Inhibitory Effects of HepG2 Cell-Derived Apolipoprotein A-I-Containing Lipoproteins on Cholesteryl Ester Accumulation in Macrophages†

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Received April 11, 1997; Revised Manuscript Received June 5, 1997⊗

ABSTRACT: We investigated the mechanisms of inhibitory effects on foam cell formation of apolipoprotein A-I-containing lipoproteins secreted by HepG2 cells (HepG2-HDL) using mouse peritoneal macrophages. When macrophages were incubated with acetylated low-density lipoprotein (acetyl-LDL) in the presence of HepG2-HDL, cholesterol ester (CE) accumulation in cells was reduced by 63%. This inhibitory capacity was almost similar to that of plasma high-density lipoprotein (HDL). When macrophages were converted to foam cells with acetyl-LDL and then reacted with HepG2-HDL or plasma HDL, the HDL-induced CE reduction was 2.2-fold greater than HepG2-HDL. Similar results were obtained using apo E-free HepG2-HDL. Since the inhibitory effect of HDL on acetyl-LDL-induced CE accumulation in macrophages is due largely to its cholesterol efflux capacity, these results suggest the presence of an additional mechanism for the inhibition of CE accumulation by HepG2-HDL. To investigate the mechanism, acetyl-LDL was reisolated from HepG2-HDL by Sephacryl S-300 gel filtration after incubation in a cell-free system. Reisolated acetyl-LDL showed a significant reduction in electrophoretic mobility. The extent of CE accumulation by reisolated acetyl-LDL was reduced by 20% compared with control acetyl-LDL. Moreover, its endocytic degradation by macrophages was reduced by 28%. HepG2-HDL also inhibited macrophage degradation of acetyl-LDL as well as oxidized LDL, a likely atherogenic lipoprotein. This inhibitory effect was ascribed to the HepG2-HDL subfraction containing pre-β HDL. Our results indicated that apo A-I-containing lipoproteins as a physiological model of nascent HDL may inhibit foam cell formation by reducing ligand activity of atherogenic lipoproteins. These data possibly suggest inhibitory function of nascent HDL for the formation of foam cells *in vivo*.

The presence of macrophage foam cells is a characteristic feature of the early stages of atherosclerosis (Ross, 1993). Cultured macrophages are known to take up chemically modified low-density lipoproteins (modified LDLs) such as acetylated LDL (acetyl-LDL) and oxidized LDL (Ox-LDL) through the scavenger receptor pathway (Brown & Goldstein, 1983; Steinberg et al., 1989). LDL is oxidatively modified by a variety of cells in the arterial wall, such as smooth muscle cells, endothelial cells, and macrophages, and changed to a ligand for the macrophage scavenger receptor (MSR) (Steinberg et al., 1989). This leads to the accumulation of intracellular cholesteryl esters (CE) or the formation of foam cells. Foam cell formation by these mechanisms is believed to play an essential role in the progression of early atherosclerotic lesions *in vivo* (Ross, 1993).

In contrast to modified LDLs, an anti-atherogenic role for high-density lipoprotein (HDL) has been emphasized, based mainly on epidemiological evidence that plasma HDL levels correlate inversely with the incidence of atherosclerotic diseases [for review, see Stampfer et al. (1991)]. The anti-atherogenic property of HDL has been mainly explained by enhancement of cholesterol efflux from peripheral cells, representing the first step in reverse cholesterol transport from peripheral tissues to the liver (Gordon & Rifkind, 1989; Johnson et al., 1991). *In vitro* experiments showed that an incubation of HDL with macrophage foam cells resulted in a significant decrease in cellular CE (Miyazaki et al., 1992; Hakamata et al., 1994). However, recent studies have uncovered other anti-atherogenic properties of HDL. These include: (i) inhibition of oxidative modification of LDL (Ohta et al., 1989a; Parthasarathy et al., 1990; Kunitake et al., 1992); (ii) induction of macrophage growth by Ox-LDL was inhibited by the presence of HDL (Sakai et al., 1994); (iii) when acetyl-LDL was incubated with discoidal reconstituted complex of dimyristoylphosphatidylcholine and apolipoprotein A-I (DMPC/apo A-I), a large amount of DMPC was transferred from DMPC/apo A-I to acetyl-LDL.

† This work was supported in part by Grants-in-Aid for Scientific Research (07557076, 08877159, 08044304) from the Ministry of Education, Science and Culture of Japan. T.K. and H.H. contributed equally to this study.

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1997.

[1] Abbreviations: LDL, low-density lipoprotein; acetyl-LDL, acetylated LDL; HDL, high-density lipoprotein; CE, cholesteryl ester; Ox-LDL, oxidized LDL; FC, free cholesterol; TG, triglycerides; PL, phospholipids; MSR, macrophage scavenger receptor; apo A-I, apolipoprotein A-I; DMPC, dimyristoylphosphatidylcholine; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; apo A-II, apolipoprotein A-II; apo Cs, apolipoprotein Cs; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein.

S0006-2960(97)00844-1 CCC: $14.00

This was accompanied by a marked reduction in the ligand activity of acetyl-LDL for MSR (Miyazaki et al., 1994). Similar results were obtained in experiments using Ox-LDL (Sakai et al., 1996).

HepG2 cells, a human hepatoblastoma-derived cell line, have been often used to characterize hepatic lipoprotein metabolism in man because of their secretion of apolipoproteins A-I, A-II, B100, C-II, C-III, and E (Rash et al., 1981; Zannis et al., 1981, 1983). With respect to HDL metabolism, HepG2 cells are able to synthesize precursor forms of apo A-I and apo E (Zannis et al., 1981, 1983; Gordon et al., 1983). HepG2-secreted HDL is characterized biochemically by a higher level of free cholesterol and phospholipid and a lower level of cholesteryl esters as compared with plasma HDL (Thrift et al., 1986) and morphologically by discoidal particles and small spherical particles (McCall et al., 1988; Cheung et al., 1989). Additionally, the apolipoprotein and lipid composition and the morphological structure of HepG2-secreted HDL were similar to those isolated from LCAT-deficient patients having immature HDL particles in plasma (Mitchell et al., 1980; Soutar et al., 1982; Ohta et al., 1994). Furthermore, HepG2 cells were reported to secrete lipid metabolism-related molecules such as lecithin:cholesterol acyltransferase (LCAT) (Chen et al., 1986), hepatic triglyceride lipase (HTGL) (Busch et al., 1990), and cholesteryl ester transfer protein (CETP) (Faust & Alberts, 1987). However, the recent report indicated that the LCAT activity in culture media of HepG2 cells was relatively low (Chen et al., 1986). From these results, it seems reasonable to expect that HepG2 cells are useful to study nascent HDL particles secreted from the liver *in vivo*.

Previous studies using HDL particles isolated from culture media of HepG2 cells have mainly focused on their morphology as well as their lipid and apolipoprotein composition (Zannis et al., 1981, 1983; Thrift et al., 1986; Forte et al., 1989). It is also important, however, to use these HDL particles to understand their anti-atherogenic properties at a cellular level since newly secreted HDL (HepG2-HDL) is physicochemically similar to interstitial HDL [for review, see Sloop et al. (1987)].

In the present study, we first isolated apo A-I-containing lipoproteins (HepG2-HDL) from culture media of HepG2 cells using anti-apo A-I immunoaffinity chromatography. We next determined the *in vitro* inhibitory effects of HepG2-HDL on acetyl-LDL-induced foam cell formation and compared them with plasma HDL. The results of the present study show that plasma HDL inhibited accumulation of CE in macrophages by cholesterol efflux, whereas HepG2-HDL inhibited CE accumulation not only by cholesterol efflux but also by reducing the ligand activity of atherogenic lipoproteins for MSR. Thus, based on these results using HepG2-HDL as newly secreted HDL particles, we suggest that nascent HDL particles may also serve as anti-atherogenic lipoprotein *in vivo*.

MATERIALS AND METHODS

Materials. Tissue culture media and reagents were obtained from Life Technologies, Inc. Silica gel on aluminum sheets for thin-layer chromatography (TLC) was obtained from Merck. $Na^{125}I$ (17 Ci/mg) and [9,10(N)-^{3}H]-oleate (370 GBq/mmol) were purchased from Amarsham. All other chemicals were of the best grade available from commercial sources.

Lipoproteins and Their Modifications. LDL ($d = 1.019-1.063$ g/mL) and HDL ($d = 1.063-1.21$ g/mL) were isolated by sequential ultracentrifugation from fresh human plasma and dialyzed against 0.15 M NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4) (solution A). Traces of apo B and E were removed from HDL by a heparin−agarose column (Murakami et al., 1987). Acetyl-LDL and Ox-LDL were prepared as described previously (Murakami et al, 1987; Sakai et al., 1994). Iodination of the two lipoproteins with ^{125}I was performed as originally described by McFarlane (1958). Protein concentrations were determined by the BCA protein assay reagent (Pierce Chemical Co.) using bovine serum albumin (BSA) as a standard (Miyazaki et al., 1992) and expressed as milligrams of protein per milliliter.

Preparation of Apo A-I-Containing Lipoproteins (HepG2-HDL) from Culture Media of HepG2 Cells. Preparation of culture medium from HepG2 cells and subsequent purification of apo A-I-containing lipoproteins using an anti-apo A-I immunoaffinity chromatography were performed according to the methods described by McCall et al. (1988) and our laboratory (Ohta et al., 1989b, 1992), respectively. Typically, human hepatoblastoma cell line HepG2 cells were seeded into 50 tissue culture flasks (175 cm^2, Falcon 3028, Oxnard, CA) in 25 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mg/mL streptomycin, 100 units/mL penicillin, and 10% heat-inactivated fetal calf serum (FCS) and incubated at 37 °C in 5% CO_2. Fourteen days after seeding at semi-confluent monolayers, each flask was washed 3 times with 25 mL of phosphate-buffered saline (PBS), and then incubated for 24 h with 20 mL of the same medium without FCS (serum-free DMEM). The culture supernatant was taken from each flask, and the combined supernatants were centrifuged at 1000*g* for 5 min at 4 °C and filtered through a 0.45 μm membrane filter (Millipore) to remove detached cells and debris, and stored at 4 °C in 1 mM EDTA prior to purification. The cells in each flask were further incubated for 24 h with 20 mL of fresh serum-free DMEM. This procedure was repeated 4 times (the total incubation time for each flask was 4 days). The supernatants from all 50 flasks (4 L in total) were combined and concentrated 100-fold using an ultrafiltration cell (Amicon, Bererly, MA) equipped with a PM-30 membrane. The concentrated solution (~100 mL) was applied on an anti-apo A-I immunosorbent column (15 × 100 mm) (Ohta et al., 1989b, 1992). The column was washed extensively with 10 mM Tris, 0.5 M NaCl, 1 mM EDTA (pH 7.5) and eluted with 0.1 M acetic acid and 1 mM EDTA (pH 3.0) at a flow rate of 20 mL/h. The effluent was immediately adjusted to pH 7.4 with 5 N NaOH, dialyzed against solution A, and concentrated to 5 mL with Centriprep-10 (Amicon). Each purification yielded approximately 8 mg of apo A-I-containing lipoproteins which was thereafter referred to as "HepG2-HDL". The total amount of HepG2-HDL used in the present study was obtained by seven sets of purification processes. For the two experiments shown in Figures 3 and 9, HepG2-HDL was further purified by a heparin−agarose column to remove apo B and E (Murakami et al., 1987). However, most of the applied protein (>95%) was recovered from the column (data not shown).

Cell Culture. Peritoneal macrophages were collected from nonstimulated male DDY mice (25−30 g) with 8 mL of ice-cold PBS, centrifuged at 200*g* for 5 min at 4 °C, and

suspended in Dulbecco's modified Eagle's medium (DMEM) containing 3% BSA, streptomycin (0.1 mg/mL), and penicillin (100 units/mL) (medium A) (Sakai et al., 1992). One milliliter of cell suspension (2×10^6 cells) was seeded onto each culture plastic dish (22 mm diameter, Corning) and incubated for 2 h at 37 °C in 5% CO_2. After washing 3 times with 1 mL of medium A, the monolayers thus formed were used in the following cellular experiments.

Assay for Cholesterol Esterification. The macrophage monolayers formed through the above process were incubated with acetyl-LDL for 18 h in the presence of 0.1 mM [^{3}H]oleate conjugated with BSA (105 232 dpm/nmol) (Goldstein et al., 1983). Cellular lipids were extracted for determination of the radioactivity of cholesteryl [^{3}H]oleate as described previously (Miyazaki et al., 1991).

Mass Determination of Cellular Cholesterol Contents. Both cellular free cholesterol and CE mass were quantified by a modification of the enzymatic fluorometric method of Heider and Boyett (1978). The enzyme mixtures were identical to those described by these workers except that the Carbowax-6000 was replaced with 0.01% Triton X-100 and enzyme concentrations were double those used originally (cholesterol oxidase, 0.16 unit/mL; cholesteryl ester hydrolase, 0.16 unit/mL) (Miyazaki et al., 1993).

Cell Association and Endocytic Degradation of ^{125}I-Acetyl-LDL and ^{125}I-Ox-LDL by Macrophages. The prepared mouse macrophage monolayers (2×10^6 cells) were incubated with ^{125}I-acetyl-LDL (644 cpm/ng of protein) or ^{125}I-Ox-LDL (632 cpm/ng of protein) for 18 h at 37 °C. Endocytic degradation was determined by trichloroacetic acid (TCA)-soluble radioactivity in the medium (Hakamata et al., 1995). The cell-associated radioactivity and cell proteins were determined after dissolving the cells with 1.0 mL of 0.1 N NaOH.

Incubation of Acetyl-LDL with HepG2-HDL and Reisolation of Acetyl-LDL from the Mixture. To characterize the interaction between acetyl-LDL and HepG2-HDL in a cell-free system, acetyl-LDL (1 mg of protein/mL) was incubated at 37 °C for 18 h with HepG2-HDL (5 mg of protein/mL) in 2 mL of solution A. The incubation mixture was applied to a gel filtration column of Sephacryl S-300 (2.4 × 150 cm, Pharmacia) and eluted with solution A at a flow rate of 10 mL/h. We referred to this eluted acetyl-LDL as reisolated acetyl-LDL. A control incubation was performed using the same protein concentration of HDL instead of HepG2-HDL.

Lipid Analysis. The lipid contents of lipoproteins were determined on a Hitachi 7450 automatic analyzer using standard enzymatic methods (Allain et al., 1974; Takayama et al., 1977; Spayd et al., 1978).

Electrophoresis. Agarose gel electrophoresis and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were performed as described previously (Suginohara et al., 1996).

Statistical Analysis. Data were expressed as mean ± SD. Differences between groups were evaluated by the Student's *t*-test. Statistical significance was inferred when the *P*-value was less than 0.05.

RESULTS

Physicochemical Properties of HepG2-HDL. We characterized the physicochemical properties of HepG2-HDL isolated by immunoaffinity chromatography. Lipid analyses revealed that the CE contents of HepG2-HDL were lower than HDL, but free cholesterol contents were higher than HDL (Table 1). However, the phospholipid contents of HepG2-HDL were similar to those of HDL. Gradient SDS−PAGE (4−20%) showed that HepG2-HDL was composed of a main band of 28 kDa and a minor band of 17 kDa, the former corresponding to apo A-I while the latter probably corresponded to apo A-II (Figure 1A, lane b). HepG2-HDL was also found to contain very small amounts of apo E and apo Cs upon 15% SDS−PAGE (data not shown). Agarose gel electrophoresis showed migration of HDL to the α position (Figure 1B, lane a), whereas HepG2-HDL migrated as two separate bands at the α and the pre-β position, respectively (Figure 1B, lane b), suggesting that HepG2-HDL was a heterogeneous lipoprotein.

Table 1: Lipid Contents of HepG2-HDL[a]

	lipid/protein weight ratio				
	TC[b]	FC[c]	CE[d]	TG[e]	PL[f]
HDL	0.37	0.06	0.31	0.09	0.49
HepG2-HDL	0.22	0.16	0.07	0.10	0.42

[a] HepG2-HDL was prepared from serum-free DMEM by an anti-apo A-I immunosorbent column (15 × 100 mm), and HDL (d = 1.063−1.21 g/mL) was isolated by sequential ultracentrifugation from fresh human plasma. The lipid content of these lipoproteins was determined as described under Materials and Methods. Experimental errors in the lipid determination were within 5%. [b] TC, total cholesterol. [c] FC, free cholesterol. [d] CE, cholesteryl ester. [e] TG, triglycerides. [f] PL, phospholipids.

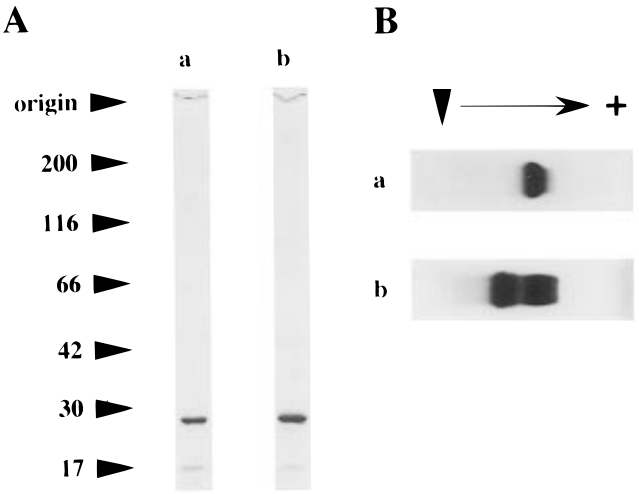

FIGURE 1: SDS−polyacrylamide gel electrophoresis (A) and agarose gel electrophoretic mobility (B) of HepG2-HDL. (A) HDL (lane a) and HepG2-HDL (lane b) (5 μg/lane) were electrophoresed on gradient SDS−PAGE (4−20%) upon nonreducing conditions and stained with Coomassie Brilliant Blue. Molecular mass (arrowheads) is indicated in kDa. (B) HDL (lane a) and HepG2-HDL (lane b) (10 μg/lane) were electrophoresed on a 1% agarose gel and stained with Coomassie Brilliant Blue. The arrowhead shows the origin, and the arrow shows the direction.

Effect of HepG2-HDL on Acetyl-LDL-Induced CE Accumulation in Mouse Macrophages. To test the inhibitory effect of HepG2-HDL on foam cell formation, we compared the effect of HepG2-HDL on acetyl-LDL-induced CE accumulation with that of HDL using the simultaneous incubation system (Hakamata et al., 1995). As shown in Figure 2, the incorporation of [^{3}H]oleate into cholesteryl [^{3}H]-oleate was inhibited, in a dose-dependent manner, in the presence of HDL. In a similar fashion, HepG2-HDL also inhibited CE accumulation in a manner indistinguishable from HDL. These results indicated that HepG2-HDL

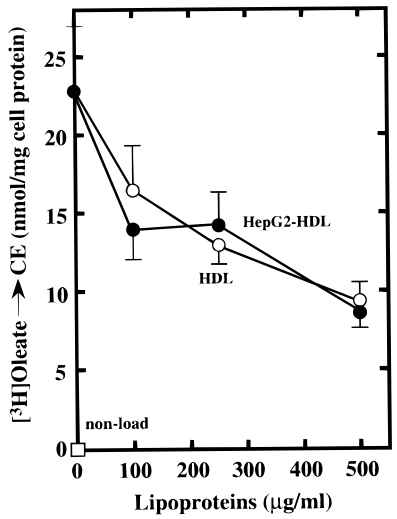

FIGURE 2: Effect of HepG2-HDL on acetyl-LDL-induced CE accumulation in mouse macrophages (simultaneous incubation system). Macrophages (2×10^6) were incubated at 37 °C for 18 h with 25 μg/mL acetyl-LDL and 0.1 mM [^{3}H]oleate in the presence or absence of the indicated concentrations of HDL (○) or HepG2-HDL (●). Cellular lipids were extracted, and the radioactivity of cholesteryl [^{3}H]oleate was determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD.

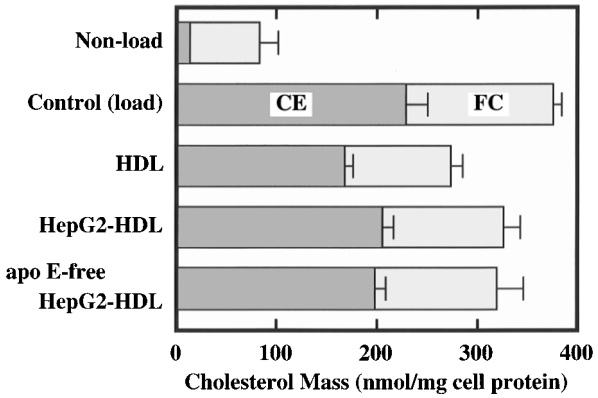

FIGURE 3: Effect of HepG2-HDL on cholesterol efflux from mouse macrophage foam cells (sequential incubation system). Macrophages (2×10^6) were converted to foam cells by 18 h incubation with 25 μg/mL acetyl-LDL. Cells were washed and then incubated for an additional 18 h with 500 μg/mL HDL, HepG2-HDL, or apo E-free HepG2-HDL. Cellular lipids were extracted, and the cholesterol mass was determined as described under Materials and Methods. Each value is the mean ± SD of quadruplicate experiments.

inhibited foam cell formation.

HepG2-HDL-Induced Cholesterol Efflux from Macrophage Foam Cells. Previous reports demonstrated that the inhibitory effect of HDL on CE accumulation in macrophages was due to the capacity of HDL to enhance cholesterol efflux (Miyazaki et al., 1994; Hakamata et al., 1995). Since the capacity of HepG2-HDL to inhibit CE accumulation in macrophages was almost similar to that of HDL (Figure 2), we hypothesized that the effect of HepG2-HDL on cholesterol efflux would be also similar to that of HDL. To test this notion, we converted macrophages to foam cells with acetyl-LDL. The cells were then incubated with HepG2-HDL or HDL using the sequential incubation system (Hakamata et al., 1995). As shown in Figure 3, the cellular CE mass was reduced by 26% with HDL. HepG2-HDL also significantly reduced cellular CE by 12%, although its CE-reducing capacity was weaker than that of HDL.

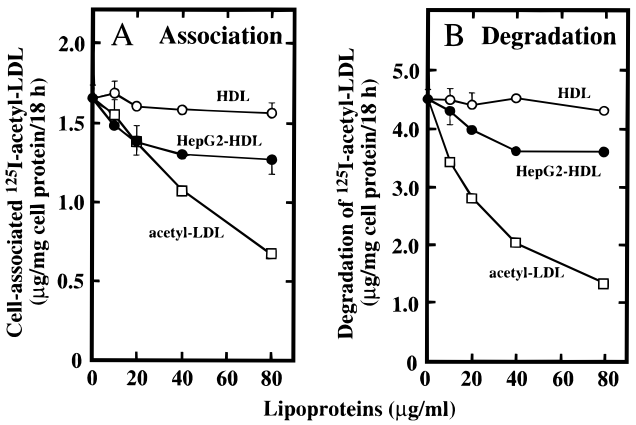

FIGURE 4: Effect of HepG2-HDL on cellular association (A) of ^{125}I-acetyl-LDL with, and endocytic degradation (B) by, mouse macrophages. Macrophages (2×10^6) were incubated at 37 °C for 18 h with 4 μg/mL ^{125}I-acetyl-LDL in the presence of the indicated concentrations of acetyl-LDL (□), HepG2-HDL (●), or HDL (○). Cellular association (A) and subsequent endocytic degradation (B) were determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD. The SD is very small and within the symbol in data without the error bars.

The main apoprotein components of HepG2-HDL were apo A-I and A-II, but very small amounts of apo E and Cs were also observed. To eliminate potential effects of apo E, apo E-containing subfractions were removed from HepG2-HDL by the heparin−agarose column. In the purified HepG2-HDL referred to as "apo E-free HepG2-HDL", no band of 33 kDa corresponding to apo E was detectable upon SDS−PAGE (data not shown). However, apo E-free HepG2-HDL was indistinguishable from HepG2-HDL in its migration pattern on agarose gel electrophoresis and its lipid composition. Apo E-free HepG2-HDL was compared in its capacity for cholesterol efflux with HepG2-HDL under identical conditions. As shown in Figure 3, their CE-reducing capacities were indistinguishable from each other.

Effect of HepG2-HDL on Cellular Association of ^{125}I-Acetyl-LDL with, and Subsequent Endocytic Degradation by, Macrophages. Although the inhibitory effect of HepG2-HDL on acetyl-LDL-induced CE accumulation was similar to that of HDL (Figure 2), its capacity for cholesterol efflux from foam cells was weaker than that of HDL (Figure 3). Therefore, there could be an additional mechanism(s) for HepG2-HDL-induced inhibition of CE accumulation. To identify such mechanism(s), we first tested the effect of HepG2-HDL on endocytic degradation of acetyl-LDL. Macrophages were incubated with ^{125}I-acetyl-LDL in the presence of HepG2-HDL. As shown in Figure 4B, the competition of degradation of ^{125}I-acetyl-LDL by unlabeled acetyl-LDL amounted to a maximum of 70%. In contrast, HDL showed no inhibitory effect. On the other hand, under these conditions, HepG2-HDL effectively reduced the degradation of ^{125}I-acetyl-LDL by up to 21%. Similar results were obtained in cellular association of ^{125}I-acetyl-LDL (Figure 4A). These findings suggest a possible interaction of HepG2-HDL with acetyl-LDL with subsequent modification of endocytic degradation of acetyl-LDL by macrophages.

Physicochemical Properties of Acetyl-LDL after Exposure to HepG2-HDL. To confirm the interaction of acetyl-LDL with HepG2-HDL in the medium, we incubated acetyl-LDL with HepG2-HDL at 37 °C for 18 h in a cell-free system

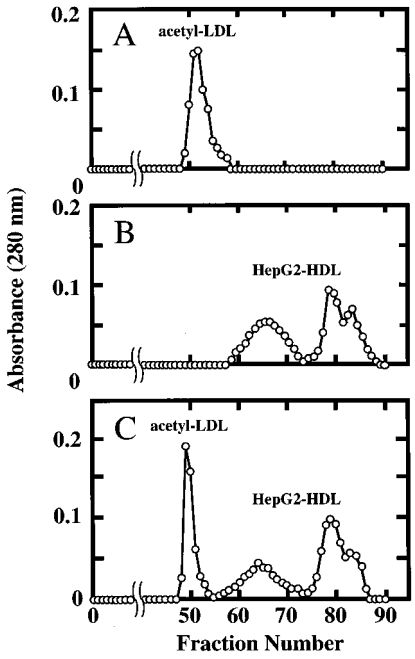

FIGURE 5: Reisolation of acetyl-LDL from HepG2-HDL by Sephacryl S-300 gel chromatography after cell-free incubation. Acetyl-LDL (1 mg/mL) was incubated with HepG2-HDL (5 mg/mL) at 37 °C for 18 h in 2 mL of 0.15 M NaCl and 1 mM EDTA (pH 7.4). The mixture was subjected to a Sephacryl S-300 gel filtration column (2.4 × 150 cm) and eluted with the same buffer as described under Materials and Methods. Protein concentration was monitored with the absorbance at 280 nm. (A) Acetyl-LDL alone, (B) HepG2-HDL alone, (C) acetyl-LDL after exposure to HepG2-HDL.

and subjected the mixture to Sephacryl S-300 gel filtration chromatography. The control experiments consisted of application of acetyl-LDL (Figure 5A) or HepG2-HDL (Figure 5B) alone to the same column. HepG2-HDL was fractionated into three separate peaks (Figure 5B), suggesting that HepG2-HDL was composed of three particles of different sizes. After cell-free incubation, the elution profiles of the mixture (Figure 5C) showed an initial major peak corresponding to acetyl-LDL, followed by three smaller peaks corresponding to those of HepG2-HDL. The first peak, termed reisolated acetyl-LDL, was used in the following experiments.

In the next step, we determined the distribution of apolipoprotein of fractionated peaks (Figure 5C) by SDS−PAGE (Figure 6A). Incubation of acetyl-LDL with HepG2-HDL did not influence the protein moieties of reisolated acetyl-LDL (Figure 6A, lane d) compared with acetyl-LDL (lane c), indicating that transfer of apolipoprotein did not occur between acetyl-LDL and HepG2-HDL. The three peaks derived from HepG2-HDL had mainly apo A-I and a small amount of apo A-II, E, and Cs (data not shown).

Acetylation of LDL increased its electrophoretic mobility from the β position to near the α position (Figure 6B, lanes a and c). In contrast, the electrophoretic mobility of reisolated acetyl-LDL after incubation with HepG2-HDL was near the pre-β position (Figure 6B, lane d), suggesting that the interaction with HepG2-HDL decreased the net negative charge of acetyl-LDL. We showed earlier that HepG2-HDL exhibited two bands on agarose gel electrophoresis whose electrophoretic mobility corresponded to α and pre-β, respectively (Figure 1B, lane b). After gel filtration, reisolated HepG2-HDL was separated into three peaks (subfractions 1, 2, and 3) (Figure 5C). A further agarose gel electrophoretic analysis revealed that subfraction 1 (fraction 64) migrated to the α position (Figure 6B, lane e) while subfraction 2 (fraction 78) and subfraction 3 (fraction 82) migrated to the pre-β position (Figure 6B, lanes f and g).

When the lipid composition of reisolated acetyl-LDL was compared with that of control acetyl-LDL, the phospholipid content of reisolated acetyl-LDL was 1.1 times higher and the free cholesterol 1.4 times higher than acetyl-LDL, but the CE content was less by about 30% (Table 2). A control incubation of acetyl-LDL with HDL did not result in any significant change in lipid contents (data not shown).

Biological Properties of Acetyl-LDL following Incubation with HepG2-HDL. The above experiments showed that treatment of acetyl-LDL with HepG2-HDL decreased its net negative charge (Figure 6B, lane d). Such a change may affect the ligand activity of reisolated acetyl-LDL for the macrophage scavenger receptor (MSR) (Goldstein et al., 1979; Brown & Goldstein, 1983). To elucidate this notion, we compared reisolated acetyl-LDL with control acetyl-LDL in the capacity to induce CE accumulation in macrophages and to be degraded by these cells. As shown in Figure 7, control acetyl-LDL caused CE accumulation in a dose-dependent manner. Acetyl-LDL reisolated after exposure to HepG2-HDL also caused CE accumulation in macrophages; however, the level of CE accumulation was 20% less than that of control acetyl-LDL. In contrast, the CE accumulation capacity of acetyl-LDL reisolated after exposure to HDL was indistinguishable from control acetyl-LDL (data not shown). Since the total cholesterol contents per protein of reisolated acetyl-LDL diminished following interaction with HepG2-HDL (Table 2), the results shown in Figure 7 were reanalyzed with cholesteryl [^{3}H]oleate as the dependent variable and cholesterol concentrations of lipoproteins as the independent variable. Such an analysis yielded a relationship similar to that shown in Figure 7. Therefore, it is likely that the capacity of acetyl-LDL for CE accumulation was reduced by the incubation with HepG2-HDL.

We next examined the endocytic degradation of ^{125}I-reisolated acetyl-LDL by macrophages. The total degradation decreased by 28%, compared with control acetyl-LDL (Figure 8B). Similar results were obtained when the cellular association of ^{125}I-reisolated acetyl-LDL was examined (Figure 8A). These results indicated, therefore, that the interaction of acetyl-LDL with HepG2-HDL in a cell-free system led physicochemically to a decrease in the net negative charge and biologically to a reduced ligand activity for the MSR.

Effect of HepG2-HDL, Apo E-Free HepG2-HDL, and Its Subfractions on the Cell Association of ^{125}I-Acetyl-LDL with, and Subsequent Endocytic Degradation by, Macrophages. We next examined the effect of subfractions of apo E-free HepG2-HDL on cell association and degradation of ^{125}I-acetyl-LDL. Apo E-free HepG2-HDL was fractionated into three peaks (subfractions 1, 2, and 3) upon Sephacryl S-300 gel filtration chromatography (data not shown). This pattern was indistinguishable from that of HepG2-HDL (Figure 5B). Apo E-free HepG2-HDL and its subfractions 1 and 2 were examined for their effects on cell association and degradation of ^{125}I-acetyl-LDL. As shown in Figure 9B, endocytic degradation of ^{125}I-acetyl-LDL by macrophages was reduced

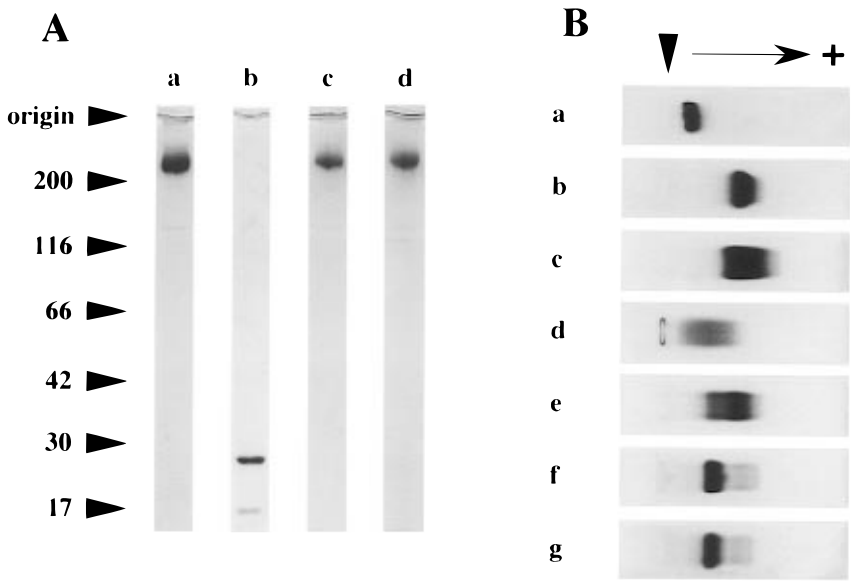

FIGURE 6: SDS−polyacrylamide gel electrophoresis (A) and agarose gel electrophoretic mobility (B) of acetyl-LDL reisolated after incubation with HepG2-HDL. Acetyl-LDL (1 mg/mL) was incubated in a cell-free system with HepG2-HDL (5 mg/mL) or HDL (5 mg/mL) and reisolated as described under Materials and Methods. Each sample was electrophoresed on gradient SDS−PAGE (4−20%) upon a nonreducing condition (5 μg/lane) (A), or on an agarose gel (10 μg/lane) (B) and stained with Coomassie Brilliant Blue. (A) Molecular mass (arrowheads) is indicated in kDa. (B) The arrowhead shows the origin, and the arrow shows the direction. Lane a, LDL; lane b, HDL; lane c, acetyl-LDL; lane d, acetyl-LDL reisolated after exposure to HepG2-HDL; lane e, subfraction 1 of reisolated HepG2-HDL (fraction 64 in Figure 5B); lane f, subfraction 2 of reisolated HepG2-HDL (fraction 78 in Figure 5B); lane g, subfraction 3 of reisolated HepG2-HDL (fraction 82 in Figure 5B).

Table 2: Lipid Contents of Acetyl-LDL Reisolated after Cell-Free Incubation with HepG2-HDL[a]

	lipid/protein weight ratio				
	TC[b]	FC[c]	CE[d]	TG[e]	PL[f]
control acetyl-LDL	1.25	0.32	0.93	0.23	0.82
reisolated acetyl-LDL	1.06	0.44	0.61	0.31	0.91

[a] Acetyl-LDL (1 mg/mL) was incubated with HepG2-HDL (5 mg/mL) at 37 °C for 18 h in 2 mL of 0.15 M NaCl and 1 mM EDTA (pH 7.4). The mixture was separated by Sephacryl S-300 gel filtration chromatography (see Figure 5), and lipid contents were determined as described under Materials and Methods. Experimental errors in the lipid determination were within 5%. [b] TC, total cholesterol. [c] FC, free cholesterol. [d] CE, cholesteryl ester. [e] TG, triglycerides. [f] PL, phospholipids.

by up to 20% by apo E-free HepG2-HDL whose inhibitory capacity was almost similar to that of HepG2-HDL (Figure 2B). Subfraction 2 was also able to reduce the degradation of ^{125}I-acetyl-LDL more effectively than apo E-free HepG2-HDL, whereas subfraction 1 did not show such an effect, indicating that subfraction 2 was responsible for the inhibitory effect of apo E-free HepG2-HDL. Similar results were obtained for their effect on the cellular association of ^{125}I-acetyl-LDL (Figure 9A).

Effect of HepG2-HDL on the Cell Association of ^{125}I-Ox-LDL with, and Subsequent Endocytic Degradation by, Macrophages. Results of previous studies suggested that Ox-LDL is a likely candidate for atherogenic lipoprotein *in vivo* based on its presence in atherosclerotic lesions in human arterial walls (Palinski et al., 1989; Ylä-Herttuala et al., 1989). Therefore, we examined whether HepG2-HDL could also reduce the endocytic degradation of Ox-LDL. For this purpose, macrophages were incubated with ^{125}I-Ox-LDL in the presence of HepG2-HDL. As shown in Figure 10B, the competition of endocytic degradation of ^{125}I-Ox-LDL was 81% by unlabeled Ox-LDL, and by a smaller but significant 23% by HepG2-HDL. In contrast, HDL failed to show such

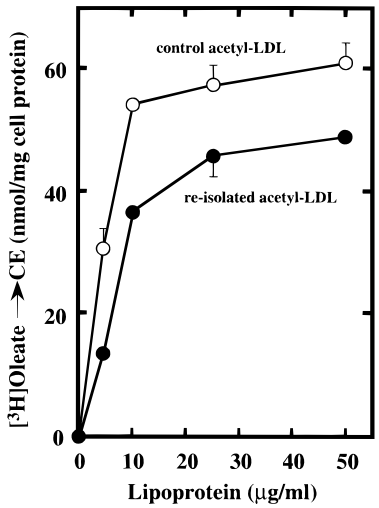

FIGURE 7: Capacity of reisolated acetyl-LDL for CE accumulation in mouse macrophages. Macrophages (2×10^6) were incubated at 37 °C for 18 h with 0.1 mM [^{3}H]oleate in the presence of the indicated concentrations of acetyl-LDL (○), or acetyl-LDL reisolated after exposure to HepG2-HDL (●). Cellular lipids were extracted, and the radioactivity of cholesteryl [^{3}H]oleate was determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD. The SD is very small and within the symbol in data without the error bars.

an effect. Similar results were obtained with respect to the cellular association of ^{125}I-Ox-LDL (Figure 10A). These results suggest that HepG2-HDL could interact with atherogenic lipoproteins in the medium and reduce their cellular uptake by macrophages.

DISCUSSION

The results of the present study could be grouped under three major findings. First, HepG2-HDL purified from culture supernatant of HepG2 cells inhibited acetyl-LDL-

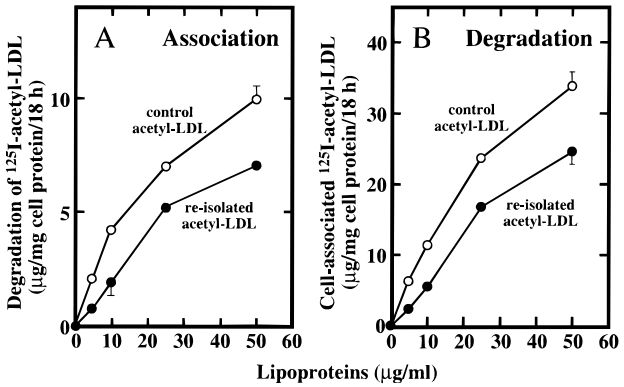

FIGURE 8: Cellular association (A) of ^{125}I-reisolated acetyl-LDL with, and subsequent endocytic degradation (B) by, mouse macrophages. Macrophages (2×10^6) were incubated at 37 °C for 18 h with the indicated concentrations of ^{125}I-acetyl-LDL (○) or ^{125}I-reisolated acetyl-LDL after exposure to HepG2-HDL (●). Cellular association (A) and endocytic degradation (B) were determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD. The SD is very small and within the symbol in data without the error bars.

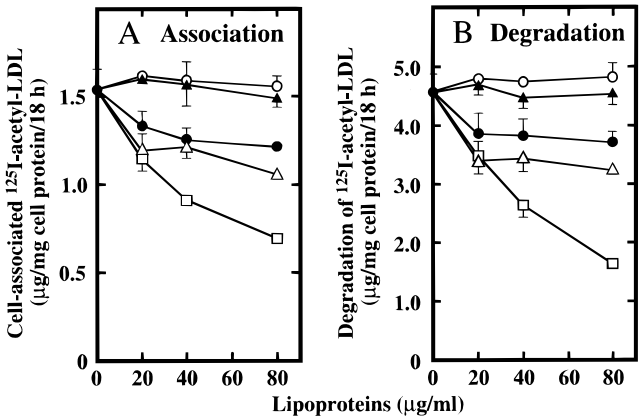

FIGURE 9: Effect of HepG2-HDL, apo E-free HepG2-HDL, and its subfractions on cellular association (A) of ^{125}I-acetyl-LDL with, and endocytic degradation (B) by, mouse macrophages. Apo E-free HepG2-HDL prepared from HepG2-HDL by a heparin−agarose column was fractionated into three peaks (subfractions 1, 2, and 3) on Sephacryl S-300 gel filtration chromatography as described under Materials and Methods. Macrophages (2×10^6) were incubated at 37 °C for 18 h with 4 μg/mL ^{125}I-acetyl-LDL in the presence of the indicated concentrations of acetyl-LDL (□), apo E-free HepG2-HDL (●), HDL (○), subfraction 1 (▲), or subfraction 2 (△). Cellular association (A) and subsequent endocytic degradation (B) were determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD. The SD is very small and within the symbol in data without the error bars.

induced CE accumulation in macrophages. HepG2-HDL directly interacted with acetyl-LDL in the medium and reduced its ligand activity for MSR. Second, the efflux capacity of HepG2-HDL to reduce cellular cholesterol mass was less than half of plasma HDL. Finally, HepG2-HDL also showed a significant inhibitory action on endocytic degradation by macrophages of Ox-LDL, a likely atherogenic lipoprotein *in vivo*. These findings suggest that HepG2-HDL may play a role in inhibiting the formation of foam cells *in vivo*, thus acting as an anti-atherogenic lipoprotein.

Three different methods, including agarose gel electrophoresis, immunoaffinity chromatography, and ultracentrifugation, are now available for isolation of HDL particles (Barrans et al., 1996). The classical method is sequential

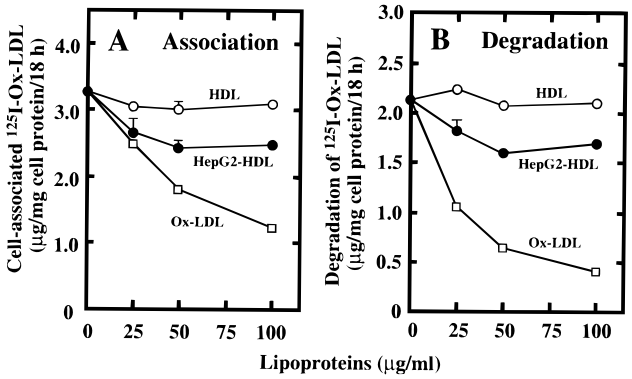

FIGURE 10: Inhibitory effect of HepG2-HDL on cellular association (A) of ^{125}I-Ox-LDL with, and endocytic degradation (B) by, mouse macrophages. Macrophages (2×10^6) were incubated at 37 °C for 18 h with 5 μg/mL ^{125}I-Ox-LDL in the presence of the indicated concentrations of Ox-LDL (□), HepG2-HDL (●), or HDL (○). Cellular association (A) and subsequent endocytic degradation (B) were determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent the SD. The SD is very small and within the symbol in data without the error bars.

ultracentrifugation, while the direct isolation of HDL from serum or plasma using immunoaffinity chromatography is increased in number in recent publications (Kunitake & Kane, 1982; McVicar et al., 1984; Cheung & Albers, 1984; Cheung, 1986). HDL isolation by ultracentrifugation causes significant losses of proteins, because several proteins associated with HDL become dissociated from HDL (Fainaru et al., 1975, 1976; Curry et al., 1976; Mahley et al., 1977; Kunitake & Kane, 1982). In contrast, the lipid and protein composition of immunoaffinity chromatographically-isolated HDL is reported to show a similar property to native HDL circulating in plasma (McVicar et al., 1984). However, it is possible that both LCAT and CETP are inactivated by elution of immunosorbent apo A-I with thiocynate or acetic acid (Cheung, 1986). Our HepG2-HDL preparation obtained by acetic acid elution, however, had no, if any, effect on LCAT and CETP activities (Ohta et al., unpublished observation).

The present results identified four physicochemical properties of HepG2-HDL, including the following: (i) it mainly contains 28 kDa and 17 kDa bands, probably corresponding to apo A-I and A-II, respectively (Figure 1A); (ii) electrophoretically, HepG2-HDL is divided into two particles corresponding to the α and the pre-β positions (Figure 1B); (iii) upon gel filtration chromatography of Sephacryl S-300, HepG2-HDL migrates as three separate peaks (Figure 5B); (iv) further experiments using electron microscopy have subsequently shown that subfraction 1 (Figure 5B) is rich in spherical particles, whereas subfraction 2 contains discoidal particles (data not shown). These characteristics, together with the chemical analysis (Table 1), suggest that the HepG2-HDL used in the present study may well resemble the nascent HDL prepared previously by ultracentrifugation from the media of HepG2 cells (Thrift et al., 1986; McCall et al., 1988). At the same time, it is highly suggested that the anti-apo A-I immunoaffinity chromatography is useful for the preparation of nascent HDL.

Our results showed that HepG2-HDL interacts with acetyl-LDL in the cell-free medium and weakens its ligand activity for MSR (Figure 8). We have recently observed a similar phenomenon with a discoidal reconstituted complex of apo A-I and dimyristoylphosphatidylcholine (DMPC) (DMPC/

apo A-I). Incubation of DMPC/apo A-I with acetyl-LDL or Ox-LDL led to a marked reduction of their ligand activity for MSR (Miyazaki et al., 1994; Sakai et al., 1996). The reducing effect of HepG2-HDL was not directly compared with that of DMPC/apo A-I in the present study. However, based on their capacity relative to HDL, the ligand activity-reducing capacity of DMPC/apo A-I is obviously much higher than that of HepG2-HDL. A possible explanation for this difference would be ascribed to the fact that acetyl-LDL reisolated after interaction with DMPC/apo A-I resulted in a marked reduction of its CE accumulation capacity compared with acetyl-LDL reisolated after exposure to HepG2-HDL (Miyazaki et al., 1994). The molecular mechanism underlying the reduction of ligand activity following interaction with DMPC/apo A-I is not clear. However, a change in the lipid moiety of LDL or modified LDLs appears to be important (Kleinman et al., 1988; Aviram et al., 1988, 1991; Gupta & Rudney, 1992). In fact, interaction between modified LDLs and DMPC/apo A-I results in the transfer of a large amount of DMPC to modified LDLs (Miyazaki et al., 1994; Sakai et al., 1996). In the present study, we also observed an increase in phospholipid contents in acetyl-LDL following exposure to HepG2-HDL (Table 2). It is likely, therefore, that this increase in phospholipid may in part be responsible for a decrease in the ligand activity for MSR. In addition, upon exposure to HepG2-HDL, acetyl-LDL increased free cholesterol but decreased CE (Table 2). These changes in the cholesterol moiety may also be involved in reducing the ligand activity (Aviram et al., 1991). Considered together, lipid transfer from HepG2-HDL or DMPC/apo A-I to acetyl-LDL might lead to a reduction of the ligand activity for MSR.

The marked difference in the reducing capacity of the ligand activity for MSR between HepG2-HDL and DMPC/apo A-I can be explained by the physicochemical characteristics of these lipoproteins. DMPC/apo A-I migrated only to the pre-β position electrophoretically and was eluted as a single peak upon Sephacryl S-300 gel filtration chromatography (Miyazaki et al., 1994), whereas HepG2-HDL migrated to the α as well as the pre-β position (Figure 1B) and was eluted as three peaks upon gel filtration (Figure 5B). Moreover, our results showed that the phospholipid content of DMPC/apo A-I was 3.4-fold greater than that of HepG2-HDL on a protein basis (Table 1). Based on these findings, the amount of pre-β HDL seems to underline the different capacities to reduce the ligand activity for MSR, implicating a potential role of pre-β HDL in HepG2-HDL-induced inhibition of CE accumulation in macrophages. Furthermore, this notion is supported by the finding that subfraction 2 of apo E-free HepG2-HDL migrating to the pre-β position effectively reduced degradation and association of ^{125}I-acetyl-LDL to the similar extent of apo E-free HepG2-HDL as well as HepG2-HDL, while subfraction 1 migrating to the α position did not reduce them at all (Figure 9A,B).

One important issue that remains to be addressed is whether HepG2-HDL has a lower cholesterol efflux capacity than plasma HDL (Figure 3). Two possible mechanisms for cholesterol efflux from cells have been proposed; one is specific efflux, and the other is nonspecific efflux (diffusional efflux). Recent reports from several laboratories have emphasized the importance of pre-β HDL in specific cholesterol efflux from cell membranes (Castro & Fielding, 1988; Huang et al., 1993; Kawano et al., 1993). These studies indicate that pre-β HDL functions as an initial acceptor of cellular cholesterol, while α-HDL plays an intermediate role by converting pre-β HDL-derived cholesterol into cholesteryl esters and accumulating them in its core [for review, see Barrans et al. (1996)]. This may well explain why the efficiency of pre-β HDL for cholesterol efflux is greater than that of HDL, but the capacity for cholesterol efflux is smaller than that of HDL (Castro & Fielding, 1988; Huang et al., 1993). Our HepG2-HDL appeared to contain some particles similar to pre-β HDL (Figure 1B, lane b), while its counterpart in plasma HDL was undetectable (Figure 1B, lane a). One simple interpretation of these results would be that HepG2-HDL reduces cellular cholesterol more effectively than plasma HDL because it contains a definite amount of pre-β HDL. However, the result we obtained in Figure 3 was opposite. There are three possible explanations for this unexpected result. First, our experiment for cholesterol efflux consists of a long-time incubation system ($>$18 h) that likely reflects the nonspecific cholesterol efflux where α-HDL can act as a main cholesterol acceptor [for review, see Fielding and Fielding (1995)]. An attempt was made to examine the cholesterol efflux capacity of HepG2-HDL using a short-time incubation. HepG2-HDL or plasma HDL, however, could not significantly reduce cellular cholesterol mass from macrophage foam cells upon 1 h incubation (data not shown). Further studies are needed to elucidate the specific efflux capacity or efficiency of HepG2-HDL or its subfractions by using the short-time incubation assay with [^{3}H]cholesteryl ester-labeled macrophages. The second possibility is the involvement of LCAT activity in HDL particles. Pre-β HDL-mediated specific cholesterol efflux is expected to depend on LCAT activity (Castro & Fielding, 1988; Miida et al., 1992; Fielding & Fielding, 1995). It was reported that the concentration of LCAT in HepG2-secreted apo A-I-containing lipoproteins was about 15% of normal plasma (Cheung et al., 1989). In this connection, our previous study showed that HDL particles isolated from LCAT-deficient subjects, which possess both α-HDL and pre-β HDL similar to HepG2-HDL, also show a lower cholesterol efflux capacity than plasma HDL (Ohta et al., 1994). As a third possibility, Arbogast et al. (1976) showed that the relative capacity of HDL to promote cholesterol efflux from cells might depend on the free cholesterol/phospholipid molar ratio (so-called FC/PL ratio) of HDL particle. Based on the data shown in Table 1, it is possible that HDL, with a calculated FC/PL ratio of 0.26, represents a better candidate to promote cholesterol efflux than HepG2-HDL (FC/PL ratio $=$ 0.79). When these results are considered together, the difference in the capacity for cholesterol efflux between HDL and HepG2-HDL could be derived either from experimental systems, LCAT activity, the FC/PC ratio, or pre-β contents of HepG2-HDL, or their combinations.

The present study provides new evidence that the anti-atherogenic effects of apo A-I-containing lipoproteins secreted by HepG2 cells may reflect the combination of two distinct pathways: one is cholesterol efflux from macrophage foam cells, and the other is the neutralizing effect on the ligand activity of modified LDLs for MSR, although the former pathway seems to be weaker than that of plasma HDL in our assay. HepG2-HDL is regarded as nascent HDL because it has not yet been exposed to intravascular processing. Such examples include HDL isolated from LCAT-

deficient subjects (Ohta et al., 1994), remodeled HDL by lipoprotein-deficient plasma and HTGL (Ohta et al., 1996), interstitial lymph HDL [for review, see Sloop et al. (1987)], and ovarian follicular fluid HDL (Jaspard et al., 1996). Since the initial event of reverse cholesterol transport expectedly occurs in the subendothelial space, it is likely that the nascent HDL particle may interact with macrophages or atherogenic lipoproteins *in vivo*. In our experience, injection of rabbit apo A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbit without a concomitant increase in the plasma HDL level (Miyazaki et al., 1995). We speculate that injected apo A-I is transferred from the intravascular space to the subendothelial space to produce its anti-atherogenic effect. Further studies examining the role of nascent HDL in the subendothelial space are, therefore, important.

ACKNOWLEDGMENT

We are grateful to Drs. Akira Miyazaki, Masakazu Sakai, Yu-Ichiro Sakamoto, Hirofumi Matsuda, Takeshi Biwa, Takashi Kawasaki, and Yuko Hasunuma in our laboratory for their collaborative endeavor throughout this study. We also thank Dr. F. G. Issa from the Department of Medicine, University of Sydney, Sydney, Australia, for careful reading and editing of the manuscript.

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BI9708444