (BSA) (0.2% w/v) was incubated with the cells in the presence of [*methyl*-^{3}H]choline (2 μCi/mL) for 24 h in order to load the cells with cholesterol and probucol (if present) and to label cellular cholesterol and choline-phospholipid in a 3.5 cm culture dish (the control and probucol-treated cells) as described in our previous paper (Hara & Yokoyama, 1991; Li et al., 1993). The cells were washed and maintained in the lipoprotein-free medium for another 24 h before any further experiment.

Measurement of Cholesterol and Phospholipid Flux. The cholesterol-loaded cells prepared with and without probucol as described above were incubated with various amounts of lipid-free apolipoproteins in 1 mL of medium A for 2−24 h. The culture medium was collected and centrifuged at 12 400*g* for 15 min to remove cell debris, and lipid was extracted as described elsewhere (Hara & Yokoyama, 1991). Lipid from the cellular fraction was extracted directly (Hara and Yokoyama, 1991). The extracted lipid was analyzed by thin-layer chromatography to measure the radioactivity of each isotope in various lipid fractions. Neither the total nor the specific radioactivity of each lipid was significantly different between control and probucol-treated cells. The amount of lipid efflux was expressed as a percentage of each lipid in the cellular pool immediately before the flux experiments, assuming cholesterol biosynthesis is negligible and showing that a change of specific radioactivity of phospholipid is insignificant (<10%) in the cholesterol-loaded cells during the efflux (Hara & Yokoyama, 1991; Li et al., 1993). Lipid efflux to lipoproteins was also observed by measuring the efflux of radiolabeled cellular lipid during the initial 8 h of the reaction. The amount of efflux was expressed as the transfer of the originally cell-associated lipid to the extracellular lipoprotein calculated from the specific radioactivity of each cellular lipid before the efflux event and the efflux of radiolabeled lipid (Czarnecka & Yokoyama, 1995, 1996). Cholesterol influx from lipoproteins to the cells was measured as the incorporation of [^{14}C]cholesterol-labeled lipoprotein into the cells. The amount of influx was expressed as the transfer of the originally lipoprotein-associated cholesterol to the cells calculated on the basis of the specific radioactivity of cholesterol in the lipoprotein before the influx event (Czarnecka & Yokoyama, 1995, 1996). The difference between the efflux and influx thus calculated was therefore considered as the net flux. Each data point was triplicated unless otherwise indicated in one series of the experiment.

Cellular Cholesterol Esterification. Acyl-CoA:cholesterol acyltransferase activity in the cells after 24 h incubation with various amounts of apoA-I was measured according to Francis et al. (1993). Briefly, after 24 h incubation with apoA-I, culture medium containing [^{14}C]oleate was applied to the cell for 2 h at 37 °C, and determined incorporation to the cellular cholesteryl ester was determined. The assay was triplicated.

Incorporation of [^{35}S]Methionine to Cellular Proteins. RPMI medium 1640 containing [^{35}S]-L-methionine (55 μCi/1 mL) was applied to the probucol-treated and control macrophages, and incubated for various times. The cells were washed with the nonlabeled medium thoroughly, and [^{35}S]-methionine uptake was measured. Furthermore, the cells were demolished by freeze and thaw, and the fragments were incubated at 37 °C for 1 h in the presence of 3.4% SDS. The fraction was centrifuged in order to remove the cell debris, and solubilized cellular proteins were precipitated by 15% trichloroacetic acid (TCA) and collected by a pulse ultracentrifuge spin. Radioactivity and protein of the TCA-precipitable fraction were measured. The assay was triplicated in one series of the experiment.

^{125}I-Apolipoprotein A-I Binding to the Cell Surface. ApoA-I was dissolved as 82 μg/mL in 10 mL of the 0.4 M glycine−0.4 N NaOH, pH 8.5, buffer (McFarlane, 1958). ^{125}I, 1 mCi, was added to the solution, and the labeling reaction was carried out and stopped by adding 50 μL of chloramine T solution (4 mg/mL) and stirring for 2 min at room temperature, followed by adding 100 μL of 16 mg/mL sodium metabisulfite and further stirring for 10 min (Greenwood & Hunter, 1963). The mixture was added to 40 mL of acetone−diethyl ether (3:1, v/v), left overnight at 4 °C, and centrifuged at 2500 rpm for 25 min to recover the pellet. The pellet was washed once more with the same organic solvent, dried by evaporation, dissolved in the PBS buffer, and finally dialyzed against the PBS buffer. The final solution was concentrated by Amicon Centriflo 25 (Amicon, Danvers, MA). The analysis of the product by electrophoresis in SDS−polyacrylamide showed that more than 80% of the total radioactivity was found with the band of apoA-I and the specific radioactivity was 127 700 cpm/μg (approximately 1 iodine molecule per 1000 apoAI molecules). The cholesterol-loaded macrophages with and without treatment with probucol were incubated in the 3.5 cm dish with various concentrations of ^{125}I-apoA-I at 0 °C for 2 h in RPMI 1640 culture medium (1 mL), pH 7.4, containing 25 mM HEPES, 0.2% (w/v) BSA, and PCSM (medium B). The cells were then washed twice by incubating at 0 °C for 2 h with 1 mL of culture medium B or medium B containing 50 μg of nonradiolabeled apoA-I. The cells were further washed with 1 mL of chilled PBS without EDTA 3 times, and cell protein was solubilized with 1 N NaOH by 1 h incubation at room temperature. The radioactivity and the protein in the NaOH solution were determined. The assay point was normally duplicated.

Table 1: Lipid Composition of the Modified LDL[a]

	free cholesterol	phospholipid	esterified cholesterol	triglyceride	probucol
LDL	11.9 ± 0.2	33.0 ± 0.6	53.2 ± 1.1	1.9 ± 0.2	0
control AcLDL	11.1 ± 0.1	33.9 ± 0.1	45.9 ± 0.1	9.0 ± 0.1	0
probucol AcLDL	12.1 ± 0.1	35.4 ± 0.4	47.8 ± 0.1	3.9 ± 0.2	0.83 ± 0.01

[a] Lipids were measured by using enzymatic assay kits for total and free cholesterol, triglyceride (including free glycerol), and choline-phospholipid. Probucol was determined by reverse-phase HPLC as described in the text. The data are expressed as the mass percentage of each lipid against total lipid including probucol. Abbreviations: LDL, LDL before the modification; control AcLDL, LDL reisolated and acetylated after incubation with the control microemulsion as described in the text; probucol AcLDL, LDL reisolated and acetylated after incubation with the microemulsion containing probucol as described in the text. The values represent the mean ±SE of the three preparations.

Analytical Methods. Quantitative measurement of protein was carried out by the method of Lowry et al. (1951) for lipoproteins, apolipoproteins, and cells. Cholesterol, cholesteryl ester, and choline phospholipid were measured by using the enzymatic−fluorescent method (Hara & Yokoyama, 1991). The content of probucol in lipoproteins and in the cells was determined for the duplicated aliquots by high-performance liquid chromatography (TSK ODS-80TM, 4.6 × 150 mm) in acetonitrile−hexane−0.1 M ammonium acetate (180:13:7) with 10 μg of 2-pentanone [3,5-di-*tert*-butyl-4-hydroxyphenyl)mercaptole] as an internal standard (Satonin & Coutant, 1986). The probucol measurement was carried out by Dr. Nobutoshi Sugiyama at the Tokyo Research and Development Center of Dai-ichi Pharmaceutical Co.

All the experiments were repeated at least 3 times, and the data demonstrated as the figures and tables represent a typical set.

RESULTS

Characterization of Probucol-Containing Lipoprotein and Probucol-Treated Cells. After incubation of LDL with the microemulsions, the electrophoretic mobilities of LDLs and their apoB were intact regardless of the incorporation of probucol (data not shown). Table 1 shows the typical chemical composition of acetylated LDL prepared with and without probucol. As a result of incubation with the lipid microemulsion in the presence of the lipid transfer activity, the triglyceride content of the control acetylated LDL was somewhat higher than that of the original LDL, and this increase was also to the same extent in the probucol-incorporated acetylated LDL. In these particular preparations, the probucol content in the acetylated LDL was 0.8% of the lipid mass (2.0% of the cholesterol mass) or 24 probucol molecules per 1 LDL particle that contains 458 and 1120 free and esterified cholesterol molecules, respectively (Shen et al., 1977). This was roughly 5 times higher than the peak of probucol concentration in human plasma lipoproteins when 3 g of probucol was given to the patient (Satonin & Coutant, 1986). Change of the electrophoretic mobility of LDL by acetylation was not affected by the presence of probucol in LDL (data not shown). The acetylated LDLs were used in order to load cholesterol and probucol to macrophages as described under Experimental Procedures. Figure 1 shows the uptake of cholesterol and probucol by the cell during incubation with probucol-containing acetylated LDL up to 48 h. Probucol in the cell increased in a time-dependent manner in parallel with the increase of total cellular cholesterol. The maximum mass ratio of probucol to cholesterol was 1.6 ± 0.2% in this particular case (6 μg/mg of cell protein), of the same order as that in the probucol-carrying acetylated LDL. This value is lower than the cellular probucol level employed in previous reports to study its *in vitro* effect on cellular lipid metabolism, such as 11 μg/mg of cell protein (Ku et al., 1990) or 6−30 μg/mg of cell protein (8 molar % to cholesteryl ester) (McLean et al., 1992). Figure 2 demonstrates the uptake of cholesteryl ester by macrophages from probucol-carrying and probucol-free acetylated LDL as traced from [^{3}H]cholesteryl oleate in the acetylated LDL. Cholesterol accumulation was not influenced by the parallel accumulation of probucol during 24 h. The ratio of free and esterified cholesterol in the cell was also unaffected by probucol, perhaps indicating that probucol does not influence hydrolysis of cholesteryl ester and re-esterification of cholesterol after uptake by the scavenger receptor. Under such conditions that macrophages were loaded with cholesterol with and without probucol, cellular protein biosynthesis was evaluated by the uptake of [^{35}S]methionine. Intracellular probucol accumulation had no effect either on its uptake by the cells or on its incorporation into the TCA-precipitable protein fraction (Figure 3). Hereby, lipid efflux was compared between the cholesterol-loaded macrophages with and without probucol thus prepared.

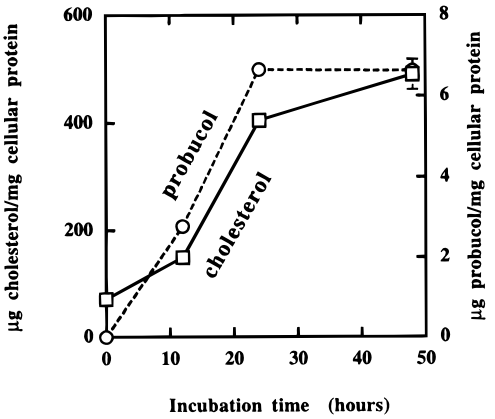

FIGURE 1: Cholesterol and probucol accumulation in macrophages. Mouse peripheral macrophages (248 ± 49.6 μg of cell protein/10 cm dish) were incubated with acetylated probucol-containing LDL, 350 μg of protein, in 7 mL of the medium up to 48 h. Cholesterol accumulation in the macrophages (□) was measured by the enzymatic fluorescence method, and probucol accumulation (○) was detected by reverse-phase HPLC as described in the text. Each data point represents the average and the error of the duplicated assay.

Cellular Lipid Efflux by Lipid-Free Apolipoproteins. Cellular lipid efflux was induced by lipid-free apolipoproteins from the cholesterol-loaded macrophages. Figure 4 shows the efflux of cholesterol, phosphatidylcholine, and sphingomyelin induced by apoA-I. Without probucol treatment, the efflux of cholesterol, phosphatidylcholine, and sphingomyelin from cholesterol-loaded macrophage was demonstrated in a dose-dependent manner, consistent with our previous works

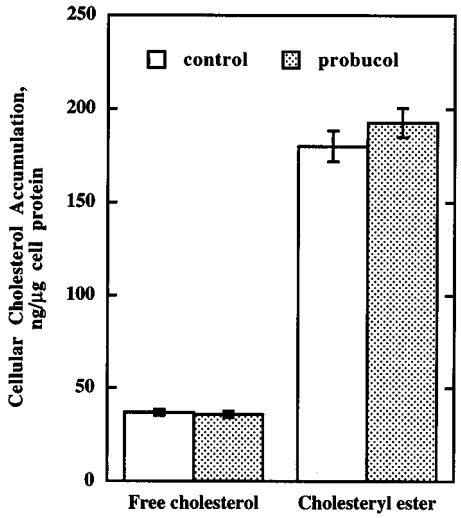

FIGURE 2: Effect of probucol on cellular cholesterol accumulation. The accumulation of cholesterol in macrophages from acetylated control LDL and acetylated probucol-containing LDL was measured. As described under Experimental Procedures, acetylated LDL with and without probucol containing radiolabeled cholesteryl ester (50 μg as LDL protein) was incubated with the cells (39.1 $\pm$ 8.8 μg of cell protein/3.5 cm dish) at 37 °C for 24 h. The cellular uptake of cholesterol originating from cholesteryl ester in the acetylated LDLs was calculated from the radioactivity in the cell and the specific radioactivity in the acetylated LDLs. Open column, loading from control acetylated LDL; stippled column, loading from probucol-containing LDL. The error bar represents the SE of the triplicated assay for each data point.

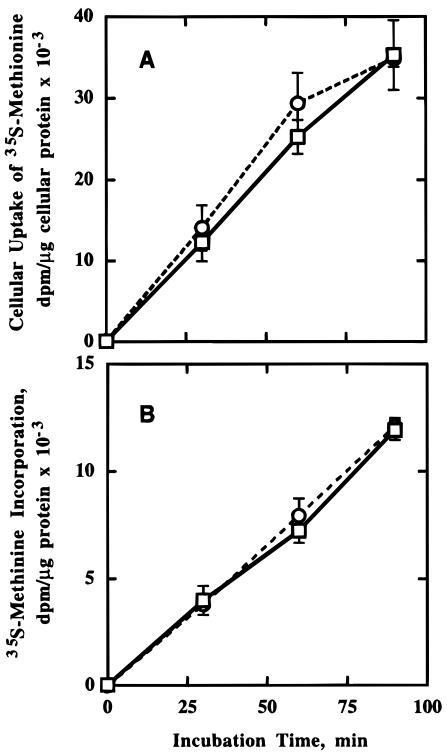

FIGURE 3: Effect of probucol on macrophages and cellular uptake of [^{35}S]methionine and [^{35}S]methionine incorporation into the cellular protein. ^{35}S-Labeled L-methionine (52.6 μCi for 40 μg of cellular protein) in methionine-deficient medium was applied to the macrophages loaded with control acetylated LDL or probucol-containing acetylated LDL (defined in Figure 2). Panel A shows [^{35}S]methionine uptake by the cells; panel B, [^{35}S]methionine incorporation into 15% TCA-precipitable protein fraction. Squares indicate the control cells (cholesterol was loaded by incubating with probucol-free acetylated LDL), and circles indicate the probucol-treated cells (cholesterol and probucol were loaded by incubating with probucol-containing acetylated LDL), in both panels. The error bar represents S. E. of the triplicated assay for each data point.

(Hara & Yokoyama, 1991; Li et al., 1993). The maximum rate can be reached by 10 μg of apoA-I in 1 mL of the medium to the extent of 30−60% of the apparent rate by HDL_3 as 100 μg of protein (Komaba et al., 1992). Probucol treatment of the cells resulted in complete inhibition of efflux of all these three lipids by apoA-I (Figure 4). Figure 5 shows the intracellular incorporation of oleate into cholesteryl ester followed by incubation of the cells with various concentrations of apoA-I. The reaction decreased as free apoA-I induced cholesterol efflux in the control cells, showing the decrease of intracellular cholesterol available to acyl-CoA: cholesterol acyltransferase, while the esterification did not change in the probucol-treated cells as no efflux is induced by free apoA-I. The inhibition of cellular lipid efflux by probucol was similarly demonstrated with other helical apolipoproteins, apoA-II and apoE, which otherwise induced cellular lipid efflux (Hara & Yokoyama, 1991) (Figure 6).

Binding of ApoA-I to the Cellular Surface. In order to examine the mechanism of the inhibition of apolipoprotein-mediated cellular lipid efflux by probucol, binding of apoA-I to the surface of the macrophage was examined. Binding of ^{125}I-labeled apoA-I was measured directly and after displacing the specific reversible binding by washing the cells with an excess amount of unlabeled apoA-I. Figure 7A shows direct results of the experiment demonstrating that the probucol-treated cell has no specific binding. Figure 7B shows the binding data after subtracting nonspecific binding from total binding. The profile of specific apoA-I binding to the control cell is superimposable to the induction of cellular lipid efflux by apoAI (Figure 4; Hara & Yokoyama, 1991; Li et al., 1993), and probucol completely inhibited this specific binding to the cell. Thus, inhibition of specific binding of lipid-free apolipoprotein results in suppression of the apolipoprotein-mediated lipid efflux. The dissociation constant and maximum binding level of apoA-I were roughly estimated as 1.7×10^{-7} M and 1.1 pmol/mg of cell protein, respectively, by a linearized plot of the specific binding data according to an equilibrium binding model (Yokoyama et al., 1980).

Cholesterol Flux between Cells and Lipoproteins. In order to examine the effect of probucol on nonspecific cholesterol exchange between the cells and lipoproteins, cholesterol efflux and influx were observed with LDL. The influx was slightly higher than the efflux of cholesterol, perhaps indicating receptor-mediated LDL uptake by the cells either by a trace amount of the LDL receptor on mouse macrophages or due to slight denaturation of LDL that may cause scavenger receptor-mediated uptake. Probucol treatment of the cells influenced neither efflux nor influx of cholesterol (Figure 8). Thus, probucol does not influence nonspecific cholesterol exchange between the cells and lipoprotein.

Finally, the effect of probucol on cholesterol efflux to HDL_3 was observed in order to examine the contribution of the apolipoprotein-mediated mechanism to the overall cellular cholesterol efflux to HDL. Figure 9 shows cellular cholesterol flux between the NEM-treated HDL and the control and probucol-treated cells to eliminate the effect of LCAT-mediated cholesterol esterification on HDL. Probucol suppressed apparent cellular cholesterol efflux to HDL by 40−70%. The two left panels in the figure represent one of these

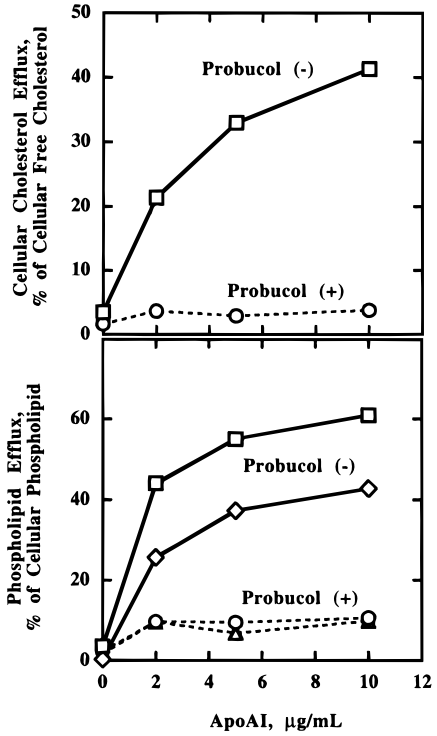

FIGURE 4: Effect of probucol on lipid-free apoA-I-mediated cellular lipid efflux. Lipid-free apoA-I was incubated with the control and probucol-treated macrophages (defined by Figure 2) for 24 h. The initial conditions of the control and probucol-treated cell were as follows: cellular protein, 86.4 ± 10.6 and 84.4 ± 12.0 μg/3.5 cm dish; cellular total cholesterol, 151.0 ± 18.4 and 154.1 ± 27.7 μg/mg of cell protein (19 and 20% is free); and cellular choline-phospholipid estimated, 52.3 ± 11.1 and 48.6 ± 6.3 μg/mg of cell protein, respectively. The efflux of cellular cholesterol and phospholipids was measured as described in the text. The top panel shows cholesterol efflux from the control cells (□) and from the probucol-treated cells (○), and the bottom panel demonstrates the efflux of phosphatidylcholine from the control and probucol-treated cells (□ and ○, respectively), and the efflux of sphingomyelin from the control and probucol-treated cells (◇ and △, respectively). The error bar represents the SE of the triplicated assay for each point.

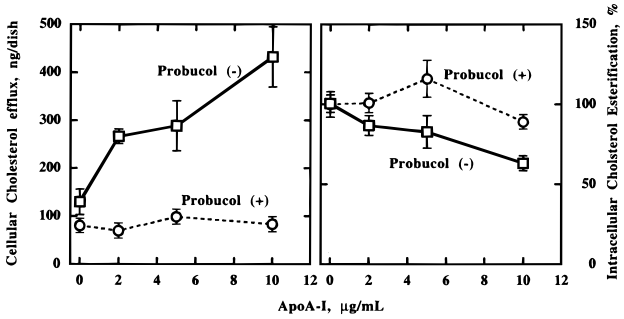

FIGURE 5: Cellular acyl-CoA:cholesterol acyltransferase activity during apoA-I mediated cholesterol efflux. Cholesterol efflux was mediated by apoA-I for 24 h incubation. The intracellular cholesterol esterification was measured as the incorporation of [^{14}C]oleic acid into cholesteryl ester as described in the text. The initial conditions of the cell were 40.5 ± 5.7 μg of protein/3.5 cm dish, cellular free cholesterol 26.7 ± 1.0 and esterified cholesterol 90.6 ± 3.3 μg/mg of cell protein for control cells, and free cholesterol 30.4 ± 1.9 and esterified cholesterol 88.3 ± 4.8 μg/mg of cell protein for probucol-treated cells. Squares indicate control cells, and circles indicate probucol-treated cells (defined by Figure 2). The error bar represents the SE of the triplicated assay for each data point.

experiments (60% inhibition of efflux by probucol in this particular experiment). On the other hand, the rate of influx of cholesterol from HDL was much slower than efflux from

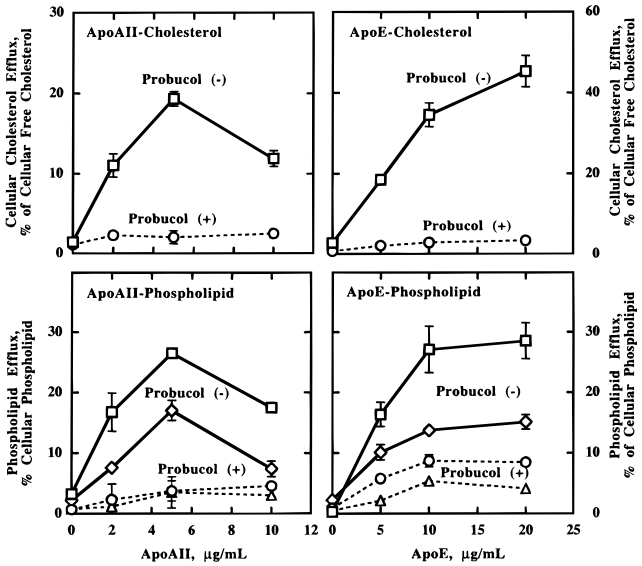

FIGURE 6: Cellular lipid efflux mediated by apoA-II and apoE. ApoA-II and apoE were incubated with the control and probucol-treated macrophages (defined in Figure 2) at 37 °C for 24 h, and the efflux of cellular lipid was measured as described in the text. The cellular portion was 40.9 ± 6.9 μg for apoA-II and 37.6 ± 5.6 μg for apoE per 3.5 cm dish. Cellular total cholesterol was 67.5 ± 11.0 and 72.1 ± 9.8 μg (34 and 33% is free), and cellular choline-phospholipid 28.3 ± 5.5 and 26.1 ± 8.8 μg, respectively. There was no significant difference between the control and probucol-treated cells in these parameters. The top two panels show cholesterol efflux from the control (□) and probucol-treated cells (○) induced by apoA-II (left) and apoE (right); the lower two panels show the efflux of phosphatidylcholine from the control and probucol-treated cells (□ and ○, respectively), and the efflux of sphingomyelin from the control and probucol-treated cells (◇ and △, respectively), induced by apoA-II (left) and apoE (right). The error bar represents the SE of the triplicated assay for each data point.

the control cells, and this rate was not affected the probucol treatment of the cells (the two panels in the center). The net cellular cholesterol efflux mediated by HDL was then estimated by subtracting the influx from the efflux as demonstrated in the right panel. While substantial net efflux was generated from the control macrophages by HDL, probucol completely inhibited the net efflux of cholesterolto HDL. This result implicates that the net cellular cholesterol efflux by HDL in the absence of LCAT is dependent upon the apolipoprotein-mediated mechanism. Figure 10 shows similar experiments to those in Figure 9 in the presence of the LCAT activity using HDL_3 untreated with NEM. A substantial portion of cellular cholesterol appearing in HDL was esterified regardless of the inhibitory effect of probucol on the efflux (the left two panels). The influx of HDL cholesterol was reduced by LCAT reaction in agreement with our previous observation (Czarnecka & Yokoyama, 1995, 1996), but probucol did not influence this result (the central two panels). Consequently, the net efflux was increased by LCAT both in the absence and in the presence of probucol (the right panel). When the increment of the net efflux by LCAT was calculated from the results in Figures 9 and 10, there was no difference between the control and probucol-treated cells (Figure 11).

We have shown that LCAT reaction induces net cellular cholesterol efflux to HDL through nonspecific cholesterol exchange reaction but not through apolipoprotein-mediated reaction (Czarnecka & Yokoyama, 1995, 1996). Thus, we

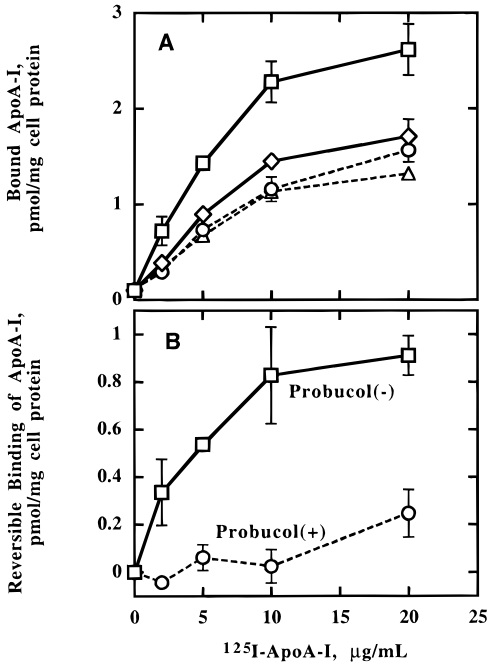

FIGURE 7: Binding of apolipoprotein to the cellular surface. ApoA-I was labeled with ^{125}I as described in the text. The labeled apoA-I was incubated with control and probucol-treated macrophages (defined by Figure 2, 158 ± 1.3 μg of cell protein/3.5 cm dish) at 0 °C for 2 h. The reversibly bound [^{125}I]-apoA-I was displaced by washing the cells with 50 μg of unlabeled apoA-I at 0 °C for 2 h twice. Panel A shows the binding of labeled apoA-I without and with displacement of reversible binding to the control cells (□ and ◇, respectively) and to the probucol-treated cells (○ and △, respectively). Panel B represents the reversible binding of apoA-I, estimated by subtracting the nondisplaceable binding from the total binding using the data in the panel A, to the control cells (□) and the probucol-treated cells (○). Each data point represents the average and the error of the duplicated assay.

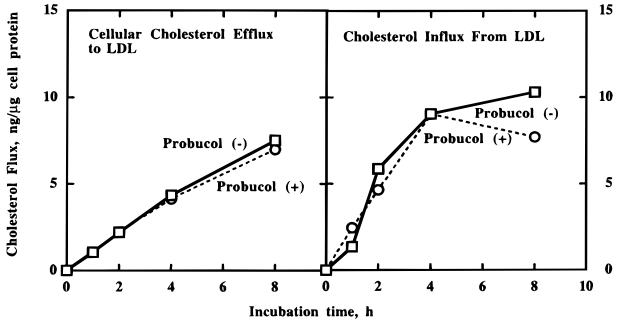

FIGURE 8: Effect of probucol on cholesterol flux between LDL and macrophages. Cellular cholesterol and LDL cholesterol influx was observed with the control and probucol-treated macrophages (defined by Figure 2, 70.6 ± 9.4 μg of protein/3.5 cm dish, cellular total cholesterol 120 ± 10 μg/mg of cellular protein), as 50 μg of protein/mL of LDL was applied to the cell at 37° C. Detail of the method is described in the text. Squares represent the data with the control cells, and circles represent the probucol-treated cells. Each data point represents the average of the duplicated assay.

conclude that nonspecific physicochemical cholesterol exchange between the cells and HDL is not affected by probucol. The effect of probucol is strictly limited to the net cellular lipid efflux mediated by helical apolipoproteins through the inhibition of specific apolipoprotein−cell binding, and it is responsible for the net cellular cholesterol efflux to HDL.

Cellular phospholipid efflux to HDL and LDL is shown in Figure 12. Consistent with cholesterol efflux, both the

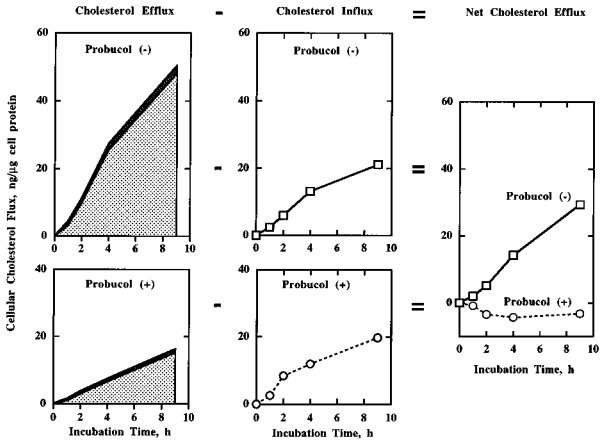

FIGURE 9: Effect of probucol on HDL-mediated cellular cholesterol flux in the absence of lecithin:cholesterol acyltransferase activity. The left two panels show cellular cholesterol efflux from macrophage [31.2 ± 4.4 and 29.5 ± 6.2 μg of cell protein/3.5 cm dish for the control and probucol-treated cells, respectively; total cellular cholesterol 125 ± 25 and 119 ± 29 (22 and 24% is free), phosphatidylcholine 27 ± 5.5 and 15 ± 5.9, and sphingomyelin 16 ± 3 and 15 ± 5 μg/mg of cell protein, in the initial condition] to the NEM-treated HDL_3 (100 μg of protein/1 mL of medium). The shadowed area represents free cholesterol, and the hatched area represents esterified cholesterol in the medium. The upper figure is the efflux from the control cell, and the lower figure is that from the probucol treated-cell. The two panels in the center show the influx of HDL cholesterol to the control and probucol-treated macrophage (lower and upper panels, respectively). The net cholesterol efflux has been obtained by subtracting cholesterol influx from the efflux rate and demonstrated in the right panel. Squares represent the net efflux from the control cell, and circles indicate that from the probucol-treated cell. Each data point represents the average of the duplicated assay.

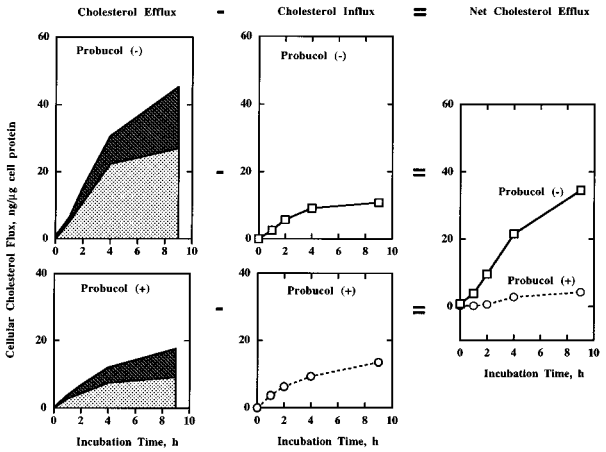

FIGURE 10: Effect of probucol on HDL-mediated cellular cholesterol flux in the presence of lecithin:cholesterol acyltransferase activity. The same experiment as that in Figure 9 was carried out with HDL_3 untreated with NEM. Symbols and arrangement of the panels are all the same as those in Figure 9.

efflux of phosphatidylcholine and the efflux of sphingomyelin to HDL were decreased by probucol, and LCAT did not influence the results. On the other hand, there was no effect of probucol on the efflux of cellular phospholipids to LDL. The results indicate that apolipoprotein-mediated cellular cholesterol efflux is accompanied by phospholipid efflux, and it is independent of nonspecific phospholipid exchange between lipoprotein and the cell membrane.

DISCUSSION

The effect of probucol treatment of the mouse peritoneal macrophage on cellular cholesterol efflux was examined.

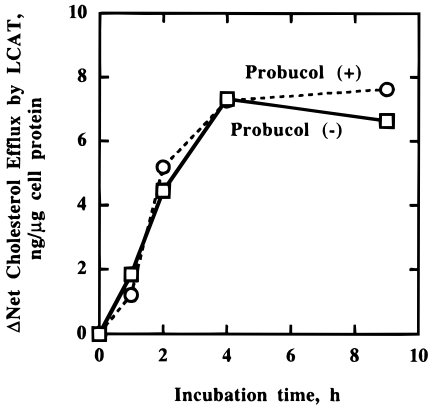

FIGURE 11: Net efflux of cholesterol from the macrophages to HDL induced by LCAT. The increment of the net efflux of cellular cholesterol by LCAT was calculated from the data in Figures 9 and 10. The increment was estimated by subtracting the net cholesterol efflux data in the absence of LCAT (the right panel of Figure 9) from those in the presence of LCAT (the right panel of Figure 10), for the control (□) and probucol-treated cells (○), respectively.

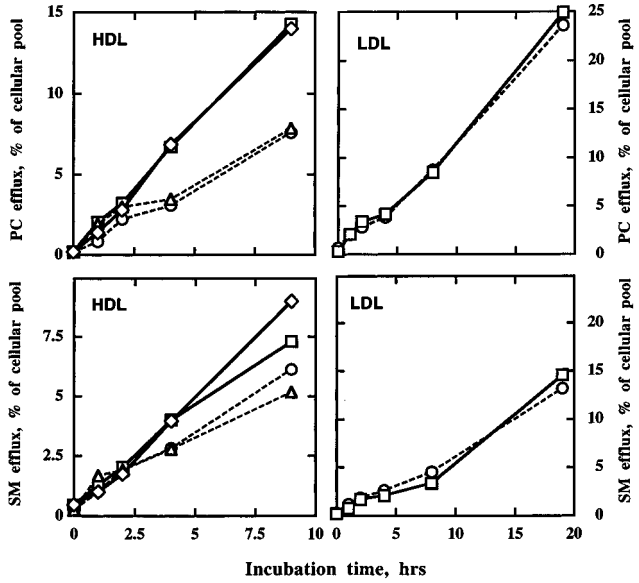

FIGURE 12: Phospholipid efflux mediated by HDL and LDL from macrophages. Phosphatidylcholine and sphingomyelin efflux were measured as described in the text. HDL (100 of μg protein/1 mL of medium) with or without LCAT or LDL (50 μg of protein/1 mL of medium) was incubated with the control and probucol-treated macrophages for 1−19 h at 37 °C. Conditions of the cells are the same as described in Figures 9, 10, and 11. The top two panels show the efflux of phosphatidylcholine (PC) to HDL (left) and LDL (right) from the control cells in the absence and presence of LCAT reaction (□ and ◇, respectively) and from the probucol-treated cells in the absence and presence of LCAT reaction (○ and △, respectively). The lower two panels show the efflux of sphingomyelin (SM) to HDL (left) and LDL (right) from the control cells in the absence and presence of LCAT reaction (□ and ◇, respectively) and from the probucol-treated cells in the absence and presence of LCAT reaction (○ and △, respectively). Each data point represents the average of the duplicated assay.

Probucol was given to the cells by the uptake of acetylated LDL containing probucol *via* the scavenger receptor pathway in this work. Cellular uptake of this drug *in vivo* is perhaps mainly by the receptor-mediated lipoprotein uptake since it is carried by plasma lipoproteins. Oxidized modification of LDL is believed to be an *in vivo* form recognized by the scavenger receptor of macrophages. However, its strong antioxidative potential would prevent similar oxidative modification *in vitro* of probucol-containing LDL to the control LDL. Thus, acetylated modification was employed to make probucol-loaded LDL recognizable by the scavenger receptor of the macrophage. The concentration of probucol in the acetylated LDL used was several times higher than what can be clinically reached (Satonin & Coutant, 1986), and therefore perhaps equivalent to the level in many of the animal experiments. The extent of probucol loading to the cell was lower than those reported in previous publications (Ku et al., 1990; McLean et al., 1992). It was also substantially lower than the highest accumulation of probucol in tissues of the probucol-fed experimental animals (40−80 μg/mg wet tissue of the aortic lesion of rabbits: personal communication by Dr. N. Sugiyama, Dai-ichi Pharmaceutical Co.).

Probucol was shown to be an inhibitor of apolipoprotein-mediated cellular lipid efflux and also to be an inhibitor of the binding of apolipoprotein to the cell surface. The profile of the specific binding of apoA-I to macrophage was identical to that of apoA-I-mediated cellular lipid efflux. The apparent affinity of apoA-I for macrophage, 1.7×10^{-7} M, was much lower than that for the lipid surface with low curvature (Tajima et al., 1983) and closer to the apparent affinity observed for the interaction between LDL and the LDL receptor (Brown & Goldstein, 1986). Interestingly, the roughly estimated value of the maximum binding of apoA-I was also of the same order as that of LDL against the LDL receptor of human skin fibroblast (Brown & Goldstein, 1986) and much lower than that of helical apolipoproteins toward the lipid surface (Tajima et al., 1983; Yokoyama et al., 1985). These data provided supportive evidence for the hypothesis that a specific cell surface binding site is involved in the apolipoprotein-mediated cellular lipid efflux pathway (Li et al., 1995; Czarnecka & Yokoyama, 1996).

Nonspecific lipid exchange between macrophage and LDL caused no net cholesterol flux, and probucol did not influence this reaction. On the other hand, the apparent efflux of cellular cholesterol to HDL in the absence of LCAT was substantially reduced by probucol, but the cholesterol influx from HDL was not affected at all. The efflux was suppressed by probucol to the same level as the influx, so that no net efflux of cholesterol was generated from the macrophage to HDL in the presence of probucol in the cell. Thus, HDL behaves like a lipid microemulsion or LDL in terms of cholesterol efflux when the apolipoprotein-mediated efflux was inhibited by probucol (Figure 8; Li et al., 1993, 1995; Li & Yokoyama, 1995). Therefore, it is conceivable that the net efflux of cellular cholesterol to HDL in the absence of LCAT is mediated by the apolipoprotein-dependent mechanism which accounts for the portion of the efflux to HDL suppressed by probucol.

The data with HDL and LDL showed that probucol does not influence nonspecific cholesterol exchange between the cell and lipoproteins. LCAT induces net cellular cholesterol efflux through nonspecific cholesterol exchange (Czarnecka & Yokoyama, 1995, 1996), and in fact probucol does not interfere with the LCAT-mediated net cellular cholesterol efflux (Figure 11). Thus, net cellular cholesterol efflux can still be carried out by a nonspecific pathway even in the absence of apolipoprotein−cell interaction and with the lack of HDL-generation with the cellular lipids.

McLean et al. (1992) reported that probucol increased the fluidity of cholesteryl ester droplets and cholesterol-enriched

cell membrane when it is present only in 1−2 mass % of cholesterol, the same extent of loading as achieved in this paper. In the same paper, they also showed that probucol increased the nonspecific cholesterol efflux to phospholipid vesicles from Fu5AH cells when loaded with probucol at up to several times higher extent than the level employed in this study. Cholesterol efflux from this cell line seems to depend largely on a nonspecific cholesterol exchange mechanism since no large net cholesterol efflux was generated by HDL (Karlin et al., 1987). Thus, probucol may influence nonspecific cholesterol exchange with the cells when loaded higher than this study. Zambon et al. reported that in human monocyte-derived macrophages probucol had little effect on the induction of intracellular sterol translocation and cellular cholesterol efflux by HDL (1992). Since the cellular content of probucol was not determined in these experiments, the true reason for the discrepancy between these data and the present report is not known.

It is of interest and importance to know how the inhibition of apolipoprotein-mediated cellular lipid efflux is dependent on cellular levels of probucol. The method to answer this question is under development in our laboratory by preparing LDL with various contents of probucol. We preliminarily observed that the efflux was shut down in an all-or-none manner even with the lower variation of cellular probucol content within the same order of magnitude as used in this paper.

A potential problem may rise from the labeling procedure for cellular cholesterol via the uptake of esterified [^{3}H]-cholesterol in acetylated LDL. Although the same distribution of radioisotope between free and esterified cholesterol in the control and probucol-treated cells indicates that hydrolysis of the lipoprotein cholesteryl ester and its re-esterification are not influenced by probucol, there still may be the possibility that the details of this process are not exactly the same in the presence of probucol, and free cholesterol generated from cholesteryl ester in the acetylated LDL in such condition is not transferred to the pool available for the efflux. However, the apolipoprotein-mediated efflux of cellular phospholipid and the binding of apoA-I to the cell were also completely blocked by probucol. On the other hand, nonspecific efflux of the similarly labeled cellular free cholesterol and phospholipid were unaffected by probucol. Therefore, it is unlikely that the intracellular distribution of the labeled free cholesterol is the cause of the apparent inhibition of the apolipoprotein-mediated cellular lipid efflux by probucol.

In conclusion, probucol was shown to be a potent tool to differentiate apolipoprotein-mediated cellular lipid efflux from nonspecific cholesterol exchange between the cell and lipoprotein. Since specific binding of helical apolipoprotein to the cell surface is blocked by probucol, the site of binding can be identified by using this model system. Further details of the mechanism for probucol to inhibit apolipoprotein binding remain to be studied. The amount of probucol in the cell is only 1 molar % of total cellular cholesterol, but it would still be possible that probucol in this amount may have significant influence on general physicochemical properties of the membrane (McLean et al., 1992). Thus, it is unknown whether this inhibition of the binding is caused by specific interaction of probucol with the apolipoprotein binding site, by a change of the physicochemical properties of the membrane, or by some other factors such as redistribution of free cholesterol among various cell membranes. It is also unknown whether or not the effect of probucol presented here is related to its antioxidative effect.

It is an interesting analogy between the probucol-treated macrophage and fibroblasts from homozygotes of Tangier Disease that there is a defect in the cellular interaction with apolipoproteins for lipid efflux (Francis et al., 1995). This coincidence may indicate that generation of HDL by the apolipoprotein−cell interaction is a major source of plasma HDL and reduction of plasma HDL level by probucol is related to the lack of HDL generation by this mechanism. In an *in vivo* study on the HDL-lowering effect of probucol performed in the transgenic mice of human apoA-I, it was also implicated that probucol mainly decreases HDL production although a slightly higher catabolic rate of HDL may partly contribute to the decrease of HDL (Hayek et al., 1991). Thus, our results seem to be consistent with the *in vivo* observation. It remains to be answered why probucol apparently regresses xanthoma in spite of the suppression of cellular cholesterol efflux.

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