Cholesterol Is Poorly Available for Free Apolipoprotein-Mediated Cellular Lipid Efflux from Smooth Muscle Cells†

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ABSTRACT: To study the mechanism for resistance of smooth muscle cells (SMC) to cholesterol efflux caused by lipid-free apolipoproteins [Komaba, A., et al. (1992) *J. Biol. Chem. 267*, 17560–17566], the efflux of phospholipids and cholesterol was induced from mouse peritoneal macrophages (MP) and rat aortic SMC by phospholipid/triglyceride microemulsion, by human plasma high- and low-density lipoproteins (HDLs and LDLs), and by lipid-free human apolipoprotein (apo) A-I. The efflux of both lipids by the lipid microemulsion showed essentially the same kinetic profile for these two types of cells except that the rate of phospholipid efflux was 5–6 times slower by weight than cholesterol in both cases. The same ratio of cholesterol to phospholipid was also found in the efflux to LDLs. Lipid-free apoA-I mediated cellular cholesterol efflux, but the rate was much slower from SMC than from MP. However, the rate of apoA-I-mediated phospholipid efflux was similar between these two cells generating HDL-like particles, resulting in a high phospholipid:cholesterol ratio, (4–5):1 by weight, in the lipid efflux from SMC, in contrast with (0.8–1):1 in the lipid efflux from MP. When standardized for the cellular free cholesterol, the V_{max} of cholesterol efflux induced by lipid-free apoA-I was 10 times slower from SMC than from MP, but only by at most 2-fold slower when lipid microemulsion was the acceptor. Thus, free cholesterol of SMC is less available than that of MP for free apolipoprotein-mediated generation of HDLs with cellular lipids. ApoA-I enhanced both cholesterol and phospholipid efflux from SMC to the lipid microemulsions, being consistent with the model that apoA-I in the aqueous phase mediates the enhancement [Hara, H., & Yokoyama, S. (1992) *Biochemistry 31*, 2040–2046]. The phospholipid:cholesterol ratio increased as apoA-I enhanced the lipid efflux, suggesting that the increase of the phospholipid:cholesterol ratio may indicate the contribution of the lipid-free apolipoprotein-mediated reaction. This ratio was higher in cellular lipid efflux from SMC to HDL than that to microemulsion and also than that from MP to HDL.

One of the important initiation factors of atherosclerotic vascular lesion is intracellular accumulation of cholesterol in certain types of cells in the arterial wall. These are identified as foam cells in microscopic observation due to the presence of intracellular lipid droplets composed predominantly of the acyl ester of cholesterol. Macrophages are among those playing such a role actively at the very initial stage of the development of the vascular lesion as well as cutaneous and tendinous xanthomas (Gerrity, 1981a,b). Cholesterol ester accumulation in this type of cell seems reversible to some extent, being demonstrated clinically by means of reducing the level of plasma cholesterol and other measures (Yamamoto et al., 1989), and also in in vitro experiments by exposing the cells to high-density lipoproteins (HDLs)[1] (Burns & Rothblat, 1969; Werb & Cohen, 1971; Stein et al., 1976; Ho et al., 1980). On the other hand, vascular smooth muscle cells are thought to play an important role in advancing the stage of vascular lesion (Geer, 1965; Ross, 1986), and the foam cells derived from them may be relatively resistant to removal of intracellularly accumulated cholesterol by HDL (Tabas & Tall, 1984; Savion & Kotev-Emeth, 1989). Low-density lipoproteins (LDLs) do not remove cholesterol so much as HDLs in vitro, so that, hence, HDL has been thought to play a key role in cellular cholesterol removal in vivo as well (Ho et al., 1980).

The mechanism of cellular cholesterol efflux has not been fully understood. Cellular free cholesterol is exchangeable with extracellular pools such as the lipoprotein surface by such nonspecific mechanisms as its diffusion through an aqueous phase, so that its net efflux can be induced by a gradient of cholesterol between the cell surface and extracellular pools (Rothblat & Phillips, 1982; Johnson et al., 1986, 1988; Karlin et al., 1987). Specific binding sites on the cell surface to HDL apoproteins may play a role in the efflux by mobilizing cholesterol from its intracellular pool to the cell surface (Slotte et al., 1987; Aviram et al., 1989; McKnight et al., 1992). Cellular cholesterol appears in the preβ-HDL fraction prior to other HDLs or other lipoproteins when fibroblasts are incubated with human plasma, suggesting that the pathway for cellular cholesterol removal is independent of nonspecific diffusion/exchange (Castro & Fielding, 1988; Miida et al., 1990).

We have demonstrated that many lipid-free apolipoproteins with amphiphilic α-helical segments, such as apolipoproteins (apo) A-I, A-II, A-IV, E, and apolipophorin III from insect (*Manduca sexta*) hemolymph, mediate the net efflux of cholesterol and phospholipids from macrophages, generating HDL-like particles with preβ mobility and resulting in reduction of intracellularly accumulated cholesterol (Hara & Yokoyama, 1991; Hara et al., 1992). It has also been shown that this pathway is consistent with a model that preβ-HDL is generated in situ by this mechanism and mediates the

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[1] Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; apo, apolipoprotein.

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efflux of cholesterol to other lipoproteins in plasma rather than accepting cholesterol from the cells with priority (Hara & Yokoyama, 1992). The concentration of apolipoproteins required for the reaction was very low, such as 1/500 of the plasma apoA-I concentration for the K_m. Therefore, the reaction has physiological relevance in such terms that lipid-free apolipoproteins may be present in low concentrations in the interstitial fluid or that transfer of certain apolipoproteins may occur from the lipoprotein to the cell surface.

More interestingly, we have discovered that smooth muscle cells are highly resistant to net cholesterol efflux by this lipid-free apolipoprotein-mediated mechanism, in comparison to macrophages and fibroblasts, while its cellular cholesterol was exchangeable with extracellular pools with only slightly lower rates than those of other cells by nonspecific mechanisms (Komaba et al., 1992). An apparent affinity of apolipoproteins for the smooth muscle cell surface, however, might be even higher than that for macrophages judging from the lower K_m of apolipoproteins for the cholesterol efflux reaction (Komaba et al., 1992). This finding was the first evident hint for the possible resistance of smooth muscle cells to regression of atherosclerosis. In the present paper, we describe the results of further investigation of this specific resistance of smooth muscle cells to cholesterol efflux. We show that smooth muscle cells are as reactive as macrophages to lipid-free apoA-I for cellular phospholipid efflux. However, cellular cholesterol is extremely poorly available for this reaction, resulting in its very poor net efflux through this mechanism.

EXPERIMENTAL PROCEDURES

Lipoproteins, Apolipoproteins, and Lipid Microemulsion. HDL and LDL were isolated from human plasma at densities of 1.063–1.21 and 1.006–1.063 g/mL, respectively, in NaBr. Lipoprotein preparations were dialyzed against 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and stored at 4 °C under argon. The purity of lipoproteins was confirmed in 0.5% agarose gel electrophoresis, and their lipid and apolipoprotein compositions were verified by using enzymatic lipid assay kits, by protein determination (Lowry et al., 1951), and by electrophoresis in polyacrylamide gels in sodium dodecyl sulfate (Laemmli, 1970). ApoA-I was isolated from the human HDL preparation and dissolved in an aqueous solution as described previously (Yokoyama et al., 1982). LDL containing [1,2-^{3}H]cholesteryl oleate (45.4 Ci/mmol, purchased from Amersham Canada) was prepared according to the method previously described (Hara & Yokoyama, 1991; Nishikawa et al., 1986). The labeled LDL was either acetylated or cationized by the method described by Basu et al. (Hara & Yokoyama, 1991; Komaba et al., 1992; Basu et al., 1976). A lipid microemulsion (with an average diameter of 26 nm) of triolein (Sigma, >99%) and egg phosphatidylcholine (Avanti) was prepared and isolated according to the method previously described (Tajima et al., 1983). The weight ratio of triglyceride to phospholipid in the final preparation was 1.25 to 1.

Loading Cells with Radiolabeled Cholesterol and Labeling of Cellular Choline-Phospholipids. Mouse peritoneal macrophages were obtained by peritoneal lavage and loaded with radiolabeled cholesterol according to the procedure previously described (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992) by incubating the cells with the acetylated LDL containing radiolabeled cholesteryl oleate for 24 h in 1 mL of RPMI 1640 medium (FLow Laboratories) containing 2 mg of bovine serum albumin together with [*methyl*-^{3}H]-choline chloride (15 Ci/mmol, Amersham) and then washing and incubating without lipoprotein for another 24 h (Hara & Yokoyama, 1991, 1992; Hara et al., 1992). Smooth muscle cells were prepared from the thoracic aorta of a Sprague-Dawley rat (Komaba et al., 1992; Gunther et al., 1980) and loaded with radiolabeled cholesterol by incubating in minimum essential medium with the cationized LDL containing the labeled cholesteryl ester for 1 week and with the radiolabeled choline for the last 24 h of this period in the same medium, washing, and incubating without lipoprotein for another 24 h (Komaba et al., 1991).

Incubation of Cholesterol-Loaded Cells with Lipoproteins, Lipid Microemulsions, and ApoA-I. The cholesterol-loaded cells prepared as above were incubated with 1 mL of RPMI 1640 (macrophages) or minimum essential medium (smooth muscle cells) without bovine serum albumin in the presence of HDL, LDL, lipid microemulsion, and apoA-I. After incubation for 24 h unless otherwise specified, the culture medium was removed and centrifuged at 3000*g* for 2 min to remove cellular remnants (Komaba et al., 1992). The lipids were extracted from the medium and from the cells as previously described (Hara & Yokoyama, 1991; Komaba et al., 1992). Cellular cholesterol was measured by an enzymatic fluorescence method (Heider & Boyett, 1978). The assay mixture contained 0.05 IU of bovine pancreatic cholesterol esterase (Sigma), 0.05 IU of cholesterol oxidase from Brevibactellium (Sigma), 0.5 IU of hoarseradish peroxidase (Sigma), and 0.4 mg of *p*-hydroxyphenylacetate in 0.5 mL of buffer containing 0.05% Triton X-100 and 0.1% cholic acid. Total cholesterol in the sample was measured after hydrolysis of cholesteryl ester with 0.05 N NaOH (Komaba et al., 1992) with excitation at 321 nm and emission at 407 nm. The specific radioactivity of cellular free and esterified cholesterol was determined respectively for several representative samples using the same assay method as used for total cholesterol after lipids were extracted and separated by thin-layer chromatography. Both specific radioactivities were always in good agreement within an error of 20% in macrophages and smooth muscle cells [consistent with the results by Hara and Yokoyama (1991) using gas chromatography assay for macrophages]. Therefore, free and esterified cholesterols in the cells and medium were calculated by using the radioactivity in each fraction and the specific radioactivity of cellular total cholesterol (Komaba et al., 1992). Phosphatidylcholine and sphingomyelin in the medium were also measured in a similar manner based on the specific radioactivity of each phospholipid determined for the cellular lipid extract by organic phosphorus assay (Hara & Yokoyama, 1991; Ames & Dubin, 1960) and the radioactivity of the thin-layer chromatography fractions (Hara & Yokoyama, 1991). Cellular proteins was determined by Lowry's method (Lowry et al., 1951). Lipid efflux values estimated by this method were within an error of 10% in duplicated assays, so that the data shown in the figures without error bars all represent a single assay point. The smooth muscle cell culture medium after incubation with 10 μg of apoA-I for 24 h was analyzed by sucrose density gradient ultracentrifugation. The radioactivity of phosphatidylcholine in each fraction was determined after lipid was extracted and separated by thin-layer chromatography (Hara & Yokoyama, 1991).

Cholesterol Oxidation by Extracellular Cholesterol Oxidase. Cellular free cholesterol was oxidized by extracellular cholesterol oxidase according to the method described by Lange and Ramos (1983) in order to assess its accessibility from the cellular surface. Both macrophages and smooth muscle cells were loaded with radioactive cholesterol as described above, respectively. After washing, 24-h blank-incubation, and a

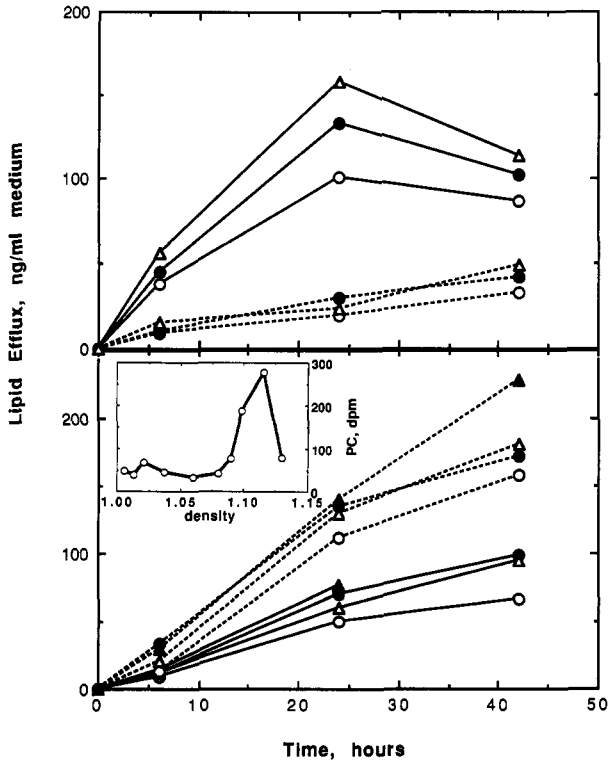

FIGURE 1: Time course of lipid efflux from smooth muscle cells. The cells were loaded with cholesterol by incubating with cationized LDL as described under Experimental Procedures, giving the initial conditions for lipid efflux: cell protein, 167 μg/dish; phosphatidylcholine, 1.76 μg/dish; sphingomyelin, 1.40 μg/dish; free and esterified cholesterol, 1.54 and 1.75 μg/dish, respectively. Cellular lipid efflux was induced (solid lines, cholesterol; dashed lines; phosphatidylcholine) by lipid microemulsion [upper panel; 100 (○), 200 (●), and 400 μM (△) phosphatidylcholine concentration in the medium] and by lipid-free apoA-I [lower panel; 1 (○), 2 (●), 5 (△), and 10 μg/mL (▲) in the medium]. The inset of the lower panel is the result of density gradient ultracentrifugation analysis for phosphatidylcholine in the medium of smooth muscle cells after incubation with 10 μg of apoA-I for 24 h. Lipid was extracted from each fraction and separated by thin-layer chromatography to obtain the radioactivity in phosphatidylcholine.

final washing at 4 °C, the cells were treated with 1% glutaraldehyde for 15 min at 0 °C. The cells were washed with the 310 mM sucrose solution and prewarmed at 37 °C for 15 min, and cholesterol oxidase was added at a final concentrationn of 3 IU (1 mL of medium)$^{-1}$ dish^{-1}. The reaction was terminated at 3 min by removing the medium and extracting the cellular lipid (Hara & Yokoyama, 1991) because the rapid phase of the reaction was complete within 2 min and no further significant oxidation was observed at least up to 10 min in our preliminary time course experiments for both macrophages and smooth muscle cells, being consistent with the results by Lange and Ramos (1983) using fibroblasts. Radioactivities of cholesteryl ester, cholestone, and cholesterol were determined after the lipids were separated by thin-layer chromatography using petroleum ether/diethyl ether/acetic acid, 80:20:1 (v/v).

RESULTS AND DISCUSSION

Lipid Efflux from Smooth Muscle Cells. Typical kinetic profiles of the efflux of lipid from smooth muscle cells and macrophages with various inducers are shown in the figures. Figure 1 demonstrates the time course of the efflux of cholesterol and phosphatidylcholine from smooth muscle cells induced by the lipid microemulsion and apoA-I. As shown in the top panel, cholesterol efflux seems to reach a plateau for all three different concentrations of the emulsion after 24-h incubation, suggesting that the efflux and back-influx of cholesterol may be reaching equilibration. In this particular condition, cholesterol in the medium was 0.1–0.2% of phospholipid by weight in the microemulsion while cholesterol efflux accounted for more than 10% of the total cellular free cholesterol pool. On the other hand, the apparent efflux of prelabeled cellular phosphatidylcholine continuously increased up to 43-h incubation but to much less extent than cholesterol compared on the basis of weight. The bottom panel of Figure 1 shows the efflux of the lipids induced by lipid-free apoA-I in the medium. In a previous paper, we showed that the efflux of cholesterol from smooth muscle cells by apolipoproteins was very little in comparison to macrophages and fibroblasts (Komaba et al., 1992). This observation was reproduced in Figure 1, showing the very low rate of cholesterol efflux. However, to our surprise, the rate of phospholipid efflux was much greater than that of cholesterol. Both cholesterol and phosphatidylcholine efflux increased almost linearly up to 43 h. As shown in the inset of the bottom panel, density gradient ultracentrifugation revealed that cellular phosphatidylcholine removed by apoA-I formed particles in the medium at a density of 1.10–1.12 g/mL, in agreement with the results of the cholesterol peak in the same analysis (Komaba et al., 1992). The efflux of cholesterol to the microemulsion and apoA-I was already shown to increase continuously during the initial 24 h of incubation time in our previous work (Hara & Yokoyama, 1991; Hara & Yokoyama, 1992). Therefore, 24-h incubation was chosen for further experiments in order to investigate the lipid efflux.

Figure 2 demonstrates the increase of lipid efflux from smooth muscle cells depending on the concentration of microemulsion, apoA-I, and HDL in the medium. By microemulsion (top panel), the efflux of cholesterol, phosphatidylcholine, and sphingomyelin showed essentially the same kinetic profile, but the rate was much greater for cholesterol than either phospholipid. The estimated K_m value was (8 ± 3) ~ 10^{-5} M phosphatidylcholine. In contrast, phosphatidylcholine efflux by apoA-I from smooth muscle cells was much greater than that of cholesterol (middle panel). Lipid-free apoA-I was shown to induce cholesterol efflux from smooth muscle cells, generating HDL-like particles, but with a much slower rate than that observed with macrophages and fibroblasts (Komaba et al., 1992). Indeed, the apparent V_{max} of cholesterol efflux was only 3% of the total cellular free cholesterol, and the K_m for the reaction was about 1.5 μg/mL apoA-I in this particular experimental condition, consistent with our previous data (Komaba, 1992). The rate of phosphatidylcholine efflux was, however, about 3 times greater than that of cholesterol with approximately the same K_m value of apoA-I. Lipid efflux from smooth muscle cells to HDL is shown in the bottom panel of Figure 2. The ratio of phospholipid efflux to cholesterol efflux was higher than that observed with the lipid microemulsion but much lower than that with apoA-I.

Lipid Efflux from Macrophages. Figure 3 represents efflux of lipid from macrophages. The profile of cholesterol efflux to the microemulsion (top panel) was reproducible from our previous work (Hara & Yokoyama, 1992) with an apparent K_m value of (4.5 ± 1.2) × 10^{-5} M phosphatidylcholine and a V_{max} rate around 25% of the initial cellular free cholesterol pool in this particular experimental condition. The efflux of phosphatidylcholine and sphingomyelin to the emulsion was much lower than that of cholesterol. These results were all similar to those with smooth muscle cells shown in Figure 2

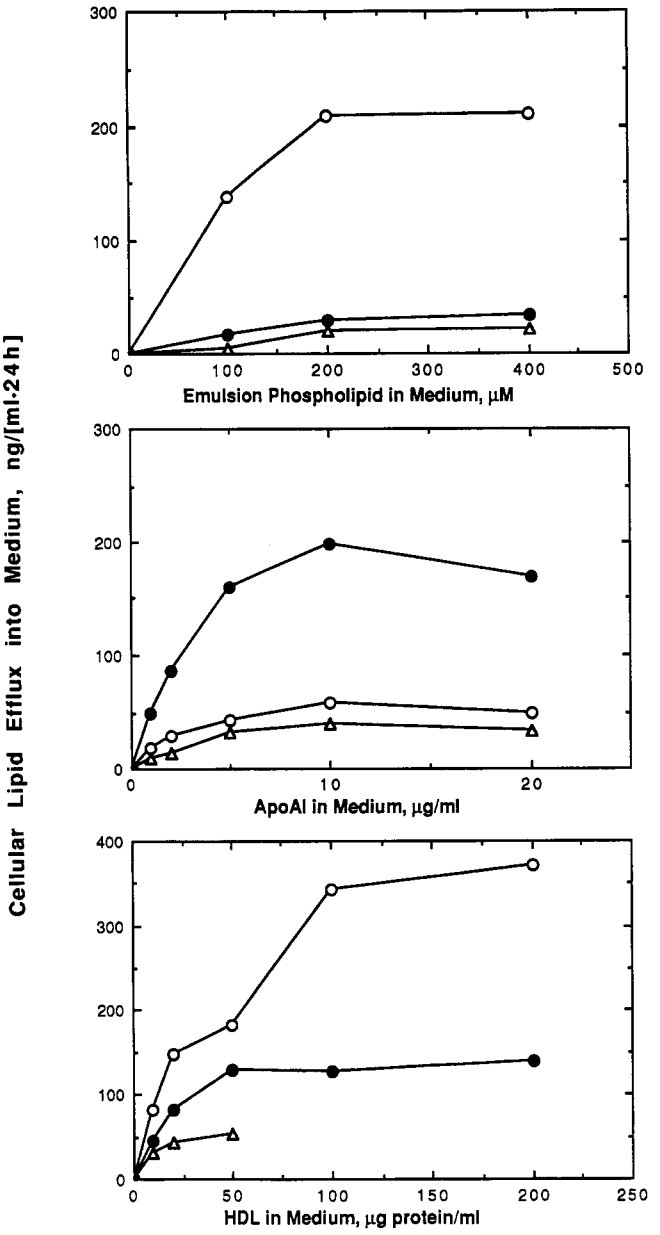

FIGURE 2: Cellular lipid efflux from smooth muscle cells. The cells were loaded with cholesterol by incubating with cationized LDL as described under Experimental Procedures, giving the initial conditions for lipid efflux: cell protein, 272 μg/dish; phosphatidylcholine, 1.62 μg/dish; sphingomyelin, 0.92 μg/dish; free and esterified cholesterol, 2.03 and 1.01 μg/dish, respectively. The efflux of cellular cholesterol (○), phosphatidylcholine (●), and sphingomyelin (△) was measured in the medium induced by the lipid microemulsion (top panel), lipid-free apoA-I (middle panel), and HDL (bottom panel).

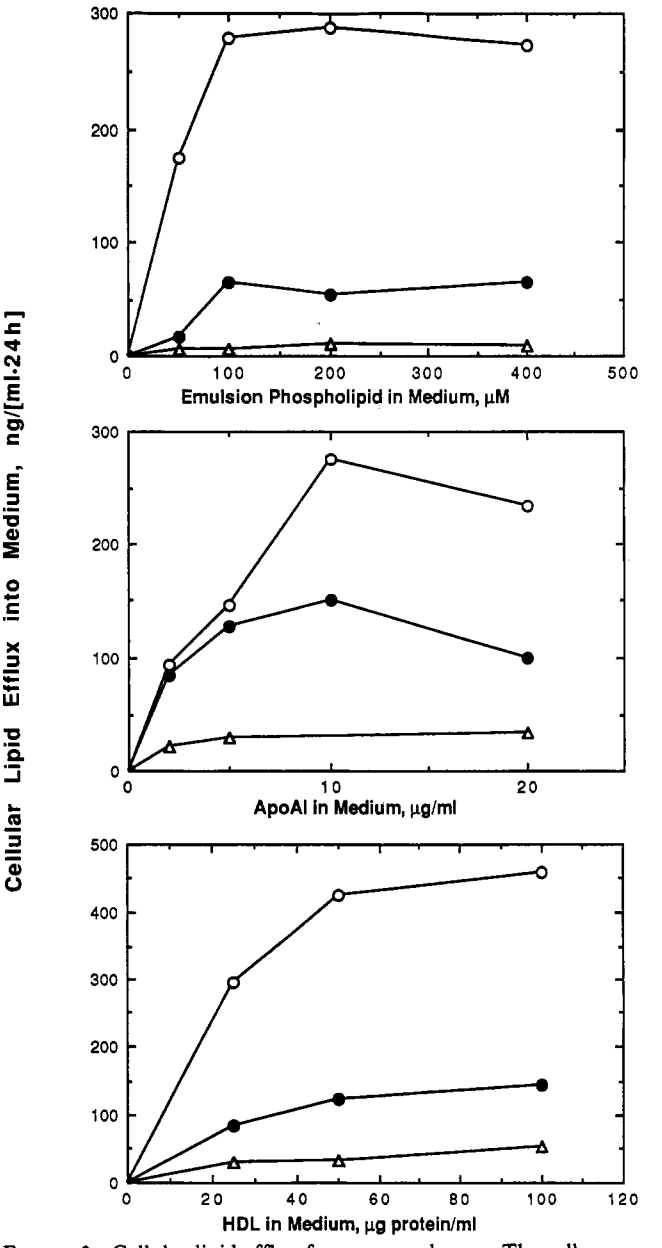

FIGURE 3: Cellular lipid efflux from macrophages. The cells were loaded with cholesterol by incubating with acetylated LDL as described under Experimental Procedures, giving the initial conditions for the efflux: cell protein, 26 μg/dish; phosphatidylcholine, 0.80 μg/dish; sphingomyelin, 0.5 μg/dish; free and esterified cholesterol, 1.30 and 2.71 μg/dish, respectively. The efflux of cellular cholesterol (○), phosphatidylcholine (●), and sphingomyelin (△) was measured in the medium induced by the lipid microemulsion (top panel), lipid-free apoA-I (middle panel), and HDL (bottom panel).

with a slightly higher rate of cholesterol efflux standardized for the cellular free cholesterol pool. In the lipid efflux induced by lipid-free apoA-I generating HDL-like lipoprotein (Hara & Yokoyama, 1991), a significant amount of phospholipids was found with cholesterol in the efflux, as shown in the middle panel of Figure 3, in contrast to the microemulsion. The absolute V_{max} efflux rate of phospholipid was in a similar range to the V_{max} of phospholipid efflux from smooth muscle cells by apoA-I (Figure 2). The weight ratio of phosphatidylcholine plus sphingomyelin to cholesterol in the efflux was about 1, in good agreement with our previous data (Hara & Yokoyama, 1991), that is, much less than the ratio of (4–5):1 observed in the lipid efflux from smooth muscle cells (Figure 2). The lipid efflux profile from macrophage to HDL was essentially the same as the efflux from smooth muscle cells in terms of the rate and the ratio of phospholipids to cholesterol in the lipid efflux (bottom panel of Figure 3).

Phospholipid:Cholesterol Ratio in Cellular Lipid Efflux. The results of the experiments are summarized in Table I for the weight ratio of cellular phospholipids to cholesterol in the lipid efflux induced by various factors. The phosphatidylcholine:cholesterol ratio was a more reproducible parameter than the sphingomyelin:cholesterol ratio probably because a lower content of sphingomyelin in cells caused greater experimental errors. To lipid microemulsion, the rate of apparent efflux of phosphatidylcholine was only 13–28% of cholesterol by weight from smooth muscle cells and 10–29% from macrophages. Sphingomyelin efflux was 10–50% of phosphatidylcholine. This ratio should be considered as the relative rate of nonspecific exchange of these lipids between

Table I: Phospholipid:Cholesterol Ratio in Lipid Efflux from Cultured Cells[a]

		smooth muscle cells			macrophages	
efflux inducer	expt[b]	PC:Chol	SM:Chol	expt	PC:Chol	SM:Chol
apoA-I	1	2.10 ± 0.42	0.43 ± 0.12	1	0.43 ± 0.17	0.15 ± 0.05
	2	3.20 ± 0.39	0.65 ± 0.20	2	0.69 ± 0.20	0.24 ± 0.10
	3	2.09 ± 0.45				
	4	6.02 ± 1.10				
emulsion	1	0.13 ± 0.04	0.013 ± 0.004	1	0.29 ± 0.12	0.16 ± 0.08
	2	0.15 ± 0.014	0.077 ± 0.030	2	0.10 ± 0.01	0.036 ± 0.017
	3	0.28 ± 0.09		3	0.18 ± 0.03	0.031 ± 0.009
	4	0.27 ± 0.11		4	0.23 ± 0.05	0.105 ± 0.032
LDL	1	0.17 ± 0.04		1	0.14 ± 0.03	0.086 ± 0.023
HDL	1	0.83 ± 0.08	0.45 ± 0.12	1	0.30 ± 0.012	0.100 ± 0.016
	2	0.90 ± 0.26				

[a] Efflux of cellular free cholesterol, phosphatidylcholine (PC), and spingomyelin (SM) induced by apoA-I, lipid microemulsion, and lipoproteins was measured in the medium according to the method described under Experimental Procedures, and the ratios of PC and SM to cholesterol were calculated as weight/weight. For smooth muscle cells, Figure 1 corresponds to apoA-I experiment 3 and emulsion 3, and Figure 2 corresponds to apoA-I expt 2, emulsion expt 2, and HDL expt 1. For macrophage, Figure 3 corresponds to apoA-I expt 2, emulsion expt 3, and HDL expt 1. Data represent mean ± SE for three to five experimental points in each series. Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; Chol, cholesterol. [b] Experiment series number.

Table II: Accessibility to Cellular Cholesterol by Extracellular Probes[a]

	smooth muscle cells	macrophages
V_{max}/cellular free cholesterol[b]		
microemulsion	18.2 ± 1.6 (3)[c]	43.3 ± 12.4 (3)
HDL	36.9 ± 2.4 (3)	72.5 ± 8.4 (3)
apoA-I	3.5 ± 0.5 (3)	35.9 ± 5.9 (3)
cholesterol oxidation[d]	27.3 ± 2.3 (6)	42.2 ± 4.8 (6)

[a] The values are the percent of the initial cellular free cholesterol pool. [b] Apparent V_{max} rate of cellular free cholesterol efflux per 24 h standardized as percent of the initial cellular free cholesterol pool. [c] Number of experiments. [d] Percent oxidation of cellular free cholesterol in 3 min by extracellular cholesterol oxidase probes.

the cell surface and lipoprotein surface because back-influx of radioactivity is still negligible at this point. The same ratio of phospholipids to cholesterol was also found in the lipids that efflux from these cells to LDL, showing that there seems to be no specific contribution of apoB to the lipid exchange between LDL and these cells.

Probing Surface Cholesterol. In order to approach a possible mechanism for the minimal availability of cholesterol in apoA-I-mediated lipid efflux (generation of HDL-like particles), the accessibility of cellular cholesterol was assessed using extracellular cholesterol oxidase as a probe, and the results were compared with the data of cholesterol efflux (Table II). Since the rapid phase of the oxidation was shown to be completed within the initial 2 min of the reaction under the conditions used, oxidized cholesterol was measured at 3 min. More cellular free cholesterol was oxidized by cholesterol oxidase in macrophages than in smooth muscle cells. However, this difference was not more than the relatively small difference observed between these cells in the V_{max} of cholesterol efflux standardized for the cellular free cholesterol pool to the lipid microemulsion or HDL. Thus, the extremely poor availability of cellular cholesterol for the lipid efflux by the lipid-free apoA-I-mediated reaction with smooth muscle cells cannot be explained by the general accessibility of cellular cholesterol by such an extracellular probe.

Role of Apolipoprotein in Lipid Efflux to Lipoprotein. As indicated in the results described above, the phospholipid: cholesterol ratio in the lipid efflux induced by apoA-I was greater than that to the lipid microemulsion or to LDL. From macrophages, the total ratio of phosphatidylcholine and sphingomyelin to cholesterol in the efflux by apoA-I was about 1:1, being consistent with our previous data (Hara & Yokoyama, 1991). This ratio was much greater in the lipid efflux from smooth muscle cells by apoA-I because phospholipid efflux was not much different between macrophage and smooth muscle cells while cholesterol efflux was very low as was shown in our previous paper (Komaba et al., 1992) and in this paper again.

The phospholipid:cholesterol ratio in the lipid efflux to HDL was higher than that to the emulsion or LDL but lower than that by apoA-I, especially with smooth muscle cells (Table I). This may suggest that HDL apolipoproteins play a role in inducing more phospholipid efflux relative to cholesterol. In order to know whether lipid-bound or lipid-free apolipoproteins mediate enhancement of phospholipid efflux from the cells, the effect of apoA-I on the lipid efflux from smooth muscle cells was observed. Figure 4 demonstrates the results of the experiments. Cholesterol efflux increased as a sigmoidal function of apoA-I concentration in the medium as demonstrated in previous work (Hara & Yokoyama, 1992). This curve was shown to be consistent with the model that free apoA-I in the aqueous phase but not lipid-bound apoA-I is responsible for this enhancement (Hara & Yokoyama, 1992). The extent of cholesterol efflux enhancement was about 50%. Phosphatidylcholine efflux was also enhanced as a sigmoidal function of apoA-I so that this enhancement is also consistent with the same model. The increase of phospholipid efflux by apoA-I was approximately by 200%. As a result, the ratio of phosphatidylcholine to cholesterol in the lipid efflux also increased by apoA-I (bottom panel of Figure 4). Thus, the data are consistent with a model that lipid-free apoA-I mediates the increase of the phospholipid:cholesterol ratio in lipid efflux from the cell to lipoproteins.

Thus, we conclude the following: (1) cellular cholesterol in both macrophages and smooth muscle cells is exchangeable with an extracellular pool, and this exchangeability is compatible with the accessibility of cellular cholesterol by the extracellular enzymatic probe; (2) the availability of cholesterol for apolipoprotein-mediated cellular lipid efflux is, however, extremely low in smooth muscle cells though the reaction itself it not less than in macrophages as demonstrated by a similar rate of phospholipid efflux from these cells.

These findings provide more evidence that there are at least two well-distinctive pathways of cellular cholesterol efflux. One is a nonspecific exchange of cholesterol between the cell surface and the extracellular lipid surface, mostly lipoprotein surface in vivo, by diffusion through the aqueous phase or by collision between membranes. In this pathway, specific

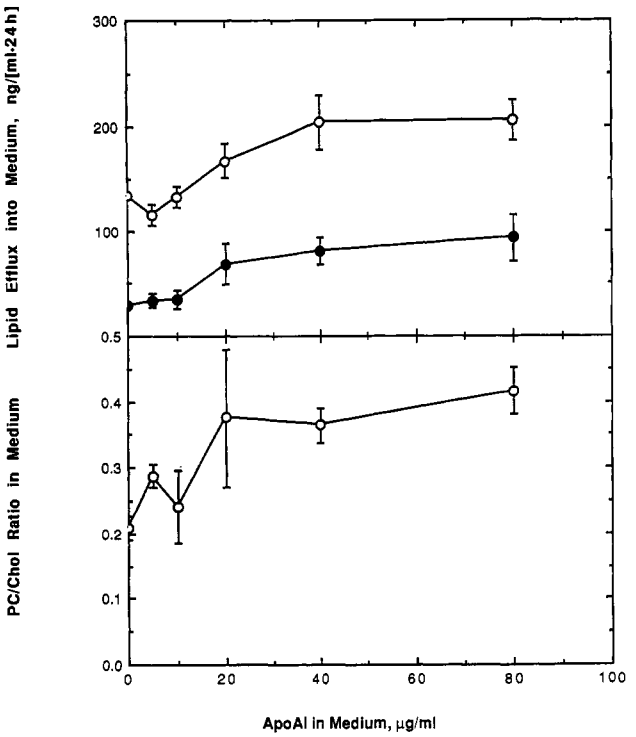

FIGURE 4: Effect of apoA-I concentration on lipid efflux from smooth muscle cells induced by the lipid microemulsion. The initial conditions are the same as those of Figure 1. Lipid-free apoA-I was added to the lipid microemulsion, 200 μM phosphatidylcholine in the medium, indicated on the abscissa, and the cellular lipid efflux was measured in the medium. The upper panel shows the efflux of cholesterol (○) and phosphatidylcholine (●), and the lower panel shows the ratio of phosphatidylcholine to cholesterol (○). The error bars indicate the standard error of three experimental points.

interaction between the acceptor lipoproteins and cell surface is unlikely to be involved (Karlin et al., 1987; Hara & Yokoyama, 1992). The rate-limiting factor would be the size of an available pool of cholesterol for this diffusion. A net decrease of cellular cholesterol by this mechanism may only be achieved when the cells are overloaded with cholesterol, causing a difference in its efflux and influx [that can be up to 30% of the apparent rate of efflux (Johnson et al., 1988)]. The other pathway is net lipid removal by apolipoproteins, generating new preβ-HDL-like particles with cellular lipids (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992). The physiological relevance of this reaction is justified by the low K_m value for the apolipoprotein concentration such as 1/1500 of plasma apoA-I for fibroblasts and smooth muscle cells and 1/500 for macrophages. There are several possibilities: (1) it may not be inconsistent with putative lipid-free apolipoproteins in interstitial fluid; (2) liberation of apolipoproteins can occur by a lipid-transfer reaction in the peripheral circulation under certain conditions (Clay et al., 1992; Ryan et al., 1992); and (3) some apolipoproteins may be transferred from the lipoprotein to the cell surface because the K_m for the efflux is much smaller than the dissociation constant of these apolipoproteins for their binding to lipoprotein-like lipid particles (Tajima et al., 1983). This pathway is consistent with the data that cellular cholesterol appears in preβ-HDL fractions prior to other lipoproteins in plasma in vitro (Castro & Fielding, 1988; Miida et al., 1990). Since nonspecific exchange of phospholipid is much slower than cholesterol, these two pathways are distinguishable by the relative amount of phospholipid to cholesterol in the lipid efflux. This was shown by enhancement of lipid efflux from smooth muscle cells to the lipid microemulsion by apoA-I (Figure 4). A higher content of phospholipid in the lipid efflux to HDL than in the nonspecific efflux to the microemulsion may also indicate the contribution of such direct interaction of apolipoproteins with the cell surface to the lipid efflux to HDL.

Cholesterol was poorly available in the lipid efflux mediated by lipid-free apolipoproteins from smooth muscle cells. The standardized V_{max} rate of nonspecific cholesterol efflux for the cellular free cholesterol pool may also be slightly lower in smooth muscle cells than in other cells. The accessibility of cholesterol from the cellular surface was estimated for smooth muscle cells and macrophages using a cholesterol oxidase probe to measure the oxidation of cellular cholesterol in the initial 3 min (Lange & Ramos, 1983). The result was consistent only with the small difference observed for the nonspecific cholesterol exchange rate between the two cells. Thus, the poor availability of cholesterol in apolipoprotein-mediated lipid efflux from smooth muscle cells was very unique and specific.

We postulate two possible mechanisms. The first possibility is that the smooth muscle cell surface does not contain so much cholesterol as other cell surfaces. By nonspecific exchange, the cell surface may just be a "window" for this reaction granted that surface cholesterol is rapidly exchangeable with other cellular free cholesterol pools. Therefore, the V_{max} rate of this exchange may not be affected by the low cholesterol content on the surface. The cholesterol oxidase method may not probe this surface pool accurately because of relatively rapid exchange of cholesterol between the pools. Apolipoproteins interact directly with the surface lipids and use these lipids to generate lipoprotein. Therefore, the lipid composition of the surface may be more directly reflected in the lipid efflux by this mechanism. The second possibility is that there is a specific cholesterol pool in the cell surface available for the apolipoprotein-mediated efflux or that there is a specific mechanism by which cholesterol is incorporated into newly generated HDL-like particles. This idea may be consistent with the recent proposal by Mahlberg and Rothblat (1992) of two distinguishable kinetic pools of cholesterol on the cell surface for the nonspecific exchange reaction. The mechanism for creating different cholesterol pools in the membrane is unknown. One of the possibilities is association of cholesterol with other specific molecular components of the membrane, such as sphingomyelin, that may lead to restriction of exchangeability of cholesterol with other membranes or to comovement of both lipids. Sphingomyelin: phosphatidylcholine weight ratio in the cells were 0.91 ± 0.21 in macrophages and 0.27 ± 0.03 in smooth muscle cells in our experimental conditions, which may have been reflected in the apoA-I-induced lipid efflux as 0.35 versus 0.20, respectively, and in the emulsion-induced efflux as 0.39 versus 0.28, respectively (Table I). However, it is difficult to discover any direct relationship between these ratios and specific poor cholesterol efflux by apoA-I from smooth muscle cells. Thus, the results of cellular lipid compositional analysis did not provide any conclusive information for discussion. In order to distinguish between these two possibilities, direct measurement of cell surface cholesterol is required.

Involvement of a specific binding site on the cellular surface in apolipoprotein-mediated lipid efflux remains unclear. There is no specificity for apolipoproteins capable of carrying out this reaction (Hara & Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992). However, the very low K_m of apolipoprotein concentration for the reaction indicates a possible specific interaction of apolipoproteins with the cell

surface. These values are significantly lower than most dissociation constants of the apolipoprotein–lipid interaction (Tajima et al., 1983; Yokoyama et al., 1985; Okabe et al., 1988). The proposed contribution of such specific binding to mobilization of cellular cholesterol to the surface (Slotte et al., 1987; Aviram et al., 1989; McKnight et al., 1992) is one of the possible factors that modulate this specific poor availability of cholesterol in the apolipoprotein-mediated lipid efflux from smooth muscle cells.

The present work confirmed our recent work that cholesterol efflux from smooth muscle cells is poor, especially by the pathway mediated by lipid-free apolipoproteins that leads to pure net lipid efflux (Komaba et al., 1992). These results indicate that atherosclerotic vascular lesions in the stage predominant in smooth muscle cells are very resistant to regression. This paper revealed that this is not because the cells are less reactive with apolipoproteins but because cellular cholesterol is not available for this reaction. Therefore, it may be possible to modulate cellular cholesterol metabolism in smooth muscle cells in order to create a cellular cholesterol pool reactive with the apolipoprotein-mediated lipid efflux and to make smooth muscle cell-dominated vascular lesions regressible.

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