Modification of Very Low Density Lipoproteins Leads to Macrophage Scavenger Receptor Uptake and Cholesteryl Ester Deposition

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Chemically modified low density lipoproteins (LDL) are recognized by the macrophage scavenger receptor and can lead to substantial cholesteryl ester accumulation in cultured macrophages. Uptake of modified lipoproteins in vivo could contribute to foam cell formation during generation of the atherosclerotic plaque lesion. In the present study, modification of human pre-beta migrating very low density lipoprotein (VLDL) by acetylation led to recognition by the macrophage scavenger receptor as demonstrated in cross-competition experiments with acetylated LDL (ALDL). Recognition by this alternative binding site was associated with increased cholesterol delivery to human macrophages as assessed by suppression of LDL receptor activity, stimulation of cholesteryl esterification rates, and accumulation of intracellular cholesteryl ester. Subfractionation of acetylated very low density lipoprotein (AVLDL) by ultracentrifugation in a discontinuous NaCl gradient demonstrated that AVLDL subfractions were equally effective in competing for 125I-ALDL uptake by macrophages when compared on the basis of particle number. These results suggest that modification of VLDL with subsequent recognition by the macrophage scavenger receptor may be a mechanism by which VLDL particles participate in macrophage cholesteryl ester overload. (Arteriosclerosis 7:191–196, March/April 1987)

Monocyte-derived macrophages play a key role in the atherogenic process.1 Along with arterial smooth muscle cells, they give rise to lipid-laden arterial plaque cells, termed foam cells.2–4 The intracellular neutral lipid within foam cells is cholesteryl ester, largely synthesized from lipoprotein-derived cholesterol. A great deal of attention has been focused on the mechanism by which lipoprotein cholesterol enters cells in such excess, especially in view of the stringent regulation of low density lipoprotein (LDL) receptor activity by cell cholesterol content.5 The existence of a distinct receptor for certain species of modified LDL, termed the scavenger receptor, has been described in human macrophages,6,7 macrophage cell lines,8 and mouse peritoneal macrophages.9 The activity of this receptor is regulated by a number of factors including cell density,10 lymphokines,11 and glucocorticoid hormones.12 This receptor is not, however, regulated by cell cholesterol content.7,9 Recognition of LDL by this receptor has been shown to require chemical modification of positively charged lysine residues of apolipoprotein B (apo B),5,7 and such modifications have included acetylation,7–9 acetoacetylation,14 or malondialdehyde derivatization.6

The observation that cell cholesterol does not modulate modified LDL uptake led to the suggestion that cholesteryl ester overload of macrophages in vivo could be accounted for by modification of LDL within the arterial wall and subsequent uptake by the scavenger receptor.13 In line with this, it has already been shown15,16 that cultured endothelial cells and arterial smooth muscle cells can modify LDL in vitro to allow its recognition by the macrophage scavenger receptor. In addition, a protein has been isolated from vascular wall plaque lesions which is recognized by this binding site.17

The properties of the scavenger receptor, large numbers of receptors per cell,18 high internalization index,18 and lack of control by cell cholesterol content,7,9 suggest that any cholesterol-containing ligand could mediate macrophage cholesteryl ester overload if it can be recognized by this binding site. One ligand of particular interest is the other major apo B-containing lipoprotein, very low density lipoprotein.

While beta migrating very low density lipoprotein (VLDL) from cholesterol-fed animals can deliver substantial amounts of cholesterol to macrophages,19 normolipemic or hypertriglyceridemic pre-beta migrating human VLDL has been much less potent in this regard.20–22 Normolipemic and hypertriglyceridemic VLDL, however, both lead to substantial macrophage triglyceride accumulation,20–23 which may occur by multiple mechanisms including receptor-mediated uptake of whole or partly catabolized VLDL particles, or by macrophage-derived lipoprotein lipase hydrolysis of VLDL triglyceride with subsequent macrophage uptake of free fatty acids.21,23 The ligand responsible for macrophage receptor recognition of normolipemic VLDL requires further investigation; however, a role for apolipo-
protein E (apo E) has been suggested. Data obtained in human skin fibroblasts, which possess only the LDL receptor, demonstrate that receptor binding for a subfraction of normolipemic VLDL (SI 20 to 60) can be mediated by apo-protein B. In the current study, we wished to examine a potential mechanism by which pre-beta migrating normolipemic human VLDL could participate in macrophage cholesteryl ester overload. Specifically, we determined whether chemical modification of normolipemic human VLDL would lead to recognition by the macrophage scavenger receptor, and evaluated the impact this might have on macrophage cholesterol homeostasis.

**Methods**

**Cell Culture**

Human monocytes were obtained by counterflow centrifugation as previously described. Monocytes were allowed to differentiate into macrophages in 20% autologous serum in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37° C in a 5% CO2 atmosphere.

**Lipoproteins**

Human LDL (d = 1.019 to 1.063) and VLDL (d < 1.006) were prepared by sequential ultracentrifugation in a fixed angle rotor of fresh plasma obtained each month from one of five normolipemic healthy donors. VLDL was further subfractioned using a discontinuous NaCl gradient in a SW41 rotor. To inhibit lipoprotein degradation during isolation, blood was drawn into EDTA (final concentration = 1 mM), and fresh plasma was brought to 10 μM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Missouri) and 10 μg/ml of aprotinin (Boehringer-Mannheim, Indianapolis, Indiana). Lipoprotein-deficient serum was prepared from pooled human serum by ultracentrifugation at d = 1.21. Lipoproteins were acetylated and iodinated as previously described. For VLDL subfractionation experiments, VLDL was acetylated prior to subfractionation. For the iodinated lipoprotein tracers, the specific activity ranged from 96 to 372 cpm/ng. Greater than 97% of total counts were precipitated by 10% (wt/vol) trichloroacetic acid, and less than 4% were extracted into chloroform/methanol.

**Electrophoresis**

Agarose gel electrophoresis of lipoproteins was performed using the Paragon Lipoprotein Electrophoresis system (Beckman Instruments, Fullerton, California). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Jones et al. Apoproteins (10 μg/lane) were separated on a 5% to 22.5% gradient gel using 3 mA for 16 hours. Gels were stained using Coomassie brilliant Blue.

**Assays**

Lipoprotein degradation was used as an index of receptor-mediated uptake and catabolism as previously described. Incubations with 125I-lipoproteins were performed under the indicated conditions at 37° C. After the times shown, an aliquot of medium was removed for the determination of cholesteryl oleate-soluble 125I that was not due to free iodine. Non-cell-associated degradation was subtracted from total degradation values in all experiments. All lipoproteins were added on the basis of protein concentration, unless otherwise indicated.

Gas-liquid chromatography was used to measure lipoprotein and cellular free and total cholesterol. Cholesteryl ester was calculated (total minus free). Cholesteryl ester synthesis was measured using 14C-oleic acid-BSA complex which was made according to the method of St. Clair et al. Cholesteryl oleate incorporation into cholesteryl ester was quantified by fractionation of cell lipid extracts on silica gel G plastic thin-layer chromatography plates (J.T. Baker) developed with petroleum ether/ethyl ether/acetone (75:25:1).

**Results**

Acetylation of VLDL led to an increased net negative charge as shown by its increased mobility on agarose gel electrophoresis (Figure 1). As has been reported for LDL, this modification also led to recognition by the macrophage scavenger receptor as indicated by the representative experiments shown in Figure 2. With 125I acetylated low density lipoprotein (ALDL) as the labeled ligand, a 15-fold excess of ALDL decreased 125I-AVLDL degradation by 50% (Figure 2A). In the reverse experiment, the addition of a 15-fold excess of ALDL reduced 125I-AVLDL degradation by 93%, indicating that the uptake of the latter particle was almost totally mediated by the scavenger receptor (Figure 2B). A 15-fold excess of ALDL decreased 125I-AVLDL degradation by 65%; substantially less suppression than that produced by unmodified ALDL. In a separate experiment, VLDL did not significantly compete for 125I-AVLDL uptake (not shown).

Recognition of AVLDL by the scavenger receptor led to a substantial increase in intracellular cholesterol delivery as reflected in three different types of assays (Figure 3). Pre-incubation of human macrophages with AVLDL vs parent VLDL was accompanied by more substantial down-regulation of the macrophage LDL receptor (Figure 3A; AVLDL vs VLDL, p < 0.001; AVLDL vs LDL, p < 0.001). This observation is similar to that reported for preincubations in acetylated LDL versus LDL. Increased stimulation of cholesteryl ester synthesis (Figure 3B) and augmented accumulation of cholesteryl ester mass (Figure 3C) were also observed. All of these measurements reflect increased intracellular delivery of lipoprotein cholesterol associated with recognition by the macrophage scavenger receptor. AVLDL stimulated increased cholesteryl ester synthesis compared to parent VLDL over short and long incubation periods (Figure 3B). In addition, AVLDL produced more cholesteryl ester accumulation after 72 hours than either unmodified VLDL or LDL (Figure 3C). Accumulation of triglyceride was not different after 24-hour pre-
Figure 1. Agarose gel electrophoresis of VLDL and acetylated VLDL and subfractions. Acetylated VLDL was subfractionated as described in Methods.

Figure 2. Cross-competition experiments for $^{125}$I-ALDL (A) or $^{125}$I-AVLDL (B) degradation by human macrophages. Each well was washed with serum-free medium and placed in 0.2% BSA in DMEM with 5 μg/ml of labeled ligand plus or minus the concentration shown of unlabeled lipoprotein (A) or 7.5 μg/ml of ligand plus or minus a 15-fold excess of the indicated unlabeled lipoprotein (B). Values are the means of triplicate determinations.

Figure 3. Ability of native and acetylated VLDL to deliver cholesterol to human macrophages. A. Macrophages were incubated for 24 hours in 5% lipoprotein-deficient serum in DMEM plus or minus 25 μg/ml of the indicated lipoprotein. The cells were then washed, and 0.2% BSA in DMEM containing 7.5 μg/ml of $^{125}$I-LDL was added for a 4-hour degradation assay. Mean ± so for triplicate determinations. B. Macrophages were washed and placed in 0.2% BSA in DMEM plus or minus 10 μg/ml of VLDL or AVLDL at 0 hours. Two hours before the times indicated on the horizontal axis, the cells were washed and $^{14}$C-oleic acid was added for a 2-hour pulse. The background esterification that occurred in the presence of DMEM at each time point was subtracted. The values shown are the average of duplicate determinations. C. Macrophages were incubated in 10% autologous serum in DMEM plus or minus 50 μg/ml of the indicated lipoprotein. After 72 hours, the cells were extracted for analysis of cholesteryl ester content. Mean ± so for triplicate determinations.
incubations in VLDL or AVLDL (not shown), perhaps reflecting the multiple mechanisms (including a dominant role for nonreceptor-mediated mechanisms) by which this accumulation can occur or the efflux of cellular triglyceride which might have readily occurred with the culture conditions employed.21-23

Human VLDL represents a heterogeneous population of particles which can be separated using ultracentrifugation in a discontinuous NaCl gradient.26, 27 The data shown in Figure 2B, that ALDL is a more effective competitor for AVLDL degradation than AVLDL itself, could potentially be explained by a greater scavenger-receptor affinity for ALDL. Alternatively, this observation could result if only a subpopulation of AVLDL particles competed for scavenger receptor binding as has been previously shown for normolipemic, native VLDL uptake by the fibroblast LDL receptor.25, 26 To address this question, we subfractionated AVLDL by the method of Lossow et al.27 and compared the ability of each subtraction to compete for 125I-ALDL degradation, and to stimulate macrophage cholesteryl ester synthesis. The triglyceride and free and esterified cholesterol composition for a representative AVLDL subtraction experiment are shown as lipid to protein ratios in Table 1. These data closely agree with data previously published by Gianrurco et al.32 for normolipemic human VLDL when the latter data are expressed as lipid to protein ratios, indicating that acetylation did not alter the lipid composition of VLDL. For the AVLDL subfractionation experiment shown, 51% of the recovered protein was in the AVLDL3 subtraction, while 13% and 36% were found in AVLDL1 and AVLDL2, respectively.

The apoprotein composition of AVLDL subfractions, as determined by polyacrylamide gel electrophoresis, is shown in Figure 4. The predominant apoprotein of each subtraction was apo B-100. AVLDL1 (Sf 100 to 400) had the most apo E (as a percentage of total protein), while AVLDL3 (Sf 20 to 80) had the least. This also is in agreement with previously reported radioimmunoassay data for normolipemic human VLDL subfractions.26 When these subfractions were incubated with macrophages at equal particle numbers, they were equally effective in competing for 125I-ALDL uptake by the scavenger receptor (Figure 5). In separate experiments (not shown) AVLDL subfractions, when incubated with macrophages at equal particle numbers, stimulated similar rates of macrophage cholesteryl ester synthesis.

Discussion

Many studies have previously documented that incubation of macrophages with chemically modified LDL can lead to substantial cholesteryl ester accumulation.7-9, 10 These observations focused attention on the scavenger receptor and the study of possible mechanisms for the generation of in vivo ligands for this receptor. Soon afterward it was reported that a protein isolated from atherosclerotic vessel wall lesions could interact with the scavenger receptor and that this protein displayed apo B immunoreactivity.17 Potential mechanisms for in situ modification of LDL in the artery wall were provided by the observation that cultured endothelial cells15, 16 or arterial smooth muscle cells15 could modify LDL to a form recognized by the scavenger receptor. Incubation of LDL with aggregating platelets leads to malondialdehyde derivatization and allows for uptake by this binding site.30 For the latter process, the nature and stoichiometry of the modification necessary for scavenger receptor recognition have been reported.13

In the current study, we have shown that chemical modification or pre-beta migrating human VLDL leads to recognition by the macrophage scavenger receptor and this produces substantial cholesteryl ester deposition. The ability of acetylated VLDL to stimulate macrophage cholesteryl esterification and accumulation while the parent VLDL is much less effective in this regard, might be partly explained by the high internalization index of the scavenger receptor.15 The observation, however, that parent VLDL particles stimulate only small amounts of cholesteryl ester accumulation even after 72 hours compared to AVLDL (Figure 3C) suggests that native VLDL uptake is regulated by cell cholesterol content in human macrophages. Similar to what has been reported for LDL, acetylation of VLDL leads to an alternative mechanism for cellular uptake that is unregulated by cell cholesterol content. The derivatized particle, with exactly the same lipid composition as the parent native particle, acquires the potential to produce substantial macrophage cholesteryl ester accumulation.

Several groups have recently reported on the effects of normolipemic human VLDL on macrophage cholesterol metabolism.20-22 Gianrurco et al.20 using unstimulated mouse peritoneal macrophages, found that normolipemic human VLDL at 43 μg protein/ml did not stimulate cholesteryl ester formation after an 18-hour incubation. On the other hand, Kraemer et al.,22 using thioglycollate-elicited...
Mouse peritoneal macrophages, and Lindquist et al.,
using unstimulated mouse peritoneal and J774 macrophages, have shown modest but definite increases in macrophage cholesteryl ester accumulation after incubations with normolipemic human VLDL. The data in Figure 3 shows that normolipemic human VLDL can indeed increase cholesterol delivery to human monocyte-macrophages as measured by down-regulation of the LDL receptor or accumulation of cellular cholesteryl mass, but in each case it is less effective than AVLDL. The difference between native and modified particles is especially evident in their abilities to stimulate cholesteryl ester formation, which was measured over a 2-hour period, and would therefore be the most sensitive and specific index of the rates of cholesterol delivery for the experiments shown.

Elevations of LDL have been strongly associated with coronary atherosclerosis. The relationship between elevated VLDL levels and atherosclerosis is clear, the relationship between elevated VLDL levels and premature clinical vascular disease remains controversial and will require further study. Additional insight regarding cellular mechanisms by which pre-beta migrating VLDL could participate in the formation of cholesteryl ester laden arterial wall cells would be of value. The present study defines a cellular mechanism by which VLDL can contribute to macrophage cholesteryl ester overload and thereby directly participate in the genesis of the atherosclerotic plaque at the vessel wall.

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196 ARTERIOSCLEROSIS Vol. 7, No 2, March/April 1987


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