Involvement of the Macrophage Low Density Lipoprotein Receptor–Binding Domains in the Uptake of Oxidized Low Density Lipoprotein

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Macrophages, unlike most other cells, possess both low density lipoprotein (LDL) and scavenger receptors. The scavenger receptor has been shown to mediate the uptake of oxidized LDL (ox-LDL), which ultimately leads to cholesterol loading of the macrophages. The present study was undertaken to define epitopes on ox-LDL that are important for lipoprotein binding to macrophages and to ascertain whether ox-LDL can bind to the LDL receptor. Monoclonal antibodies (Mabs) directed against several epitopes along the apolipoprotein B-100 (apo B-100) molecule were used. LDL (300 μg/ml) was oxidized by incubation with 10 μM CuSO₄ for 24 hours. Ox-LDL, as opposed to acetylated LDL (ac-LDL), reacted with Mabs directed against the LDL receptor-binding domains (Mabs B1B6 and B1B3). Similarly, uptake of ox-LDL but not ac-LDL by a murine J774 macrophage-like cell line was inhibited by as much as 40% after using Mab B1B6. The anti-LDL receptor antibody IgG-C7 also inhibited ¹²⁵I-ox-LDL uptake by macrophages by 60%. Chromatography on heparin-Sepharose columns of LDL that was partially oxidized for only 3 hours resulted in two fractions: an unbound fraction with characteristics similar to those of ox-LDL and a bound fraction similar to native LDL. Macrophage degradation of the unbound fraction was inhibited by Mab IgG-C7 and Mab B1B6, which are directed toward the LDL receptor and the LDL receptor-binding domains on apo B-100, respectively. When incubated with three types of macrophages, J774 macrophage cells, mouse peritoneal macrophages, and human monocyte-derived macrophages, excess amounts of unlabeled ox-LDL, like native LDL but unlike ac-LDL, substantially suppressed the uptake and degradation of ¹²⁵I-labeled LDL. Similar studies with fibroblasts, however, revealed that unlabeled LDL but not unlabeled ox-LDL or ac-LDL competed with ¹²⁵I-LDL for cellular uptake and degradation. Mab directed against epitopes on the amino terminus domain of apo B-100 (C14) demonstrates a similar immunoreactivity with ox-LDL and native LDL but a much lower reactivity with ac-LDL. Mab C14 inhibited macrophage degradation of ox-LDL by 34% but had no inhibitory effect on the uptake of native LDL or ac-LDL. Thus, the ac-LDL and LDL receptor-binding domains as well as a unique epitope on the amino terminus of apo B-100 may be involved in macrophage binding of ox-LDL. We conclude that 1) ox-LDL contains epitopes that are also present in native LDL, although considerable changes in these epitopes are suggested and 2) these epitopes are involved in the uptake of ox-LDL by macrophages. (Arterioscler Thromb 1992;12:484-493)

KEY WORDS • oxidized low density lipoproteins • monoclonal antibodies • macrophages

Macrophages, like those resident in the atherosclerotic lesion, poorly take up native low density lipoprotein (LDL), whereas several modified forms of LDL are taken up avidly by the macrophage scavenger receptor. Modification of lipoproteins includes chemical, enzymatic, and cell-mediated changes. During the oxidation process, the apolipoprotein B-100 (apo B-100) of LDL is degraded into fragments that are able to retain the conformations present in native LDL. Oxidized LDL (ox-LDL), whether induced by cells or copper ions, when incubated with macrophages results in an enhanced lipoprotein degradation. Macrophages are known to express several scavenger receptors, and recently it has been demonstrated that ox-LDL and acetylated LDL (ac-LDL) interact with similar as well as with different receptors.

Methods

Lipoprotein Preparation and Characterization

Ox-LDL was prepared from native LDL in the following manner. After 14 hours of fasting, blood was drawn from normolipidemic subjects into Na₂EDTA (1 mM). Plasma lipoproteins were separated by density...
Heparin-Sepharose Chromatography

The degree of oxidation was determined by malondialdehyde (MDA) analysis using the thiobarbituric acid reactive-substances (TBARS) assay. 19 Lipoprotein conjugated dienes were measured at 234 nm. 20 The presence of reactive amino groups was determined with trinitrobenzenesulfonic acid (TNBS) by mixing LDL (50 µg protein) with 1 ml 4% NaHCO₃, pH 8.4, and adding 50 µl 0.1% TNBS. After incubation for 1 hour at 37°C, 100 µl of 1N HCl and 100 µl of 10% sodium dodecyl sulfate (SDS) were added, and the absorbance at 340 nm was measured. TNBS reactivity was expressed as a percentage of the absorbance obtained for nonoxidized LDL.

The protein content of lipoproteins was determined with the Folin phenol reagent by the method of Lowry et al. 21 Cholesterol was analyzed by the ferric chloride assay. 22 Ac-LDL, which was employed as the control in some experiments, was prepared by acetylating LDL with acetic anhydride as described by Goldstein et al. 23 LDL was radioiodinated using the iodine monochloride method, 24 and the specific radioactivity of 125I-LDL was 160–235 cpm/ng. The iodinated LDL was found to be similar to native LDL in its electrophoretic characteristics, TNBS reactivity, and heparin binding. 125I–ox-LDL was prepared by iodination performed before the modification. This procedure did not interfere with the modification, as analyzed by the electrophoretic mobility pattern.

The electrophoretic characteristics of the lipoproteins were determined on cellulose acetate as previously described. 25 Gradient (3–12%) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the lipoproteins was performed under reducing conditions using β-mercaptoethanol. 26 Protein bands were identified by Coomassie blue staining. To determine the relative diameters of lipoproteins, electrophoresis was also performed in some experiments, was prepared as follows.

Immunoreactivity of Lipoproteins

The epitope expression on apo B-100 associated with native LDL, ox-LDL (oxidized for 24 hours at 37°C by incubation with 10 µM CuSO₄), and ac-LDL was determined using a solid-phase competitive-binding radioimmunoassay. 29 LDL, ox-LDL, and ac-LDL were assayed in competitive displacement assays on microtiter plates. The plates were coated with 150 µl of purified Mabs (10 µg/ml) and left overnight, and then the wells were blocked with 3% bovine serum albumin (BSA)-PBS. Serial dilutions of ox-LDL or ac-LDL in 1% (vol/vol) BSA-PBS were added followed by a constant amount of 125I-LDL (500 ng). After incubation for 4 hours at room temperature, the wells were washed three times with PBS, and binding (B) was determined using a gamma counter. The maximum binding (Bₒ) was obtained in wells to which no competing antigen lipoprotein had been added. The results were expressed as the B to Bₒ ratio.

Production of Monoclonal Antibodies

Female BALB/c mice were immunized with human LDL between densities 1.025–1.050 g/ml or purified apo E. Initial injections of antigen were made with Freund's complete adjuvant followed at 3–4 week intervals by injections with Freund's incomplete adjuvant. Antibody titers of mouse sera were tested 5–10 days after the last subcutaneous injection. Splenic cell fusions were performed using SP2/0-Ag14 mouse myeloma cells. Hybridoma culture supernatants were screened for immunoreactivity with their respective immunogens by either solid-phase antigen or direct-binding assays. Positive clones were subcloned, restested for activity, and expanded as ascites tumors in Pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, Mo.)-treated BALB/c mice. Monoclonal immunoglobulin G (IgG) antibodies were purified by ion-exchange chromatography on an FPLC Mono Q column (Bio-Rad).

Anti-human Mabs against apo B-100 and apo E epitopes were produced in mice using intact very low density lipoprotein (VLDL) or LDL and apo E as immunogens as previously described. 30,31 The epitopes for these Mabs had been mapped to the apo B regions of amino acid residues from the amino to the carboxyl terminus: 97–401 (C14), 401–582 (D3D5), 1,878–2,148 (D72), 3,214–3,506 (B1B6), and 3,506–3,635 (B1B3). Immunoreactivity of ox-LDL in comparison with native LDL and ac-LDL was studied.

Cells

The effects of Mabs on the uptake of radiolabeled ox-LDL and LDL by cells were determined. Three different sources of macrophages, the J774A macrophage–like cell line, mouse peritoneal macrophages (MPMs), and human monocyte–derived macrophages (HMDMs), were employed. Similar experiments were performed using human skin fibroblasts. The cells were prepared as follows.

J774A.1 macrophage–like–cell line. Murine macrophage–like J774A.1 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). For each experiment the cells were plated in 16×35-mm dishes at
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rum for 48 hours before the beginning of the experiment

DMEM containing 10% (vol/vol) lipoprotein-deficient se-

The cells were plated at 2X10^4 cells/16-mm dish in

medium containing 10% (vol/vol) autologous serum. After 18

hours of incubation at 37°C in 5% CO_2, nonadherent

containing 10% (vol/vol) autologous serum. After 18

medium, and monocytes were then placed in fresh

cells were removed by two washes with serum-free

by aspiration, and the cells were washed twice with PBS.

Cells were resuspended in RPMI-1640 culture medium

inactivated at 56°C for 30 minutes), penicillin (100

units/ml heparin) was layered over 15 ml Ficoll-Paque in

by Leucosep tubes (Pharmacia Fine Chemicals, Piscat-

23°C. The mixed mononuclear cell band was removed

after intraperitoneal injection of 4% (vol/vol) Brewer

thioglycollate.31 Peritoneal fluid from 10 mice (10–

20×10^6 cells/mouse) was pooled, and the cells were

collected by centrifugation at 1,000g for 10 minutes at

room temperature. The cells were resuspended in

DMEM containing 15% (vol/vol) horse serum (heat

inactivated at 56°C for 30 minutes), penicillin (100

units/ml), and streptomycin (100 mg/ml) to a final

concentration of 10^6 cells/ml. The cell suspension

was dispensed into 16-mm wells in plastic dishes (10^6 cells/

well) and incubated in a humidified incubator (5% CO_2/

95% air at 37°C) for 2 hours. Then each dish was

washed once with 2-3 ml DMEM to remove nonadher-

ent cells and incubated under similar conditions for 18

hours. The experiment was initiated by changing the

medium to DMEM containing 0.2% (vol/vol) BSA

without (control) or with the various lipoproteins. Each

10^6 adherent cells contained 70–100 μg protein.

**Human monocyte-derived macrophages**

Mononuclear cells were isolated by density gradient centrifugation of

blood derived from fasting normolipidemic subjects.32 Twenty milliliters of blood (anticoagulated with 10

units/ml heparin) was layered over 15 ml Ficoll-Paque in Leucosep tubes (Pharmacia Fine Chemicals, Piscata-

away, N.J.) and centrifuged at 1,000g for 15 minutes at

23°C. The mixed mononuclear cell band was removed

by aspiration, and the cells were washed twice with PBS.

Cells were resuspended in RPMI-1640 culture medium

containing 100 units/ml penicillin and 100 μg/ml strep-

tomycin and plated at a density of 5×10^6 monocytes/

16-mm well (Primaria brand, Falcon Labware, Becton

Dickinson & Co., Oxnard, Calif.) in the same medium

containing 10% (vol/vol) autologous serum. After 18

hours of incubation at 37°C in 5% CO_2, nonadherent

cells were removed by two washes with serum-free

medium, and monocytes were then placed in fresh

medium containing 10% (vol/vol) autologous serum. This

medium was changed after 5 days, and monocyte-

derived macrophages were used after 10 days of plating.

Human skin fibroblasts were used after six passages. The

cells were plated at 2×10^4 cells/16-mm dish in

DMEM containing 10% (vol/vol) lipoprotein-deficient se-

rum for 48 hours before the beginning of the experiment.

**Cellular Uptake of Lipoproteins**

Cellular uptake and degradation of the 125I-ox-LDL (obtained after 24 hours of oxidation, unless stated
differently) were determined after 24 hours of cell incubation at 37°C with the labeled lipoproteins, with or

without Mabs, by measuring the trichloroacetic acid–
soluble noniodide radioactivity in the medium.33 At

the end of the incubations the cells were washed with PBS

(three times) and dissolved in 0.1 M NaOH, and aliquots

were taken for determination of cell protein and cell-associ-ated radioactivity. In experiments in which

Mabs were tested for their ability to inhibit lipoprotein

uptake by macrophages, purified Mabs were added to

125I-lipoprotein preparations. These mixtures were

incubated for 1 hour before being applied to the cells for

cellular lipoprotein degradation analysis. MPMs and

HMDMs were also used after upregulation of their

LDL receptors by a 24-hour preincubation with 10% (vol/vol) lipoprotein-deficient serum.

**Results**

**Physicochemical Characterization of Oxidized Low Density Lipoprotein**

The effect of increasing the time of incubation with copper

ions on LDL is shown in Table 1. Conjugated dienes and TBARS increased as the oxidation time

increased, while TNBS reactivity decreased, with the

maximal effect achieved after 4 hours. The composition of ox-LDL changed as time progressed, and after 24

hours the oxidized particle contained 40% less choles-
terol relative to protein. These changes were accompa-
nied by a gradual increase in electrophoretic mobility

of ox-LDL, which had a single, broad homogeneous band

at 24 hours of oxidation. Oxidized LDL migrated as a broad homogeneous particle at all time

intervals, it may contain at certain time points before

completion of its oxidation both modified and nonmod-

ified fractions, as LDL oxidation continued for as long

as 6 hours before reaching a maximal level of TBARS.

**Table 1. Effect of Increased Periods of Oxidation Time on Low Density Lipoprotein Characteristics**

<table>
<thead>
<tr>
<th>Time of oxidation (hr)</th>
<th>Conjugated dienes (nmol/mg protein)</th>
<th>TBARS (nmol MDA/mg protein)</th>
<th>TNBS (% of control)</th>
<th>TC/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>10±3</td>
<td>0.4±0.1</td>
<td>100</td>
<td>1.83±0.11</td>
</tr>
<tr>
<td>0.5</td>
<td>25±7</td>
<td>12.4±0.3</td>
<td>72±9</td>
<td>1.80±0.12</td>
</tr>
<tr>
<td>1</td>
<td>48±13</td>
<td>16.5±0.7</td>
<td>64±7</td>
<td>1.71±0.10</td>
</tr>
<tr>
<td>2</td>
<td>75±17</td>
<td>28.7±9.2</td>
<td>38±4</td>
<td>1.50±0.09</td>
</tr>
<tr>
<td>3</td>
<td>93±12</td>
<td>32.9±1.9</td>
<td>37±4</td>
<td>1.35±0.11</td>
</tr>
<tr>
<td>4</td>
<td>150±29</td>
<td>35.1±3.1</td>
<td>35±6</td>
<td>1.30±0.08</td>
</tr>
<tr>
<td>6</td>
<td>177±31</td>
<td>42.9±4.2</td>
<td>34±5</td>
<td>1.25±0.06</td>
</tr>
<tr>
<td>24</td>
<td>195±22</td>
<td>36.0±4.0</td>
<td>35±5</td>
<td>1.08±0.06</td>
</tr>
</tbody>
</table>

Lipoproteins (300 μg protein/ml) were incubated with copper ions for the time intervals presented in the table. The conjugated dienes, thiobarbituric acid–reactive substances (TBARS), trinitrobenzensulfonic acid (TNBS), and total cholesterol (TC) to protein ratios were measured as described in "Methods." Results are given as mean±SD (n=3).

MDA, malondialdehyde.
Heparin binds to the LDL surface by interacting with the positively charged amino acid residues of apo B-100. On heparin-Sepharose chromatography of ox-LDL, the relative content of the unbound fraction increased and that of the bound lipoprotein decreased as the duration of oxidation was prolonged (Table 2). In a control experiment, ac-LDL failed to bind to heparin (data not shown). The unbound fraction obtained after chromatography of LDL that was oxidized for only 3 hours resembled nonchromatographed LDL that was oxidized for 24 hours, as shown by increased concentration of LDL conjugated dienes (by 50%), decreased TNBS reactivity (by 31%), and a reduced cholesterol to protein ratio (from 1.30 to 1.01) in comparison with the bound fraction. On lipoprotein electrophoresis, the bound and unbound fractions migrated as homogeneous fractions to 1.5±0.2 cm and 2.3±0.4 cm from the origin, respectively, similar to native and ox-LDL, which migrated to 1.4±0.2 cm and 2.4±0.7 cm from the origin, respectively. Fragmentation of the apo B-100 of ox-LDL was also a feature of the unbound fraction, whereas the bound fraction showed no evidence of apo B-100 fragmentation (data not shown).

**Immunoreactivity of Oxidized Low Density Lipoprotein**

The immunoreactivity of LDL, ox-LDL, and ac-LDL was studied using several Mabs directed against different epitopes along the apo B-100 molecule (Figure 2). Solid-phase immunoassays showed that the immunoreactivity of ox-LDL (in comparison with native LDL) with Mabs B1B3 and B1B6 (located on the carboxyl terminus of apo B-100 and related to the LDL receptor) was also a feature of the unbound fraction, whereas the bound fraction showed no evidence of apo B-100 fragmentation (data not shown).

**TABLE 2. Effect of Time of Low Density Lipoprotein Oxidation on Lipoprotein Binding to Heparin**

<table>
<thead>
<tr>
<th>Time of oxidation (hr)</th>
<th>Bound LDL/ bound + unbound LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>96±9</td>
</tr>
<tr>
<td>0.5</td>
<td>56±6</td>
</tr>
<tr>
<td>1</td>
<td>39±6</td>
</tr>
<tr>
<td>3</td>
<td>20±5</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

Low density lipoprotein (LDL) and oxidized LDL were applied to a heparin-Sepharose column (1.5×8 cm). The unbound fraction was eluted with 0.05 M NaCl, while the retained fraction was eluted with 0.8 M NaCl as described in "Methods." To determine the percentage of bound and unbound lipoprotein, a pooled-fraction protein determination was performed, and the percentage of unbound was calculated as \[
\frac{\text{unbound}}{\text{bound + unbound}} \times 100.
\]

Recovery of the lipoprotein protein from the column averaged 83% of the protein loaded. Results are given as mean±SD (n=3).

Effect of Monoclonal Antibodies on Lipoprotein Uptake by Macrophages

The ability of the Mabs to block cellular uptake of ox-LDL was studied in experiments performed on a J774 macrophage cell line. Similar to the immunoreactivity results shown in Figure 2, Mabs B1B6 and C14 (20 μg/ml) inhibited ox-LDL degradation by 40% and 30%, respectively, while Mab D72 had no effect (Figure 3). Cellular association of ox-LDL was similarly affected by all Mabs (Figure 3). The inhibitory effect was dose dependent, with a maximal effect shown at a Mab concentration of 50 μg/ml (ED50 of 30 μg/ml) and Ed50 of 28 μg/ml (ac-LDL) and native LDL were not affected by Mab C14 (20 μg/ml), whereas Mab B1B6 did not affect the degradation of ac-LDL but did reduce cellular degradation of LDL by 66% (data not shown). An anti-apo E Mab (WU-E-7) directed against the receptor-binding domain of apo E did not affect the metabolism of ox-LDL. Similar studies were also performed in the presence and absence of the anti-LDL receptor Mab, IgG-C7. LDL and ox-LDL degradations were inhibited by 80% and 60%, respectively, whereas the degradation of ac-LDL was not affected by IgG-C7. To eliminate native LDL from the ox-LDL preparation, 15.1-ox-LDL that was partially oxidized (by LDL incubation with copper ions for only 3 hours) was applied onto a heparin-Sepharose column, and the radiiodinated, unbound, oxidized fraction (I23I-ub-ox-LDL) was tested for its uptake by macrophages in the absence and presence of Mabs B1B6, C14, and IgG-C7. As shown in Figure 5, all these Mabs (at a concentration of 50 μg/ml) inhibited the degradation of 125I-ub-ox-LDL by J774 macrophages by 70–80%. These results demonstrate that epitopes present on ox-LDL, which are shared by those present on native LDL, were involved in the uptake of ox-LDL by macrophages.

**Competitive Analysis of Cellular Uptake of Oxidized Low Density Lipoprotein**

To further clarify the interaction of ox-LDL with the macrophage LDL receptor, experiments were performed using HMDMs, MPMs, and J774 macrophages as well as human skin fibroblasts. Both cell association decreased by 55% (Table 3). Very little reactivity of ox-LDL with Mab D72, located in the middle of the apo B-100 molecule (Figure 2), and with Mab D3D5, located toward the amino terminus domain of apo B-100, was noted (Table 3). The immunoreactivity curves illustrating competition toward Mab C14 (located on the amino terminal domain of apo B-100) with both LDL and ox-LDL were similar (Figure 2). LDL diluted in PBS and incubated for 24 hours in the absence of copper ions demonstrated the same immunoreactivity as native LDL (data not shown). Ac-LDL demonstrated very low immunoreactivity with Mabs B1B6, B1B3, and C14 (50% effective dose [ED50] of 30 μg/ml), macrophage C14 (35 μg/ml) and also reacted very poorly with Mab D72 (Table 3). However, with Mab D3D5 almost the same ED50 was obtained with ac-LDL as with native LDL (Table 3). These results show that ox-LDL interacted with at least three different Mabs, which reacted with epitopes located on the amino terminus and the carboxyl terminus domains of native apo B-100.

**Figure 1. Effect of increased time of in vitro low density lipoprotein oxidation on lipoprotein electrophoretic mobility on cellulose acetate.** Lipids were stained with oil red O. Lanes 1–6 represent progressive times of oxidation for 0, 1, 2, 4, 6, and 24 hours, respectively.
and degradation of $^{125}$I-LDL and $^{125}$I-ox-LDL were studied. Increasing concentrations of unlabeled LDL, ox-LDL, and ac-LDL were tested for their ability to inhibit cellular uptake of the $^{125}$I-labeled lipoproteins. The results show that in J774 macrophages but not in fibroblasts, unlabeled ox-LDL significantly competed with $^{125}$I-LDL for cellular degradation (Figures 6A and 6B). Similarly, in J774 macrophages the degradation of 10 $\mu$g/ml of $^{125}$I-LDL in the presence of 100 and 200 $\mu$g/ml of unlabeled ub-ox-LDL was reduced by 35% and 45%, respectively (data not shown). In both fibroblasts and J774 macrophages, unlabeled LDL but not ac-LDL competed with $^{125}$I-LDL for cellular uptake (Figures 6A and 6B).

When $^{125}$I-ox-LDL was used, competition experiments (Figures 6C and 6D) demonstrated that unlabeled LDL inhibited $^{125}$I-ox-LDL degradation by as much as 30% in comparison with 75% and 46% inhibition by unlabeled ox-LDL and ac-LDL, respectively (Figure 6C). In fibroblasts, however, uptake of $^{125}$I-ox-LDL could not be found (Figure 6D). Similar results were obtained for the cellular association of $^{125}$I-labeled lipoproteins in all competition studies performed either at 37°C or at 4°C (data not shown).

To determine whether the ability of ox-LDL to compete with LDL for uptake by murine J774 macrophages (but not by fibroblasts) was unique for the J774 macrophages or applied to macrophages in general, we performed similar competition experiments using MPMs and HMDMs (Figure 7). In both MPMs and HMDMs, ox-LDL effectively competed with $^{125}$I-LDL for cellular degradation (Figures 7A and 7B) and for cellular association (data not shown). $^{125}$I-LDL cellular uptake by murine J774 macrophages.
degradation was reduced by a 50-fold concentration of unlabeled ox-LDL by as much as 50% and 39% in MPMs and HMDMs, respectively. For comparison unlabeled LDL reduced $^{125}$I-LDL degradation by 70%, whereas unlabeled ac-LDL had no effect on $^{125}$I-LDL degradation in all the cells (Figures 6A, 6B, 7A, and 7B). When upregulated macrophages were used (by 24 hours of cell incubation with 10% [vol/vol] lipoprotein-deficient serum), $^{125}$I-LDL (10 μg/ml) cellular degradation rates were increased to 1,143±119 and 611±73 ng LDL protein/mg cell protein in MPMs and HMDMs, respectively, in comparison with values of 719±33 and 107±29 (n=3) in nonupregulated MPMs and HMDMs, respectively. Like the results shown for the cells that were not upregulated (Figures 7A and 7B), in the upregulated MPMs and HMDMs cellular $^{125}$I-LDL degradation was reduced by 250 μg unlabeled LDL protein/ml, to 601±33 and 303±27 ng/mg cell protein, respectively. Similarly, unlabeled ox-LDL (250 μg protein/ml) reduced the degradation of $^{125}$I-LDL to 697±31 and 351±19 ng/mg cell protein in the upregulated MPMs and HMDMs, respectively. On using $^{125}$I-ox-LDL, unlabeled LDL competed poorly for degradation by MPMs and HMDMs in comparison with a 45% reduction in $^{125}$I-ox-LDL degradation by a 50-fold concentration of unlabeled ox-LDL (Figures 7C and 7D). The addition of a 50-fold excess of unlabeled LDL, together with the excess ox-LDL, further inhibited macrophage degradation of $^{125}$I-ox-LDL by 82% and 85% in MPMs and HMDMs, respectively (data not shown). Unlabeled ac-LDL, while demonstrating greater competition than unlabeled LDL (as much as a 30% reduction in $^{125}$I-ox-LDL degradation), still competed less than unlabeled ox-LDL (Figures 7C and 7D). Similar results were
FIGURE 5. Bar graph showing effect of monoclonal antibodies (Mabs) on degradation of the oxidized low density lipoprotein (Ox-LDL) subfraction by J774 macrophages. $^{125}$I-ox-LDL (that was partially oxidized by incubation with 10 μM CuSO$_4$ for only 3 hours) was separated on a heparin-Sepharose column, and the radioiodinated, unbound (UB) lipoprotein was incubated with the cells as described in "Methods." A constant amount of Mabs (50 μg/ml) was tested for their ability to inhibit cellular degradation of $^{125}$I-ub-ox-LDL. Results are given as mean±SD (n=3).

found for cellular associations of the labeled lipoproteins (data not shown).

Discussion

Ox-LDL was previously shown to bind to the ac-LDL receptor. The present study indicates that the interaction of ox-LDL with macrophages resides in at least three domains identified by specific Mabs, which react with recognized epitopes on apo B-100. As shown by the immunoassays and by Mab inhibition of ox-LDL uptake by macrophages, these epitopes on ox-LDL apo B-100 include domains on the amino terminus of the apolipoprotein and also toward the carboxyl terminus domain. The latter epitopes correspond to the classical LDL receptor-binding domains. These domains are specific for ox-LDL, as ac-LDL, another modified form of lipoprotein that is a ligand for the macrophage scavenger receptor, shows no interaction with these Mabs, which also fail in turn to inhibit macrophage uptake of ac-LDL. Thus, despite LDL oxidation the recognized LDL receptor-binding domain is retained by ox-LDL, and an additional domain on the amino terminus appears to unravel. The binding of ox-LDL to the cells may be dependent on the presence of both receptors, acting in concert, for the uptake of ox-LDL. It is also possible that the epitopes on the carboxyl and amino termini domains on ox-LDL are in close approximation to each other and may recognize the same receptor on the macrophage.

The immunoreactivity of ox-LDL with Mabs B1B6 and B1B3, which are directed against the LDL recep-

FIGURE 6. Competition curves for J774 macrophages and human skin fibroblasts for degradation of $^{125}$I-low density lipoprotein (LDL) (panels A and B) and $^{125}$I-oxidized (Ox)-LDL (panels C and D) by unlabeled LDL, ox-LDL after 24 hours of oxidation, and acetylated (Ac)-LDL. Competition studies were performed using a constant amount of $^{125}$I-LDL or $^{125}$I-ox-LDL (10 μg/ml) and increasing concentrations of unlabeled lipoproteins. Results are mean±SD (n=3).
FIGURE 7. Competition curves for mouse peritoneal macrophages (MPM) and human monocyte-derived macrophages (HMDM) for degradation of 10 μg protein/ml of 125I-low density lipoprotein (LDL) (panels A and B) and 125I-oxidized (Ox)-LDL (panels C and D) by unlabeled LDL, αx-LDL, and acetylated (Ac)-LDL. 125I-LDL degradation in the absence of competitor was 719 and 207 ng/mg cell protein in MPMs and HMDMs, respectively. Control values for 125I-ox-LDL degradation were 3,020 and 1,005 ng/mg cell protein in MPMs and HMDMs, respectively. In cell-free control dishes, the degradation of 125I-LDL was less than 7% of the cellular degradation rate. Results are mean±SD of three experiments.

tor–binding domains on apo B-100, is 50% less than that of native LDL, suggesting considerable changes in the epitopes on ox-LDL in comparison with native LDL.

Determination of the relative purity of the ox-LDL preparation would be important, as contamination with native LDL particles could explain the results. Heparin binds to the positively charged amino acid residues of apo B-100, and at least four unique heparin-binding domains have been identified on apo B-100. Native LDL but not ox-LDL possesses these heparin-binding characteristics. These heparin-binding sites were employed to separate the oxidized particles (ub fractions) of ox-LDL from the nonoxidized retained particles. The eluted lipoprotein (ub fraction) was free of native LDL, and this lipoprotein (ub-ox-LDL) still contained the LDL receptor–binding domain.

Although several studies have demonstrated the lack of competition of unlabeled native LDL with 123I-ox-LDL for cellular uptake and degradation by macrophages, the competition of unlabeled ox-LDL with 123I-ox-LDL for macrophage uptake may be related to the increased binding capacity of ox-LDL via multiple binding domains to the macrophage receptors in comparison with the low-affinity binding of native LDL to a single LDL receptor type on macrophages. Similarly, because of its low affinity for the LDL receptor on mouse macrophages, LDL does not compete effectively with 123I-β-VLDL or apo E-containing lipoproteins for receptor binding. Because the LDL receptor could be fully downregulated after the uptake of ox-LDL by the scavenger receptor, the importance of the uptake of ox-LDL by the LDL receptor is not clear. It was reported recently that on SDS-PAGE, Western blot analysis, and enzyme-linked immunosorbent assay, ox-LDL revealed a positive interaction with Mab MB47 (which was shown to bind to the LDL receptor–binding domains). It should be taken into consideration that macrophages possess Fc receptors (which bind antibodies) and can also bind to the Mab that binds to ox-LDL. Thus, the macrophage may take up ox-LDL by phago-
cytosis. Because Mabs, as opposed to polyclonal antibodies, bind only to a single epitope, they may not be affected dramatically by the Fc receptor.

Interestingly, whereas ox-LDL was shown to bind to the macrophage LDL receptor and to the scavenger receptor, binding to the classical LDL receptor on human fibroblasts did not take place. The differential uptake of ox-LDL by the LDL receptor on macrophages as opposed to fibroblasts probably lies in the ubiquitous nature of the LDL receptor itself. LDL binds to MPMs with a 10-fold lower affinity than it does to cultured human fibroblasts. Koo et al. reported recently that the LDL receptor on MPMs differs from the same receptors on HMDMs and fibroblasts with regard to its molecular size, binding affinity to LDL, and relative insensitivity to downregulation. Using specific antibodies against the rat liver LDL receptor, Ellsworth et al. have shown that MPMs, J774 macrophages, and a mouse fibroblast cell line (3T3) all possess LDL receptors with a molecular weight about 5,000 d less than that of the rat liver LDL receptor. Hoeg et al. compared the properties of the LDL receptor in human liver cells to that in human skin fibroblasts and found striking differences relating to the number and affinity of the receptors expressed by these two tissues. The fibroblast membrane had nearly 10-fold more receptors than the hepatic membrane, and the binding affinity was increased nearly fourfold. Both MPMs and HMDMs express mainly the scavenger receptor (and to a lesser extent, the LDL receptor) and as a consequence may be less than optimal for the study of the uptake of ox-LDL through the LDL receptor. The J774A.1 macrophage-like cell line, however, exhibits a large number of LDL receptors, and thus, these macrophages are more suitable cells to study ox-LDL uptake via the LDL receptor. Both HMDMs and MPMs, when incubated with lipoprotein-deficient serum, upregulate the LDL-binding sites. The results with the upregulated cells were similar to those obtained with the cells that were not upregulated. Ox-LDL was shown to bind to the macrophage ac-LDL receptor as well as to another receptor that is unique to ox-LDL. The latter may be related to our finding of a binding domain on the ox-LDL apo B-100 amino terminus (related to Mab C14), which did not react with the ac-LDL receptor. Sparrow et al. suggested that a single receptor could have multiple binding sites, with several being available for ox-LDL. It is also possible that macrophages possess a collection of scavenger receptors with a similar basic structure and variable binding sites.

Recently, it was shown that one class of scavenger receptor recognizes ac-LDL, ox-LDL, and acidic phospholipids, whereas another type of scavenger receptor recognizes both acidic phospholipids and ox-LDL but not ac-LDL. Ox-LDL possesses multiple binding domains, and the domains on the amino residue may express similar but not identical epitopes with ac-LDL. Ox-LDL reacts with Mab C14, located on the amino terminus of apo B-100, but not with Mab D3DS5, which is located close to Mab C14. Ac-LDL reacts with Mab D3DS5 but not with Mab C14. Two types of macrophage scavenger receptors that recognize ac-LDL have recently been elucidated, but it is not clear whether they also bind ox-LDL. Macrophage uptake of LDL is dependent on the type of lipoprotein modification, such as triglyceride, cholesteryl ester, and phospholipid hydrolysis or oxidation of the LDL fatty acids or cholesterol moieties. The existence of multiple macrophage-binding sites on ox-LDL, if operable in vivo, can affect the rates of cellular uptake and processing of lipoproteins during atherogenesis.

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