Macrophage-derived Foam Cells Freshly Isolated from Rabbit Atherosclerotic Lesions Degrade Modified Lipoproteins, Promote Oxidation of Low-Density Lipoproteins, and Contain Oxidation-specific Lipid-Protein Adducts

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Abstract

Pure macrophage-derived foam cells (MFC) were isolated from the aortas of rabbits made atherosclerotic by balloon deendothelialization followed by diet-induced hypercholesterolemia. The MFC were isolated under sterile conditions using an enzymatic digestion procedure and discontinuous density gradient centrifugation. The purity of the MFC preparations was verified immunocytochemically with the macrophage specific monoclonal antibody RAM-11. MFC plated in medium containing 0.5% FCS for 24 h contained ~ 600 μg cholesterol per mg cell protein, 80% of which was esterified cholesterol. The MFC specifically degraded low density lipoprotein (LDL), acetyl-LDL, copper oxidized LDL, and beta--very low density lipoprotein (β-VLDL) at rates comparable to mouse peritoneal macrophages (MPM) in 5-h assays. MFC within sections of the atherosclerotic lesions from the balloon-damaged (MFC)–infected rabbits, expressed modified epitopes found in oxidized-LDL. These data provide direct evidence that arterial wall macrophages express modified LDL receptors in vivo, contain epitopes found in oxidized-LDL and are capable of oxidizing LDL even when maximally loaded with cholesterol. (J. Clin. Invest. 1991. 87:90–99.) Key words: macrophages + foam cells + lipoprotein oxidation + scavenger receptors + atherosclerosis + cholesterol-fed rabbits

Introduction

It is now well documented that macrophage-derived foam cells (MFC) constitute a significant percentage of the cells within atherosclerotic lesions in both humans and most animal mod-}

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1. Abbreviations used in this paper: LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; MFC, macrophage-derived foam cells; MPM, mouse peritoneal macrophage; TBARS, thiobarbituric acid reactive substance; WHHL, Watanabe Heritable Hyperlipemic rabbit.

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(Sigma Chemical Co.) and 1.0 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) for 1 h at 37°C. The enzyme/tissue mixture was then filtered through nylon (100-μm mesh) and fresh enzyme cocktail was added to the same tissue suspension (additional 1 h). The released cells were then pelleted at 500 g, resuspended in HBSS containing the glucose and amino acids and purified with a discontinuous density gradient of metrizamide (Sigma Chemical Co.) (30% cushion, 10% top, centrifuged at 1,200 g in a swinging bucket rotor for 15 min at 10°C). The isolated cells were washed, plated, and maintained in Opti-MEM I reduced serum medium (Gibco, Grand Island, NY) containing 0.5% FCS overnight. Viability was assessed by the ability of the cells to exclude Trypan blue and averaged > 90%. The plating efficiency of the isolated foam cells was extremely variable, ranging from < 50% to > 90% adherence after the overnight incubation. However, many of the most buoyant cells appeared to float in place but were actually firmly attached by means of membrane processes that attached to the plastic. These cells also remained viable as they were capable of excluding Trypan blue throughout the course of the studies.

To assess the purity of the foam cell preparations, we plated the cells on glass coverslips or in chamber slides (Lab Tek, Nunc, Inc., Naperville, IL), fixed with cold methanol for 30 min and immunostained with the monoclonal antibody RAM-11 (8), which is specific for rabbit macrophages, as well as monoclonal antibody HHF-35 (Enzo Biochem, Inc., New York, NY) (23), which recognizes muscle actin and therefore is specific for smooth muscle cells. All immunostaining procedures were done with an avidin-biotin system conjugated with either horseradish peroxidase or alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA). All experimental procedures with rabbits were conducted under a protocol approved by the Animal Care and Use Committee of the University of California San Diego.

Detection of oxidized lipid-protein adducts. To determine whether the atherosclerotic lesions in the balloononed, cholesterol-fed rabbits as well as the MFC isolated from the same lesions, contained oxidation-specific lipid-protein adducts, we fixed small segments of the abdominal aorta in formal sucrose containing 50 μM butylated hydroxytoluene (BHT) and embedded in paraffin. Serial 5-μm-thick sections were cut from the segments of aorta and immunostained using a panel of recently described antibodies specific for epitopes characteristic of those generated during the oxidative modification of LDL (12-15). Antibodies utilized included (a) guinea pig polyclonal antisera MALT-2 and the mouse monoclonal antibody MDA-2, both generated against autologous malondialdehyde-conjugated LDL (MDA-LDL) and (b) guinea pig polyclonal antiserum HNE-7 and mouse monoclonal antibody N59 generated against 4-hydroxy-2-nonenal-conjugated LDL (4-HNE-LDL) and (c) mouse monoclonal antibody OLF4-3C10, generated against fragments of apoprotein B resulting from the Cu²⁺-oxidation of LDL (12-15). Additional sections were stained with nonspecific sera (goat or horse). In addition, MFC isolated and cultured on chamber slides in the presence of BHT, were fixed with methanol and immunostained as above.

Lipoprotein degradation assays. MPM were elicited by intraperitoneal injection of 2 ml thiglycollate medium (Difco Laboratories, Detroit, MI) 3 d before harvesting. The macrophages were plated in 24-well clustered dishes at a density of 1 × 10⁶ cells/well in RPMI 1640 medium containing 10% FCS. After 2 h, nonadherent cells were removed and the cells washed with PBS. To maximize the number of LDL receptors, we incubated the cells overnight in RPMI 1640 containing 5 mg/ml lipoprotein-deficient serum (LPDS). The rabbit arterial foam cells were plated at 5 × 10⁵ cells/well in Opti-MEM I containing 0.5% FCS. Nonadherent cells were removed after an overnight incubation and before the addition of the labeled lipoproteins.

LDL (d = 1.019-1.063) and LPDS (d > 1.215) were isolated by preparative ultracentrifugation from fresh human plasma using antioxident and protease inhibitors as described (12, 13). The lipoproteins were dialyzed extensively against PBS containing 0.01% EDTA (pH 7.4), β-VLDL (d < 1.006) was isolated by preparative ultracentrifugation from the plasma of cholesterol-fed rabbits. Acetylated LDL was prepared according to the method of Basu et al. (24), and copper (Cu²⁺) oxidized LDL was prepared as described by Steinbrecher et al. (25). Modification of the lipoproteins was checked by agarose electrophoresis, thiobarbituric acid reactive substance (TBARS), reactivity and enhanced uptake in MPM compared to native LDL. Lipoproteins were radiolabeled with carrier-free sodium 125I-iodide (Amersham Corp., Arlington Heights, IL) by the method of Salacinski et al. (26) using the solid-phase oxidizing agent, iodogen (Pierce Chemical Co., Rockford, IL). Specific activities varied from 30–400 CPM/mg of LDL protein.

The degradation of 125I-LDL, 125I-[acetyl-LDL], 125I-[β-VLDL], 125I- Cu²⁺-oxidized LDL was determined as described by Goldstein et al. (27). Each cell monolayer received 0.5 ml Opti-MEM medium containing 0.5% FCS and the indicated concentration of labeled lipoprotein. To determine nonspecific degradation, unlabeld lipoprotein, at a concentration 25 times that of the labeled lipoprotein, was added to each well. After incubation at 37°C for 5 h, the medium was removed and the amount of 125I-labeled TCA soluble (noniodide) material was determined.

Determination of cellular cholesterol content. The monolayers of cells used in the degradation assays were washed with PBS, and solubilized in 0.2 ml of 0.2 M NaOH. Aliquots were removed for gamma counting and for protein determination by the method of Lowry et al. (28). Before reading the optical density, we added 2 ml of chloroform to extract the lipids. The chloroform fraction was assayed for total and free cholesterol according to the enzymatic method of Gamble et al. (29). In order to compare the cholesterol content of the isolated MFC with that of MPM maximally loaded with acetylated LDL, the MPM were incubated with 100 μg/ml acetylated-LDL for 3 d and refed for an additional 3 d. The mouse cells were then treated identically to the MFC as described above.

Analysis of the capacity to induce LDL oxidation. For the measurement of the capacity to oxidize LDL, the rabbit arterial MFC were plated at 5 × 10⁵ cells/dish in 35-mm culture dishes in RPMI medium containing 0.5% FCS. Resident MFC were obtained via lavage, and were plated at a density of 2.5 × 10⁶ cells/dish (this excess of cells yields a continuous monolayer after an overnight incubation). Confluent cultures of rabbit aortic endothelial cells (EC) (in 60-mm dishes) were also used as positive controls (25). After the overnight incubation, the medium containing any nonadherent cells was removed and the cells were incubated at 37°C with 2 ml of Ham's F-10 medium containing 200 μg of 125I-LDL for 24 h. The medium was then analyzed for TBARS after the method of Paton and Kurtz (30). An aliquot containing 5 μg of the 125I-LDL that had been incubated with the cells was tested for degradation using a fresh culture of resident MFC as described above.

Results

Yield and purity of the isolated foam cell preparations. To maximize the yield of isolated MFC, the rabbits were subjected to Fogarty balloon denudation of the endothelial lining of their aortas 1 wk after placing the animals on a high-cholesterol diet (2%). The combination of removal of the endothelium and a marked hypercholesterolemia (plasma cholesterol levels > 1,000 mg/dl during the 12 wk before sacrifice) yielded lesions that consisted almost entirely of MFC. This is demonstrated in Fig. 1, where serial sections of the abdominal aorta of one of the animals have been immunostained with antibodies specific for macrophages or smooth muscle cells.

By starting with atherosclerotic tissue that contained very few smooth muscle cells, and using discontinuous density gradient centrifugation to separate the isolated cells on the basis of size and buoyant density, we obtained cultures of pure MFC that exhibited no contamination with smooth muscle cells. The final yield was generally between 1–2 × 10⁶ pure MFC per gram of aorta. Fig. 2 demonstrates that the majority of the isolated cells were large, round, and intact (excluded Trypan
blue) and adherent to the plastic (Fig. 2 A). They contained massive amounts of neutral lipid, as shown with Oil Red O staining (Fig. 2 B), and consisted of cells that were immunoreactive only with the macrophage-specific antibody RAM-11 (Fig. 2, C and D), and not with the smooth muscle specific antibody HHF-35 (Fig. 2 E).

**Cholesterol content of the isolated foam cells.** Fig. 3 demonstrates that the isolated MFC contained massive amounts of cholesterol (~ 600 \( \mu \)g/mg cell protein) that was predominantly esterified cholesterol (> 80% of the total cholesterol). In contrast, when MPM were fed acetyl-LDL for up to 6 d, the amount of cholesterol accumulated by the cells was considerably less than that in the arterial macrophages in vivo. In addition, it appeared that a smaller percentage of the total cholesterol which accumulated in the MPM was stored as esterified cholesterol.

**Presence of oxidation specific lipid-protein adducts.** To determine whether the isolated MFC and the atherosclerotic lesions induced by the combination of ballooning and cholesterol feeding contain oxidation-specific lipid-protein adducts, such as have been previously demonstrated in atherosclerotic lesions from the WHHL rabbit (12, 13, 15, 31, 32), we immunostained isolated foam cells as well as serial sections of the lesions with a panel of both polyclonal and monoclonal antibodies that recognize MDA- and 4-HNE-lysine adducts and oxidation specific apoprotein B fragments (OLF4-3C10). Fig. 4 shows that the atherosclerotic lesions from which the MFC were subsequently isolated contained macrophages which stained with all of the antibodies tested. Fig. 5 demonstrates that the isolated MFC still retained their immunoreactivity with several of the oxidation-specific antibodies and exhibited the same type of punctate and annular staining patterns we have previously observed in macrophages in vivo (12, 15). The immunostaining shown in both Figs. 4 and 5 was specific as the use of nonspecific sera (goat or horse) (Figs. 4 K and 5 E) yielded sections devoid of staining.

**Capacity to degrade LDL, acetyl-LDL, oxidized LDL, and \( \beta \)-VLDL.** Fig. 6 demonstrates that the isolated MFC degraded very little native LDL, but were capable of degrading considerable amounts of acetyl-LDL, Cu\(^{2+}\) oxidized LDL, and \( \beta \)-VLDL in 5-h assays. The degradation of these ligands is highly specific as the addition of 25-fold excess of the unlabelled ligands reduced the amounts of degradation products by 60-90%. In comparison, MPM also degraded very little native LDL, and comparable amounts of acetyl-LDL, Cu\(^{2+}\) oxidized LDL and \( \beta \)-VLDL.

**Capacity to oxidize LDL.** Table I shows the results of two separate experiments comparing the ability of MFC and other cell types to oxidize LDL. Although plated at a considerably lower density than the other cell types (5 \( \times \) 10\(^4\) vs. 2.5 \( \times \) 10\(^5\)),
Figure 4. Macrophages within atherosclerotic lesions from ballooned, cholesterol-fed rabbits contain oxidation specific lipid-protein adducts. Immunocytochemical staining of sections of the abdominal aorta containing atherosclerotic lesions, with antibodies generated against malondialdehyde conjugated LDL (Mal-2 and MDA-2), 4-hydroxynonenal conjugated LDL (HNE-7), Cu⁺⁺ oxidized LDL (OLF4-3C10), macrophages (RAM-11), and smooth muscle cells (HHF-35). (A and B) Guinea pig polyserum Mal-2. (C and D) Mouse monoclonal antibody MDA-2. (E and F) Guinea pig polyserum HNE-7. (G and H) Mouse monoclonal antibody OLF4-3C10. (I) RAM-11. (J) HHF-35. (K) Nonspecific (goat) serum. The final magnification of panels A, C, E, G, I–K: 220; bars, 100 μm. (B, F, H) Magnification 2,200; bars, 10 μm. (D) Magnification 890; bar, 10 μm. All staining utilized an avidin-biotin-alkaline phosphatase procedure as described in Methods.
enzymatic digestion for up activity using also and accumulate protein metabolism. this ever, cells with previously cells arterial tion of rabbit aortas cholesterol fed animals first indication isolated, capacity bit aorta rich MFC Discussion that observed and degraded was aortic amount of the isolated LDL still capable of inducing comparable amounts of LDL lipid peroxidation as the MPM and the rabbit aortic EC. In addition, the LDL that was oxidized by the MFC was recognized and degraded by MPM at a rate comparable to that observed with LDL incubated with the MPM or rabbit EC.

Discussion

These studies have demonstrated for the first time that cholesteryl-ester rich MFC freshly isolated from atherosclerotic rabbit aorta have the capacity to degrade oxidized LDL, and retain the capacity to oxidize LDL. Further, the demonstration that the MFC and the atherosclerotic lesions from which they were isolated, contain oxidation-specific lipid-protein adducts, is the first indication that lipoprotein oxidation may also occur in cholesterol fed animals analogous to that reported in WHHL rabbit aortas (12, 13, 15, 31, 32).

There have been several different approaches to the isolation of foam cells from the artery wall. Primary cultures of arterial cells that have migrated out of tissue explants have been previously used to demonstrate the existence of arterial foam cells with characteristics similar to macrophages (33). However, this study did not look at parameters associated with lipoprotein metabolism. The capacity of rabbit foam cells to bind and accumulate β-VLDL, and acetyl-LDL was first ascertained also using explant techniques (34, 35). More recently, the same activity was demonstrated in cells that had been isolated by enzymatic digestion from human lesions and grown in culture for up to 2 wk (36). However, all three of these latter studies focused only on the qualitative demonstration of the uptake of fluorescently labeled lipoproteins.

The yield of cells using explant or seeding techniques is generally insufficient to perform quantitative in vitro assays, and there is a significant waiting period for the establishment of the cultures (up to 2 wk), a time during which specific receptors may be induced or down-regulated and phenotypic modulation may occur (37). Further, the resulting cultures are of mixed cell types when atherosclerotic tissue is used as the source of the explants.

Arterial foam cells can also be isolated using enzymatic digestion and gradient centrifugation procedures (19–22, 38). These techniques have previously been used to demonstrate the presence of foam cells exhibiting characteristics of macrophages (19, 21), to study the enzyme profiles and distribution of lipids within intracellular organelles (20, 22), and the capacity of the different arterial cells for synthesizing prostaglandins (22, 38). Once again, none of the studies cited looked at lipoprotein-cell interactions.

The advantages of this combined enzymatic and gradient centrifugation approach are the much greater yield of isolated cells, and the speed at which cells can be obtained (hours), and the purity of the resulting cell populations. The yield of pure foam cells in the current studies (1–2 × 10⁶ cells per gram or ~ 5–10 × 10⁶ cells per experiment) has allowed us to study the capacity of purified arterial macrophage-derived foam cells to metabolize modified lipoproteins using quantitative in vitro binding and degradation techniques. The speed at which we isolate and plate the cells (maximum of 24 h from the time of sacrifice to the time of utilization) should minimize induction

Figure 4 (Continued)
of receptor expression compared to previously used procedures which required more extensive time in culture. Thus, the activity exhibited by the isolated cells may be more reflective of their capacities in vivo.

However, by using digestive enzymes, there is always the question as to whether the enzymes may have damaged or altered the surface properties of the isolated cells. Fowler et al. (35) demonstrated that foam cells isolated using collagenase and elastase retain Fc and C3 receptors on their membranes and are capable of phagocytosing IgG coated, Fc-bound erythrocytes. The present studies have further demonstrated that the enzymatic digestion procedure did not compromise the capacity of isolated MFC to specifically bind and degrade lipopro-
teins, indicating that the LDL receptor, that form of the LDL receptor that specifically binds β-VLDL (39), and the scavenger receptors, (including the putative oxidized-LDL receptor [40]) were still expressed after enzymatic digestion and 18–24 h in culture.

An additional advantage to studying MFC is their content of cholesterol. It is clear from the data presented in Fig. 3, that attempts to load MPM with acetyl-LDL over a 6-d period did not yield cells with a comparable content or distribution of cholesterol. Loading with acetyl-LDL does not lead to storage of an equivalent percentage of cholesteryl ester as is observed with the isolated MFC. Thus, it is possible that a higher content of free cholesterol in the in vitro systems may yield different

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**Figure 5.** Isolated foam cells contain oxidation specific lipid-protein adducts. Immuncytochemical staining of the isolated foam cells with monoclonal antibodies generated against malondialdehyde conjugated LDL and 4-hydroxynonenal conjugated LDL. (A and B) Mouse monoclonal antibody MDA-2. (C, D, F) Mouse monoclonal antibody NA59. (E) Nonspecific (goat) serum. The final magnification of A, C, and E: 440; bars, 10 μm. Final magnification of B, D, and F: 2,200; bars, 10 μm. All staining utilized an avidin-biotin-alkaline phosphatase procedure as described in Methods.
information about the effects of lipoprotein derived cholesterol (stored predominantly as cholesteryl-esters in vivo) on a variety of cellular functions (41).

Clearly, cellular functions such as the capacity for binding and degrading modified lipoproteins and the ability to oxidize LDL do not appear to be compromised by the intracellular accumulation of even large amounts of esterified cholesterol. As shown in Fig. 6, the MFC can still bind and degrade small amounts of LDL, and larger amounts of acetyl-LDL, oxidized-LDL, and β-VLDL in short term assays. Thus, the present in vivo observations are consistent with previous studies of foam cells derived from explanted atherosclerotic lesions (30-32). The present studies specifically document that oxidized LDL is taken up and degraded by arterial foam cells and provides quantification of lipoprotein degradation whereas previous studies were purely descriptive. The results from the present studies are also consistent with in vitro observations that the acetyl-LDL receptor and LDL/β-VLDL receptors are expressed by macrophages (9, 10) and are not down-regulated by cholesterol loading (10).

Because the MFC retain their capacity to induce the oxidation of LDL (Table I) and β-VLDL (data not shown) and contain immunoreactive oxidation specific lipid-protein adducts (Fig. 5), some of the accumulated lipoprotein-derived lipid may be stored in an oxidized form. This would be consistent with recent in vitro observations of Sparrow et al. (40) that oxidized-LDL appears to accumulate within macrophages in an undegraded form. The retention of oxidized lipoproteins may have an impact on the long-term capacity of the cells to further degrade native or modified lipoproteins (42).

The direct comparison of the capacity of several different cell types to oxidize LDL is somewhat misleading (Table I). Although the MFC were plated at 20% of the density of the MPM or EC, they were still able to effect as much lipid peroxidation in the LDL present in the medium as the other cell types when the data were expressed as the amount of MDA produced per milligram of LDL added. If the activity was normalized on the basis of cell protein, it would appear that the MFC have greater capacity for oxidizing LDL than the other cell types. However, because the continuous accumulation of oxidized-LDL may be toxic to the cells (43) it would be necessary to determine the rate of loss of cells during the incubation period. At present, the low yield of foam cells does not allow us to make this type of measurement, although it would be of interest to determine whether the large amount of lipid stored within the MFC make them more or less resistant to the cytotoxic effects of oxidized LDL.

The presence of immunoreactive oxidation-specific lipid-protein adducts in both the atherosclerotic lesion and in the isolated MFC from the cholesterol-fed rabbits indicates that the cells contained the epitopes in vivo and that the oxidation did not occur during the isolation procedure. Although the antibod-

Table I. Comparison of the Capacity of Different Cell Types to Oxidize LDL and the Degradation of the Oxidized-LDL by Mouse Peritoneal Macrophages

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<td>nmol MDA/mg LDL protein</td>
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<td>µg/mg cell protein</td>
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<td>Mouse peritoneal Mφ</td>
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<td>Isolated foam cells</td>
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Monolayers of cells were incubated with 200 µg/ml of human 125I-LDL in Ham's F-10 medium for 24 h at 37°C. The medium was then analyzed for TBARS activity as described under Methods. 5 µg of the 125I-LDL that had been incubated with the cells was added to fresh MPM and degradation products were measured as described in Methods. Values shown are the average of triplicate wells except for isolated foam cells where values are the average of duplicate wells. All cell types were plated at 2.5 x 10⁶ cells/well except for isolated foam cells where cells were plated at 5 x 10⁵ cells/well.
ties used in this study were generated against oxidized-LDL, they are not specific for lipid-protein adducts derived entirely from lipoproteins, i.e., they recognize MDA-lysine residues present on a variety of different proteins (14). However, almost certainly some of the lipid-protein adducts recognized by the antibodies in atherosclerotic lesions and specifically in macrophages, are of lipoprotein origin because (a) macrophages in other organs, such as spleen and liver, do not stain with these antibodies, nor does normal aortic tissue, (b) LDL can be gently extracted from the same lesions that contain apo B and apo B fragments that are immunoreactive to the same antibodies (12, 13), and (c) we have consistently observed an inverse relationship between the distribution of immunoreactive apo B and oxidized epitopes in areas of lesions containing macrophages (15, 31). This suggests that the macrophages may effect the oxidation of LDL followed by the rapid uptake and further degradation of the apoprotein component. The present observations of macrophage associated oxidation-specific lipid-protein adducts are consistent with our earlier studies of the distribution of oxidation specific lipid-protein adducts in atherosclerotic lesions from the WHHL rabbits (15). Those studies demonstrated punctate and annular intracellular staining patterns in macrophase-derived foam cells within both fatty streaks and more advanced lesions. The present observations indicate that the oxidative process may not be limited to lesions derived from a predominantly LDL hypercholesterolemia as occurs in the WHHL rabbit. Cholesterol feeding of rabbits results predominantly in the elevation of β-VLDL and our results clearly document that there is a marked accumulation in the aorta of oxidation specific lipid-protein adducts. This is consistent with the studies of Parthasarathy et al. which have shown that oxidation of β-VLDL enhances its uptake in macrophages (42). Thus, oxidation of lipoproteins may be a generalized phenomenon underlying the formation of macrophase-derived foam cells.

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