Oxidative Modification of Beta-Very Low Density Lipoprotein
Potential Role in Monocyte Recruitment and Foam Cell Formation

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Oxidative modification of low density lipoprotein (LDL) generates a form that is degraded much more rapidly by macrophages and may thus be more atherogenic than unoxidized LDL. Recently, we provided evidence that oxidative modification of LDL may play a significant role in the generation of fatty streaks in the LDL receptor-deficient rabbit. The major lipoprotein in cholesterol-fed animals is the β-VLDL. Since β-VLDL is avidly taken up by macrophages, it could lead to foam cell formation without the need for oxidative modification or modification of other kinds. However, the present studies show that β-VLDL can be oxidized by incubation with endothelial cells or with copper ions. Oxidized β-VLDL was degraded by macrophages at about twice the rate of unoxidized β-VLDL, and it stimulated cholesterol esterification twice as much as unoxidized β-VLDL. The degradation of oxidized β-VLDL was inhibited either by oxidized LDL itself or by oxidized LDL but not by unoxidized β-VLDL. β-VLDL was chemotactic for human monocytes and contained significant amounts of lysophosphatidylcholine, previously shown to be a chemotactic agent. In summary, oxidized LDL is degraded by macrophages proportionately more than oxidized β-VLDL as compared to the unmodified lipoproteins. However, the twofold increase may, nevertheless, be significant in the atherogenicity of β-VLDL.

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The lipid-laden foam cells of the early atherosclerotic lesion represent primarily tissue macrophages originating from circulating monocytes. Plasma cholesterol, which in humans is present chiefly in the low density lipoprotein (LDL) fraction, is the source of most of the cholesterol ester in foam cell lesions. LDL is degraded in most cells mainly by way of the LDL receptor of Brown et al. The fact that severe atherosclerosis develops in animals and in humans that have deficiencies of functional LDL receptors has focused attention on alternative pathways for LDL uptake. As a result, it has been suggested that LDL in vivo may be modified to a form that is degraded by cells by way of mechanisms independent of the LDL receptor. It has been reported that a number of modified forms of LDL are degraded by macrophages via alternative receptors, resulting in cholesterol ester accumulation. We have shown that LDL oxidatively modified by incubation with specific cells or with redox metals may influence the atherogenic process not only by recruiting and retaining monocytes in the artery wall but also by promoting lipid accumulation in these cells. Our recent work showed that probucol acting as an antioxidant specifically inhibited the rate of degradation of LDL in fatty streak lesions of Watanabe heritable hyperlipemic (WHHL) rabbits and slowed lesion development. These results and those of Kita et al. support the possibility that oxidized LDL is a more atherogenic form of LDL.

In cholesterol-fed animals, a large fraction of the plasma cholesterol is present in lipoproteins that are of very low density (VLDL) and have β electrophoretic mobility (β-VLDL). The atherosclerotic lesions in cholesterol-fed rabbits are similar in nature to those found in WHHL rabbits, and both involve macrophage foam cells, suggesting that β-VLDL may itself be atherogenic. Indeed, β-VLDL is avidly taken up by tissue macrophages in culture, resulting in foam cell formation. This rapid uptake is probably the result of the high affinity with which β-VLDL binds to the LDL receptor on the macrophage and not, as previously thought, due to a specific receptor for β-VLDL. Reduction of plasma cholesterol concentrations by treatment with drugs, including probucol, decreases atherosclerosis in cholesterol-fed rabbits. However, the fact that probucol is an antioxidant and the observation that cholesterol feeding increases lipid peroxidation in vivo raises the possibility that the antiatherogenic activity of probucol in rabbits may have been, in part, mediated by inhibition of oxidation of β-VLDL. In other words, oxidation may potentiate the already high rate of degradation of β-VLDL by macrophages and may help recruit macrophages by generating chemotactic activity, as it does in LDL. In the present studies, we show that β-VLDL can, indeed,
undergo in vitro oxidative modification analogous to that of LDL during incubation with endothelial cells and that this modification increases the rate at which it is degraded by macrophages.

After these studies had been completed, Haratz et al.26 reported that incubation of rabbit lipoproteins of density 1.006 to 1.019 with cultured bovine smooth muscle cells oxidatively modifies it to a form degraded several-fold more rapidly by the J774 line of macrophages or by resident mouse peritoneal macrophages. The present studies also show that β-VLDL as isolated from fresh plasma is chemotactic for monocytes, probably because it contains significant quantities of lysophosphatidylcholine (lyso PtdCho), which has previously been shown to be a chemotactic lipid.27 Thus, β-VLDL in cholesteryl-fed rabbits may be atherogenic, not only by virtue of its direct uptake into macrophages, but also due to: 1) its oxidation to a form taken up even more rapidly by the macrophage and 2) its high content of lyso PtdCho acting as a chemotactic factor.

Methods

Materials

Carnegie-free Na15221 and 14C-oleic acid were purchased from Amersham (Arlington, IL). Ham's F-10 and Dulbecco's modified Eagle's (DME) medium were purchased from Whittaker M. A. Bioproducts and Gibco Laboratories, respectively. Fetal bovine serum was supplied by Hyclone Laboratories (Logan, UT). Phenylmethylsulfonylfluoride (PMSF) and benzamidine hydrochloride were from Behringer Diagnostics (La Jolla, CA). p-Phenylalanine-2-propyl-l-arginine chloromethyl ketone (PPACK), gentamycin sulfate, and lipid-free bovine serum albumin were from Sigma Chemical Company (St. Louis, MO). Protocoll4,4'-[isopropylidenediathio][bis[2,6-di-t-butylphenol]] was provided by Merrell Dow Pharmaceuticals (Indianapolis, IN). Cholesterol was purchased from Aldrich Chemical Company (Milwaukee, WI). Complete rabbit chow containing 2% cholesterol (wt/wt) was from ICN Biochemicals (Cleveland, OH).

Animals

All procedures relating to the use of animals were in compliance with National Institutes of Health guidelines on the care and use of animal subjects and were approved by the university's Committee on Animal Subjects. Female New Zealand White rabbits were obtained from Holbert's Rabbitry (Spring Valley, CA). When studied, the 17 rabbits weighed 3.0±0.2 kg. All rabbits were initially maintained on a standard cholesterol-free diet. After 2 weeks, that diet was replaced with a low-fat diet containing 2% cholesterol. After 11 weeks, the diet of five rabbits was supplemented with 1% probucol; another five animals whose diet was treated with vehicle only (diethyl ether) served as controls. After 5 weeks of further treatment with the cholesterol diet containing probucol or vehicle, 30 ml of blood was collected from the central artery of the ear of each rabbit directly into 0.015 volume of 0.4 M EDTA (pH 7.4). Plasmas were pooled according to treatment group, and β-VLDL was isolated from each pool as described below. Four other animals were fed the probucol-supplemented diet beginning after 15 weeks of cholesterol feeding; the remaining three were treated with vehicle only. After 5 weeks of treatment, blood was collected as before from these animals and the plasma again pooled by treatment group before lipoprotein isolation.

Lipoproteins

The d<1.006 fraction, principally β-VLDL, was obtained from the plasma of hypercholesterolemic rabbits by centrifugation for 15 hours at 50,000 rpm in a Ti 60 rotor. They were recentrifuged at d<1.006 for 16 hours at 50,000 rpm. In one of the two cases, the plasma was adjusted to 100 μg/ml gentamycin sulfate, 50 μg/ml chloramphenicol, 1 μM PPACK, 2 mM benzamidine, and 1.5 mM PMSF before isolation of β-VLDL. The presence of these antibiotic and antiproteolytic agents did not affect the behavior of the β-VLDL in the modification experiments.

LDL (d=1.019 to 1.063) from human plasma isolated by ultracentrifugation as described previously.28 The lipoproteins were radiiodinated as described earlier.29 Acetyl LDL was prepared as described earlier.30 The acetyl LDL sample tested showed a 2.4 times increase in electrophoretic mobility on agarose gel as compared to the unmodified lipoprotein. All lipoprotein samples were dialyzed against phosphate-buffered saline (PBS) containing 0.01% EDTA at 4°C. Residual EDTA was removed by dialysis against PBS without EDTA before modification trials.

Albumin treatment of the lipoprotein was carried out by incubation of 2 mg of β-VLDL with 200 mg of fatty acid-free albumin in 20 ml of DME medium at 37°C for 24 hours. The lipoprotein was then resolubilized at density (d=1.10) by ultracentrifugation.

Cells

Rabbit aortic endothelial cells (a cell line developed by Vincent Buonasera) were grown in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 15% fetal bovine serum as described. Resident peritoneal macrophages were harvested from female SwissWebster mice by peritoneal lavage and were used for lipoprotein degradation studies.40 Human circulating monocytes were isolated as described by Kumagal et al.41 Monocyte chemotaxis was determined in a 48-well modified Boyden chamber, and the results are expressed as chemotactic index (CI), defined as the ratio between the number of cells migrating under the influence of the chemotactic factor and the number migrating in control medium without any chemoattractant.21

Oxidative modification of LDL and β-VLDL was performed by incubating 200 μg of the lipoproteins in Ham's F-10 medium with confluent rabbit aortic endothelial cells or with copper (5 or 10 μM) in a volume of 2 ml at 37°C for 24 hours.18,19

Macrophage degradation of the lipoproteins was measured as follows: 5 μg of the oxidized lipoprotein was added to cultured macrophages (1×10⁶ cells in a 24-well plate in 0.5 ml of DME medium), and this was incubated at 37°C for 5 hours. The medium was then analyzed for trichloroacetic acid (TCA) soluble radioactivity as described.18 Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive products (TBARS) in the
medium with fresh tetramethoxypropane as standard.\textsuperscript{42} Incorporation of \textsuperscript{14}C-oleate into macrophage cholesterol esters was measured as follows: Macrophages (\(1 \times 10^6\)) were first incubated with 5 mg/ml lipoprotein-deficient serum overnight. The medium was removed, and the cells were then incubated in a total volume of 1 ml, with 0.2 mM \textsuperscript{14}C-oleate (1 \(\mu\)Ci) in the presence of 0.6 mg/ml of bovine serum albumin (BSA) and 50 \(\mu\)g of native or oxidized lipoprotein at 37°C for 24 hours. The medium was removed, and the cells were then repeatedly extracted (5 x 2 ml) with 3:2 heptane/isopropyl alcohol (vol/vol). The combined extracts were dried under nitrogen, and cholesterol esters were separated by silica gel thin-layer chromatography with 80:20:0.7 petroleum ether/diethyl ether/acetic acid (vol/vol/vol) as the solvent system. Radioactivity was then determined using a toluene-based scintillation fluid.

\(\beta\)-VLDL lipids were extracted by the method of Bilgh and Dyer.\textsuperscript{43} Lyso PtdCho and protein content were determined as described.\textsuperscript{16} Plasma probucol concentrations were determined by high-performance liquid chromatography as described.\textsuperscript{42}

**Results**

In the present studies, the \(\beta\)-VLDL fraction used was the d<1.006 fraction, which was washed at d=1.006. No attempts were made to separate the hepatic \(\beta\)-VLDL from chylomicron remnants. As shown in Table 1, incubation of \(\beta\)-VLDL with endothelial cells or with 5 \(\mu\)M copper ion resulted in oxidation of the lipoprotein. Small amounts of TBARS were associated with unincubated native \(\beta\)-VLDL; this increased six- to ninefold upon incubation with endothelial cells or copper, indicating extensive lipid peroxidation. The oxidized \(\beta\)-VLDL showed increased electrophoretic mobility on

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**Table 1. Oxidative Modification of \(\beta\)-VLDL from Rabbits Fed Cholesterol**

| \(\beta\)-VLDL                      | TBARS (nmol/mg) | Mac degn (\(\mu\)g/STV/mg) | \(^{13}\)C-choleate (nmol/mg/mac protein) |
|------------------------------------|----------------|-----------------------------|-------------------------------------------|
| From untreated rabbits             |                |                             |                                           |
| Nonincubated control               | 12.61          | 1.66                        | 66.2                                      |
| After incubation with endothelial cells | 78.56          | 4.32                        | 147.9                                     |
| After incubation with copper ions  | 116.68         | 4.56                        | N.D.                                      |
| From probucol-treated rabbits      |                |                             |                                           |
| Nonincubated control               | 11.92          | 1.65                        | 46.2                                      |
| After incubation with endothelial cells | 17.63          | 1.55                        | 33.5                                      |
| After incubation with copper ions  | 23.56          | 1.38                        | N.D.                                      |

\(\beta\)-VLDL = \(\beta\) very low density lipoprotein. N.D. = not determined. TBARS = thiobarbituric acid-reactive substances, Mac degn = macrophage degradation, chol = cholesterol.

\(\beta\)-VLDL from control or probucol-treated rabbits were incubated with cultured rabbit aortic endothelial cells or copper acetate (5 \(\mu\)M) in 2 ml of Ham's F-10 medium at 100 \(\mu\)g/ml for 24 hours. TBARS, macrophage degradation, and oleate incorporation were determined as described in the text. Values are averages of four determinations from duplicates of two separate experiments.

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*Figure 1.* 3% to 14% gradient SDS-PAGE and agarose gel electrophoretic mobilities of native and oxidatively modified \(\beta\)-VLDL. SDS-PAGE: Lane A. Native LDL. Lane B. Native \(\beta\)-VLDL. Lane C. Cu-\(\beta\)-VLDL. Agarose gel electrophoresis: Lane D. Native \(\beta\)-VLDL. Lane E. Cu-\(\beta\)-VLDL. SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis, \(\beta\)-VLDL = \(\beta\) very low density lipoprotein.
Table 2. Monocyte Chemotactic Activity and Lyso PtdCho Content of Unoxidized and Oxidized β-VLDL from Cholesterol-fed Rabbits

| Sample                        | CI     | Lyso PtdCho (nmol/mg) |
|-------------------------------|--------|-----------------------|
| Freshly isolated β-VLDL       | 12.83  | 238.3                 |
| Albumin-treated β-VLDL        | 4.45   | 80.7                  |
| Lipid extracted from albumin  | 13.70  | 190.8                 |
| Albumin-treated β-VLDL        |        |                       |
| subsequently oxidized with    |        |                       |
| copper (10 µM)                | 13.65  | 156.4                 |
| Albumin-treated β-VLDL        |        |                       |
| subsequently oxidized by      | 10.80  | N.D.                  |
| incubation with endothelial   |        |                       |
| cells                         |        |                       |

N.D. = Not determined. CI = Chemotactic index. PtdCho = Phosphatidylcholine. β-VLDL = β very low density lipoprotein.

Treatment of the lipoprotein with albumin and the determinations of chemotaxis and lyso PtdCho were performed as described in the text. Values are averages from duplicates from a representative experiment. β-VLDL as isolated contained 518 nmol of PtdCho/mg protein.

the degradation of oxidized β-VLDL even at concentrations 50 times that of the labeled ligand. This finding suggested that oxidized β-VLDL and acetyl LDL are perhaps degraded by two separate pathways. Recent observations from our laboratory based on the incomplete competition between oxidized LDL and acetyl LDL have shown that a second receptor was probably involved in the uptake and degradation of oxidized LDL by the macrophages and that that receptor did not recognize acetyl LDL.44 Figure 2B shows the reciprocal competitions with labeled copper-oxidized LDL. In these experiments, only oxidized LDL and oxidized β-VLDL, but not the unoxidized lipoproteins, competed for the degradation of oxidized LDL. As expected, oxidized LDL was a more effective competitor for macrophage degradation of labeled oxidized LDL than was oxidized β-VLDL. However, oxidized LDL also competed for macrophage degradation of labeled oxidized β-VLDL to an even greater extent than did unlabeled oxidized β-VLDL. These results suggest that macrophage degradation of oxidized LDL and oxidized β-VLDL occurs via the same receptor and that the receptor is distinct from the LDL receptor.

Oxidation of LDL is associated with an increase in its content of lyso PtdCho,18,19 and we recently demonstrated that lyso PtdCho accounts for a part of the monocyte chemotactic activity associated with oxidized LDL.27 In the present studies, we found that even native β-VLDL as freshly isolated was chemotactic for circulating human monocytes (Table 2) and that it already contained considerable amounts of lyso PtdCho much more than that found in normal VLDL.18,19 The concentration of lyso PtdCho in normal VLDL also is very low.45 When native β-VLDL (100 µg/ml) was first incubated with lipid-free albumin (10 mg/ml, 24 hours) and reisolated by centrifugation, most of its chemotactic activity was lost and the activity was recovered in the lipids associated with the albumin. Lyso PtdCho as determined by TLC was the major lipid component transferred to the albumin. Oxidation of this albumin-treated β-VLDL by incubation with

LDL nor unoxidized β-VLDL competed effectively for the degradation of labeled oxidized β-VLDL by mouse peritoneal macrophages. In contrast, oxidized LDL and oxidized β-VLDL both competed effectively for the degradation of labeled oxidized β-VLDL. Acetyl LDL competed poorly for

Figure 2B. Competition of various lipoproteins for the degradation of 125I-labeled Cu-oxidized lipoproteins by mouse peritoneal macrophages. A. 125I-labeled Cu-β-VLDL. B. 125I-labeled Cu-LDL. Mouse peritoneal macrophages (1×10^5 cells/16-mm, 24-well dishes) were incubated with 10 µg 125I-labeled Cu-oxidized lipoprotein with the indicated concentrations of unlabelled competing lipoproteins in a total volume of 1 ml DME medium for 5 hours at 37°C. Unlabeled lipoproteins were native LDL (●), native β-VLDL (▲), Cu-oxidized LDL (○), Cu-oxidized β-VLDL (●), and acetyl LDL (▲). In the absence of competitors, macrophages degraded 5.56 µg Cu-β-VLDL protein or 11.18 µg Cu-LDL protein per 5-hour incubation per milligram cell protein. Each point is the mean of duplicate determinations from one representative experiment of a set of four experiments. β-VLDL = β very low density lipoprotein, LDL = low density lipoprotein, DME = Dulbecco's modified Eagle's medium.
endothelial cells or with copper regenerated its chemotactic activity and increased its lyso PtdCho levels toward the level found in the untreated, freshly-isolated preparation.

**Discussion**

While unmodified β-VLDL is itself capable of generating foam cells by specific interaction with macrophages, the possibility that its potential in this regard may be enhanced by post-secretory modification has not been ruled out. The present studies show that β-VLDL is effectively oxidized by endothelial cells and by copper ions (in the absence of cells), generating levels of TBARS comparable to those generated in similar amounts of LDL incubated under similar conditions. This oxidized β-VLDL was degraded by mouse peritoneal macrophages at a rate two- to threefold greater than that for the untreated β-VLDL. The results of Haratz et al. are similar, with only an occasional instance of enhancement greater than threefold. Thus, the effect of oxidation on subsequent macrophage degradation of β-VLDL is less striking than the effect of oxidation on macrophage degradation of LDL. Nevertheless, even a two- to threefold increase in rate of macrophage degradation might have significance in the deposition of cholesterol in the developing fatty streak lesion. It should be noted, however, that the lipoprotein fraction used by Haratz et al. was the fraction with density between 1.019 and 1.063 (lipoprotein "remnants" or intermediate density lipoprotein fraction). In contrast, most of the previous studies of β-VLDL have involved the use of the fraction with density less than 1.006 which, in cholesteryl-fed animals, consists mostly of lipoproteins with beta mobility and with a very high cholesterol/protein ratio. The oxidation of β-VLDL may have another significance. Ball and coworkers have reported that the accumulation of ceroid pigment in cultured macrophages occurs only when the LDL being taken up has been previously oxidized; uptake of acetylated LDL, even though it is taken up at a comparable rate, does not cause ceroid accumulation. It would be of interest to determine whether uptake of oxidized β-VLDL also can cause accumulation of ceroid.

We have previously reported that oxidized LDL is chemotactic for human circulating monocytes and that the activity is in large part attributable to the presence of lyso PtdCho generated during the oxidative modification. The present results show that β-VLDL—even as it is freshly isolated—is chemotactic for monocytes and that the chemotactic activity is attributable to lyso PtdCho associated with the β-VLDL. The origin of this lyso PtdCho in β-VLDL is not clear. Apoprotein B-containing lipoproteins are reported to have a high affinity for lyso PtdCho, and so the lipoproteins would be expected to carry much of it no matter what the origin. It should be noted that a large excess (100-fold) of lipid-free albumin was able to remove most of the lyso PtdCho from β-VLDL. Whether or not this material plays a role in recruitment of monocytes depends upon the concentrations within the intimal space relative to the concentrations of lyso PtdCho in the plasma compartment. Berliner et al. have shown that incubation of β-VLDL (but not LDL) with cultured endothelial cells increases the chemotactic activity of the incubation medium for monocytes. The nature of the chemotactic activity was not established, but it seems unlikely that it was lyso PtdCho, since an antioxidant was present during the incubation with endothelial cells.

The possibility that oxidation of lipoproteins plays a role in the atherogenesis in cholesteryl-fed animals deserves further study, especially in vivo. In has been reported that cholesterol feeding enhances lipid peroxidation. Also, probucol has decreased the severity of lesions in cholesteryl-fed animals. However, the cholesterol levels in the probucol-treated animals were decreased and the question of whether the antioxidant effect added to the hypcholesterolemic effect cannot be decided without more careful investigation.

In conclusion, the results provided evidence that oxidation of β-VLDL may augment the atherogenic potential of the lipoprotein in two ways: 1) Oxidation may further enhance the macrophage degradation and cholesterol esterification in the cell, and 2) More importantly, oxidation may account for the presence of monocyte/macrophages in the developing lesion. While hypercholesterolemia by itself may alter the chemotactic responsiveness of monocytes, lyso PtdCho associated with β-VLDL appears to be a potential candidate for the enhanced chemotactic stimulus. The levels of lyso PtdCho, both in plasma and arterial lesions, are certainly elevated during atherosclerosis; however, it remains to be established whether lyso PtdCho plays a role in the recruitment of monocytes during the initiation of early atherosclerotic lesions.

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Index Terms: atherosclerosis • monocytes • chemotaxis • lyso PtdCho • cholesterol-fed rabbit