Lipolytic remnants of human VLDL produced in vitro: effect of HDL levels in the lipolysis mixtures on the apoCs to apoE ratio and metabolic properties of VLDL core remnants

Byung Hong Chung[1,*] and Nassrin Dashti[†]

Atherosclerosis Research Unit, Department of Medicine* and Department of Nutrition Science,[†] University of Alabama at Birmingham, Birmingham, AL, 35294

Abstract **To determine the role of high-density lipoprotein (HDL) as an acceptor of lipolytic surface remnants of very low density lipoprotein (VLDL) in the metabolism of VLDL core remnants, we examined the effect of HDL levels in the VLDL lipolysis mixture on *1*) the morphology and the apoCs to E ratio in VLDL core remnants and *2*) the metabolic properties of VLDL core remnants in human hepatoma cell line HepG2 and human hepatocytes in the primary culture. Normolipidemic VLDL was lipolyzed in vitro by purified bovine milk lipoprotein lipase (LpL) in a lipolysis mixture containing a physiologic level of VLDL and albumin (30 mg VLDL-cholesterol (CH)/dl and 6% albumin) in the absence and presence of either a low HDL level (VLDL-CH:HDL-CH = 3:1) or a high HDL level (VLDL-CH:HDL-CH = 1:4). Lipolysis of VLDL in either the absence or presence of HDL resulted in the hydrolysis of >85% of VLDL-triglycerides (TG) and the conversion of VLDL into smaller and denser particles. In the absence of HDL, heterogeneous spherical particles with numerous surface vesicular materials were produced. In the presence of low or high HDL, spherical particles containing some or no detectable vesicular surface components were produced. The apoCs to apoE ratios, as determined by densitometric scanning of the SDS polyacrylamide gradient gel, were 2.89 in control VLDL and 2.27, 0.91, and 0.22 in VLDL core remnants produced in the absence and in the presence of low and high HDL levels, respectively. In vitro lipolysis of VLDL markedly increased binding to HepG2 cells at 4°C and internalization and degradation by human hepatocytes in primary culture at 37°C. However, the HDL-mediated decrease in the apoCs to apoE ratio had a minimal effect on binding, internalization, and degradation of VLDL core remnants by HepG2 cells and human hepatocytes in primary culture. In order to determine whether HepG2 bound VLDL and VLDL core remnants are deficient in apoCs, ^{125}I-labeled VLDL and VLDL core remnants were added to HepG2 culture medium at 4°C. The bound particles were released by heparin, and the levels of ^{125}I-labeled apoCs and apoE, relative to apoB, in the released particles were examined. When compared with those initially added to culture medium, the VLDL and VLDL core remnants released from HepG2 cells had a markedly increased (113%) level of apoE and a reduced (30–39%), but not absent, level of apoCs. We conclude that apoCs, as a minimum structural and/or functional component of VLDL and VLDL core remnants, may not have an inhibitory effect on the binding of VLDL or VLDL core remnants to hepatic apoE receptors.**—Chung, B. H., and N. Dashti. **Lipolytic remnants of human VLDL produced in vitro: effect of HDL levels in the lipolysis mixtures on the apoCs to apoE ratio and metabolic properties of VLDL core remnants.** ***J. Lipid Res.*** **2000.** 41: **285–297.**

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Human VLDL in circulating blood, secreted from the liver as TG-rich particles, contains nonexchangeable apoB-100 as the structural protein component and exchangeable apoC-I, C-II, C-III, and E as the functional protein components (1). It is well established that apoC-II on the VLDL surface acts as a specific protein co-factor necessary for the hydrolysis of VLDL-TG by LpL (2), while apoE acts as a specific ligand for the binding of VLDL and their remnants to lipoprotein receptors of hepatic cells (3–7). A number of studies have shown that apoCs have an inhibitory effect on the binding of apoB- and/or apoE-containing lipoproteins to lipoprotein receptors of hepatic and nonhepatic cells (4, 5, 8–10). An initial step in the catabolism of VLDL involves the hydrolysis of the TG moiety of VLDL by LpL bound to the vascular endothelium (11). After an initial lipolysis, the apoE-deficient VLDL core remnants are further modified, perhaps by the combined action of LpL and hepatic lipase as well as lipid transfer proteins, to become low density lipoproteins (LDL), while the VLDL

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; VLDL, Very low density lipoprotein; LpL, lipoprotein lipase; CH, cholesterol; TG, triglyceride.

[1] To whom correspondence should be addressed.

core remnants containing apoE are believed to be removed mostly by the liver through the apoE-mediated binding to LDL receptors, LDL receptor-related proteins, and/or heparan sulfate proteoglycan on hepatocytes (6, 7, 12, 13).

As apoCs play a direct inhibitory role in the binding of apoE-containing lipoproteins to lipoprotein receptors (4, 5, 8–10), the efficient removal of VLDL core remnants by the liver would require the dissociation of apoCs. The dissociation of apoCs from VLDL occurs only when VLDL-TG is hydrolyzed by LpL, where the degree of the dissociation of apoCs from the VLDL core remnants is proportional to the degree of lipolysis (14–16). A number of studies have shown that lipolytic surface remnants, which are surface components of VLDL comprised of phospholipids, free cholesterol, apoCs and/or apoE, generated during the lipolysis of VLDL were transferred to HDL (17–19). When VLDL was lipolyzed in the absence of HDL, lipolytic surface remnants were reported to be dissociated from the core remnants and recovered as vesicular and/or discoidal particles in the HDL density regions, but VLDL core remnants retained a significant portion of surface remnants containing apoCs (20). Whether the levels of HDL in plasma affect the degree of dissociation of lipolytic surface remnants from the core remnants has not been fully examined previously.

Most of the previous studies evaluating the inhibitory effect of apoCs on the binding of apoE-containing lipoproteins to lipoprotein receptors have measured the effect of exogenously added purified apoCs (4, 5, 8, 10). Whether apoCs as structural components of apoE-containing lipoproteins have an inhibitory effect on the binding of lipoproteins to lipoprotein receptors is not currently known.

In this study, we have determined the influences of HDL as an acceptor of lipolytic surface remnants, on the density, morphology, apoCs to apoE ratio, and metabolic properties of the VLDL core remnants produced by in vitro lipolysis.

MATERIALS AND METHODS

Materials

Fresh normolipidemic plasma was obtained from the Alabama Regional Blood Bank, Birmingham, AL. LpL was isolated from fresh raw bovine milk and purified by the heparin-agarose affinity chromatographic method (21). Fatty acid-poor bovine serum albumin was purchased from Boehringer Mannheim Biochemical Co., Indianapolis, IN, and Sigma Chemical Co., St. Louis, MO. Human hepatoma cell line HepG2 cells were obtained from the American Type Culture Collection, Rockville, MD. Cultured human primary hepatocytes in 12-well culture plates, derived from adult males, were purchased from Clonetics Corporation, San Diego, CA.

Preparation and characterization of VLDL and lipolytic remnants of VLDL

VLDL was isolated from normolipidemic fasting plasma by ultracentrifugation in a Beckman 50.2 Ti fixed-angle rotor at 45,000 rpm for 16 h. Isolated VLDL was washed once by a modified method of short single vertical spin density gradient ultracentrifugation (22). Modified density gradient ultracentrifugation involves the formation of a three-layer density gradient, consisting of 4 ml density-adjusted VLDL (d 1.21 g/ml) in the bottom, 3 ml KBr solution (d 1.12 g/ml) in the middle, and 4 ml normal saline on the top in a Beckman SW 41 swingout rotor tube, and ultracentrifugation of the density gradient samples for 3 h at 40,000 rpm. The VLDL that floated to the top of the tubes was collected and dialyzed against buffered saline (0.01 m Tris-0.15 m NaCl, pH 7.4) for 16 h. HDL and lipid-free plasma fractions were isolated from VLDL-depleted plasma fraction by the sequential ultracentrifugation method (23).

Lipolysis of VLDL was performed in vitro by incubating isolated VLDL in a lipolysis mixture containing a physiologic level of VLDL (30 mg VLDL-CH/dl) and albumin (6%) in the absence or presence of a low HDL level (VLDL-CH:HDL-CH = 3:1) or high HDL level (VLDL-CH:HDL-CH = 1:4) with purified bovine milk LpL (10 μl LpL/ml mixture) for 90 min at 37°C. The above lipolysis mixtures also contained 0.5% lipoprotein-free plasma (d > 1.21g/ml) fraction as a source of lipid transfer factors. Mixtures containing heated LpL served as controls. The change in the density of VLDL and/or HDL in the lipolysis mixture after lipolysis was examined by the lipoprotein cholesterol autoprofiler method developed in this laboratory (22).

Lipolysis mixtures were subjected to the short single-spin density gradient ultracentrifugation (22) to separate VLDL or VLDL core remnants from surface remnants, LpL, and/or albumin. Twenty-four fractions were collected from each density gradient tube by puncturing the bottom of the tube using a density gradient fractionator (Hoeffer Scientific Instrument, San Francisco). The peaks of VLDL, core or surface remnants of VLDL and/or HDL in the density gradient fractions were located after the measurement of the cholesterol level in each fraction. Fractions containing VLDL, VLDL core or surface remnants and/or HDL were pooled, and lipoproteins in the pooled fractions were isolated by differential ultracentrifugation after adjusting the density to d 1.063g/ml or d 1.21 g/ml with KBr (23).

The morphology of VLDL, core and surface remnants of VLDL, and HDL was examined by negative staining with 2% potassium phosphotungstate and by examining the grid on a Phillips 400 transmission electron microscope. The apolipoprotein composition of VLDL and VLDL core remnants was examined after the separation of individual apoproteins by SDS gradient gel electrophoresis and subsequent densitometric scanning of the gel. The chemical compositions of VLDL and VLDL core remnants were determined by measuring the concentration of proteins, phospholipids, total and unesterified cholesterol, and TG of purified samples. The concentrations of the above lipids were measured by enzymatic assay kits (Kit Nos. 276-64909, 274-47109, 432-40201, and 990-54009) obtained from Wako Diagnostics Inc., Richmond, VA, and protein level was determined by the method of Lowry et al. (24). A portion of isolated VLDL and VLDL lipolytic core remnants was radiolabeled with ^{125}I by the procedure of McFarlane (25). To remove free iodine, the radiolabeled VLDL and VLDL core remnants were passed through a prepacked desalting column (Bio-Rad Lab., Hercules, CA) and subsequently dialyzed against buffered saline. More than 99% of radiolabeled VLDL and VLDL core remnants were precipitated with 10% trichloroacetic acids (TCA). Approximately 15–18% of ^{125}I radioactivities in radiolabeled VLDL and VLDL core remnants was associated with the lipid moiety.

Binding, internalization, and degradation of VLDL and VLDL core remnants in HepG2 cells and human hepatocytes in primary culture

The binding of VLDL and VLDL core remnants to lipoprotein receptors at 4°C and their metabolism (internalization and deg-

radation) at 37°C were studied in HepG2 cells and human hepatocytes in primary culture after the incubation of cells with ^{125}I-labeled VLDL and VLDL core remnants.

HepG2 cells

The human hepatoma HepG2 cells were seeded into 24-well (16 mm diameter) cell culture plates and maintained in minimal essential medium (MEM) supplemented with 2 mm glutamine, 1 mm sodium pyruvate, kanamicin (50 μg/ml), and 10% fetal bovine serum (FBS) at 37°C in 95% air and 5% CO_2 atmosphere as previously described (26). Twenty-four hours before the experiments, the maintenance medium was removed, cells were washed with phosphate-buffered saline (PBS), and MEM containing 10% lipoprotein-deficient FBS was added. In binding studies, HepG2 cells were pre-chilled for 30 min at 4°C in MEM supplemented with 20 mm HEPES, pH 7.4. This medium was replaced with the same medium containing various concentrations (2.5–40 μg/ml) of ^{125}I-labeled control VLDL or VLDL core remnants, and cells were incubated for 3 h at 4°C. After the incubation, the medium was removed and the cells were washed 3 times with 2 ml ice-cold PBS containing 0.2% BSA and 3 times with PBS alone. The washed cells were dissolved in 0.7 ml of 0.1 N NaOH and analyzed for radioactivity and protein content. The effect of increasing concentrations (2.5–40 μg/ml) of unlabled VLDL core remnants produced in the absence or presence of low and high HDL levels on the binding of ^{125}I-labeled VLDL core remnants produced in the absence of HDL to HepG2 cells at 4°C was determined as previously described (26, 27).

Human primary hepatocytes

Cultured human primary hepatocytes in 12-well culture plates were purchased from Clonetic Inc., San Diego, CA. Hepatocytes were maintained in serum-free hepatocyte maintenance culture medium (Clonetic Inc., San Diego, CA) containing 10^{-7} m insulin, 10^{-7}m dexamethasome, gentamicin (50 μg/ml), and amphotericin (50 μg/ml) at 37°C in 95% air and 5% CO_2 atmosphere. For the experiment, culture medium was replaced with fresh human hepatocyte maintenance medium containing various levels (2–20 μg/ml) of ^{125}I-labeled VLDL and VLDL core remnants, and cells were incubated at 37°C for 6 h. After the incubation, the culture medium was removed and cells were washed 4 times with PBS. Cells adhering to culture plates were detached by addition of trypsin (0.25–0.5 ml) and incubation at 37°C for approximately 30 min. Trypsin neutralizing solution (Clonetic Inc., San Diego, CA) was added to the culture plates, and the cell suspension was transferred into conical micro-centrifuge tubes, and cells were pelleted by centrifugation at 10,000 rpm for 10 min. The radioactivities released by trypsin and recovered in the supernate, which represents the surface bound ^{125}I-labeled lipoproteins, were determined. Cells were dissolved in 0.1 N NaOH, and the levels of radioactivities associated with cells, which represents the internalized ^{125}I-labeled lipoproteins, and protein content of cells were determined. The extent of degradation of ^{125}I-labeled VLDL and VLDL core remnants was determined by measuring the noniodide ^{125}I-labeled protein degradation products in culture medium according to the method described by Bierman, Stein, and Stein (28). Briefly, 20% trichloroacetic acid (TCA) was added to cell-free culture medium containing added carrier proteins (0.5% BSA) to provide a final TCA-concentration of 5%, and the mixtures were incubated at 4°C for 30 min. After removal of protein precipitates by centrifugation, 10 μl of 40% KI and 40 μl of 30% H_2O_2 were added to 1 ml TCA-soluble fraction, and the mixtures were incubated at room temperature for 30 min. The mixtures were then extracted with 2 ml chloroform to remove free iodine. A 0.5-ml aliquot of upper layer was counted. Nanograms of VLDL and VLDL core remnants bound, internalized, and degraded per mg of cell proteins were calculated based on specific activities of radiolabeled samples and percent TCA-soluble non-iodide radioactivities.

Determination of the ratios of ^{125}I-labeled apoCs or apoE to apoB on VLDL and VLDL core remnants added to culture medium or displaced from HepG2 cells by heparin

In certain experiments, ^{125}I-labeled VLDL or VLDL core remnants bound to the cell surface of HepG2 cells at 4°C were released by heparin. The relative ratios of apoCs or apoE to apoB as well as apoCs to apoE ratio on the particles released from the cells were determined and compared with those added to the culture medium. Briefly, HepG2 cells were seeded into 100 mm diameter culture dishes and grown as described above. After 4 days in culture, the maintenance medium was removed and cells were washed with PBS. Serum-free MEM containing 20 mm HEPES (pH 7.4) was added, and cells were incubated at 4°C with ^{125}I-labeled VLDL and VLDL core remnants (50 μg/ml), produced in the absence or in the presence of a HDL high level, by the same procedures described above. After the removal of culture medium, cells were washed 4× with cold PBS. The ^{125}I-labeled lipoproteins bound to the cell surface were released by incubating washed cells with medium containing sodium heparin (10 mg/ml) as described by Goldstein, Basu, and Brown (29). The levels of ^{125}I radioactivities in culture medium of pre- and post-incubation, PBS wash, and heparin wash were measured. The residual ^{125}I-radioactivities associated with cells after the heparin treatment were also measured after dissolving the cell monolayer in 0.1 N NaOH. In order to prevent the association of VLDL or lipolytic VLDL core remnants released from cells with added heparin, sodium chloride at 1.0 m final concentration was added to the medium. The ^{125}I-labeled lipoproteins released from the cells were isolated by differential ultracentrifugation after adjustment of their density to d 1.063 g/ml, dialyzed, and concentrated by lyophilization. After the dissolution of the lyophilized samples in SDS buffer, total radioactivities of samples were measured. Samples containing 25,000–50,000 cpm of ^{125}I-labeled VLDL and VLDL core remnants released from the cells were loaded onto a preformed 10-well, 4–20% Tris-glycine SDS gradient gel (Novex Co., San Diego, CA). In order to identify the exact position of ^{125}I-labeled apoB, apoCs, and apoE bands in the gel, cold (unlabeled) VLDL was loaded into a well adjacent to wells containing each ^{125}I-labeled sample. After electrophoresis, the gels were stained with rapid colloidal Coomassie blue stain (Novex Co., San Diego, CA). After staining of the gels, the major apolipoprotein bands (apoB-100, apoE, and apoCs) were clearly visible in lanes containing cold VLDL, but little or no apoprotein band was visible in lanes containing ^{125}I-labeled VLDL or VLDL core remnants. The ^{125}I-labeled apoB-100, apoE, and apoC bands corresponding to stained apoB, apoE, and apoCs bands were excised from the gel, and the levels of their ^{125}I radioactivities were measured. The levels of radioactivity associated with apoB, apoE, and apoCs of control ^{125}I-labeled VLDL and VLDL core remnants added to the culture medium were also determined as described above. The ratios of ^{125}I-labeled apoCs or E to ^{125}I-labeled apoB or apoCs to apoE of VLDL and VLDL core remnants were calculated.

RESU LTS

Changes in density, morphology, and composition of VLDL after in vitro lipolysis of VLDL

Lipolysis of normolipidemic VLDL by purified bovine milk LpL in a lipolysis mixture containing a physiologic level of VLDL and albumin in the presence or absence of

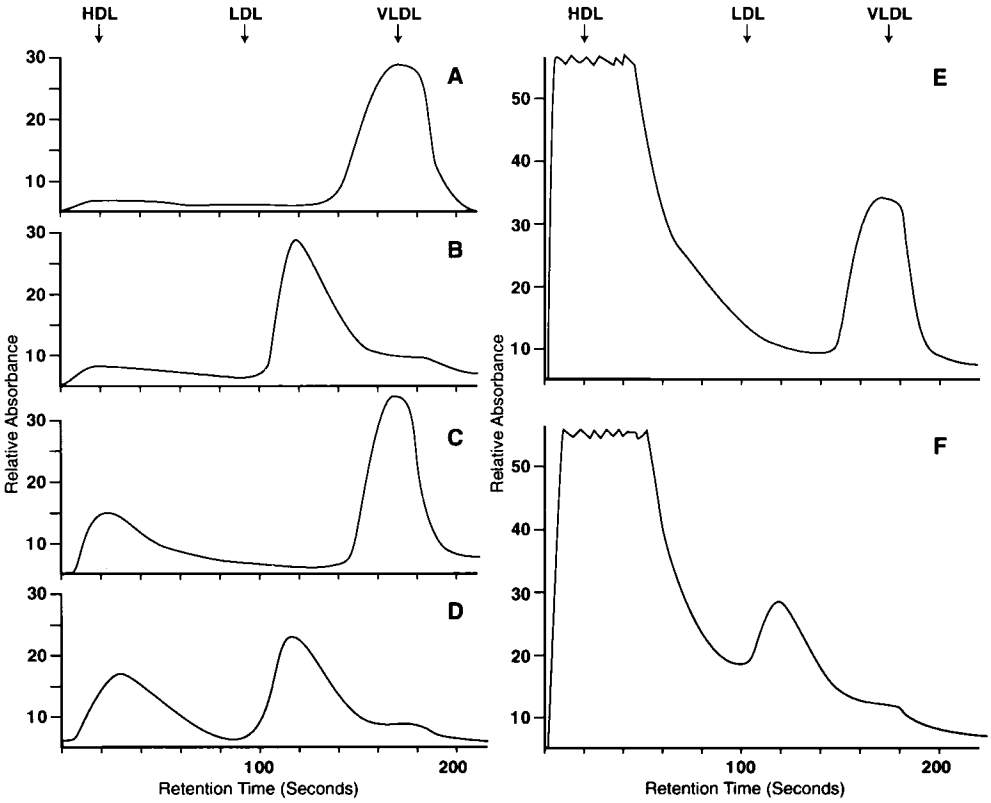

Fig. 1. Change in the density of VLDL following in vitro lipolysis of VLDL in the absence and presence of a low or a high HDL level. VLDL isolated from normolipidemic plasma was lipolyzed in vitro, and the change in banding density of VLDL in the density gradient tubes was determined by the procedures described in Methods. Density profiles A–F are pre- and postlipolysis mixtures containing VLDL and albumin only (profiles A and B), pre- and postlipolysis mixtures containing VLDL, albumin, and a low HDL level (profiles C and D), and pre- and postlipolysis mixtures containing VLDL, albumin, and a high HDL level (profiles E and F), respectively.

HDL resulted in the hydrolysis of >85% of VLDL-TG and the conversion of most of VLDL into particles with density ranges of intermediate density lipoproteins (IDL) and LDL (**Fig. 1** and **Table 1**). Thus, lipolysis of VLDL converted TG-rich VLDL into denser CH-rich and TG-poor particles (Table 1, Fig. 1). In the density gradient tubes, the VLDL core remnants banded at a much lighter density region than plasma LDL (Fig. 1), suggesting that VLDL core remnants may be larger or richer in lipids than plasma LDL. The presence of HDL in the lipolysis mixture had little or no effect on the extent of change in the density of VLDL after lipolysis (Fig. 1). The computer-aided deconvolution of the lipoprotein cholesterol profiles (22) of pre- and postlipolysis VLDL showed that lipolysis of VLDL in the absence of HDL resulted in the transfer of most (80%) of VLDL-cholesterol to the VLDL core remnant peak banded in the IDL-LDL density region; only a small portion of VLDL-cholesterol (11%) was transferred into the HDL density region (Fig. 1, profiles A and B). As compared to the lipolysis of VLDL in the absence of HDL, the lipolysis of VLDL in the presence of a low HDL level caused a transfer of a greater portion of VLDL cholesterol into the HDL density fraction (18% vs. 11%), and thus, the levels of VLDL cholesterol recovered in the IDL and LDL density fraction was somewhat less in the presence of a low HDL than in the absence of HDL (75% vs. 80%) (Fig. 1, profiles A–D).

TABLE 1. Composition of control VLDL (A) and VLDL core remnants produced in the absence of HDL (B) or in the presence of a low HDL (C) or a high HDL (D) level

Samples	Proteins	FC	CE	PL	TG
			% of total mass		
A	9.0 ± 0.8	7.0 ± 1.0	8.8 ± 1.8	16.0 ± 1.3	59.2 ± 3.2
B	21.4 ± 3.8	17.1 ± 1.4	24.1 ± 4.8	25.9 ± 2.9	11.5 ± 1.8
C	22.5 ± 2.7	15.2 ± 1.2	25.7 ± 2.3	25.3 ± 1.8	11.3 ± 2.6
D	22.3 ± 3.1	13.2 ± 1.6	27.4 ± 2.8	25.4 ± 1.6	11.7 ± 2.7

Values are means + SD of triplicates.

Figure 2 shows the negative-stain electron micrographs (EM) of control VLDL and VLDL core remnants produced in the absence and presence of HDL. The VLDL core remnants produced in the absence of HDL were mixtures of spherical particles, core-depleted particles to which excess vesicular surface remnants were attached and aggregated vesicular particles (Fig. 2, panels A and B). The mean particle diameters of spherical core remnant particles were smaller than those of control VLDL (288 Å vs. 475 Å), but the vesicular particles were much larger than those of control VLDL (864 Å vs. 475 Å). The

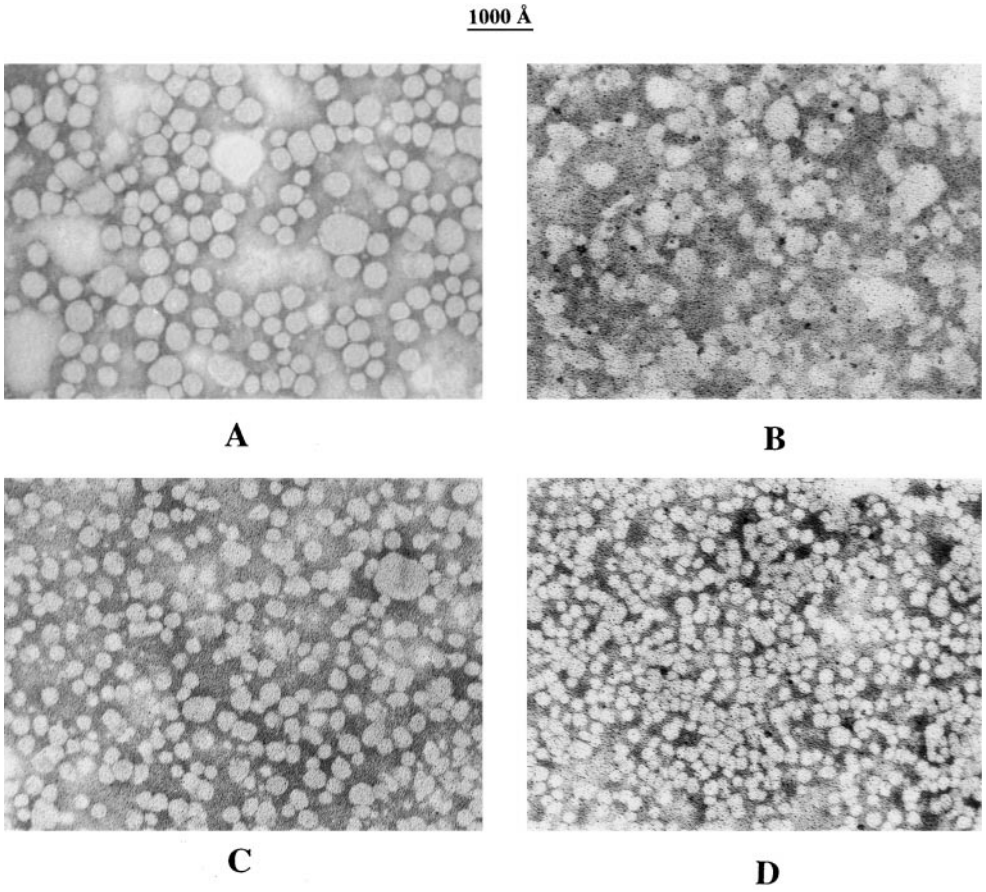

Fig. 2. Electron micrographs of control VLDL and VLDL core remnants produced in the absence of HDL or in the presence of a low or a high HDL level. Panels A–D are control VLDL (A), VLDL core remnants produced in the absence of HDL (B), and VLDL core remnants produced in the presence of a low HDL level (C) or a high HDL level (D). Particle diameter means and standard deviations of spherical particles (n = 100) on panels A–D are 475 ± 168 Å (A), 288 ± 60 Å (B), 282 ± 53 Å (C), and 240 ± 38 Å (D), respectively, and vesicular particles (n = 36) on panel B were 864 ± 225 Å.

VLDL core remnants produced in the presence of either a low or a high HDL level were mostly spherical particles (panels C and D), but the levels of HDL in the lipolysis mixture affected the sizes of VLDL core remnants; the VLDL core remnants produced in the presence of a high HDL level were more uniform and smaller than those produced in the presence of a low HDL level (240 Å vs. 282 Å) (Fig. 2, panels C and D). It is evident that some of VLDL core remnants produced in the presence of a low HDL retained electron luscent vesicular materials on the surface (Fig. 2, panel C).

The SDS gradient gel electrophoregrams revealed that VLDL core remnants produced in the absence of HDL retained most of the VLDL apoCs, but those produced in the presence of a high HDL level retained little or no VLDL apoCs (**Fig. 3**, top, lanes A, B, and D). The VLDL core remnants produced in the presence of a low HDL level retained apoCs much more than those produced in the presence of a high HDL level but much less than those produced in the absence of HDL (Fig. 3, top, lanes A–D). The densitometric scanning of the SDS gradient gels revealed that the approximate ratios of apoCs to apoE were 2.89 in control VLDL and 2.27, 0.91, and 0.22 in VLDL core remnants produced in the absence of HDL and in the presence of a low and a high level of HDL, respectively (Fig. 3, bottom). The above data suggest that the levels of HDL in the lipolysis mixture influenced the extent of the dissociation of lipolytic surface remnants containing apoCs from the core remnants. It should be noted that apoA-I was not detectable in control VLDL but was the major apolipoprotein constituent of VLDL core remnants when they were produced in the presence of either a low or a high HDL level (Fig. 3, top). This data indicates that apoA-I on HDL transfers to VLDL core remnants. The densitometric scans of the gels (Fig. 3, bottom) indicated that the ratios of apoE to apoA-I in the VLDL core remnants produced in the presence of a low or a high HDL level were similar (1.2–1.3), indicating that the level of HDL in the lipolysis mixture may not influence the extent of transfer of apoA-I from HDL to the core remnants. The chemical composition of VLDL core remnants showed that the presence of HDL in the lipolysis mixtures lowered the content of unesterified cholesterol in the core remnant particles in a concentration-dependent manner (Table 1).

In another series of experiments, the VLDL core rem-

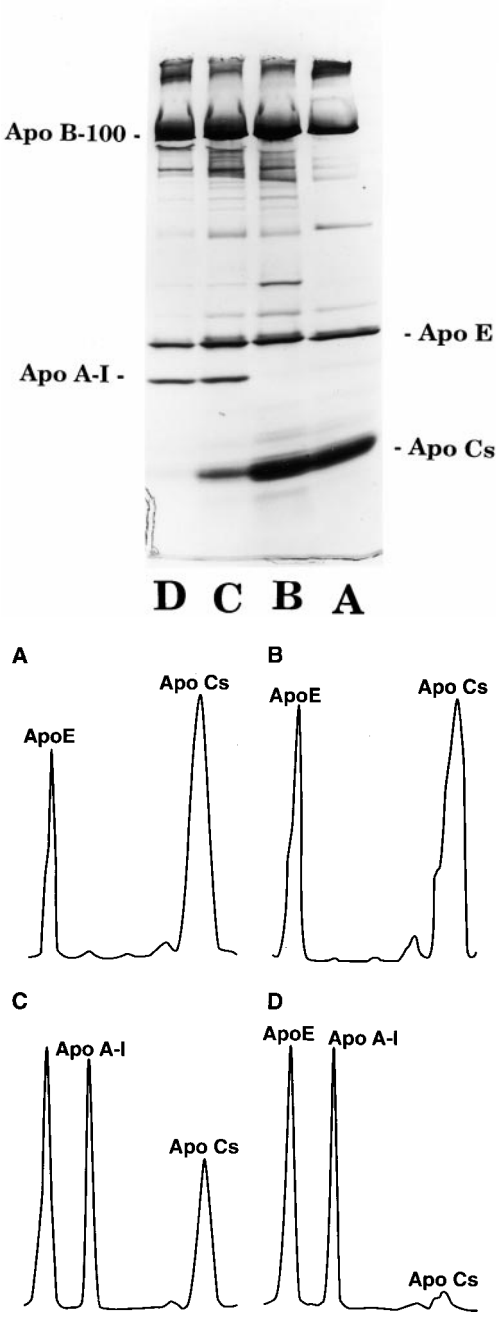

Fig. 3. SDS gradient gel electrophoregrams and densitometric scans of SDS gel of control VLDL and VLDL core remnants produced in the absence of HDL or in the presence of a low or a high HDL level. Panels A–D of Fig. 3, top (SDS gradient gel electrophoregrams) and Fig. 3, bottom (gel scans) are control VLDL (A), VLDL core remnants produced in the absence of HDL (B) and VLDL core remnants produced in the presence of a low HDL level (C) or a high HDL level (D).

nants having excess surface remnants (apoCs and vesicles), produced in the absence of HDL, were treated with a 4-fold excess amount of HDL, and potential dissociation of surface remnants from core remnants by HDL was examined. As shown in **Fig. 4**, HDL treatment converted VLDL core remnants having apoCs and vesicular material on the surface into smaller spherical particles containing little or no vesicular material and apoCs (EM and SDS gel electrophoregram of panels A and B). ApoA-I appeared in the VLDL core remnants after treatment with HDL (Fig. 4, gels on panels A and B). The above data suggest that HDL may promote the dissociation of lipolytic surface components from core remnants by acting as an acceptor of lipolytic surface remnants.

As in vitro lipolysis of VLDL in the absence of HDL resulted in transfer of a small portion (11%) of VLDL cholesterol into the HDL density fraction (Fig. 1, profiles A and B), we examined further the morphology and apolipoprotein composition of VLDL remnant particles recovered in the HDL density region as well as those recovered from the IDL-LDL density region (**Fig. 5**). The VLDL remnants recovered in the IDL-LDL density region again showed a mixture of spherical particles and spherical particles with excess vesicular surface materials containing apoB, apoE, and apoCs as their major apolipoprotein constituents (Fig. 5, top, panel A). The lipolytic remnants of VLDL recovered in the HDL density region were mostly vesicular particles containing apoA-I and apoCs as their major apolipoprotein constituents; a faint apoE band but little or no apoB-100 band was detected in these vesicular particles (Fig. 5, top, panel B). Although we could not detect a clear apoA-I band in control VLDL (data not shown), apoA-I became a major apolipoprotein constituent of the vesicular particles recovered in the HDL density region and a minor apolipoprotein component of spherical remnants recovered in the IDL-LDL density region (Fig. 5, top, panel B of SDS gel electrophoregram). The apoA-I on these lipolytic remnants was likely derived from apoA-I contaminants in commercial albumin added to the lipolysis mixture as an acceptor of free fatty acids. Deckelbaum, Olivecrona, and Fainaru (30) have reported that high grade commercial albumin preparations were contaminated with variable amounts of apoA-I, and that the lipolysis of VLDL in the presence of such albumin preparations resulted in the appearance of apoA-I on the lipolytic surface remnants recovered in the HDL density region as well as LDL-like VLDL core remnants recovered in the VLDL-LDL density regions.

In a further study, we have examined whether the vesicles accumulated in the HDL density region when VLDL was lipolyzed in the presence of a high HDL level. As shown in Fig. 5, bottom, the HDL density fraction separated from the lipolysis mixture containing VLDL and a high HDL level consisted exclusively of spherical HDL particles that have a mean diameter much larger than those HDL particles in the prelipolysis mixtures (132 Å vs. 88 Å); no vesicular particle was detectable in this fraction (Fig. 5, bottom). These data suggest that vesicular surface remnants of VLDL produced during lipolysis may not be stable and thus may be absorbed into HDL.

Binding, internalization, and degradation of control VLDL and lipolytic core remnants produced in the absence or presence of HDL in cultured HepG2 cells and human primary hepatocytes

It has been previously demonstrated that the time dependency of the binding of ^{125}I-labeled VLDL and LDL to

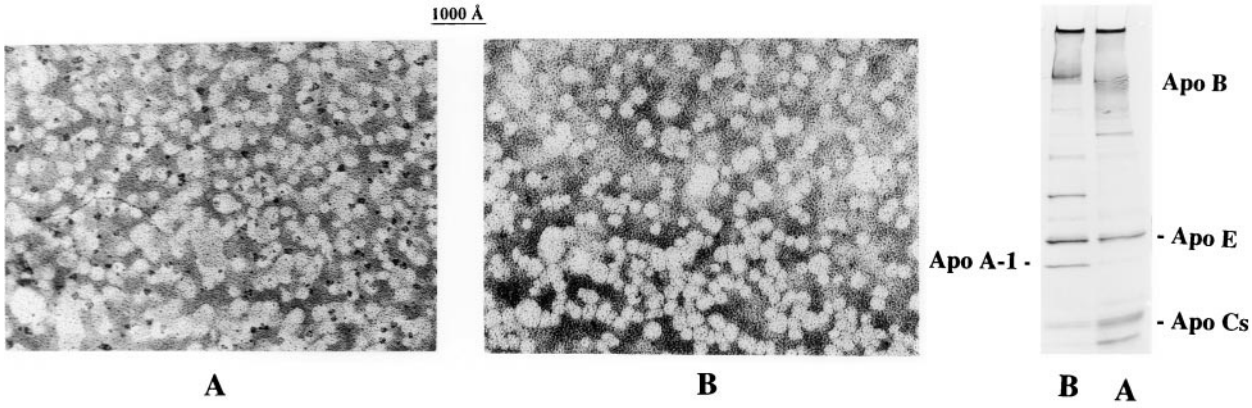

Fig. 4. Effect of the treatment of VLDL core remnants produced in the absence of HDL with 4-fold excess levels of HDL on the change in their morphology and apolipoprotein composition; A: before treatment; B: after treatment. Particle diameter means and standard deviations of spherical particles (n = 100) on panels A and B are 279 ± 53 Å and 284 ± 65 Å, respectively.

HepG2 cells at 4°C appeared to be saturable and reached near completion within 3 h (26, 27). Therefore, HepG2 cells were incubated with increasing concentrations of ^{125}I-labeled control VLDL, VLDL core remnants produced in the absence and presence of a low or high HDL level, and LDL for 3 h at 4°C to evaluate the potencies of these lipoproteins for binding to lipoprotein receptors in these cells. The binding of ^{125}I-labeled control VLDL, VLDL core remnants, and LDL to HepG2 cells was a curvilinear function of substrate concentration, i.e., it did not reach a plateau but continued to increase up to 40 μg/ml used in this study. The binding affinity of VLDL for lipoprotein receptors on HepG2 cells was 2.8-fold lower than that of LDL at a protein concentration of 40 μg/ml (**Fig. 6**). However, lipolysis of VLDL markedly increased the binding of VLDL to HepG2 cells; at a protein concentration of 40 μg/ml, the binding of VLDL core remnants to HepG2 cells was about 2.5-fold higher than that of plasma LDL and 7-fold higher that of control VLDL (Fig. 6). It should be noted that the binding of control VLDL to HepG2 cells was about 2.8-fold less than that of LDL, but the number of VLDL particles bound to HepG2 cells may be approximately equal to that of LDL as the protein content of VLDL is less than one-half of that of LDL. Because the protein content of VLDL core remnants is similar to that of LDL (Table 1), the number of VLDL core remnant particles bound to HepG2 cells will be about 2.5-fold greater than plasma LDL. The extent of the binding to HepG2 cells of VLDL core remnants produced in the absence or presence of a low or high HDL level was similar (Fig. 6) in spite of the marked difference in the apoCs to apoE ratios of these remnants (Fig. 3). Competition study showed that the binding of ^{125}I-labeled VLDL core remnants produced in the absence of HDL to HepG2 cells was somewhat more effectively inhibited by unlabeled VLDL core remnants produced in the presence of a high HDL level than by those produced in the absence of HDL (**Fig. 7**).

The binding, uptake, and degradation of ^{125}I-labeled VLDL and VLDL core remnants, produced in the absence and presence of HDL, by human primary hepatocytes at 37°C are shown in **Fig. 8**. The levels of ^{125}I-labeled VLDL core remnants bound to the cell surface and internalized were 2- to 3-fold greater than those of control VLDL (Fig. 8). The extent of degradation of ^{125}I-labeled VLDL core remnants was also greater than that of control VLDL, however, this increase was substantially less than that observed for internalization. It should be noted that as compared to lipolytic VLDL core remnants produced in the absence of HDL, those produced in the presence of HDL bound less on the surface but were degraded to a greater extent. The above data suggest that the presence of apoCs on the core remnants may retard the internalization and/or degradation of VLDL core remnants.

Ratio of ^{125}I-labeled apoCs or apoE to apoB on VLDL and VLDL core remnants added to the culture medium and released from HepG2 cells by heparin

To determine whether ^{125}I-labeled VLDL or VLDL core remnants bound to HepG2 cells have an apoCs to apoE ratio similar to that of VLDL and VLDL core remnants added to the culture medium, ^{125}I-labeled VLDL and VLDL core remnants bound at 4°C to HepG2 cells were released by heparin as described in Methods. The level of ^{125}I-labeled apoCs or apoE relative to ^{125}I-labeled apoB in the released VLDL and VLDL core remnants was determined and was compared with those added to the culture medium. Determination of the levels of ^{125}I radioactivities in culture medium of pre- and postincubation, PBS wash, heparin wash, and dissolved cells indicates that HepG2 cells bound about 0.45% of ^{125}I-labeled control VLDL and 1.9–2.3% of VLDL core remnants added to the culture medium, and that 65–78% of cell-bound ^{125}I radioactivity of VLDL and VLDL core remnants were released by heparin. The residual ^{125}I radioactivities still bound to HepG2 cells after heparin wash appear to be VLDL or VLDL core remnant particles since we have observed that the percent ^{125}I radioactivity extractable by chloroform–methanol 2:1 mixture was approximately equal to that of VLDL or VLDL core remnants added to the culture dish (data not shown). The levels of ^{125}I radioctivities bound to HepG2

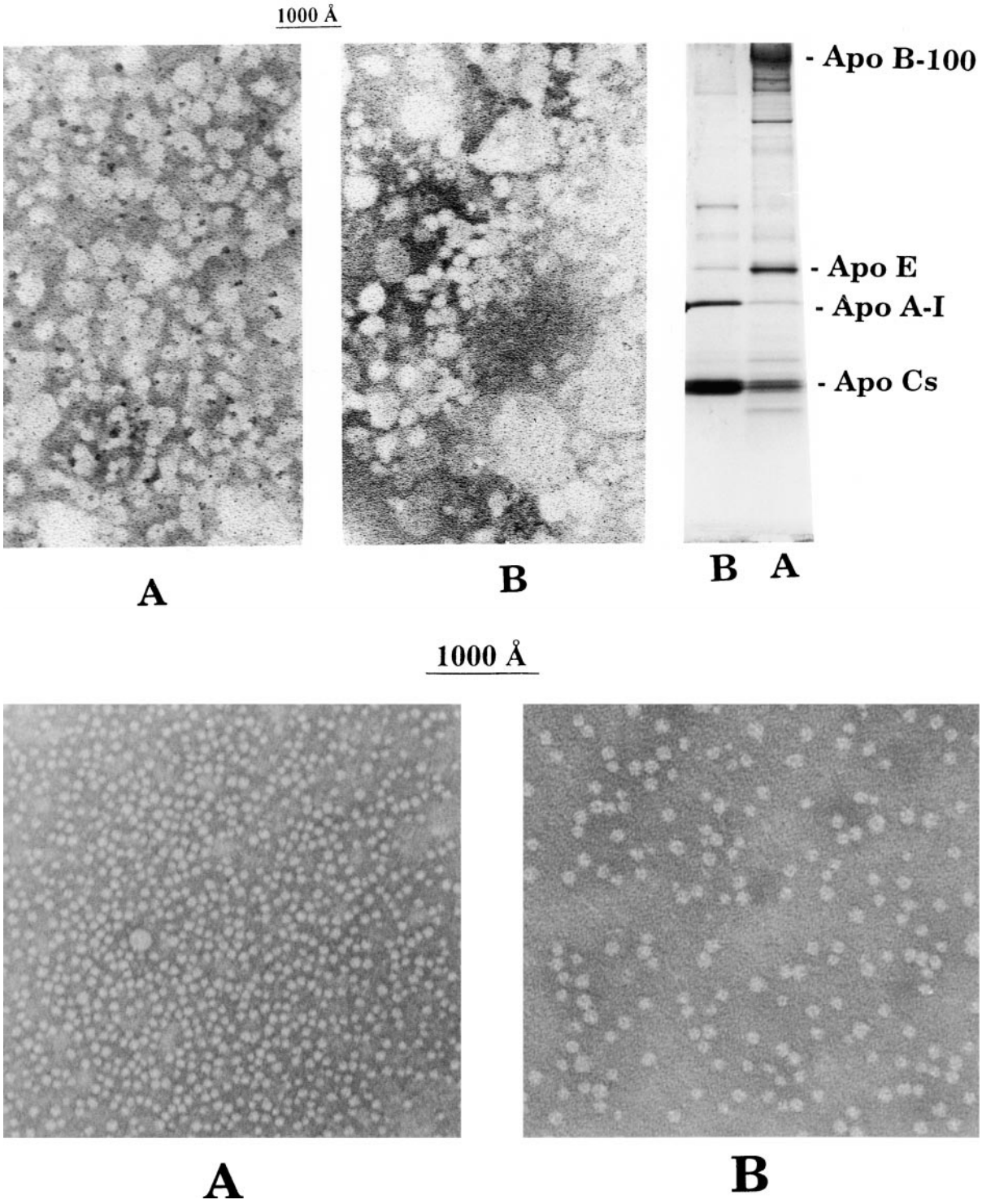

Fig. 5. Top: Electron micrographs and SDS gradient gel electrophoregrams of VLDL core remnants and surface remnants produced in the absence of HDL. Lipolytic remnants of VLDL were produced after in vitro lipolysis of VLDL in the absence of HDL. Morphology and apolipoprotein composition of the lipolytic remnants recovered in the VLDL-LDL density regions (A) and in the HDL density region (B) of the density gradient tubes were examined. Particle diameters of vesicles on panel B were ranged 185 to 1950 Å. Bottom: Electron micrographs of HDL density fraction separated from prelipolysis and postlipolysis mixture containing VLDL, albumin, and a high HDL level. Panels A and B are HDL isolated from the mixtures of prelipolysis and postlipolysis, respectively. The particle diameter means and standard deviation of HDL on panels A and B were 88 ± 25 Å and 132 ± 32 Å, respectively.

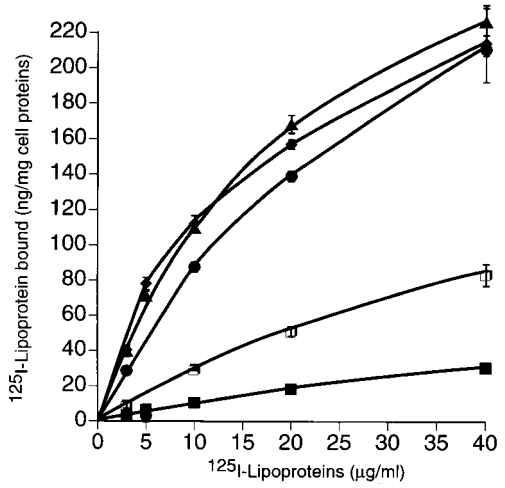

Fig. 6. Binding of ^{125}I-labeled control VLDL, LDL, and VLDL core remnants produced in the absence or presence of a low or a high HDL level by HepG2 cells as a function of lipoprotein concentration. Cells were incubated with increasing concentrations of control VLDL (■–■), LDL (□–□), or VLDL core remnants produced in the absence of HDL (●–●) or in the presence of a low HDL (▲–▲) or a high HDL level (◆–◆) at 4°C, and the amount of ^{125}I-labeled lipoproteins bound to cells was determined. Values are mean ± SD of triplicates.

cells at 37°C for 3 h were about 2-fold higher than those bound at 4°C, and less than 40% of cell-bound ^{125}I radioactivities was releasable by heparin. Kane et al. (31) have previously estimated the content of soluble apoproteins (apoCs and E) in VLDL by measuring the level of VLDL proteins soluble in 0.45 m tetramethyl urea. We have observed that the percent of VLDL or VLDL core remnant ^{125}I radioactivities which were soluble in 0.45 m tetramethyl urea was not appreciably altered following their incubation with HepG2 cells at 4°C or 37°C (data not shown). This observation suggests that excess surface remnants associated with core remnants, which are readily transferable to HDL in vitro (Fig. 4), may not be readily transferable to cell membranes of Hep G2 cells.

Table 2 shows the relative distribution of ^{125}I radioactivity among apoB, apoE, and apoC moieties of VLDL and VLDL core remnants added to the culture medium and released from HepG2 cells by heparin. As compared to control VLDL and apoC-containing VLDL core remnants added to the culture medium (preincubation), those released from HepG2 cells by heparin had a decreased apoC but increased apoE content, as evidenced by 31–39% decreases in the apoCs to apoB ratio and a 113% increase in the apoE to apoB ratio (Table 2); thus, the apoCs to apoE ratios were 3.2- to 3.4-fold lower in VLDL and VLDL core remnants released from Hep G2 cells than in those added to the culture medium (Table 2). The apoE to apoB ratio of apoC-deficient VLDL core remnants, produced in the presence of a high HDL level, was also higher in particles released from HepG2 cells by heparin than in those added to the culture medium (0.35 vs. 0.23). These data suggest that HepG2 cells preferentially bind a subpopulation of VLDL or VLDL core remnant particles with increased apoE and/or decreased apoC content, and that the presence of apoCs as a minimum structural component of VLDL may not have an inhibitory effect on the binding of VLDL core remnants to hepatic apoE receptors.

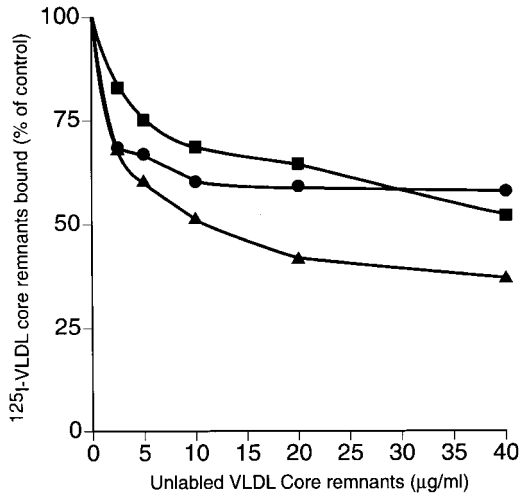

Fig. 7. Effect of unlabeled VLDL core remnants produced in the absence or presence of a low and a high HDL level on the binding of ^{125}I-labeled VLDL core remnants produced in the absence of HDL to HepG2 cells at 4°C. Cells were incubated with 2.5 μg of ^{125}I-labeled VLDL core remnants produced in the absence of HDL and increasing concentratons (2.5–40 μg) of unlabled VLDL core remnants produced in the absence of HDL (●–●) or in the presence of a low HDL level (■–■) or a high HDL level (▲–▲) to HepG2 cells, and the extent of binding of ^{125}I-labeled VLDL remnants to cells were determined. Values are mean of triplicates.

DISCUSSION

A number of studies have shown that the lipolysis of VLDL occurs in either the absence or presence of HDL or plasma in the lipolysis mixture (14–17, 19, 20, 32, 33). Consistent with these reports, the present study further showed that lipolysis of VLDL by purified LpL in either the absence or presence of HDL resulted in the hydrolysis of most of VLDL-TG and the conversion of most of VLDL into particles with a density greater than that of VLDL but less than that of plasma LDL (Table 1, Fig. 1). The presence of HDL and its levels in the lipolysis mixtures, however, affected the morphology and apolipoprotein composition of VLDL core remnants (Figs. 2 and 3). The VLDL core remnants produced in the absence of HDL retained a major portion of their surface remnants as evidenced by the presence of vesicular material and apoCs on the VLDL core remnants (Figs. 2 and 3). The retention of vesicular material and apoCs on the VLDL core remnants was lessened or prevented by the presence of a low or high HDL level during the lipolysis of VLDL (Figs. 2 and 3). Lakashmanan et al. (34) have shown previously that VLDL and chylomicron remnants generated in the perfused heart system in the absence of HDL contained apoCs as a major apolipoprotein component. However, apoCs were not de-

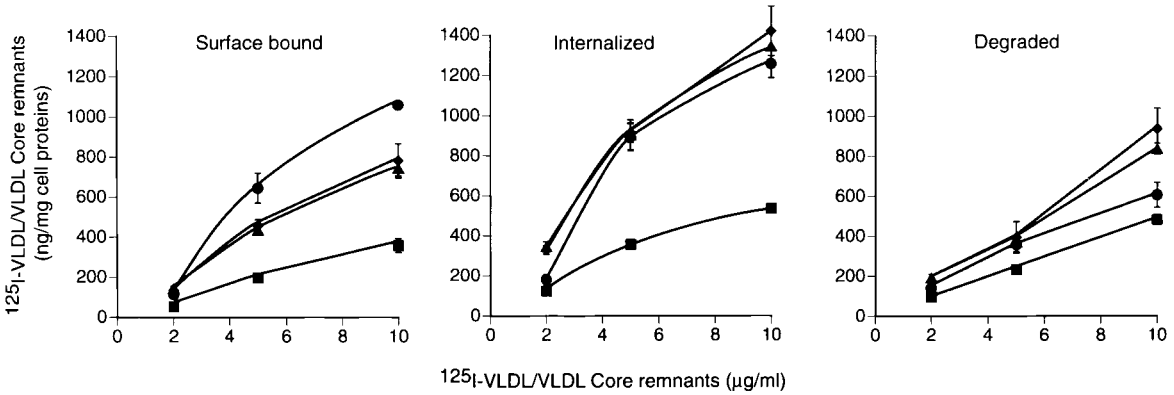

Fig. 8. Binding, internalization, and degradation of ^{125}I-labeled control VLDL and VLDL core remnants produced in the absence or in the presence of a low or a high HDL level by cultured human primary hepatocytes as a function of lipoprotein concentration. Cells were incubated for 6 hr at 37°C with increasing concentrations (2–10 μg) of ^{125}I-labeled control VLDL (■–■) and VLDL core remnants produced in the absence of HDL (●–●) or in the presence of a low HDL level (▲–▲) or a high HDL level (◆–◆), and the extent of binding, internalization, and degradation of ^{125}I-labeled lipoproteins were measured.

tectable in the VLDL or chylomicron remnants when HDL was included in the perfusion medium. These observations suggest that level of HDL in circulating blood may be an important factor that regulates the extent of retention of lipolytic surface remnants containing apoCs by the core remnants of TG-rich lipoproteins in vivo.

We found that VLDL core remnants produced in the presence of a high HDL level (VLDL-CH / HDL-CH = 1:4) were smaller and more uniform in size than those produced in the absence or presence of a low HDL level (Fig. 2), suggesting that the removal of excess surface remnants can reduce the particle size of VLDL core remnants. However, the VLDL core remnants containing no detectable vesicular surface material and apoCs were still more buoyant than plasma LDL (Figs. 1 and 3), suggesting that these particles may contain excess core lipids as compared with LDL. Deckelbaum et al. (20) previosuly reported that LDL-like VLDL core remnants produced after in vitro lipolysis of VLDL are larger than LDL because the number of CE molecules in the VLDL core remnant particles is twice that of LDL. Hepatic lipase has been implicated in the conversion of VLDL core remnants into LDL (13), but a recent study by Murdoch et al. (32) revealed that the hepatic lipase in combination with LpL was not effective in the complete conversion of VLDL into LDL. Murdoch and Beckenridge (33) have shown that the transfer of lipolytic surface remnants of VLDL into HDL during lipolysis was markedly enhanced by the addition of purified lipid transfer proteins to the lipolysis mixture. We have included lipoprotein-free plasma proteins as a source of lipid transfer protein in all lipolysis mixtures. Whether lipid transfer protapoA-Ieins can promote the dissociation of lipolytic surface remnants in the absence of its acceptor (HDL) is not clear as the absence of HDL in the lipolysis mixutre resulted in the retention of most of the VLDL apoCs by the VLDL core remnants (Fig. 3).

A number of studies have clearly demonstrated that apoCs have an inhibitory effect on the apoE-mediated binding of VLDL to lipoprotein receptors on the liver or in cultured cells in vitro and on the removal of VLDL from circulating blood in vivo when the level of apoCs relative to that of apoE on the surface of lipoproteins was ab-

TABLE 2. Distribution of ^{125}I-radioactivity among apoB, apoE, and apoC moieties and ratios of ^{125}I-labeled apoCs or apoE to apoB or apoCs to apoE of VLDL and VLDL core remnants added to the culture medium of HepG2 cells and released from HepG2 cells by heparin

	% Distribution of ^{125}I Radioactivities			Ratios of Apolipoproteins		
Lipoproteins	ApoB	ApoE	ApoCs	Cs/B	E/B	Cs/E
Added						
A. VLDL	67.8 ± 3.5	10.3 ± 0.7	21.9 ± 3.1	0.32	0.15	2.12
B. VLDL core remnants (VLDL + BSA + LpL)	71.6 ± 3.8	11.7 ± 1.4	16.7 ± 2.7	0.23	0.16	1.42
C. VLDL core remnants (VLDL + BSA + HDL)	76.6 ± 3.4	17.8 ± 2.5	5.6 ± 2.5	0.07	0.23	0.31
Released						
A. VLDL	64.8 ± 4.9	21.0 ± 3.0	14.2 ± 4.5	0.22	0.32	0.67
B. VLDL core remnants (VLDL + BSA + LpL)	67.4 ± 6.8	23.0 ± 6.2	9.6 ± 1.1	0.14	0.34	0.41
C. VLDL core remnants (VLDL + Alb + HDL + LpL)	70.9 ± 3.9	25.5 ± 2.8	3.6 ± 2.1	0.05	0.35	0.14

Values are mean ± SD of triplicates. VLDL core remnants were produced after in vitro lipolysis of VLDL in the absence of HDL (B) or in the presence of a high HDL level (C).

normally increased either by the addition of purified apoCs in vitro (4, 5, 8, 9) or by overexpression of apoCs gene in vivo (35, 36). Sehayek and Eisenberg (9) have proposed that the inhibitory effect of apoCs on the apoE-dependent uptake of lipoproteins is predominantly due to the interaction of apoCs with apoE at the lipoprotein surface, where exogenously added apoCs mask apoE or alter the conformation of apoE. On the other hand, Weisgraber et al. (37) have proposed that the apoC-mediated inhibition of the binding of apoE-containing lipoproteins to the LDL receptor-related proteins is a result of the displacement of apoE on VLDL by exogenously added apoCs. Whether apoCs as an essential structural component of VLDL have such an inhibitory effect on the binding of VLDL to apoE receptors is not currently clear. Borensztajn and Kotlar (38, 39) have reported that the hepatic uptake of chylomicron remnants containing normal levels of apoCs or E is not determined by the relative ratio of apoCs to apoE but by the alteration of surface lipid compositions of chylomicrons.

In this study, we could produce VLDL core remnants having different apoCs to apoE ratios by the HDL-mediated dissociation of apoCs from the VLDL core remnants instead of adding exogenous apoCs or apoE. Although VLDL core remnants produced in the presence of a low or high HDL level had an apoCs to apoE ratio about 3- to 10-times lower than that on VLDL core remnants produced in the absence of HDL (Fig. 3), the ability of VLDL core remnants having either a high or low apoCs to apoE ratio to bind to lipoprotein receptors in HepG2 cells or to be metabolized by human hepatocytes in a primary culture was about equal (Figs. 6 and 8). This indicates that apoCs to apo E ratio of the VLDL core remnants may have no influence on their ability to bind to lipoprotein receptor on HepG2 cells or human hepatocytes. The VLDL core remnants with a low apoCs to apoE ratio, however, contained apoA-I, derived from HDL, as a major apolipoprotein constituent of the remnants (Fig. 3). Whether the presence of apoA-I on the VLDL core remnants can influence the ability of VLDL core remnants to bind to lipoprotein receptors on HepG2 cells or to human primary hepatocytes is not known. Arbeeny and Eder (40) reported previously that placing diabetic rats on an atherogenic diet resulted in marked hypercholestolemia and appearance of IDL particles containing apoB, apoE, and apoA-I as their major apolipoprotein species. These investigators (40) found that the rate and extent of removal by perfused liver of IDL containing apoA-I were identical to IDL having no detectable apoA-I. Thus, they concluded that apoA-I does not modulate the uptake of apoB- and E-containing lipoproteins by liver (40). Cultured HepG2 cells possess receptors that are capable of binding and degrading VLDL, LDL, and HDL (26, 27, 41). The competition studies demonstrated that unlabeled HDL as well as VLDL and LDL can effectively compete with ^{125}I-labeled VLDL for binding to HepG2 cells (26). The inhibitory effect of various unlabled HDL on the binding of ^{125}I-labeled VLDL to HepG2 cells was correlated significantly with levels of apoE but not with the levels of apoA-I or apoA-II on HDL (26). Although these observations (26, 40) suggest that apoA-I appearing on the VLDL core remnants may not have any influence on the binding of VLDL core remnants to the apoE receptors on HepG2 cells or human primary hepatocytes, further study must to be done to determine the potential effect of apoA-I on the binding of VLDL core remnants to lipoprotein receptor as apoA-I can act as a ligand for binding to HDL receptors (42).

Our study showed that receptor-bound VLDL and VLDL core remnants released by heparin had a reduced level, but were not devoid, of apoCs (Table 2). The presence of apoCs on the VLDL or VLDL core remnants bound to lipoprotein receptors on HepG2 cells indicates further that apoCs, as a minimum structural or functional component of VLDL or VLDL core remnants, may not have an inhibitory effect on the ability of these particles to bind to lipoprotein receptors. VLDL and VLDL core remnants released from HepG2 cells, however, had a markedly increased content of apoE with a reduced content of apoCs (Table 2), suggesting that lipoprotein receptors on HepG2 cells preferentially bind a subpopulation of VLDL enriched in apoE. It is well known that human VLDL consists of two major subfractions, based on the apoE contents (43, 44). Huff and Telford (44) reported that a VLDL subfraction enriched in apoE, which constitutes 58–75% of human VLDL, had a apoE to apoC ratio 2- to 8-fold higher than the other subfraction poor in apoE. In experimental animals, the fractional catabolic rate of apoB on the apoE-rich VLDL subfraction was nearly 2-fold greater than that of the apoE-poor VLDL subfraction (44). Although we have not separated and examined the metabolic properties of the two VLDL subfractions presented in our VLDL samples, the VLDL and VLDL core remnants bound to lipoprotein receptors on HepG2 cells would likely be an apoE-rich subfraction of VLDL and VLDL core remnants as the apoE to apoB ratio in the VLDL and VLDL core remnants released from HepG2 was twice that of VLDL and VLDL core remnants added to the culture medium (Table 2). The present study showed that the lipolysis-mediated changes in size and lipid composition of VLDL had a marked influence on the ability of VLDL to bind to HepG2 cells or to be metabolized by human liver cells with a minimal change in the apoCs to apoE ratio (Figs. 6 and 8). Our observations support a hypothesis that the hepatic discrimination for the removal of TG-rich lipoproteins is determined by the alteration in lipid composition but not by the relative amount of apoE and Cs on the surface of lipoproteins (38, 39).

We have shown in this study that in vitro lipolysis of VLDL in the absence of HDL resulted in an association of most of their surface remnants containing apoCs with core remanats (Fig. 3), and that some of these surface remnants can be dissociated from the core remnants and form vesicular particles (Fig. 5). The accumulation of such vesicular remnants could be prevented by including proper levels of HDL in the lipolysis mixtures (Figs. 3 and 5). A number of studies (45, 46) have shown that heparin-induced lipolysis in hypertriglyceridemic subjects resulted

in the accumulation of flattened vesicles in circulating blood. Similar vesicular particles was also shown to appear in circulating blood when a large dose of chylomicrons was injected into the rat (47) or when plasma HDL level was markedly lowered by the targeted mutation of phospholipid transfer proteins in mice (48). We have previously reported that the lipolysis of hypertriglyceridemic serum, but not normolipidemic serum, resulted in the accumulation of aggregate vesicular particles containing apoCs and apoA-I in plasma (49). These in vivo and in vitro observations (45–49) suggest that vesicular remnants could be accumulated in circulating blood in vivo when the blood HDL level is not sufficiently high enough to accept all lipolytic surface remnants produced during the catabolism of TG-lipoproteins. Because the vesicular particles produced during lipolysis of VLDL contained apoCs and apoA-I but little or no detectable ligand apolipoproteins (apoB or apoE) responsible for their binding to cellular lipoprotein receptors (Fig. 5), it is not currently clear how these vesicular remnants will be removed from circulation when such remnants are chronically produced due to a low HDL level. We have previously shown that vesicular particles produced during in vitro lipolysis of hypertriglyceridemic serum are morphologically and compositionally similar to those liposome-like extracellular lipid particles extracted from human atherosclerotic plaques (49). It is plausible that vesicular particles produced during the lipolysis of TG-rich lipoproteins could serve as a precursor of extracellular vesicular particles in atherosclerotic lesions. Although we observed in this study that HDL-mediated depletion of apoCs on VLDL core remnants had no significant effect on their ability to bind to lipoprotein receptors on cultured HepG2 cells or human primary hepatocytes (Figs. 7 and 8), an earlier study by Lakshmanan et al. (34) revealed that apoC-deficient VLDL or chylomicron remnants generated in the perfused heart system in the presence of HDL had 3- to 4-fold greater potency to inhibit hepatic fatty acid synthesis than apoC-containing VLDL or chylomicron remnants generated in the absence of HDL. We postulate that one possible role of HDL in protecting against the development of atherosclerosis is its ability to prevent the accumulation of lipolytic surface remnants, a potential precursor of vesicular particles in atherosclerotic lesions, in circulating blood and to prevent the potential divergent metabolism of core remnants of TG-rich lipoproteins due to the presence of excess surface remnants.

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