Apolipoprotein E and Lipoprotein Lipase Secreted From Human Monocyte-Derived Macrophages Modulate the Uptake of Very Low Density Lipoproteins

ヒト単球由来マクロファージから分泌されるアポリポタンパクEとリポタンパクリパーゼによる超低比重リポ蛋白の取りこみと調節

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Introduction

Involvement of monocytes/macrophages in the development of atherosclerosis has been indicated by several lines of evidence. Foam cells observed in atheromatous fatty streaks are derived from monocytes/ macrophages through interaction with plasma lipoproteins (1, 2). Adhesion and sebsequent migration of circulating monocytes into endothelium occurs as an initial event which leads to fatty streak formation (3, 4).

Because the atheromatous lesion contains predominantly cholesteryl ester as lipids, considerable attention has been paid to the uptake of cholesteryl ester-rich lipoproteins such as LDL, ß-VLDL and modified-LDL. On the other hand, macrophages take up triglyceride-rich lipoproteins avidly, resulting in both triglyceride and cholesterol ester accumulation in vitro (5). However, little is known about the mechanism behind triglyceride-rich lipoprotein uptake. In the uptake of triglyceride-rich lipoproteins by macrophages, not only the lipoprotein receptor but also secretory products modifying the lipoproteins could play a major role, since macrophages secrete proteins which can alter the binding affinity of triglyceride-rich lipoproteins for lipoprotein receptors; lipoprotein lipase (6-10) and apolipoprotein E (apo E)[1] (11, 12).

Lindqvist et al. showed that hydrolysis of triglyceride-rich lipoproteins by lipoprotein lipase is essential for the uptake of the lipoproteins by macrophages (13). However, its precise mechanism has not been well clarified yet. Furthermore, although apo E secreted by macrophages has been studied from the standpoint of cholesterol efflux from the cells (11, 12, 14, 15), there have been no reports addressing its role in the uptake of the lipoproteins. We therefore attempted to show the precise action of lipoprotein lipase as a precursor-product relationship of lipoproteins containing apo B100, and to test the hypothesis that both lipoprotein lipase and apo E are essential for facilitating the uptake of lipoproteins, especially triglyceride-rich lipoproteins.

Materials and methods

Materials: Sodium [^{125}I]iodide and ^{125}I-protein A were purchased from ICN (Irvine, CA), and [^{35}S] methionine and glycerol tri[9,10(n)-^{3}H]oleate from Amersham/Searle Corp. (Arlington Heights, IL). Benzamidine, bovine serum albumin (BSA, essentially fatty acids free), dimethyl sulfoxide, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was obtained from Gibco (Grand island, NY) and was heat-inactivated (56 °C, 30 min) prior to use. Heparin Sepharose CL6B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), DE52 from Whatman (Kent, England), Nitroplus 2000 (0.45 μm) from Micron Separations Inc. (Westboro, MA), Aqualuma Plus from Lumac (Landgraaf, the Netherlands) and Silica gel GF plates (Cat. No. 02011) from Analtech (Newark, DE). Other chemicals were of analytical grade.

A patient with apo CII deficiency—An 8-month-old male was diagnosed to have apo CII deficiency. The complete absence of apo CII in his plasma was demonstrated by radioimmunoassay, isoelectric focusing (Fig.1A), two-dimensional electrophoresis (Fig. 1B) and its immunoblot (16). Plasma triglyceride and cholesterol concentrations were 12,020 and 680 mg/dl, respectively.

Patients with type III hyperlipoproteinemia with E2/2 phenotype—Three patients with type III hyperlipoproteinemia were used for experiments (M.T.: 50-year-old female, S.A.: 47-year-old female and K.M.: 51-year-old male). Their plasma triglyceride concentrations were 271, 2700, 751 mg/dl, respectively, and their plasma cholesterol concentrations were 425, 1180, 449 mg/dl, respectively. Agarose gel electrophoresis of plasma lipoproteins revealed a broad-ß band. By isoelectric focusing of their VLDL, the apolipoprotein phenotype of all three subjects was determined to be E2/2.

Lipoprotein preparation—Blood of healthy volunteers was collected into tubes containing EDTA (1 mg/ml) after overnight fasting. Benzamidine and NaN_3 were added to plasma to give final concentrations of 300 μg/ml and 0.01 % (w/v), respectively. Human

VLDL (d < 1.006 g/ml) and LDL (d 1.019-1.063 g/ml) were separated by preparative ultracentrifugation using a Beckmann 50.2 Ti rotor in a L8-55 Ultracentrifuge (Palo Alto, CA) (17). After re-ultracentrifugation, lipoprotein solution was dialyzed against 5 l x 2 of dialyzing buffer containing 150 mM NaCl, 0.01 % (w/v) EDTA, 0.01 % (w/v) NaN_3, and 2 mM sodium phosphate, pH 7.4. Before experiments, lipoprotein solution was dialyzed against 2 l of buffer containing 150 mM NaCl and 2 mM sodium phosphate, pH 7.4, and filtered through a 0.45 μm Acrodisc (Gelman Sciences, Ann Arbor, MI). VLDL prepared were used within 2 days and LDL within 3 days. Lipoproteins were radioiodinated with ^{125}I by the McFarlane monochloride procedure as modified for lipoproteins (18). VLDL-triglycerides were radiolabeled with glycerol tri-[^{3}H]oleate in vitro according to Fielding et al. (19). Samples were filtered (0.45 μm, Gelman) immediately before use. Specific activities of the radioiodinated samples were 70-200 cpm/ng protein.

Apolipoprotein preparation—VLDL prepared from type V hyperlipoproteinemic subjects were dialyzed against 5 l of 20 mM NH_4HCO_3 and delipidated by ethanol/ether. The apo VLDL was dissolved in 10 ml of 8 M urea, 50 mM NaCl, and 2 mM sodium phosphate, pH 7.4 and applied to a heparin-Sepharose CL6B column (2 x 10 cm). The void volume fraction was applied to a DEAE cellulose column (2 x 20 cm) and eluted by the buffer containing 6 M urea 5 mM Tris-HCl, pH 7.4 with gradient NaCl concentrations from 10 mM to 165 mM. Fractions of the first peak contained apo CII. The purities of these apolipoproteins were checked by SDS polyacrylamide gel (20) and urea gel electrophoresis.

Culture of human monocyte-derived macrophages—Phosphate buffered saline (PBS) without Ca^{2+} was added to the blood cell pellets obtained from healthy subjects to give an initial volume of blood. After swirling completely, this solution was overlaid on the surface of Lymphoprep (Daiichi Pharmaceutical, Co., Tokyo) and centrifuged at 400 x g for 30 min at room temperature. The mononuclear cells at the interface between Lymphoprep and PBS were collected and suspended in PBS without Ca^{2+} and centrifuged

at 400 x g for 12 min at 4 °C. The pellets were washed twice with PBS at 160 x g for 10 min. The washed mononuclear cells were adjusted to a concentration of 2-3 x 10^6 cells/ml of RPMI-1640. One ml per each well (well diameter 22 mm) of 12-well plates or 5 ml per each dish (60 x 15 mm) was incubated at 37 °C at the atmosphere of 5 % CO_2 and 95 % air.

After 2 h, the non-adherent cells were removed and the adherent cells were gently washed with prewarmed PBS and incubated with RPMI-1640 supplemented with 10% autologous serum unless otherwise stated. The medium was replaced by fresh medium twice a week and the cells were used for experiments 2 weeks after the mononuclear cell preparation. Two days before the experiments, the cells were washed with PBS and the medium was replaced by 0.5 ml and 3 ml of medium containing 5 mg/ml of lipoprotein deficient serum (LPDS), respectively. Apo E secreted from macrophages was measured by radioimmunoassay as described previously (21).

Culture of human skin fibroblasts—Human skin fibroblasts were cultured from forearm skin biopsy specimens of healthy volunteers. The cells were maintained in MEM supplemented with 10 % FCS and used for experiments between the 5th and 15th passage. For the experiments, approximately 2 x 10^4 cells were seeded into each well (well diameter: 35 mm) of 6-well plates with 2 ml of complete medium containing 10 % FCS. When the cells were approximately 75 % confluent (3-5 days), the cells were washed with PBS and the medium was replaced by 2 ml of medium containing 5 mg/ml of human LPDS for 48 h.

Density gradient ultracentrifugation—Five μg/ml of ^{125}I-VLDL in RPMI-1640 containing LPDS or BSA were incubated with macrophages at 37 °C. After varying time intervals, the medium was collected. After low-speed centrifugation, EDTA, NaN_3, and PMSF were added to the medium to give final concentrations of 0.01 % (w/v), 0.01 % (w/v), and 1 mM, respectively. The density gradient was made by a stepwise compilement of three different density layers (top: d = 1.00; middle: d = 1.0315; bottom: d = 1.063). The volume of each layer was 3.7 ml and its density was adjusted by a concentrated stock

solution of NaCl. The middle layer contained 0.5 ml of the sample medium. The tubes were ultracentrifuged in a Beckmann SW-41 rotor at 40,000 rpm for 22 h at 12 °C (22). One ml of the top layer was isolated by tube slicing. The remaining solution was fractionated by aspiration from the top to the bottom into 10 fractions of 1 ml. The density of each fraction was measured with electric conductivity meter.

After measuring the radioactivity (A) of each fraction , 50 µl of concentrated LDL solution (2 mg/ml) as the carrier and 0.95 ml of isopropanol was added to it and mixed thoroughly (23). After incubation at room temperature for 1 h, 0.8 ml of the mixture was taken to measure its radioactivity (B). The remaining mixture was centrifuged at 10,000 x g for 10 min at room temperature and 0.8 ml of the supernatant was taken to measure its radioactivity (C). The radioactivity in apo B was calculated as A x (B - C)/B .

Degradation and binding of lipoproteins by cells—The degradation products of radioiodinated lipoproteins were measured according to Goldstein and Brown. (24). For the binding assay, the cells were preincubated with RPMI-1640 containing 5 mg/ml of LPDS for 48 h. To measure 4 °C binding, the cells were precooled at 4 °C for 30 min. Thereafter, the cells were washed with ice-cold PBS and incubated with ice-cold radioiodinated lipoproteins with and without excess unlabeled lipoproteins dissolved in binding buffer (MEM for fibroblasts/RPMI-1640 for macrophages, 5 mg/ml of BSA, and 10 mM HEPES at pH 7.4). After 2-3 h on a rocking platform on ice, the medium was removed and the cells were washed with buffer A (150 mM NaCl, 2 mM $CaCl_2$, 2 mg/ml BSA, and 10 mM Tris-HCl at pH 7.4) rapidly three times, 10 min twice, and finally rapidly rinsed with buffer A without BSA. The cells were dissolved in 1 ml of 0.1 N NaOH for measuring their radioactivity. Their protein concentrations were measured by the method of Lowry et al. (25). To measure 37 °C binding, the binding buffer contained $NaHCO_3$ instead of HEPES. The cells were incubated with radioiodinated lipoproteins for 5 h at 37 °C unless otherwise indicated. After incubation, the medium was collected for measuring

the amounts of degradation products and the cells were cooled to 4 °C and washed as mentioned above.

Incorporation of VLDL-triglycerides into cells—Macrophages were incubated with apo CII deficient VLDL labeled with glycerol tri[^{3}H]oleate in RPMI-1640 containing 5 mg/ml of free fatty acid-free BSA with and without indicated amounts of apo CII for 12 h at 37 °C. The cells were washed rapidly twice with buffer A and once with buffer A without BSA. The cells were incubated with 1 ml of isopropanol/hexane (2/3) for 30 min at room temperature and rinsed with another 0.5 ml. The organic solvent for lipid extraction was combined and evaporated under N_2 stream. The dried lipids were dissolved in hexane and spotted on the silica GF thin layer chromatography and developed by heptane/diethyl ether/acetic acid (90/30/1). After the lipids were stained with iodine vapor, the silica gel containing triglycerides was scraped and its radioactivity was counted in 10 ml of Aqualuma Plus.

Immunoblotting analysis of apo E isoform—Lipoproteins in solution were precipitated by the phosphotungstate procedure as described by Steinmetz et al. (26). Thereafter, isoelectric focussing was performed as described (15), using a slab gel instead of a disk gel. Proteins were transferred to Nitroplus 2000 according to the method of Burnette (27). After the nitrocellulose sheet was incubated with the antiserum (2 μl/ml) and then with ^{125}I-protein A (2,000 cpm/ng, 0.5 μg/ml), autoradiography was performed.

Results

Lipolysis and degradation of VLDL obtained from apo CII deficiency by macrophages—In order to see the action of lipoprotein lipase secreted from macrophages, VLDL from a patient with apo CII deficiency (VLDLc) were incubated with macrophages and the effects of apo CII on the conversion of VLDLc to IDL and LDL were studied. The changes in apo B radioactivities in each fraction of density gradient ultracentrifugation are shown in Fig. 1 AB. The rate constant of the disappearance of ^{125}I-apo B in VLDLc (b: $VLDL = VLDL_0 \times e^{-bt}$) was 7 times greater in the presence of apo CII than that in its absence. In the absence of apo CII, VLDLc were converted only slightly (Fig. 2A). In the presence of apo CII, VLDLc were converted extensively to both IDL and LDL (Fig. 2B). The degradation of ^{125}I-VLDLc by the cells in the presence of apo CII was twice as great as that in its absence (Fig. 2C).

The degradation of ^{125}I-labeled VLDLc by macrophages was enhanced by apo CII in a dose dependent manner up to 4-fold (Fig. 3A). Furthermore, we measured the degradation of ^{125}I-labeled VLDLc at varying lipoprotein concentrations with and without apo CII (Fig. 4A). The increases in the degradation of ^{125}I-VLDLc were curvilinear with a maximal velocity at 10 μg/ml of VLDLc protein. The addition of apo CII resulted in an increase in the degradation of VLDLc. From Lineweaver-Burk plot analysis of these results, the addition of apo CII increased the Vmax for ^{125}I-VLDLc uptake and degradation 2.3-fold, from 226 to 520 ng/mg. The increased Vmax by the addition of apo CII suggested the presence of a mechanism causing the lipolysed VLDL by macrophages to be endocytosed more efficiently than the native VLDL.

In the study using triglyceride-labeled VLDL, the incorporation of tri-[^{3}H]oleate of VLDLc into the cells was also enhanced by the addition of apo CII in a dose-dependent manner up to 6-fold (Fig. 3B). The incorporation increased curvilinearly with an increase in VLDLc protein concentration. The addition of apo CII increased the Vmax for the

incorporation of [^{3}H]triglycerides 9.6-fold, from 25 to 241 μg/mg (Fig. 4B). Thus, the uptake of VLDL proteins was associated with the more prominent incorporation of triglycerides from VLDL into the cells.

The effects of apo E in LPDS on the degradation of VLDL by macrophages—When the proteins secreted by macrophages were radiolabeled by [^{35}S]methionine and the medium was ultracentrifuged at the density of 1.25 g/ml, the lipoprotein fraction ($d < 1.25$ g/ml) contained apo E as a major and single constituent (Fig. 5). Apo E is known to be released from lipoprotein particles by ultracentrifugal force (21) and incorporated into lipoproteins when incubated with lipoproteins (28-30). In fact, when VLDL were incubated with macrophages in the presence of [^{35}S]methionine and the medium was centrifuged by density gradient centrifugation, a substantial portion of apo E was distributed in the VLDL fraction (Fig. 6).

We found appreciable amounts of apo E (approximately 1 mg/dl) in LPDS, measured by single radial immunodiffusion measurement (Daiichi Pharmaceutical Co.). To remove apo E, LPDS was applied to heparin Sepharose CL6B affinity chromatography. We studied the effects of apo E in LPDS on the degradation of ^{125}I-VLDL in the culture of macrophages (Fig. 7). The degradation of ^{125}I-VLDL in the LPDS without apo E was about 50 % that of the LPDS with apoE. These results indicated that VLDL could accept apo E from LPDS and gain a consequent higher affinity for the LDL receptors than the original VLDL, and then such VLDL could be taken up and degraded by macrophages.

Binding of VLDL incubated with macrophages for lipoprotein receptors on fibroblasts and macrophages—When monolayers of macrophages were incubated with varying amounts of ^{125}I-VLDL at 37 °C, the cell association of ^{125}I-VLDL increased in a saturable fashion. In parallel with the cell association, ^{125}I-VLDL were degraded by a saturable process. The amounts of cell association and degradation of ^{125}I-VLDL by macrophages were about one fifth of those by fibroblasts on cellular protein basis (data not shown).

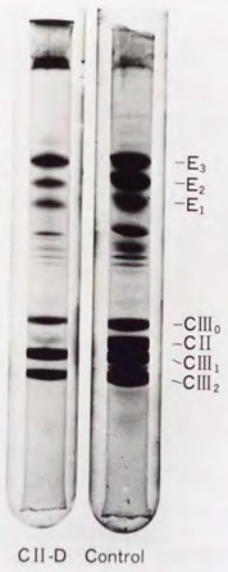
We next carried out competition experiments to compare the binding affinity of VLDL incubated with macrophages, with VLDL without incubation. The VLDL incubated with macrophages showed 17-fold greater ability to compete the binding of ^{125}I-LDL to fibroblasts at 4 °C than the native VLDL, with 50 % inhibition occurred at 0.12 and 2 μg/ml, respectively (n = 7) (Fig. 8). These results indicated that the incubation with macrophages increased the affinity of VLDL for the LDL receptors on fibroblasts. During the incubation of VLDL with macrophages, 23 % of triglycerides in the VLDL were lipolysed and the density gradient ultracentrifugation revealed a minimal shift in its density.

Concentrations of apo E in medium containing 2 mg/ml of BSA after 24 h-incubation with macrophages at 37 °C were 0.13 - 0.43 μg/ml (n = 4), whereas those at 4 °C were less than 0.01 μg/ml. The secretions of lipoprotein lipase from macrophages were also negligible at 4 °C compared to those at 37 °C. Thus, we performed the experiments at 37 °C to estimate the influence of secretory products of macrophages on the VLDL uptake. We compared the binding of ^{125}I-LDL to the LDL receptors on macrophages competed by unlabeled VLDL at 4 °C, with that at 37 °C. As shown in Fig. 9, unlabeled VLDL inhibited the binding and degradation of ^{125}I-LDL more strongly than LDL. At 4 °C, the VLDL showed a 2-fold greater affinity in inhibiting the binding of ^{125}I-LDL to macrophages than LDL (Fig. 9A). At 37 °C, the VLDL showed a 30-fold greater affinity in inhibiting the degradation of ^{125}I-LDL than LDL (Fig. 9B). This apparent increase in the affinity of VLDL at 37 °C could be caused by the modification of VLDL by secretory products of macrophages.

The role of apo E secreted by macrophages for VLDL uptake—The degradation of ^{125}I-LDL by fibroblasts was displaced by VLDL obtained from a subject with E3/3 phenotype (E3/3-VLDL) more strongly than ß-VLDL obtained from a subject with E2/2 phenotype (E2/2-ß-VLDL) (Fig. 10). We then compared the affinity of E3/3-VLDL incubated with macrophages obtained from a subject with E2/2 phenotype (E2/2-macrophages) to that with

macrophages obtained from a subject with E3/3 phenotype (E3/3-macrophages) in fibroblasts (Fig. 11). After the incubation with E3/3-macrophages, the ability of the E3/3-VLDL to inhibit the ^{125}I-LDL binding increases by a factor of 10, from 50 to 5 μg/ml to give 50 % inhibition. On the other hand, incubation with E2/2-macrophages decreased the ability of E3/3-VLDL to inhibit the binding, with 50 % inhibition occurred at 80 μg/ml .

During the incubation of E2/2-ß-VLDL with E3/3-macrophages, apo E secreted by E3/3-macrophages was incorporated into E2/2-ß-VLDL as shown in Fig. 12. The amounts of the incorporated apo E3, which were derived from the cells during the 48 h of the culture, were comparable to those of apo E2 originally present in E2/2-ß-VLDL. Such amounts were presumed to be sufficient for altering the binding affinity of VLDL (31). To see the contribution of the macrophages's own secreted apo E to their uptake of VLDL, we compared the degradation of either E3/3-VLDL or E2/2-ß-VLDL between E3/3- and E2/2-macrophages in the culture of 12 h , as shown in Table 1. The amounts of ^{125}I-labeled E2/2-ß-VLDL bound and degraded by E3/3-macrophages was about twice and three times, respectively, as much as those by E2/2-macrophages. Additionally, the amounts of ^{125}I-labeled-E3/3-VLDL bound and degraded by E3/3-macrophages were also greater than those by E2/2-macrophages, although the difference was less striking and not statistically significant. Of particular interest, the uptake and degradation of ^{125}I-labeled E2/2-ß-VLDL by E2/2-macrophages were the smallest of all the combinations. Incorporation of apo E3 secreted from E3/3-macrophages into E2/2-ß-VLDL might increase their binding affinity for the LDL receptors, resulting in facilitation of the uptake of the lipoproteins.



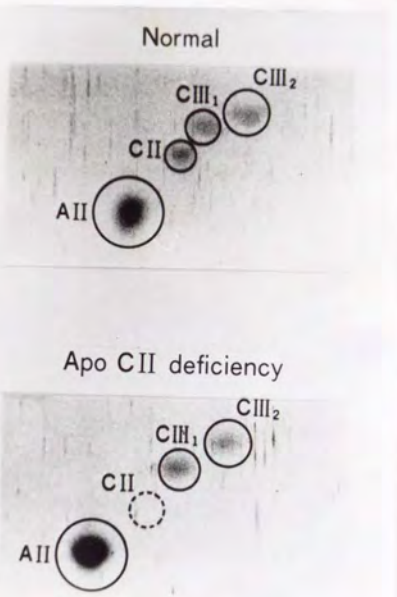


Fig. 1A **Isoelectric focusing of apoproteins of the d < 1.006 g/ml plasma lipoprotein fraction from a normal subject (right) and the patient with apo CII deficiency (left).** The lipoprotein fraction containing 200 μg of apoproteins was subjected to analysis.

Fig. 1B. **Two-dimentional gel electrophoretogram of plasma from a control subject (above) and the patient (below).** 2 μl of plasma pretreated with a detergent solution were placed on polyacrylamide gels and electrofocused. Apolipoproteins were visualized silver stain.

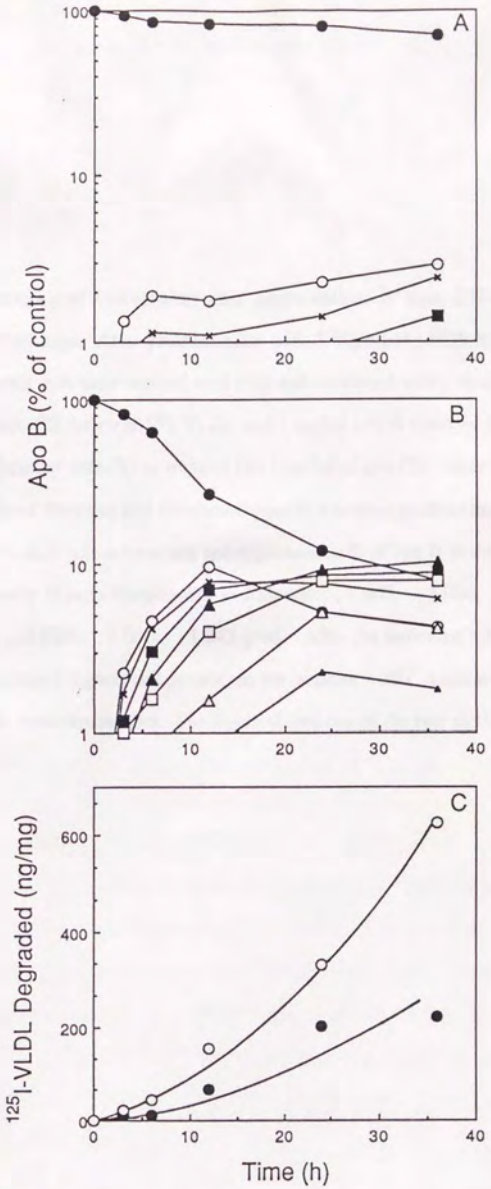

A
B
C
100
10
1
0
10
20
30
40
Apo B (% of control)
600
400
200
125I-VLDL Degraded (ng/mg)
Time (h)

Fig. 2 **Time course of conversion and degradation of apo CII-deficient ^{125}I-VLDL by macrophages.** After preincubation with 5 mg/ml of LPDS for 48 h, the cells cultured in a 35-mm dish were washed with PBS and incubated with a medium containing 5 μg protein/ml apo CII deficient ^{125}I-VLDL and 7 mg/ml LPDS obtained from a subject with apo CII deficiency with (B) or without (A) 1 μg/ml of apo CII. After the indicated time, the media were removed and ultracentrifuged in a density gradient manner. Apo B content in each fraction was determined and expressed as % of apo B in the unincubated VLDL. The density of each fraction was as follows: , 1.003; , 1.006; , 1.009; , 1.013; , 1.021; , 1.028; , 1.036; , 1.043 g/ml. After the indicated time, the amounts of ^{125}I-VLDL degraded (ng/mg cell protein) in the medium with ( ) and without ( ) 1 μg/ml of apo CII were determined. The figure shows one of the two similar experiments.

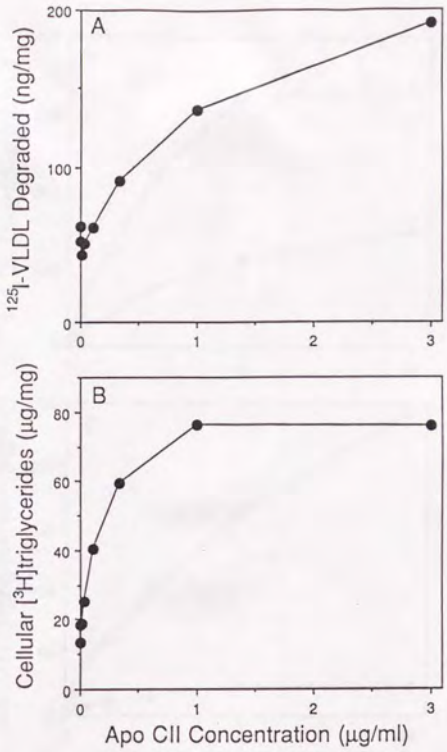


Fig. 3 **Dose dependent effects of apo CII on degradation of apo CII-deficient ^{125}I-VLDL (A) and incorporation of VLDL-[^{3}H]triglycerides by macrophages (B).** After preincubation with 5 mg/ml of LPDS for 48 h, the cells were washed by PBS and incubated with medium containing 5 mg/ml of BSA and 5 µg/ml of either ^{125}I-VLDLc or [^{3}H]triglyceride-labeled VLDLc with increasing concentrations of apo CII. After 12 h, the amounts of ^{125}I-VLDL degraded (ng/mg cell protein) and [^{3}H]triglycerides in the cells (µg/mg cell protein) were measured. Each point represents the mean of triplicate wells.

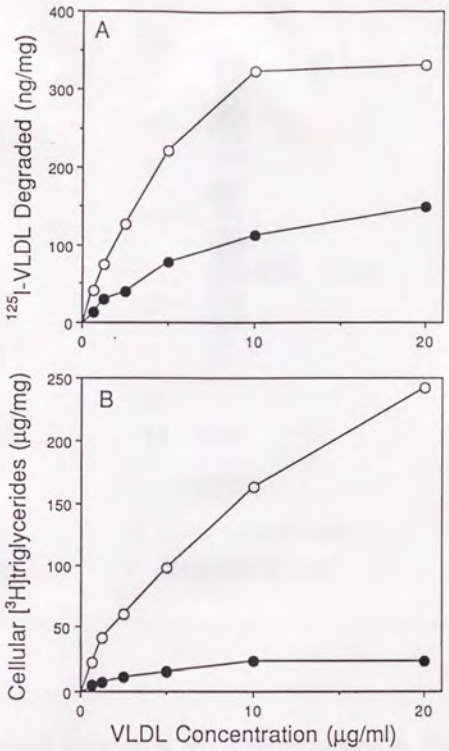


Fig. 4 **Protein degradation (A) and cellular triglyceride incorporation (B) in macrophages incubated with apo CII-deficient VLDL.** After preincubation with 5 mg/ml of LPDS for 48 h, the cells were washed by PBS and incubated with medium containing 5 mg/ml of BSA and increasing concentrations of either ^{125}I-VLDLc or [^{3}H]triglyceride-labeled VLDLc with (○) or without (●) 2 μg/ml of apo CII. After 12 h, the amounts of ^{125}I-VLDL degraded (ng/mg cell protein) and [^{3}H]triglycerides in the cells (μg/mg cell protein) were measured. Each point represents the mean of duplicate wells.

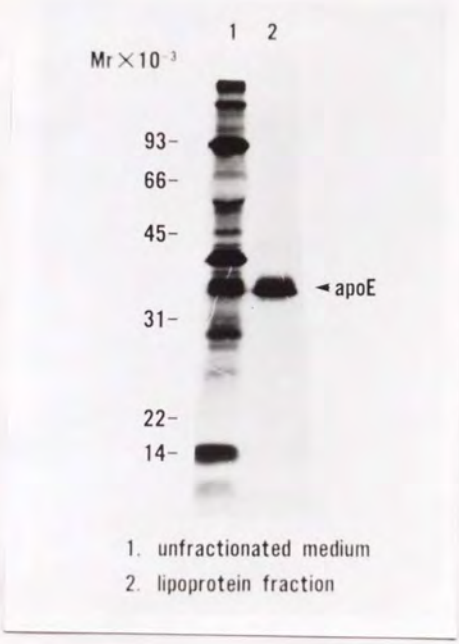


Fig. 5 **Synthesis and secretion of apo E by macrophages.** Two ml of medium containing 40 μCi/ml of [^{35}S]methionine were incubated with monolayer of macrophages cultured in 30 mm dishes for 24 h. After the density was adjusted to 1.25 g/ml with KBr, one ml of the medium were ultracentrifuged at 38,000 rpm for 40 h at 12 °C in a 40.3 rotor (Beckman Instruments, CA). The original medium and the lipoprotein fraction were dialysed against 5 L of 10 mM of NH_4HCO_3 and were lyophylized. These samples were subjected to SDS-polyacrylamide gel electrophoresis in reducing condition. The gel was treated with EN^{3}HANCE (New England Nuclear, MA) and the proteins were visualized by fluorugraphy.

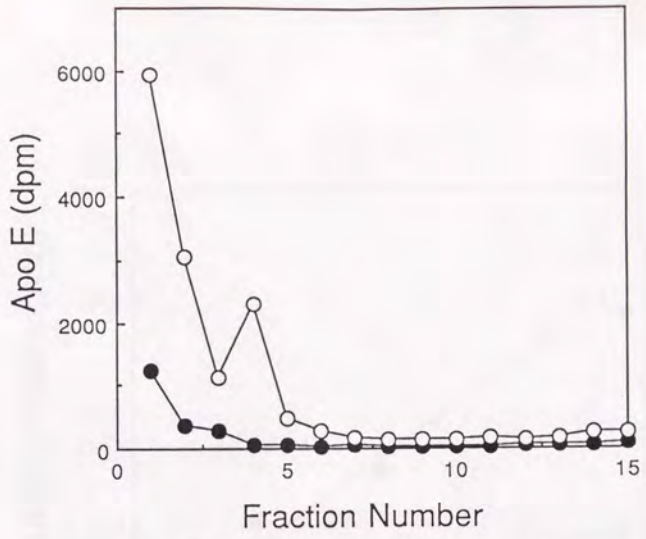


Fig. 6 **Distribution of ^{35}S-labeled apo E by density gradient ultracentrifugation after incubation of VLDL with macrophages in the presence of ^{35}S-methionine.** Two hundred and fifty µl of 100 µg protein/ml of VLDL in 10 % LPDS were incubated with monolayers of macrophages cultured in a 16-mm well in the presence of 40 µCi/ml of [^{35}S]methionine for the indicated time: 1 h (●) and 8 h (○). The medium was centrifuged to remove cell debris and dialysed against 5 l of 150 mM NaCl and 0.01 % EDTA and 2 mM sodium phosphate, at pH 7.4. After the volume and density of the medium were adjusted to 300 µl and 1.34 g/ml, respectively, each sample was placed at the bottom of the density gradient layer from 1.21 to 1.006 g/ml and centrifuged at 26,500 rpm for 24 h at 4 °C in an SW 27 rotor (Beckman Instruments, CA). The sample was fractionated into 25 fractions. Two hundred µl of each fraction were mixed with 4 ml of Aqualuma Plus to measure its ß-counts. SDS/PAGE of the samples revealed that more than 90% of the ^{35}S-labeled proteins in each fraction were apo E.

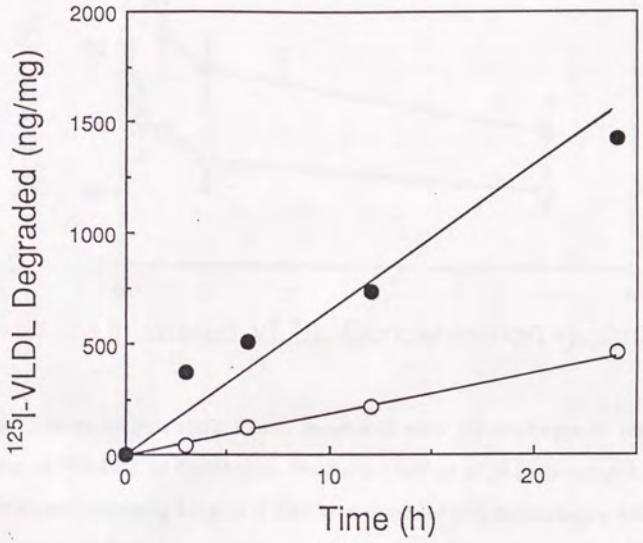


Fig. 7 **Effect of apo E in LPDS on degradation of ^{125}I-VLDL by macrophages.** After preincubation with 5 mg/ml of LPDS, macrophages were incubated in a medium containing 5 μg protein/ml of ^{125}I-VLDL and 5 mg/ml of LPDS with (●) and without (○) apo E. After the indicated time, the degradation products in the medium were measured. Each point represents the mean of duplicate wells.

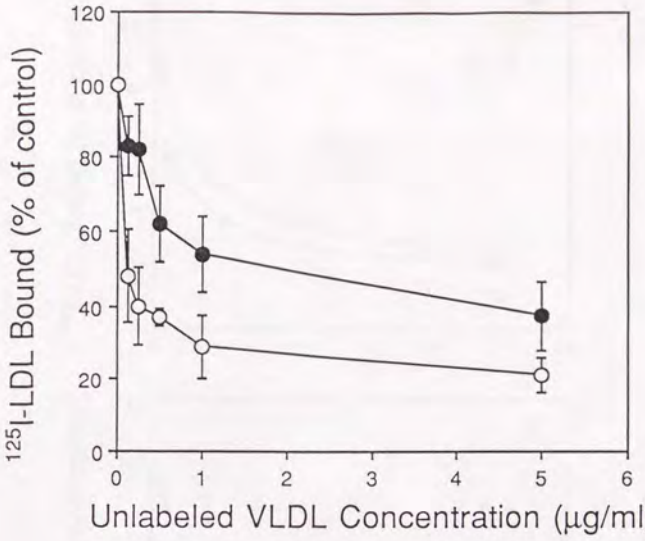


Fig. 8. **Ability of unlabeled VLDL incubated with macrophages to inhibit the binding of ^{125}I-LDL to fibroblasts.** Four and a half ml of 50 μg protein/ml of VLDL in RPMI-1640 containing 5 mg/ml of BSA were incubated with macrophages, which were obtained from subjects with apo E3/3 phenotype and cultured in a 60-mm dish, and another 4.5 ml was incubated in a 60 mm dish without cells as no cell control for 24 h. The medium was removed and centrifuged at 3,500 rpm for 15 min at 4 °C. The medium was diluted with RPMI buffered with HEPES containing 5 mg/ml of BSA. Each monolayer of fibroblasts received 0.5 ml of medium containing 2 μg protein/ml of ^{125}I-LDL and the indicated concentrations of either unlabeled VLDL of no cell control (●) or unlabeled VLDL incubated with macrophages (○). After incubation for 3 h at 4 °C, the amounts of ^{125}I-LDL bound to the cells (ng/mg cell protein) were determined. Each point represents the mean ± S.E. of seven separate experiments. The control (100%) value was 20.0 ± 6.7 ng bound/mg cell protein.

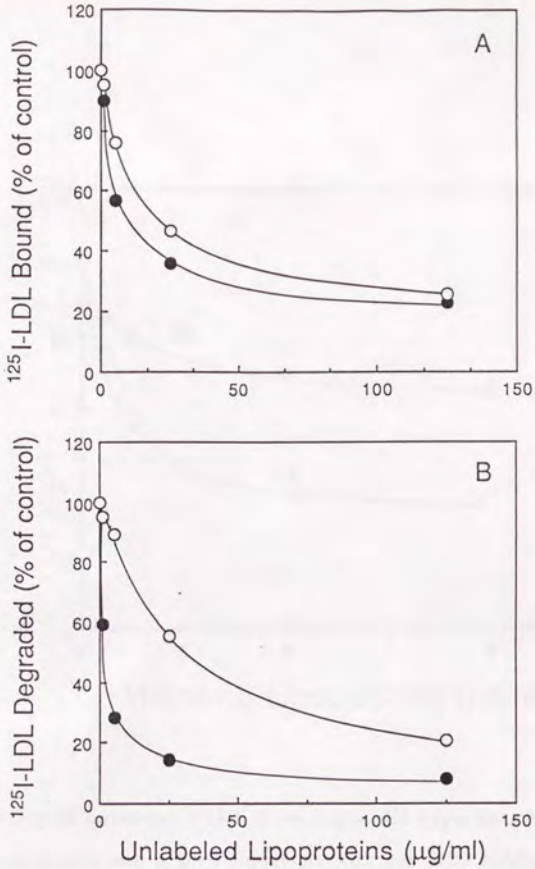


Fig. 9 **Abilities of unlabeled LDL and VLDL to inhibit the binding at 4 °C and degradation at 37 °C of ^{125}I-LDL by macrophages.** After preincubation with 5 mg/ml of LPDS, the monolayers of macrophages were incubated with 5 μg protein/ml of ^{125}I-LDL and the indicated concentrations of unlabeled LDL (○) and VLDL (●) at 4 °C (A) and 37 °C for 6 h (B). The cell-associated ^{125}I-LDL (ng/mg cell protein) and the amounts of ^{125}I-LDL degraded (ng/mg cell protein) were measured. The control (100%) values were 11.1 (A) ng bound/mg cell protein and 157 (B) ng degraded/6 h/mg cell protein.

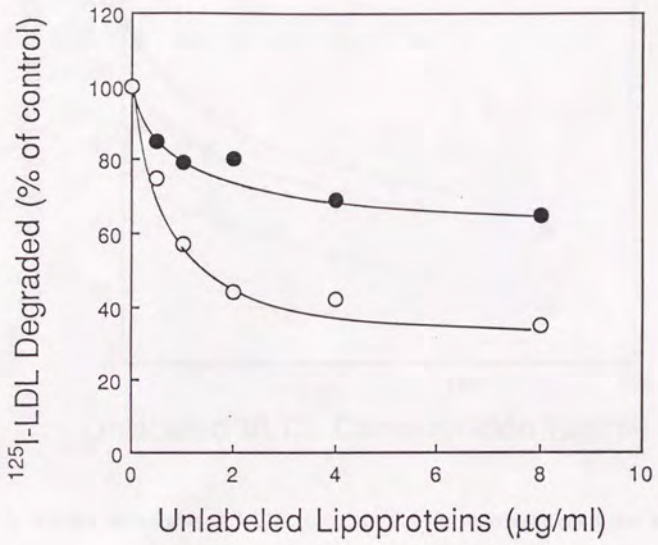


Fig. 10 **Ability of unlabeled VLDL from a type III hyperlipoproteinemic with apo E2/2 phenotype and a normolipidemic with apo E3/3 subject to inhibit the degradation of ^{125}I-LDL by fibroblasts.** After preincubation with 5 mg/ml of LPDS for 48 h, fibroblasts were incubated with 2 μg protein/ml of ^{125}I-LDL and the indicated concentrations of unlabeled VLDL from a type III hyperlipoproteinemic subject (M. T.) with apo E2/2 phenotype (●) and normolipidemic subject with apo E 3/3 phenotype (○) in medium containing 5 mg/ml of BSA at 37 °C for 6 h. The amounts of ^{125}I-lipoproteins degraded (ng/mg cell protein) were measured. The control (100%) value was 31 ng degraded/6 h/mg cell protein.

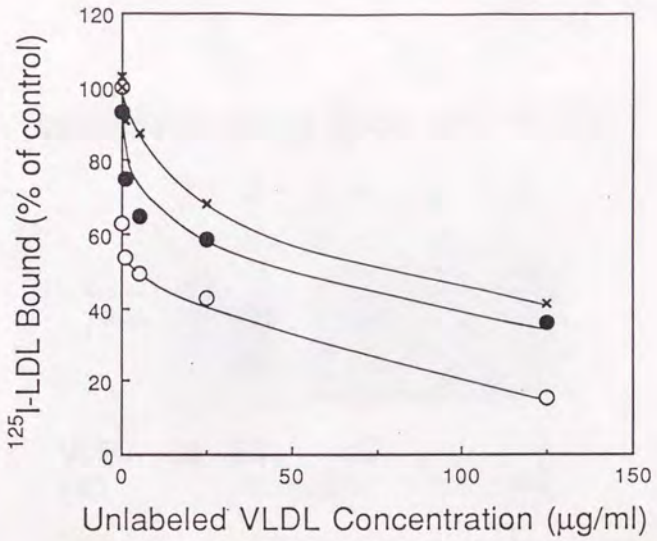


Fig. 11 **Ability of unlabeled VLDL incubated with macrophages from apo E3/3 (○) and apo E 2/2 (×) phenotype to inhibit binding of LDL to fibroblasts.** 1 ml of 500μg/ml of VLDL from apo E3/3 phenotype in RPMI-1640 containing 5 mg/ml of BSA was incubated with monolayers of macrophages obtained from either a normolipidemic with apo E 3/3 or a type III hyperlipidemic with apo E2/2 phenotype (M.T.) in 35 mm dishes and another 1 ml was incubated in a dish without cells (●) at 37 °C for 24 h. The triglyceride contents in the VLDL after the incubation with apo E3/3, apo E2/2, and no macrophages were 2.52, 2.64, and 3.10 mg/ml, respectively. After preincubation with 5 mg/ml of LPDS for 48 h, fibroblasts were incubated at 4 °C for 3 h in a medium containing 2 μg/ml of ^{125}I-LDL and 5 mg/ml of BSA and the indicated amounts of VLDL. The amounts of cell associated ^{125}I-LDL (ng/mg cell protein) were measured. The control (100%) value was 95 ng bound/mg cell protein.

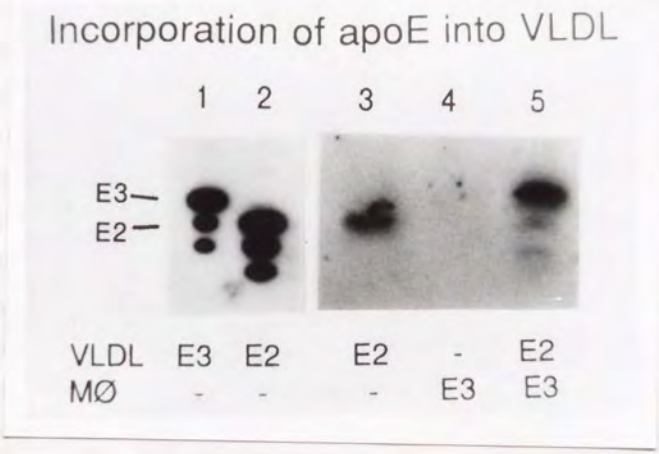


Fig. 12 **Incorporation of Apo E secreted by macrophages into VLDL.** Monocytes were prepared from a subject with apo E3/3 phenotype and cultured in RPMI-1640 containing 10 % autologous serum. ß-VLDL were isolated from a subject with apo E2/2 phenotype (S.A). 2 ml of a medium containing 5 mg/ml of BSA with and without 5 µg/ml of the ß-VLDL were incubated with the E3/3-macrophages grown in 60 mm dish for 48 h. The lipoproteins in the medium were precipitated by phosphotungustate method for isoelectric focussing followed by immunoblotting. Lane 1, E3/3-VLDL; lane 2, E2/2-VLDL; lane 3, the unincubated ß-VLDL; lane 4, the condition media without ß-VLDL; lane 5, the conditioned media with ß-VLDL.

Table I

Degradation of ^{125}I-VLDL by macrophages obtained from normolipidemics with apo E3/3 and type III hyperlipidemics with apo E 2/2 phenotype (M.T. , S.A. and K.M.).

Monocytes were prepared from subjects with both apo E 3/3 and apo E 2/2 phenotypes and cultured in RPMI containing 10 % FCS. VLDL were also prepared from both subjects with apo E 3/3 and apo E 2/2 phenotypes. The medium was replaced every 4 days. After 14 days, the cells were incubated in the medium containing 5 mg/ml of LPDS for an additional 48 h. The monolayers of the cells were incubated at 37 °C for 12 h in 0.5 ml of medium containing the indicated concentrations of ^{125}I-VLDL and 5 mg/ml of BSA. Nonspecific binding and degradation were determined by the addition of a 40-fold excess of unlabeled LDL. The ^{125}I-VLDL degraded were measured. The % values of the ^{125}I-labeled E3/3-VLDL degraded by E 3/3-macrophages were calculated at each VLDL concentration and expressed as mean ± SE (n = 3). The values were compared with each other by analysis of variance. The 100 % values were indicated in parenthesis as the mean ± S.D..

Apo E phenotypes		^{125}I-VLDL Bound		^{125}I-VLDL Degraded	
VLDL	Macrophages	A	B	A	B
		% of E3/3 v.s. E3/3			
E2/2	E2/2	44±18	49±17	17±14	23±19
E2/2	E3/3	131±6[a]	120±10[a]	64±16[a]	65±10[a]
E3/3	E2/2	111±12[a]	78±34	54±7[a]	80±13[a]
E3/3	E3/3	100[a]	100	100[a]	100[a]
		(30.3 ± 6.5)	(61.7 ± 15.9)	(63.1 ± 18)	(158 ± 54)

A: ^{125}I-VLDL concentration of 3.1 μg/ml

B: ^{125}I-VLDL concentration of 12.5 μg/ml

[a]Significantly different from the top row (E 2/2-VLDL by E 2/2-macrophages) ($p < 0.05$).

Discussion

The present study established the pivotal roles of apo E as well as lipoprotein lipase secreted from macrophages in the uptake of triglyceride-rich lipoproteins, mediated mainly by LDL receptor. Some studies suggested that the receptor for ß-VLDL is responsible for VLDL uptake (32). However, our competition study demonstrated that not only the binding but also the degradation of VLDL were well competed by LDL in the macrophages. In addition, recent studies concluded that ß-VLDL are recognized and taken up by classical LDL receptor on macrophages in both human monocyte-derived and mouse peritoneal macrophages (33-35).

Macrophages secrete lipoprotein lipase into culture medium, but its precise action on lipoproteins containing apo B has not been well elucidated yet. We observed a precursor-product relationship of lipoproteins containing apo B100, degradation of VLDL-protein and incorporation of VLDL-triglycerides by macrophages, using VLDL obtained from apo CII deficiency. VLDL-triglycerides were hydrolyzed and their densities were converted from lower to higher as in blood circulation. It is interesting to note that we can demonstrate the lipolytic process in vitro with macrophages. The uptake and degradation of VLDL took place in parallel with the lipolysis of VLDL. The experiment using apo CII-deficient VLDL showed an increase in Vmax of the degradation of VLDL by the addition of apo CII. From these results, lipolysis was suggested to initiate the modification of VLDL which, in turn, causes the enhanced uptake of VLDL through receptor-mediated endocytosis. Furthermore, we propose another modification of VLDL: accepting apo E from macrophages that was possibly enhanced after the initiation of lipolysis.

We found appreciable amounts of apo E in LPDS prepared by ultracentrifugation. It is possible that apo E in LPDS can modify the VLDL uptake process in macrophages, because apo E can be incorporated into VLDL when incubated with lipoproteins or injected in vivo (28-30), and because lipoproteins containing apo E have a high affinity for

lipoprotein receptors. To confirm the possibility, the macrophages were cultured under apo E-depleted LPDS. In the absence of apo E in LPDS, the VLDL were degraded less than in the presence of apo E. We suggest that in the presence of apo E, apo E is incorporated into VLDL from LPDS and that such VLDL gain a higher affinity for receptors than the original VLDL, resulting in the enhanced uptake of VLDL by macrophages.

In fact, the incubation of VLDL with macrophages at 37 °C markedly increased their ability to bind with LDL receptors, because macrophages produce apo E during incubation. Furthermore, when the abilities of both unlabeled VLDL and LDL to inhibit the binding and degradation of ^{125}I-LDL were determined both at 37 °C and at 4 °C, VLDL were demonstrated to have much greater ability in inhibiting the degradation of ^{125}I-LDL at 37°C than that at 4°C. This indicated the significant role of secretory products from macrophages in the VLDL uptake by macrophages and subsequent formation of foam cells. As discussed earlier, lipolysis is not all the modifications of VLDL made by the incubation with macrophages. Apo E secreted by macrophages can alter the binding property of VLDL during the incubation. Soltys et al. suggested the importance of apo E in the VLDL uptake by macrophages from the data, showing a high significant correlation between the number of moles of apo E per VLDL particle and cholesterol esterification in macrophages (36).

In addition, we confirmed the significance of apo E in the VLDL uptake, using VLDL and macrophages from subjects of E2/2 or E3/3 phenotypes. Apo E2 has markedly reduced the binding ability to LDL receptors, since Cys is substituted for Arg_{158}, a residue in the binding domain critical for receptor binding (37,38). The apo E phenotype determined in plasma is common to all tissues in human and the apo E phenotype secreted by macrophages must be identical to the plasma apo E phenotype. As shown in Fig. 7, the newly secreted apo E was incorporated into the VLDL, when the VLDL were incubated with macrophages. The VLDL incubated with E2/2-macrophages did not show an increase

in the affinity for the LDL receptors, whereas the incubation with E3/3-macrophages did (Fig. 11), indicating that the receptor active protein was incorporated into VLDL and raised their ability of binding to the LDL receptors. These results confirm the crucial role of apo E secreted by macrophages in facilitating the VLDL uptake.

To summarize the observations noted above: 1) lipolysis of VLDL by lipoprotein lipase secreted by macrophages enhances the uptake of VLDL, 2) VLDL are taken up by LDL receptor, 3) incubation of VLDL with macrophages increases their affinity for LDL receptor, and 4) apo E secreted by macrophages is incorporated into VLDL and alters their affinity for LDL receptor. Although we do not present direct evidence as to whether lipolysis relates to the incorporation of apo E into VLDL or whether these processes are independent of each other, we will propose a possible mechanism. According to the study by Ishikawa et al., a change in apo B expression induced by lipolysis is required for the binding of apo E to VLDL (39). This appears to be consistent with the study of Schonfeld et al. showing that lipolysis produces changes in the immunoreactivity of apo B and cell reactivity to VLDL (40). We suggest that apo E binds to partially lipolysed VLDL more easily than non-lipolysed VLDL. In addition to the preference of apo E to lipolysed VLDL, dynamic changes occurring on the lipoprotein surface during lipolysis may facilitate the exchange of apolipoproteins including apo E.

The secretion of apo E by macrophages was found by Basu et al., and their role in lipoprotein metabolism has been interpreted as a theory of reverse cholesterol transport (11, 12, 14, 15). There are two distinct families of HDL, HDL with apo E and HDL without apo E, and they have completely different metabolic fates (41). In the reverse cholesterol transport system, apo E secreted by macrophages is incorporated into HDL (HDL with apo E) and plays a role of cholesterol transport from peripheral tissues to the liver by delivering HDL containing apo E to the liver receptors. This theory described one of the aspects of apo E functions: redistribution of lipids among cells of different organs. Recent findings

that an increase in secretion of apo E after peripheral nerve injury (42, 43) suggested another aspect of apo E functions: redistribution of lipids among cells within an organ or tissue and delivery of lipids to apo E secreting cells themselves.

The role of apo E on the metabolism of lipoproteins containing apo B100 is quite analogous to HDL. Recent studies demonstrated the presence of two distinct populations of VLDL, B particles and B, E particles (44). B,E particles are endocytosed by LDL receptors in the liver much more rapidly than B particles (45). By injecting apo E into WHHL rabbits, we demonstrated the incorporation of apo E into VLDL and the increased clearance of these lipoproteins from blood (46). We suggest here that the secreted apo E from macrophages capture VLDL and deliver them back to the cells. This is the first study to demonstrate the evidence of an autocrine-like function of apo E secreted from macrophages.

An epidemiological study showed less incidence of ischemic heart disease in subjects with apo E2 than those with apo E4 (47). This observation is explained by the significant association of the apo E isoform with the levels of plasma LDL-cholesterol. Subjects with apo E2 have lower plasma LDL-cholesterol levels than those with apo E3, and subjects with apo E4 have the highest. Our study presents another explanation for this fact. The monocyte/ macrophages in the arterial walls from subjects with apo E2 are more resistant to foam cell formation by VLDL and/or their remnants than those from either apo E3 or apo E4, because apo E2 secreted by the cells do not promote VLDL uptake.

Summary

We investigated the roles of lipoprotein lipase and apolipoprotein E (apo E) secreted from human monocyte-derived macrophages in the uptake of very low density lipoproteins (VLDL). Apo CII-deficient VLDL were isolated from a patient with apo CII deficiency. The lipolytic conversion to higher density and the degradation of the apo CII-deficient VLDL by macrophages were very slight, whereas the addition of apo CII enhanced both their conversion and degradation. This suggests that the lipolysis and subsequent conversion of VLDL to lipoproteins of higher density are essential for the VLDL uptake by macrophages. VLDL incubated with macrophages obtained from subjects with E3/3 phenotype (E3/3-macrophages) showed a 17-fold greater affinity in inhibiting the binding of 2 μg/ml of ^{125}I-LDL to fibroblasts than native VLDL, whereas the incubation of VLDL with macrophages obtained from a subject with E2/2 phenotype (E2/2-macrophages) did not cause any increase in their affinity. Furthermore, 3 μg/ml of ^{125}I-VLDL obtained from a subject with E3/3 phenotype were degraded by E3/3-macrophages to a greater extent than by E2/2-macrophages (2-fold), indicating that VLDL uptake is influenced by the phenotype of apo E secreted by macrophages. From these results, we conclude that both lipolysis by lipoprotein lipase and incorporation of apo E secreted from macrophages alter the affinity of VLDL for the LDL receptors on the cells, resulting in facilitation of their receptor-mediated endocytosis.

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References

1. Poole, J. C. F., and Florey, H.W. (1958) J. Pathol. Bacteriol. 75, 245-252.

2. Fowler, S., Shio, H., and Haley, N. J. (1979) Lab. Invest. 41, 372-378.

3. Gerrity, R.G.,m Naito, H.K., Rochardson, M., Schwartz, C.J. (1979) Am. J. Pathol. 95, 775-785.

4. Faggiotto, A., Ross, R., Harker, L. (1984) Arteriosclerosis 4, 323-340.

5. Bates, S. R., Murphy, P. L., Feng, Z., Kanazawa, T., and Getz, G. S. (1983) Arteriosclerosis 4, 103-114.

6. Khoo, J. C., Mahoney, E. M., and Witztum, J. L. (1981) J. Biol. Chem. 246, 7105-7108.

7. Chait, A., Iverius, P., Brunzell, J. D. (1982) J. Clin. Invest. 69, 490-493.

8. Mahoney, E. M., Khoo, J. C., and Steinberg, D. (1982) Proc. Natl. Acad. Sci. USA 79, 1639-1642.

9. Mori, N., Yamada, N., Ishibashi, S., Shimano, H., Fujisawa, M., Takaku, F., and Murase, T. (1987) Biochim. Biophys. Acta. 922, 103-110.

10. Ishibashi, S., Mori, N., Murase, T., Shimano, H., Gotohda, T., Kawakami, M., Akanuma, Y., Takaku, F., and Yamada, N. (1989) Arteriosclerosis 9:650-655.

11. Basu, S. K., Brown, M. S., Ho, Y. K., Havel, R. J., and Goldstein, J. L. (1981) Proc. Natl. Acad. Sci. USA 78, 7545-7549.

12. Basu, S. K., Ho, Y. K., Brown, M. S., Bilheimer, D. W., Anderson, R. G. W., and Goldstein, J. L. (1982) J. Biol. Chem. 257, 9788-9795.

13. Lindqvist, P., Ostlund-Lindqvist, A., Witztum, J. L., Steinberg, D., and Little, J. A. (1983) J. Biol. Chem. 258, 9086-9092.

14. Basu, S. K., Goldstein, J. L., and Brown, M. S. (1983) Science 219, 871-872.

15. Gordon, V., Innerarity, T, L., and Mahley, R. W. (1983) J. Biol. Chem. 258, 6202-6212.

16. Ohno, M., Ishibashi, S., Nakao, K., Nazue, T., Nonomura, K., Yamada, N., Aburatani, H., Shimano, H., and Murase, T. (1989) Eur. J. Pediatr. 148, 550-552.

17. Havel, R. J., Eder, H. A., and Bragdon, J. J. (1955) J. Clin. Invest. 34, 1345-1353.

18. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) Biochim. Biophys. Acta 260, 212-221.

19. Fielding, C. J. (1979) Biochim. Biophys. Acta 573, 255-265.

20. Laemmli, U. K. (1970) Nature 227, 680-685.

21. Yamada, N., Murase, T., Akanuma, Y., Itakura, H., and Kosaka, K. (1982) Horm. Metabol. Res. 14, 303-306.

22. Yamada, N., Shames, D. M., and Havel, R. J. (1987) J. Clin. Invest. 80, 507-515

23. Yamada, N., and Havel, R. J. (1986) J. Lipid. R. 27, 910-912.

24. Goldstein, J. L., and Brown, M. S. (1974) Proc. Natl. Acad. Sci. USA. 71, 4288-4292.

25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.

26. Steinmetz, A., Jakobs, C., Motzny, S., and Kaffarnik, H. (1989) Arteriosclerosis 9, 405-411.

27. Burnette, W.N. (1980) Anal. Biochem. 112, 195-203.

28. Windler, E., Chao, Y. S., and Havel, R. J. (1980) J. Biol. Chem. 255, 8303-8307.

29. Innerarity, T. L., Arnold, K. S., Weisgraber, K. H., and Mahley, R. W. (1986) Arteriosclerosis 6, 114-122.

30. Imaizumi, K., Fainaru, M., and Havel, R. J., (1978) J. Lipid Res. 19, 712-722.

31. Eisenberg, S., Friedman, G., and Vogei, T. (1988) Arteriosclerosis 8, 480-487.

32. Kraemer, F. B., Chen, Y. I., Lopez, R. D., and Reaven, G. M. (1983) J. Biol. Chem. 258, 12190-12197.

33. Koo, K., Warnette-Hammond, M. E., and Innerarity, T. L. (1985) J. Biol. Chem.

261, 11194-11201.

34. Ellsworth, J. L., Kraemer, F. B., and Cooper, A. D. (1986) J. Biol. Chem. 262, 2316-2325.

35. Koo, K., Warnette-Hammond, M. E., Garcia, Z., Malloy, M. J., Utauy, R., East, C., Bilheimer, D. W., Mahley, R. W., and Innerarity, T. L. (1988) J. Clin. Invest. 81, 1332-1340.

36. Soltys, P. A., Gump, H., Hennessy, L., Mazzone, T., Carey, K. D., McGill, Jr., H. C., Getz, G. S., and Bates, S. R. (1988) J. Lipid Res. 29, 191-201.

37. Rall. S. C., Weisgraber, K. H., and Mahley, R. W. (1982) J. Biol. Chem. 257, 4171-4178.

38. Weisgraber, K. H., Innerarity, T. L., and Mahley, R. W. (1982) J. Biol. Chem. 257, 2518-2521.

39. Ishikawa, Y., Fielding, C. J., and Fielding, P. E. (1988) J. Biol. Chem. 263, 2744-2749.

40. Schonfeld, G., Patsch, W., Pfleger, B., Witztum, J. L., and Weidman, S. W. (1979) J. Clin. Invest. 64, 1288-1297.

41. Weisgraber, K. H., and Mahley, R. W. (1980) J. Lipid Res. 21, 316-325.

42. Ignatius, M. J., Gebricke-Harter, P. J., Skene, J. H. P., Schilling, J. W., Weisgraber, K. H., Mahley, R. W., and Shooter, E. M. (1986) Proc. Natl. Acad. Sci. USA 83, 1130-1134.

43. Ishibashi, S., Yamada, N., Oka, Y., Shimano, H., Mori, N., Yoon, T. H., Shimada, M., Kanazawa, Y., Akanuma, Y., Murase, T., and Takaku, F. (1988) Biochem. Biophys. Res. Commun. 155, 283-288.

44. Fielding, P. E., and Fielding, C. J. (1986) J. Biol. Chem. 261, 5233-5236.

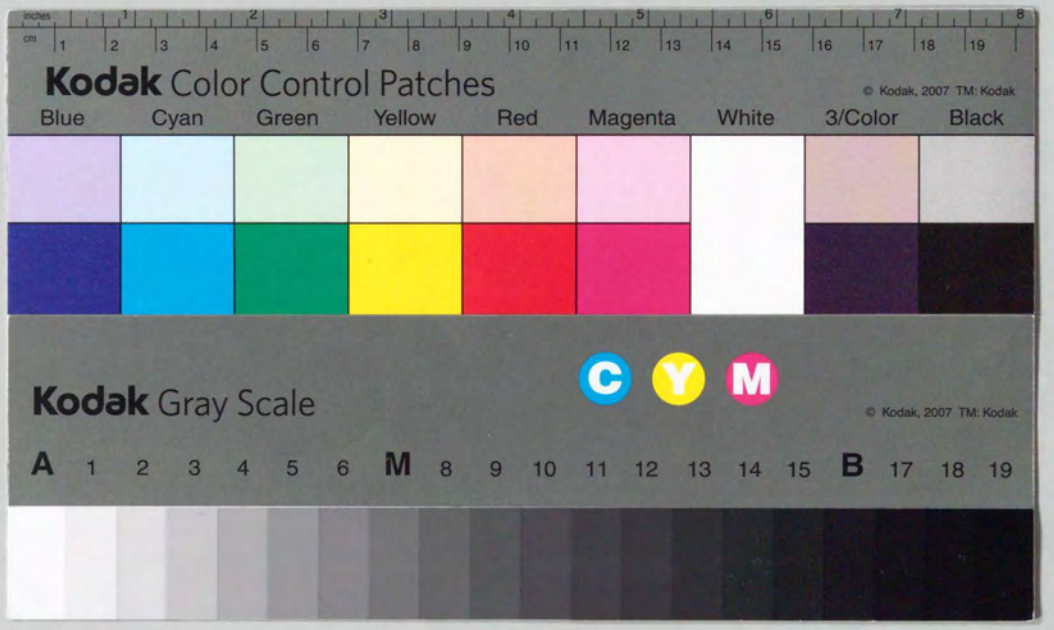
45. Yamada, N., Shames, D. M., and Havel, R. J. (1986) Proc. Natl. Acad. Sci. USA 83, 3479-3483.

46. Yamada, N., Shimano, H., Mokuno, H., Ishibashi, S., Gotohda, T., Kawakami, M., Watanabe, Y., Akanuma, Y., Murase, T., and Takaku, F (1989) Proc. Natl. Acad. Sci. USA 86, 665-669.

47. Davignon, J., Gregg, R. E., and Sing, C. F. (1988) Arteriosclerosis 8, 1-21.

Foot notes

[1]The abbreviations used here are apo, apolipoproteins; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; VLDLc, VLDL obtained from a subject with apo CII deficiency; ß-VLDL, ß-migrating very low density lipoproteins; LPDS, lipoprotein-deficient serum; BSA, bovine serum albumin; FCS, fetal calf serum; PRMI-1640, Roswell Park Memorial Institute-1640; MEM, modified Eagle's medium; PBS, phosphate buffered saline; PMSF, phenylmethylsulphonyl fluoride; WHHL, Watanabe heritable hyperlipidemic.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