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APO E-MEDIATED UPTAKE AND DEGRADATION OF NORMAL VERY LOW DENSITY LIPOPROTEINS BY HUMAN MONOCYTE/MACROPHAGES: A SATURABLE PATHWAY DISTINCT FROM THE LDL RECEPTOR*

Patsy Wang-Iverson[1,2], Henry N. Ginsberg[1], Linda A. Peteanu[1], Ngoc-Anh Le[3], and W. Virgil Brown[1]

Departments of Medicine[1], Biochemistry[2], and Biomathematics[3]
Mount Sinai School of Medicine, New York, N.Y. 10029

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SUMMARY: Normal human fasting very low density lipoproteins (n-VLDL; d<1.006 g/ml) were demonstrated to be taken up and degraded by human monocyte-macrophages via a saturable process distinct from the previously described LDL and scavenger receptors. Through the use of apolipoprotein-phospholipid complexes, apolipoprotein E (apoE) was identified as the ligand mediating recognition of n-VLDL by this receptor.

Although macrophages have long been recognized as precursors to foam cells (1,2), only recently have research efforts focused upon the elucidation of the mechanisms of lipid accumulation. The pioneering studies of Brown and Goldstein et al. (3) describing the presence of high affinity receptors for low density lipoproteins (LDL; d: 1.020-1.063 g/ml) on human skin fibroblasts led to the subsequent examination of macrophages for the presence of LDL receptors which might mediate intracellular cholesteryl ester (CE) accumulation. Murine macrophages were found not to possess the classic LDL receptors, and this lipoprotein (LP) did not induce significant CE accumulation in these cells (4). Other laboratories later reported the presence of saturable LDL receptors on human monocyte-derived macrophages (5-7), but efficient regulation of this pathway prevented

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Address reprint requests to Dr. Patsy Wang-Iverson, Annenberg 24-50, Mount Sinai School of Medicine, New York, New York 10029.

Abbreviations: apo, apolipoprotein; LP, lipoprotein; n-VLDL, normal very low density lipoproteins (d<1.006 g/ml); β-VLDL, beta-VLDL (d<1.006 g/ml); LDL, low density lipoproteins (d: 1.020-1.063 g/ml); MDA-LDL, malondialdehyde-LDL; HDL, high density lipoproteins (d: 1.070-1.21 g/ml); apoE-DMPC, apoE-dimyristoylphosphatidylcholine; PC, phosphatidylcholine; CE, cholesteryl ester; TG, triglyceride.

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CE accumulation. Both murine and human macrophages, however, possessed scavenger receptors capable of recognizing modified LDL (4,5,8,9), and excessive intracellular CE accumulation can occur via this pathway. To date, neither the physiological LP substrate for this pathway, nor the ligand triggering receptor recognition has been described. A lack of specificity of the scavenger receptor for a particular apolipoprotein is suggested by its recognition also of maleylated albumin (10).

Recently, several laboratories reported that macrophages of both murine and human origin possess specific receptors for beta-very low density lipoproteins (β-VLDL), cholesterol rich VLDL with beta mobility, which can cause excess intracellular CE accumulation (11-14). In the mouse, these receptors are distinct from the previously described scavenger receptors (11,12). In the human, the β-VLDL receptors appear to be different from both the LDL and scavenger receptors, although β-VLDL also can be taken up via the LDL receptor (13). The apolipoprotein specificity of the β-VLDL receptors remains controversial and a subject of current investigation by numerous laboratories. Recognition of β-VLDL by macrophage receptors has been attributed to the presence of apo B-48 (13,14) as well as the absence of the C apolipoproteins (11). Other laboratories have proposed that apoE, which is increased in hypertriglyceridemic VLDL and β-VLDL, does not play a role in particle recognition by the receptor (13,15).

Despite the controversy surrounding the identity of the ligand, it is clear that β-VLDL is recognized by a specific macrophage receptor. In contrast, there is conflicting evidence concerning even the existence of macrophage receptors for normal VLDL (n-VLDL). An absence of murine macrophage receptors for n-VLDL was inferred from earlier studies reporting the inability of this LP class to stimulate intracellular accumulation of triglyceride (TG) and CE or incorporation of ^{14}C-oleate into intracellular CE (11,16). However, direct interaction between n-VLDL and macrophages was not examined in these studies. Normal VLDL may not deliver a significant amount of lipid to a cell, because they carry relatively less lipid than β-VLDL or HTG-VLDL, but they still may interact with the cells via specific receptors. Recently, by monitoring the uptake and degradation of ^{125}I-n-VLDL, several laboratories (17-19) reported the presence of specific receptors for normal human VLDL on the surface of murine macrophages; in two of the studies, cross-competition was observed with β-VLDL (17,18).

Previous studies focusing upon the interaction of LPs with macrophages have demonstrated that not all data obtained with murine macrophages can be extrapolated to human macrophages; the former do not possess LDL receptors (4), whereas the latter do (5-7). Furthermore, Innerarity et al. (20) have observed that LPs from one species may not bind to cells from a different species. In the present study, we sought 1) to determine whether normal fasting human VLDL were capable of interacting with human monocyte-macrophages via a saturable process, and if so, 2) to identify the ligand recognized by the receptor. We now report that normal human VLDL obtained from fasting individuals can be taken up and degraded by human monocyte-derived macrophages via two distinct saturable processes: the LDL receptor plus a separate pathway that recognizes apolipoprotein E.

MATERIALS AND METHODS

Subjects

Venous blood was obtained after a 14 hr overnight fast from normolipidemic volunteers in accordance with the guidelines of the Mount Sinai Medical Center Research Advisory Committee. All individuals had normal hematocrits, white blood cell and differential counts, and plasma lipid profiles. The plasma triglyceride and cholesterol levels were < 100 mg% and < 200 mg%, respectively.

Isolation and Culture of Monocytes

White cells isolated from venous blood anticoagulated with EDTA (1 mg/ml) were fractionated by Ficoll-hypaque (d:1.077 g/ml) density gradient centrifugation (21). The mononuclear cells, consisting of lymphocytes and monocytes, were removed from the interface, washed, and suspended in RPMI 1640/20% AB serum, 100 u/ml penicillin, 100 ug/ml streptomycin, 1.6 mM glutamine, 15 mM Hepes, pH 7.4 (Medium I). The cells were diluted to a concentration of 1×10^6/ml monocytes as estimated with a Coulter channelyzer, and 0.5 ml aliquots were plated in 24 mm wells (Linbro; Flow Laboratories, McLean, VA). After adherence for 60-120 min at 37° in 5% CO_2, the non-adherent lymphocytes were removed by 3 washes with RPMI 1640, followed by the addition of fresh Medium I. The medium was changed on the next day and on alternate days thereafter. Monocyte-derived macrophages (>98% pure) were used for experiments between 7 and 14 days after plating, unless otherwise indicated.

Lipoproteins and Apolipoprotein-phospholipid complexes

Lipoproteins were isolated from plasma (1 mg EDTA/ml) obtained from normal fasting volunteers. VLDL ($d < 1.006$ g/ml), LDL (d:1.020-1.063 g/ml), and HDL (d:1.070-1.21 g/ml) were isolated by sequential centrifugation as described previously (22). Each isolated LP fraction was washed free of albumin and concentrated by re-centrifugation. VLDL were obtained by centrifugation in swinging bucket rotors. Under these conditions, the isolated n-VLDL contained no apo B-48. The isolated LPs were radioiodinated (23,24) and dialyzed against 0.15M NaCl/.01% EDTA to remove free $Na^{125}I$. Lipid labelling (25) in the radioiodinated preparations was less than 10% for VLDL and less than 5% for LDL and HDL. Low density lipoproteins were modified with malondialdehyde (26). The extent of modification, which always exceeded 50 mmol MDA/mol LDL, was quantified with the thiobarbituric assay (27).

ApoE-phospholipid complexes (apoE-DMPC) were prepared as described previously (28). ApoA-I-DMPC complexes were prepared by previously published methods (29). Unilamellar phosphatidylcholine (PC; egg lecithin, Sigma Chemical Co., St. Louis, MO)

vesicles of 100-200nm in diameter were prepared by detergent dialysis followed by gel filtration on Sepharose CL-2B (30). Prior to the degradation experiments, the LPs were diluted in RPMI 1640/0.2% BSA (Medium II) and filtered through Millex GV filters (0.22 um; Millipore Corp., Bedford, MA).

Assays

Uptake and degradation of ^{125}I-LPs were quantified as the amount of ^{125}I-trichloroacetic acid (TCA)-soluble (noniodide) material recovered in the culture medium (31). After removal of the medium, the cell monolayers were incubated with 1 mg/ml heparin to remove surface adsorbed radioactivity and then rinsed 4 times with ice cold 0.15M NaCl to remove extracellular protein. The cells then were scraped into 0.15M NaCl and lysed by freezing and thawing. Aliquots of the cell lysate were removed for the determination of cell-associated radioactivity and cellular protein mass. The nature of the cell-associated radioactivity was determined by lipid extraction (25) of an aliquot of the cell sonicate. The protein content of the cells and lipoproteins was estimated by the method of Lowry et al. (32). Apolipoprotein E mass was quantified by specific radioimmunoassay, as described elsewhere (33).

RESULTS

Normal human whole VLDL isolated from fasting plasma were taken up and degraded by normal human monocyte-derived macrophages via a saturable process, as demonstrated by the inhibition of ^{125}I-n-VLDL (10 ug/ml) degradation by increasing concentrations of non-radiolabeled n-VLDL (Fig. 1). Ninety percent suppression of ^{125}I-n-VLDL degradation was obtained with 190 ug/ml VLDL protein. In contrast, LDL were able to suppress ^{125}I-n-VLDL degradation by only 66%, even at a concentration of 300 ug/ml. This observation suggested that n-VLDL might interact with human macrophages via other pathways in addition to the LDL receptor. High density lipoproteins (HDL) also were able partially to suppress ^{125}I-n-VLDL degradation, although 300 ug/ml HDL protein only

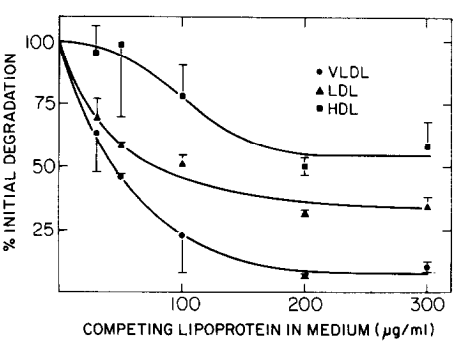

Figure 1. Specificity of n-VLDL interaction with human monocyte/macrophages. ^{125}I-n-VLDL (358 cpm/ng) were added in Medium II at 10 ug/ml to monolayers of human macrophages cultured for 8 days in 24 mm wells. Increasing concentrations of non-radiolabeled LPs were added to a final volume of 0.5 ml and incubated for 4 hr at 37° in 5% CO_2. The initial rate of ^{125}I-n-VLDL (10 ug/ml) degradation in the absence of non-radiolabeled LPs was 354 ± 94 ng/mg cell protein/4 hr. The n-VLDL preparation contained 15% apoE, and the LDL preparation (d:1.020-1.063 g/ml) contained 0.3% apoE..

reduced VLDL degradation by 40%. ^{125}I-HDL from the same source, however, were not taken up and degraded by human macrophages via a saturable process (data not shown). MDA-LDL, which are taken up and degraded via scavenger receptors (5) did not inhibit ^{125}I-n-VLDL degradation by human macrophages (data not shown), thus ruling out the possibility of uptake of these particles via the scavenger receptor.

To determine whether uptake and degradation of n-VLDL might be mediated by receptors distinct from previously described LDL receptors present on human macrophages (5-7), degradation of ^{125}I-n-VLDL was examined utilizing macrophages obtained from an individual homozygous for familial hypercholesterolemia previously characterized as LDL receptor negative (personal communication: Joseph Goldstein, Dallas, Texas). These cells demonstrated normal uptake and degradation of ^{125}I-n-VLDL; 30-fold excess n-VLDL caused an 80% inhibition of ^{125}I-n-VLDL degradation (Table 1). In contrast, they exhibited no measurable degradation of ^{125}I-LDL (data not shown). In this experiment, the cell-associated ^{125}I-n-VLDL contributed 80% and 72% of the sum of the degradation product and cell-associated LP for the FH and control macrophages, respectively.

To test a possible role for apoE in mediating recognition of the particles by human monocyte-macrophages, degradation of ^{125}I-n-VLDL was examined in the presence of increasing concentrations of apoE-DMPC. A concentration dependent inhibition of ^{125}I-n-VLDL degradation was observed, with 80% inhibition achieved at 30-fold excess non-

Table 1

Uptake and degradation of ^{125}I-n-VLDL by monocyte/macrophages from a normal individual and an individual homozygous for familial hypercholesterolemia

Additions	Subject 1	Subject 2
	^{125}I-n-VLDL degradation	
^{125}I-n-VLDL (2.5 ug/ml)	152 ± 48	137 ± 21
+n-VLDL (75 ug/ml)	30 ± 29	23 ± 3
	(20%)	(17%)
	Cell-associated ^{125}I-n-VLDL	
^{125}I-n-VLDL (2.5 ug/ml)	624 ± 153	359 ± 23
+n-VLDL (75 ug/ml)	313 ± 25	104 ± 13

Monocyte/macrophages were isolated from an individual homozygous (Subject 1) for familial hypercholesterolemia (FH) and from a normal individual (Subject 2). After 8 days in culture, the macrophages were assayed for their ability to take up and degrade ^{125}I-n-VLDL (354 cpm/ng). The degradation rate and the cell-associated ^{125}I-n-VLDL are expressed as ng/mg cell protein/4 hr. ± S.D.

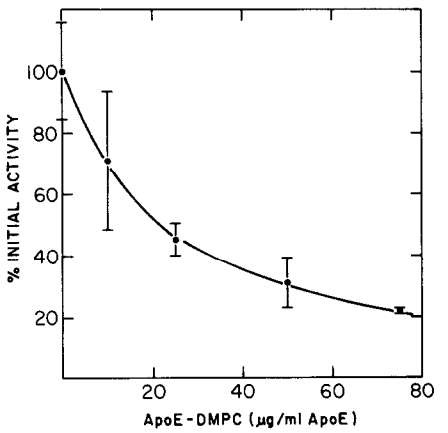

Figure 2. Inhibition of ^{125}I-n-VLDL degradation by apoE-DMPC complexes. Degradation of ^{125}I-n-VLDL (2.5 ug/ml; 389 cpm/ng) by human macrophages (22 days) was quantified in the presence of increasing concentrations of apoE-DMPC. The initial rate of degradation of ^{125}I-n-VLDL was 137 $\pm$ 21 ng/mg cell protein/4 h. The n-VLDL preparation contained 2% apoE.

radiolabelled apoE-DMPC (Fig. 2). A higher concentration of apoE-DMPC complex was required to achieve 80% suppression of ^{125}I-n-VLDL degradation than would be anticipated based upon apoE content. However, it must be noted that normal fasting VLDL consist of particles with an average diameter of 80 nM, whereas the apoE-DMPC complexes are less than 8 nM in diameter (34). Thus, the higher concentration of apoE-DMPC complex required to effect significant inhibition of ^{125}I-n-VLDL degradation may reflect the significant difference in volume of the two particles (1000-fold).

The specificity for apoE was further documented by a lack of comparable inhibition of ^{125}I-n-VLDL degradation by apoA-I-DMPC (Fig. 3A). PC vesicles devoid of any protein exhibited an inhibitory effect similar to that observed with apoA-I-DMPC, suggesting that the latter, at higher concentrations, may exert a non-receptor mediated inhibiton of ^{125}I-n-VLDL degradation via lipid-lipid interaction with the cell surface (35). In these series of experiments the cell-associated ^{125}I-n-VLDL contributed 63% of the sum of the degradation products and the cell-associated LPs. The relative amount of cell-associated radioactivity decreased in the presence of non-labelled particles (Fig. 3B), in parallel with the degradation products quantified in the medium (Fig. 3A).

DISCUSSION

Although earlier studies of apoB flux in normolipidemic subjects suggested a quantitative conversion of apoB from n-VLDL to LDL (36), recent studies carried out in

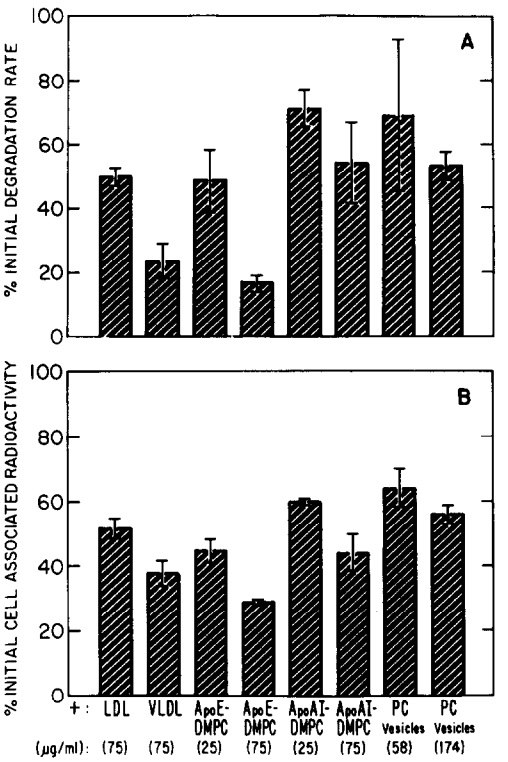

Figure 3. Specificity of apoE-DMPC inhibition of ^{125}I-n-VLDL degradation by human monocyte/macrophages. After incubation for 4 hr at 37°, degradation of ^{125}I-n-VLDL (2.5 ug/ml; 354 cpm/ng) in the medium (A) and cell-associated ^{125}I-n-VLDL (B) were quantified in the presence of non-radiolabeled LDL, n-VLDL, apo E-DMPC, apo AI-DMPC and PC vesicles, respectively, at the concentrations indicated in the figure. The concentrations of apoE-DMPC and apoA-I-DMPC are expressed in terms of the apolipoprotein mass. The PC vesicles were added at concentrations comparable to those for DMPC; the ratio of DMPC to apoE and apoA-I was 2.3:1. The initial rate of ^{125}I-n-VLDL degradation in the absence of non-radiolabeled particles was 116 ± 21 ng/mg cell protein/4 hr. The initial amount of cell-associated ^{125}I-n-VLDL quantified in the absence of non-radiolabelled particles was 198 ± 20 ng/mg cell protein/ 4 hr, which account for 63% of the sum of the degradation products and the cell-associated ^{125}I-n-VLDL. The n-VLDL preparation contained 2% apoE.

our laboratory have demonstrated that a significant portion of the apoB in n-VLDL may be removed directly from the circulation without conversion to LDL (37). The major site for the direct removal of these VLDL remnant particles may be the liver, which has different receptors for LPs: the classic LDL receptor plus a separate receptor that recognizes apoE (38,39). This latter pathway appears to provide the major mechanism for the direct removal of remnant particles in the normal physiology of plasma lipoprotein metabolism (38,39).

Our current studies demonstrating the presence of human macrophage receptors that recognize apoE suggest that VLDL remnant particles containing this apolipoprotein

also can be taken up by extrahepatic tissue via a specific apoE receptor distinct from the LDL receptor. These extrahepatic receptors may play a minimal role in normolipidemic individuals, where the liver may be the predominant site for the removal of remnant particles. However, in various disorders of LP metabolism, plasma apoE levels as well as remnants may become elevated (40,41;unpublished observation), thus possibly exceeding the capacity of the liver receptor. Under these circumstances, extrahepatic apoE receptors may assume a more important role in the uptake of apoE containing particles. The clearance by macrophages of mildly elevated levels of LP remnant particles via recognition of apoE may serve to maintain homeostasis. But in situations where circulating apoE-containing remnant levels become markedly elevated, the macrophage clearance mechanism may become impaired, thus resulting in the formation of foam cells frequently associated with atherosclerosis.

A potentially important function for the macrophage apoE receptor in the pathophysiology of Type III hyperlipoproteinemia is further emphasized by earlier studies from other laboratories. It has been documented that mutations in apoE result in decreased binding to the liver receptor (42). In contrast, the murine macrophage VLDL receptor, which recognizes both n-VLDL and β-VLDL, does not appear to distinguish among VLDL with different isoforms of apoE (14,17,19). If human macrophages exhibit similar properties, then these observations, in conjunction with our identification of apoE as a ligand recognized by a human macrophage receptor distinct from the LDL receptor, would suggest that the liver and macrophage apoE receptors may recognized different epitopes on apoE. This difference in the properties of the two receptors may then contribute to the amplication of the pathophysiology of Type III hyperlipoproteinemia.

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