Human Monocyte–Derived Macrophages Express an ≈120-kD Ox-LDL Binding Protein With Strong Identity to CD68

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Abstract A protein that specifically binds oxidized LDL (Ox-LDL) has recently been characterized in mouse peritoneal macrophages and identified as macrosialin, a protein with a molecular weight of 95 kD. First, the present work shows that human monocyte–derived macrophages express a membrane protein with a molecular weight of ≈120 kD that selectively binds Ox-LDL. Second, we tested whether this ≈120-kD Ox-LDL binding protein had any relation to CD68, the human homologue of macrosialin. The following evidence was obtained to support the role of CD68 as an Ox-LDL binding protein: (1) Ligand blots with Ox-LDL and Western blots with Ki-M6, an anti–human CD68 monoclonal antibody, revealed a single band with a molecular weight of ≈120 kD under reducing and nonreducing condition. (2) The expression patterns of the ≈120-kD Ox-LDL binding membrane protein and of CD68 paralleled each other during monocyte/macrophage differentiation. (3) Digestion with *N*-glycosidase F demonstrated that both CD68 and the Ox-LDL binding protein are glycoproteins; both showed a similar shift of ≈18 kD in apparent molecular weight. (4) CD68, probed with monoclonal antibody Ki-M6, and the ≈120-kD Ox-LDL binding protein were coprecipitated with EBM11, another anti-CD68 antibody. About 5000 molecules of CD68 are expressed on the cell surface of human macrophages. Ligation of ^{125}I–Ki-M6 to cells leads to its internalization and degradation. This capacity would be sufficient to allow for the specific uptake and degradation of Ox-LDL. Taken together, these data support a role for CD68 as a specific Ox-LDL binding protein in human monocyte–derived macrophages. (***Arterioscler Thromb Vasc Biol.* 1997;17:3107-3116.**)

Key Words • atherosclerosis • macrophages • oxidized LDL • CD68

There is strong evidence that oxidative modification of LDL plays a critical role in atherogenesis.[1] Oxidation converts LDL into a particle with new biological properties, referred to as Ox-LDL, which can contribute to the progression of atherosclerosis in various ways. Ox-LDL can induce the transformation of macrophages into lipid-laden foam cells.[2] Ox-LDL is a chemotactic agent for monocytes and reduces the motility of macrophages,[3] which then become resident in the arterial intima. Ox-LDL is cytotoxic to cells[4] by damaging the endothelium, which thereby favors platelet adhesion.[5] In advanced atherosclerotic lesions, the cytotoxicity of Ox-LDL may even result in irreversible cell necrosis.[5] Oxidation of LDL can occur in vivo,[6] and investigations were initiated to identify those receptors that might interact with Ox-LDL and mediate their biological properties.

First, scavenger receptor type A, formerly the Ac-LDL receptor, was described at the molecular level and characterized as a receptor that binds and promotes internalization of Ox-LDL.[7] This receptor can account for 30% to 70% of the total uptake of Ox-LDL by macrophages.[8-11] Other membrane proteins have been described in macrophages that could also possibly interact with Ox-LDL, such as the Fc-γRII-B2 receptor,[12] CD36 and its mouse homologue,[13] the closely related SR-B1,[14] and mouse macrosialin.[15] The role of Fc-γRII-B2 in the uptake of Ox-LDL is unclear, since monoclonal antibodies against Fc-γRII-B2 do not block internalization of Ox-LDL by macrophages.[13] SR-B1 was recently identified as a receptor for HDL, thus allowing for the selective uptake of HDL cholesteryl esters.[16] Several studies have examined the contribution of CD36 to Ox-LDL binding and uptake in macrophages. ^{125}I–Ox-LDL binding and degradation are partially blocked by monoclonal antibodies against CD36 in human monocyte–derived macrophages[17] and THP-1 cells.[13] No measurable internalization or degradation of Ox-LDL could be detected in CHO cells after stable transfection with human CD36 cDNA, although the binding of Ox-LDL to these cells was blocked by a monoclonal antibody against CD36.[18] Specific Ox-LDL binding to monocyte-derived macrophages from CD36-deficient subjects is only ≈60% that of control.[19] Importantly, CD36 has been resolved from the Ox-LDL binding activity during purification of a 94- to 97-kD Ox-LDL binding protein from mouse macrophages.[18] The peptide sequence analysis of tryptic fragments from this 94- to 97-kD protein revealed that it is identical to mouse macrosialin.[15] The latter was recently characterized as a receptor for Ox-LDL in mouse macrophages.[20] On the basis of its amino acid sequence, mouse macrosialin is 72% identical and 81% similar to human CD68.[21] CD68,

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Selected Abbreviations and Acronyms

Ac-LDL = acetylated LDL
BCA = bicinchoninic acid
CHO = Chinese hamster ovary
HRP = horseradish peroxidase
Ox-LDL = oxidized LDL
PAGE = polyacrylamide gel electrophoresis
SR-B1 = scavenger receptor B1
TCA = trichloroacetic acid

with a molecular weight of ≈120 kD, is an integral membrane glycoprotein predominantly expressed in tissue macrophages and some tumor cells.[22] CD68 has a polypeptide backbone of ≈35 kD. The largest contribution to the mass of mature CD68 comes from O-linked and N-linked glycans.[23] CD68 is routinely used as a macrophage marker in immunohistochemistry, but its exact function remains to be established.

In the present work we identified an ≈120-kD membrane protein from human monocyte–derived macrophages that specifically binds Ox-LDL and shares characteristics with CD68. This protein was defined as a selective Ox-LDL binding protein, since it does not bind Ac-LDL in contrast to the class A scavenger receptor, which does bind both types of modified lipoproteins. Immunoprecipitation from cell lysates with EBM11, an anti-CD68 monoclonal antibody, allowed for the isolation of an ≈120-kD protein that binds Ox-LDL and is recognized by Ki-M6, another monoclonal antibody raised against CD68. Together our data support a role for CD68 as an Ox-LDL binding protein in human monocyte–derived macrophages. In addition, CD68 was shown to be expressed on the cell surface of human macrophages to such an extent that it could account for the specific uptake of Ox-LDL by macrophages.

Methods

Chemicals

Ficoll-Paque, lentil lectin Sepharose 4B, and PD-10 columns were purchased from Pharmacia Biotech. M199 culture medium, penicillin, streptomycin, PBS, and pyruvate were from GIBCO BRL, Life Technologies Ltd. [^{125}I]NaI (carrier-free in 0.1 mol/L NaOH) and the enhanced chemiluminescence (ECL) detection kit were purchased from Amersham International. XAR-5 film was from Kodak. Human serum for mixed lymphocyte cultures, human serum albumin (fraction V), and PMSF were purchased from Sigma Chemical Co. Nitrocellulose membranes (Optitran BA-S 85) were from Schleicher & Schuell. Nonfat (skim) milk powder, BHT, Nonidet P-40, methyl-α-D-mannopyranoside, aprotinin from bovine lung, and leupeptin were purchased from Fluka. *N*-Glycosidase F (EC 3.2.2.18; 3.5.1.52, protease-, sialidase-, endoglycosidase F–, β-galactosidase–, β-glucosidase–, α-, and β-mannosidase–, β-*N*-acetylhexosaminidase–, and α-L-fucosidase–free), *O*-glycosidase (EC 3.2.1.97), *n*-octyl-β-D-glucoside, and DTT were purchased from Boehringer Mannheim. BCA reagent, CHAPS, and IodoGen were from Pierce. All other reagents and solvents of the highest purity available were purchased from Fluka or Sigma.

Antibodies

Mouse IgG1 and goat anti-mouse IgG–agarose were purchased from Sigma. Human IgG1 was obtained from The Binding Site. Mouse anti-human CD68 monoclonal antibodies Ki-M6 and Ki-M7 (both IgG1) were from Biomedicals. Mouse anti-human CD68 monoclonal antibodies EBM11 (IgG1) and PG-M1 (IgG3) were from Dako. Polyclonal rabbit anti-human apolipoprotein B100 antibody was from Calbiochem. HRP-conjugated goat anti-rabbit antibody and HRP-conjugated goat anti-mouse antibody were from Bio-Rad Laboratories. Mouse anti-human CD36 monoclonal antibody FA6-152, mouse anti-human CD16 monoclonal antibody 3G8, and mouse anti-human CD32 monoclonal antibody 2E1 were obtained from Biodesign International.

Isolation of Human LDL, Iodination, and Chemical Modification

Plasma from the blood of healthy volunteers collected into tubes containing EDTA were obtained at the Blutspendezentrum in Basel and stored at −20°C. LDL was isolated by sequential ultracentrifugation at a density of $1.019<d<1.063$ g/mL.[24] Prior to chemical modification, LDL was iodinated with ^{125}I at a specific activity of 250 to 400 counts per minute per nanogram apolipoprotein by using the ICl procedure.[25,26] LDL and ^{125}I-LDL were acetylated by repeated additions of acetic anhydride.[27] LDL and ^{125}I-LDL were oxidized in the presence of 10 μmol/L Cu^{2+} as described previously.[28] Modification of LDL and ^{125}I-LDL was routinely verified by agarose electrophoresis on the Paragon Electrophoretic System (Beckman), and R_f values were 0.12±0.01 (n=4), 0.44±0.01 (n=4), 0.48±0.02 (n=5), 0.41±0.02 (n=3), and 0.46±0.02 (n=3) for LDL, Ac-LDL, Ox-LDL, ^{125}I–Ac-LDL, and ^{125}I–Ox-LDL, respectively. All lipoprotein preparations were sterilized by filtration through a 0.45-μm membrane, stored at 4°C in the presence of 40 μmol/L BHT, and used within 3 weeks. Native LDL was already protected against oxidation with BHT during the isolation and was used within 1 week after preparation.

Iodination of Monoclonal Antibody Ki-M6

Albumin-free Ki-M6 (156 μg solubilized in 50 μL PBS without Ca^{2+} or Mg^{2+} [PBS^{--}]) was iodinated in an IodoGen-coated tube during an incubation of 5 minutes on ice. Iodinated Ki-M6 and unbound ^{125}I were separated by gel filtration over a PD-10 column, which had been preequilibrated with ≈70 mL PBS^{--} containing 10 mmol/L NaI and 0.05% NaN_3. Radiolabeled Ki-M6 was eluted with PBS^{--}/0.05% NaN_3 and concentrated using Amicon-30 filter units. The specific activity of ^{125}I-Ki-M6 was 4800 cpm/ng protein, whereas 1.1% of the radioactivity was TCA soluble.

Culture of Human Macrophages

Fresh, EDTA-treated leukocyte concentrates from the blood of healthy volunteers (≈40 mL) were obtained at the Blutspendezentrum in Basel and diluted with 40 mL PBS^{--} containing 0.2% BSA, pH 7.4. Mononuclear cells were isolated by Ficoll-Paque centrifugation.[29,30] Monocytes were seeded at a density of $\approx 5\times10^5$ cells in 48-well plates (Costar) in M199 medium containing 10% human serum. After washing out the cell debris and lymphocytes, the remaining adherent cell population consisted of >95% monocytes as judged by nonspecific esterase staining.[30] Long-term culture of monocytes resulted in extensive spreading of cells, increased phagocytic activity, and elevated scavenger receptor expression (especially of scavenger receptor type II), which are features typical of mature macrophages.[30,31] Human monocyte–derived macrophages were used after 7 to 8 days of differentiation and are referred to as human macrophages.

Detergent Solubilization of Human Macrophages

The following procedure was performed at 4°C with precooled solutions. About 1 to 5×10^7 cells were washed in PBS with Ca^{2+} and Mg^{2+} (PBS^{++}). Cellular proteins were solubilized on ice for 1 to 2 hours in 300 to 500 μL of 1% Triton X-100 buffered with 20 mmol/L HEPES (pH 7.5) containing 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2 \cdot 6H_2O$, 1 mmol/L EGTA, 10% glycerol, and a protease inhibitor mixture (1 mmol/L EDTA, 10 μg/mL aprotinin, and 10 μg/mL leupep-

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tin). Insoluble material was removed by centrifugation in an Eppendorf centrifuge at 15 000g for 5 minutes. Protein content of the preparation was measured using the BCA reagent.

Preparation of Macrophage Membranes and Solubilization

For radioactive ligand blotting experiments, cell membranes were first prepared from human macrophages cultured in 10-cm-diameter dishes and then solubilized in 40 mmol/L *n*-octyl-β-D-glucoside.[32]

Ligand Blotting

Solubilized proteins were separated on a 7.5% polyacrylamide gel under nonreducing conditions as described by Laemmli.[33] Proteins were then electroblotted at 150 mA for 3 hours to nitrocellulose membranes by using the Mini Trans-Blot Cell System (Bio-Rad). Cooling units of the system were changed every hour. To prevent nonspecific binding of the ligands, the nitrocellulose membranes were incubated overnight at 4°C in a solution containing 50 mmol/L Tris, 5% (wt/vol) skim milk, 90 mmol/L NaCl, 2 mmol/L $CaCl_2$, and 0.05% NaN_3, pH 7.4. Ligand blotting was performed with 10 μg/mL unlabeled or 7.5 μg/mL iodinated LDL, Ac-LDL, or Ox-LDL in the absence or presence of different competitors in incubation buffer containing 50 mmol/L Tris, 1% (wt/vol) skim milk, 90 mmol/L NaCl, and 2 mmol/L $CaCl_2$. After incubation at room temperature for 90 minutes, the nitrocellulose membranes were washed 4× for 10 minutes each in the incubation buffer and again 4× for 10 minutes each in PBS^{--}/1% BSA, pH 7.4. ^{125}I-LDL, ^{125}I–Ac-LDL or, ^{125}I–Ox-LDL bound to the nitrocellulose was detected by autoradiography on XAR-5 film after 7 to 14 days of exposure.

In nonradioactive ligand blots LDL, Ac-LDL, or Ox-LDL was probed with rabbit polyclonal anti–apolipoprotein B100 antiserum (100-fold dilution in PBS^{--}/1% BSA) at 25°C for 1.5 hours. Next, a secondary HRP-conjugated goat anti-rabbit IgG antibody (7500-fold dilution in PBS^{--}/1% BSA) was coupled to the primary antibody at 25°C for 1 hour. Detection of the secondary antibody was performed by enhanced chemiluminescence (ECL kit). In separate control dot-blot experiments, the polyclonal anti–apolipoprotein B100 showed identical cross-reactivity toward LDL, Ac-LDL, and Ox-LDL.

Western Blotting

Separately, strips of nitrocellulose membranes corresponding to those prepared for ligand blotting were used for Western blotting. After an overnight incubation at 4°C in blocking buffer containing PBS^{--}/1% BSA and 0.05% NaN_3, the strips were incubated at room temperature with 1 μg/mL Ki-M6 in PBS^{--}/1% BSA for 1 to 2 hours. After extensive washing in PBS^{--}/1% BSA containing 0.05% Tween-20, bands were detected using HRP-conjugated goat anti-mouse IgG antiserum (7500-fold dilution in PBS^{--}/1% BSA) and enhanced chemiluminescence.

Glycosidase Digestion

Twenty micrograms of protein from cell lysates of human macrophages containing the protease inhibitor cocktail were mixed with buffers and reagents at the following final concentrations: 0.2 mol/L sodium phosphate buffer, pH 7.4; 2 mmol/L EDTA; and 6 U/mL *N*-glycosidase F or 50 mU/mL *O*-glycosidase in a total volume of 30 μL. Digestions were performed at 37°C for 2 hours and terminated by adding an equal volume of concentrated Laemmli buffer. The samples were heated at 95°C for 10 minutes and directly applied onto a 7.5% polyacrylamide gel. Control digestions were performed in the absence of glycosidases.

Immunoprecipitation

Immunoprecipitations with Ki-M6 were carried out essentially as previously described[34] with some modifications as follows. All buffers contained 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 2 mmol/L PMSF. Cell lysates were prepared in 20 mmol/L Tris, 0.5% Triton X-100, 2% Nonidet P-40, 1 mmol/L $CaCl_2$, and 1 mmol/L $MnCl_2$, pH 7.5. The solubilized proteins (1.45 mg/mL in 2 mL) were applied onto a 1-mL packed lentil lectin Sepharose column. After additional washes, the specifically bound material was eluted with 1 mol/L α-methyl mannoside, 20 mmol/L Tris, and 1% Nonidet P-40, pH 7.5, and dialyzed overnight in 20 mmol/L Tris and 0.5% Nonidet P-40, pH 7.5, at 4°C. Prior to the immunoprecipitation, the dialyzed, prepurified lysates were precleared twice with goat anti-mouse IgG–agarose at 4°C for 45 minutes. The immunoprecipitation was carried out at 4°C by addition of 15 μg EBM11 to 100 μL of precleared lysate. After an incubation of 2 hours, 20 μL of packed goat anti-mouse IgG–agarose was added, and the incubation proceeded for another hour. Immunoprecipitates were washed in 20 mmol/L Tris containing 1% Nonidet P-40, pH 7.4 (5× 5 minutes each in a total volume of 1.6 mL washing buffer), boiled in DTT-containing sample buffer prior to SDS-PAGE, and electroblotted onto nitrocellulose membranes. Supernatants were concentrated four-fold by using Microcon-30 microconcentrators (Amicon) prior to SDS-PAGE and electroblotting. Control immunoprecipitation experiments were performed in the absence of primary antibody EBM11 and processed further as above.

Binding of ^{125}I–Ki-M6 and ^{125}I–Ox-LDL to Human Macrophages

Human macrophages were incubated with 300 μL of medium M199–2% human serum albumin (pH 7.4) containing increasing concentrations of ^{125}I-Ki-M6 or ^{125}I-Ox-LDL. After 4 hours of incubation at 4°C, unbound radioactivity was removed by two washes with ice-cold PBS^{++} containing 0.2% BSA, followed by two washes with ice-cold PBS^{++}, pH 7.4. Bound radioactivity was measured as previously described.[30] Nonspecific binding was defined as the binding of radiolabeled ligand in the presence of excess unlabeled Ki-M6 (1 μmol/L) or excess unlabeled Ox-LDL (200 μg/mL=67 nmol/L). The affinity constant (K_d) and the maximal binding capacity (B_{max}) were calculated by direct-fitting analysis using LIGAND software.[35]

Cell Association and Degradation of ^{125}I–Ki-M6 and ^{125}I–Ox-LDL in Human Macrophages

In competition experiments, 4 nmol/L ^{125}I–Ki-M6 was incubated in medium M199–2% human serum albumin at 37°C for 4 hours. Cell association and degradation were assessed as described.[30,36] Degradation was defined as the TCA-soluble, nonchloroform-extractable radioactivity of the medium. Nonspecific cell association and degradation of Ki-M6 was defined as the association or degradation of the radiolabeled ligand measured in the presence of 20 nmol/L unlabeled Ki-M6. Cell association and degradation of ^{125}I-Ox-LDL (1.7 nmol/L=5 μg/mL) were measured as described.[30,36] Nonspecific cell association and degradation of Ox-LDL was defined as the association or degradation of the radiolabeled ligand measured in the presence of 33 nmol/L (=100 μg/mL) unlabeled Ox-LDL. Control incubations were performed in wells containing no cells, and the corresponding values were subtracted from all experimental values.

Results

Specific Interaction of Ox-LDL With an ≈120-kD Membrane Protein From Human Macrophages

To characterize proteins that bind Ox-LDL in human macrophages, Ox-LDL ligand blotting experiments were

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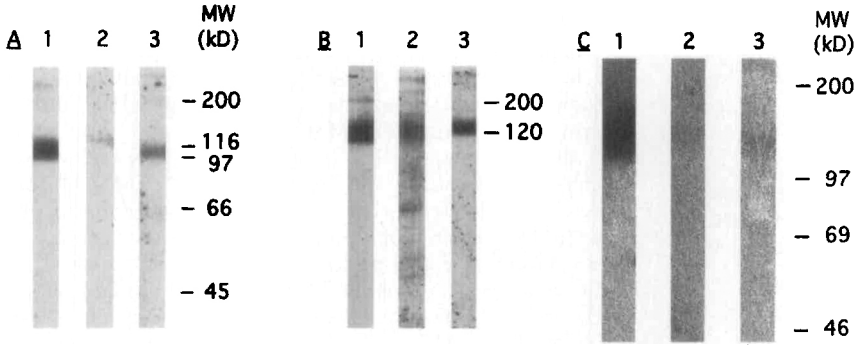

FIG 1. Ligand blots with unlabeled and radiolabeled Ox-LDL, Ac-LDL, and LDL. A, Cell lysates from human macrophages cultured for 8 days were subjected to SDS-PAGE (20 μg protein per lane) followed by electroblotting onto nitrocellulose membranes. Nitrocellulose membrane strips were incubated with 10 μg/mL lipoprotein (lane 1, Ox-LDL; lane 2, Ac-LDL, and lane 3, LDL) and then with anti-apolipoprotein B100 antiserum. Detection of bands was performed by ECL following incubation with the corresponding HRP-conjugated secondary antibody. B, Ox-LDL ligand blotting with cell lysates from human macrophages (15 μg protein per lane) prepared from three different donors. Experiment was performed as in A. C, Membrane lysates from human macrophages cultured for 7 days were subjected to SDS-PAGE (140 μg protein per lane) followed by electroblotting onto nitrocellulose membranes. Nitrocellulose membrane strips were incubated with 7.5 μg/mL ^{125}I-labeled lipoproteins (lane 1; ^{125}I–Ox-LDL; lane 2, ^{125}I–Ac-LDL; and lane 3; ^{125}I-LDL). Bound radioactivity was visualized by autoradiography, and the ≈120-kD region was excised for counting. Background radioactivity was determined by counting an excision of a blot sample of equal size from the 46- to 69-kD region and subtracted from all experimental values.

performed with cell lysates from human monocyte–derived macrophages. Using an ECL detection method, we found that Ox-LDL binds to a protein with a molecular weight of ≈120 kD (Fig 1A). Ac-LDL and LDL showed weak binding to this protein (Fig 1A). The ECL detection method was about 10 times more sensitive than direct radioactive Ox-LDL ligand blotting, thus also allowing for the detection of a 220- to 240-kD protein that binds Ox-LDL (Fig 1B), which may correspond to a dimer of the ≈120-kD protein or to the scavenger receptor type A.[37,38] A 220- to 240-kD Ox-LDL binding protein could be detected in 14 of 20 ligand-blot experiments performed with cell lysates from different macrophage preparations. The apparent relative contribution of the ≈120-kD protein versus the 220- to 240-kD protein was not affected when other detergents, such as CHAPS, Triton X-100, and *n*-octyl-β-D-glucoside, were used to solubilize the macrophages.

CD36 has been proposed as a binding protein for Ox-LDL in rat and mouse macrophages.[13,17] Hence, Western blots with monoclonal mouse anti-human CD36 antibody FA6-152 followed by ECL detection revealed a protein band with a molecular weight of ≈90 kD (data not shown), ie, significantly lower than the ≈120-kD Ox-LDL binding protein, suggesting that the ≈120-kD Ox-LDL binding protein cannot be CD36. Interestingly, it was found that COS cells transfected with CD36 exhibited an Ox-LDL binding activity that was inhibited by monoclonal antibody FA6-152.[39] This discrepancy with our data might be due to poor recognition of Ox-LDL by CD36 in ligand blotting, as opposed to cell binding experiments.

To further characterize the ≈120-kD protein, ligand blotting experiments were performed with ^{125}I–Ox-LDL, ^{125}I–Ac-LDL, and ^{125}I-LDL (Fig 1C). ^{125}I–Ox-LDL exhibited a binding activity of 90 to 95 ng apolipoprotein per milligram protein to the ≈120-kD protein (lane 1), whereas the binding of ^{125}I–Ac-LDL (lane 2) and ^{125}I-LDL (lane 3) was ≈15 times less. No binding of ^{125}I–Ac-LDL or ^{125}I–Ox-LDL could be measured at 220 to 240 kD, most probably because of insufficient sensitivity of the method.

The ability of native and Ox-LDL to compete for the binding of ^{125}I–Ox-LDL to the ≈120-kD protein was analyzed (Fig 2). As expected, LDL did not significantly inhibit the binding of ^{125}I–Ox-LDL to the ≈120-kD protein. In contrast, LDL oxidized for only 1 hour inhibited the ^{125}I–Ox-LDL ligation by ≈30%. Further oxidation of LDL led to increased blockade of the binding of ^{125}I–Ox-LDL to the ≈120-kD protein, ie, >50% inhibition with LDL oxidized for 20 hours.

The binding characteristics of the ≈120-kD membrane protein for Ox-LDL were determined by incubating the blots with increasing concentrations of ^{125}I–Ox-LDL, followed by excision and direct counting of the radioactivity. Fig 3 shows that ^{125}I–Ox-LDL bound to the ≈120-kD membrane protein in a saturable manner, with a K_d of ≈7 nmol/L (≅23 μg/mL) and a B_{max} of ≈17 fmol/mg protein (≅52 ng/mg protein). Taken together, these data suggest that this ≈120-kD membrane protein specifically binds Ox-LDL in a saturable manner and that minimal oxidation is sufficient to produce an LDL particle that is recognized by the protein.

Recognition of a Human Macrophage ≈120-kD Protein by Ki-M6, an Anti-CD68 Monoclonal Antibody

Macrosialin, the mouse homologue of human CD68, was recently characterized as an Ox-LDL binding pro-

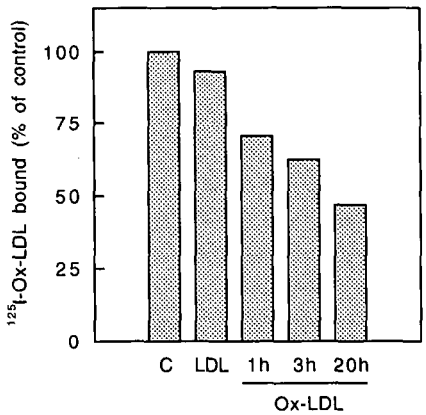

FIG 2. Inhibition of binding of ^{125}I–Ox-LDL to the ≈120-kD protein by unlabeled Ox-LDL. Membrane lysates from human macrophages cultured for 7 days were subjected to SDS-PAGE (170 μg protein per lane) followed by electroblotting onto nitrocellulose membranes. Nitrocellulose membrane strips were incubated with 7.5 μg/mL ^{125}I–Ox-LDL (fully oxidized for 20 hours) in the absence (C) or presence of a 20-fold excess of LDL or of LDL oxidized for 1, 3, or 20 hours at 37°C. Binding values were calculated as described in Fig 1C.

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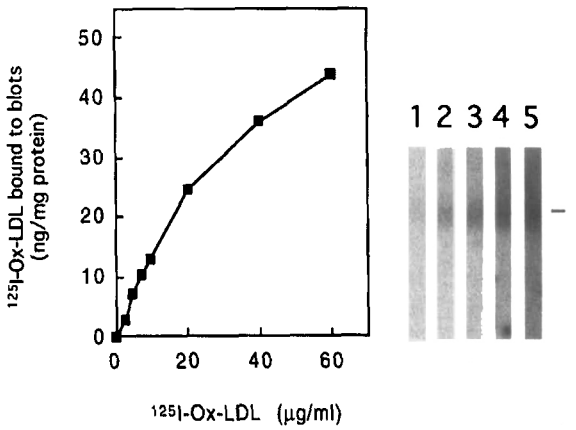

FIG 3. Saturation of ^{125}I–Ox-LDL binding to the ≈120-kD protein. Cell lysates from human macrophages cultured for 7 days were subjected to SDS-PAGE (170 μg protein per lane) followed by electroblotting onto nitrocellulose membranes. Nitrocellulose membrane strips were incubated with increasing concentrations of ^{125}I–Ox-LDL at 37°C (right panel, from left to right: 5, 10, 20, 40, or 60 μg/mL). The ≈120-kD region (see mark) was excised to quantify the radioactivity bound to the strip. Background radioactivity was determined by counting a blot sample of equal size from the 69- to 97-kD region and subtracted from all values.

tein in mouse macrophages.[15] CD68 is found predominantly in macrophages, and its reported molecular weight is ≈120 kD, ie, almost identical to the molecular weight of the Ox-LDL binding protein that we identified in human monocyte–derived macrophages. On the basis of this set of data, we hypothesized that the ≈120-kD Ox-LDL binding protein and CD68 might be identical. To test this hypothesis, we performed parallel Western blot experiments with Ki-M6 and ligand-blot experiments with Ox-LDL. Cell lysates were submitted to electrophoresis as before. After electroblotting, the nitrocellulose membrane of each lane was cut vertically in half. One half of each strip was incubated with 1 μg/mL Ki-M6 and the other half with 10 μg/mL Ox-LDL. Because of its high sensitivity, the ECL detection method was used here and in subsequent experiments. Fig 4 shows that under nonreducing conditions, Ki-M6 cross-reacted with a protein with a molecular weight of

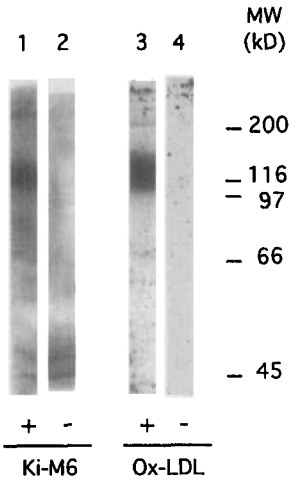

FIG 4. Combined Ki-M6 Western blots and Ox-LDL ligand blots. Cell lysates from human macrophages cultured for 8 days were subjected to SDS-PAGE (20 μg protein per lane under nonreducing conditions and using five-well combs) followed by electroblotting onto nitrocellulose membranes. Every membrane region corresponding to one SDS-PAGE lane was cut into two vertical strips. One half was incubated without or with 1 μg/mL Ki-M6 (lanes 1 and 2). The other half was incubated without or with 10 μg/mL Ox-LDL and then with anti–apolipoprotein B100 antiserum (lanes 3 and 4). Detection of bands was performed by ECL following incubation with the corresponding HRP-conjugated secondary antibodies.

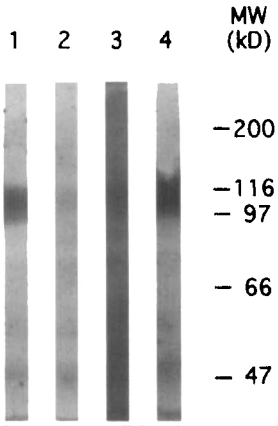

FIG 5. Western blots with anti-CD68 antibodies PG-M1, EBM11, Ki-M7, and Ki-M6. Cell lysates from human macrophages cultured for 8 days were subjected to SDS-PAGE (150 μg protein per lane) followed by electroblotting onto nitrocellulose membranes. Nitrocellulose membrane strips were incubated with 1 μg/mL of the different antibodies (lane 1, PG-M1; lane 2, EBM11; lane 3, Ki-M7; and lane 4; Ki-M6) at room temperature. Detection of the primary antibodies was performed by ECL following incubation with a corresponding secondary HRP-conjugated antibody.

≈120 kD (lane 1), identical to that of the Ox-LDL binding protein (lane 3). Control incubations, in which (1) Ki-M6 was omitted (lane 2), (2) an irrelevant mouse IgG was used (data not shown), and (3) Ox-LDL was omitted during ligand blotting (lane 4), demonstrated the specificity of the immunostaining. Since Ki-M6 showed no cross-reactivity to any other protein, it is likely that the antigen that is recognized at ≈120 kD is CD68. Both ≈120-kD bands identified by Ox-LDL binding and Ki-M6 Western blotting remained unaffected by DTT reduction. Other anti-CD68 antibodies, such as Ki-M7, EBM11, and PG-M1, showed weaker signals (Fig 5), and Ki-M6 was therefore selected for subsequent experiments.

CD68 and the ≈120-kD Ox-LDL Binding Protein Are Glycoproteins

As a glycoprotein, CD68 is sensitive to glycosidase digestion.[23] Hence, we determined whether the ≈120-kD Ox-LDL binding protein was also effected by glycosidase digestion. A 2-hour treatment of detergent lysates with *N*-glycosidase F resulted in an increase in electrophoretic mobility very similar for both CD68 and the ≈120-kD Ox-LDL binding protein and in broadening of both bands (Fig 6). The reduction in apparent molecular weight amounted to ≈18 kD for both CD68 and the Ox-LDL binding protein. In control 2-hour incubations in which *N*-glycosidase F was omitted, no change in electrophoretic mobility was observed. *O*-Glycosidase treatment of lysates did not affect the electrophoretic mobility of both CD68 and the Ox-LDL binding protein (data not shown).

CD68 and the ≈120-kD Ox-LDL Binding Protein Levels During Monocyte-Macrophage Differentiation

Freshly isolated monocytes expressed low amounts of CD68 and low amounts of the ≈120-kD Ox-LDL binding protein as well (Fig 7). During differentiation of monocytes into macrophages, expression of CD68 as well as that of the ≈120-kD Ox-LDL binding protein was dramatically increased (16-fold increase compared with expression levels in monocytes). In addition, the intensity of high-molecular-weight bands that bound Ox-LDL were increased but were not cross-reactive with Ki-M6. The highest level of expression was achieved after 8 days of differentiation, after which time the binding activity of Ki-M6 and Ox-LDL declined. Thus,

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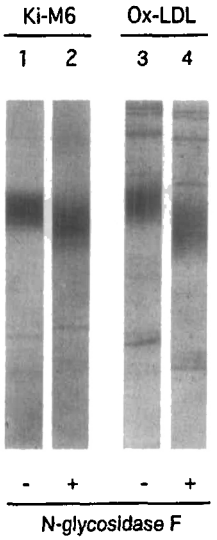

FIG 6. Effects of *N*-glycosidase F on CD68 and the ≈120-kD Ox-LDL binding protein. Cell lysates from human macrophages cultured for 8 days were incubated in the absence or presence of 6 U/mL *N*-glycosidase F at 37°C for 2 hours. Enzymatic digestion was stopped by addition of an equal volume of Laemmli buffer and heated at 95°C for 5 minutes. Control and treated lysates were subjected to SDS-PAGE (12 μg protein per lane using five-well combs) followed by electroblotting onto nitrocellulose membranes. Every membrane region corresponding to one SDS-PAGE lane was cut into two vertical strips and treated with Ki-M6 (lanes 1 and 2) or Ox-LDL (lanes 3 and 4) as described in Fig 4.

the time course of CD68 expression paralleled that of the Ox-LDL binding protein during differentiation of monocytes to macrophages for as long as 21 days in culture.

Immunoprecipitation of the Ox-LDL Binding Protein With Monoclonal Antibody EBM11

The above data, together with data from the literature,[15] suggest that CD68 and the ≈120-kD Ox-LDL binding protein might be the same molecular entity. To further substantiate this assumption, immunoprecipitation experiments were performed with antibodies to CD68, and precipitated proteins were tested for their ability to bind Ox-LDL. Repeated attempts to immunoprecipitate CD68 from human macrophage lysates or prepurified lysate preparations with Ki-M6 failed. Another monoclonal antibody, EBM11, has been reported to immunoprecipitate CD68 efficiently.[34] Although the latter antibody did not provide a clear signal on Western blots from human macrophage lysates (Fig 5), we performed immunoprecipitation experiments with EBM11 according to the method of Micklem et al,[34] which was slightly modified and optimized for low amounts of proteins. Fig 8 shows that the immunoprecipitate obtained using EBM11 yielded an ≈120-kD protein that was recognized by Ki-M6 (lane 1) and exhibited strong Ox-LDL binding activity (lane 2). No bands were detected when Ki-M6 and Ox-LDL were omitted during Western and ligand blotting, thus demonstrating the specificity of the immunostaining (data not shown). The band found at ≈50 kD resulted from cross-reactivity of the secondary HRP-conjugated antibody with reduced EBM11. Prior to SDS-PAGE, the supernatant of the immunoprecipitation with EBM11 was concentrated four times. Low amounts of the ≈120-kD Ox-LDL binding protein were found in this concentrated supernatant (lane 4), even though there was no protein cross-reactive to Ki-M6 (lane 3). This Ox-LDL binding activity in the supernatant may have resulted from incomplete immunoprecipitation or from a CD68 subpopulation that binds Ox-LDL but is not recognized by Ki-M6. Another Ox-LDL binding protein that colocalizes with CD68 cannot be entirely excluded. In addition, the sensitivity of CD68 detection with Ki-M6 might be less than that for Ox-LDL. When EBM11 was omitted during the procedure, the immunoprecipitate contained no protein cross-reactive to Ki-M6 (lane 5), and no Ox-LDL binding activity at ≈120 kD (lane 6) or at any other molecular weight. In contrast, the supernatant from the control immunoprecipitation performed in the absence of EBM11 yielded all the Ki-M6 cross-reactive protein (lane 7) as well as all of the Ox-LDL binding activity at ≈120 kD (lane 8). These data strongly suggest that CD68, immunoprecipitated with EBM11 and probed with Ki-M6, and the ~120-kD Ox-LDL binding protein are the same molecular entity.

Cell Surface Expression of CD68 and its Uptake in Human Macrophages

Although CD68 is associated with cellular membranes, it is not clear whether it is expressed at the cell surface, even in small amounts, to mediate internalization of Ox-LDL. So far, macrosialin and CD68 are

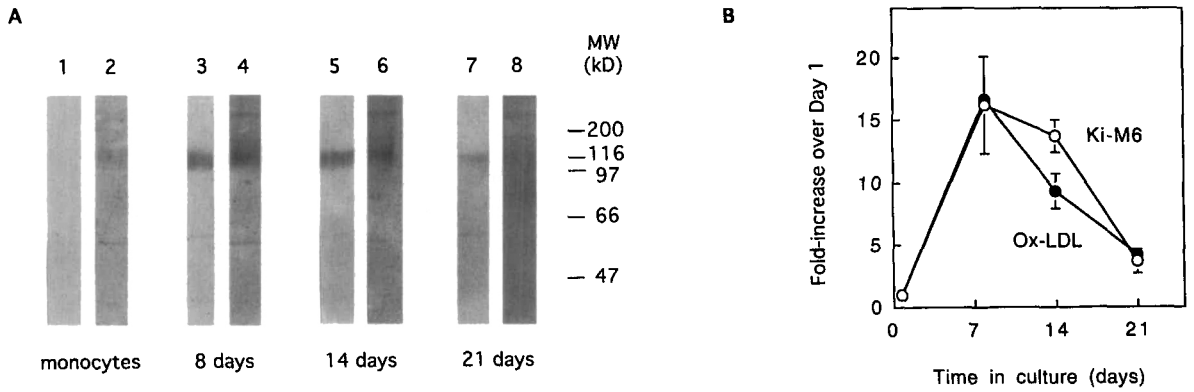

FIG 7. Expression pattern of CD68 and of the ≈120-kD Ox-LDL binding protein during monocyte/macrophage differentiation. A, For each time point, cell lysates were subjected to SDS-PAGE (30 μg protein per lane using five-well combs) followed by electroblotting onto nitrocellulose membranes. Every membrane region corresponding to one SDS-PAGE lane was cut into two vertical strips and treated with either Ki-M6 (lanes 1, 3, 5, and 7) or Ox-LDL (lanes 2, 4, 6, and 8) as described in Fig 4. B, Quantification of Ki-M6 binding (○) and of Ox-LDL binding (●) was performed by densitometric scanning of unsaturated films using a Personal Densitometer (Molecular Dynamics). Peaks were measured using Image Quant software (version 3.2). Each point represents the mean±SD of four different analyses.

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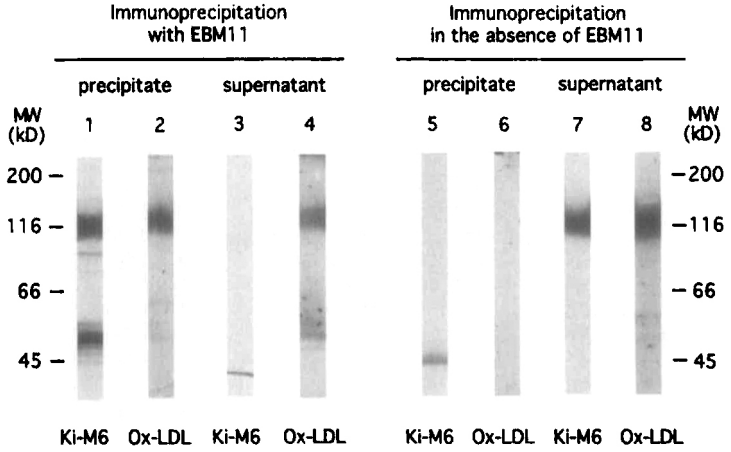

FIG 8. Coimmunoprecipitation of CD68 and the ≈120-kD Ox-LDL binding protein with antibody EBM11. A pool of cell lysates from human macrophages cultured for 8 days was prepurified on a lentil lectin Sepharose column. Prepurified eluates were precleared twice, and 100-μL aliquots were incubated in the absence or presence of 15 μg EBM11 at 4°C for 2 hours. Next, 20 μL packed goat anti-mouse IgG-agarose were added to each sample and incubated at 4°C for an additional hour. Immunoprecipitates and supernatants (4× concentrated) were boiled in DTT-containing Laemmli buffer and subjected to SDS-PAGE followed by electroblotting onto nitrocellulose membranes. Each strip was prepared and processed as described in Fig 4.

thought to be localized mainly in endosomal/lysosomal structures.[40,41] Electron microscopy and flow cytometry showed only low amounts of macrosialin and CD68 on the cell surface,[20,42] thus raising doubts about a potential role for CD68 in mediating binding and uptake of Ox-LDL. Ox-LDL is not such a convenient ligand to use to resolve this question because it is also a ligand for other cell-surface receptors such as scavenger receptor type A. To circumvent this difficulty, we substituted Ox-LDL with radiolabeled Ki-M6 and applied the antibody to human macrophages under various conditions to determine whether it bound to the cell surface and whether it was processed intracellularly.

Human monocyte-derived macrophages were incubated with increasing concentrations of ^{125}I-Ki-M6 at 4°C for 4 hours, after which time specifically bound radioactivity was measured. Human macrophages exhibited saturable binding for ^{125}I-Ki-M6 (Fig 9). Analysis of the binding data revealed that one high-affinity binding site was involved, with a K_d of 8.2±2.1 nmol/L and a B_{max} of 87±5 fmol/mg protein (Table). Coincubation of ^{125}I-Ki-M6 with blocking anti-Fc receptor antibodies or with excess human IgG did not affect ^{125}I-Ki-M6 binding

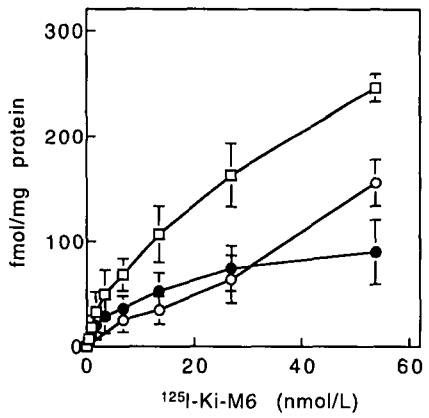

FIG 9. Binding of increasing concentrations of ^{125}I-Ki-M6 to human macrophages. Binding at different concentrations of ^{125}I-Ki-M6 to human macrophages cultured for 8 days was measured after incubation at 4°C for 4 hours. Specific binding (●) was calculated by subtracting nonspecific binding (○) from total binding (□). Nonspecific binding of ^{125}I-Ki-M6 was defined as the binding of the radiolabeled ligand in the presence of 1 μmol/L unlabeled Ki-M6. Each point is the mean±SEM of three independent experiments.

(data not shown), suggesting that the Fc receptors were not involved and that CD68 was solely responsible for the binding of ^{125}I-Ki-M6. Assuming that one antibody binds one molecule of CD68 and considering that 10^6 human macrophages represent 0.1 mg protein (data not shown), a single human macrophage would express ≈5000 molecules of CD68 at the cell surface. Binding of ^{125}I-Ox-LDL to human macrophages occurred with a K_d of 7.3±3.8 nmol/L and a B_{max} of 211±3 fmol/mg protein (Table 1). Under the conditions used, binding of ^{125}I-Ox-LDL would be mediated by CD68 as well as by other receptors, eg, scavenger receptor type A.

To verify that cell-surface expression of CD68 was not an artifact derived from nonviable and lysed cells that were exposing antigen of intracellular origin, we determined whether binding of Ki-M6 was actively coupled to internalization and lysosomal degradation. Internalization and degradation do not occur in nonviable cells. Cell association and degradation were measured at a nonsaturating concentration of 4 nmol/L of ^{125}I-Ki-M6 at 37°C (Fig 10A). Cell association was efficiently coupled to degradation: during a 4-hour incubation, 91±3 fmol ^{125}I-Ki-M6/mg protein became associated to these cells while ≈6 times more was degraded (525±19 fmol/mg protein). A fivefold excess of unlabeled Ki-M6 blocked both cell association and degradation of ^{125}I-Ki-M6 by 66% and 79%, respectively. Consistent with the binding data at 4°C, excess IgG had no effect on either cell association or degradation of ^{125}I-Ki-M6, thus showing that ^{125}I-Ki-M6 uptake and degradation were not mediated by Fc receptors (data not shown). When ^{125}I-Ki-M6 was coincubated with 100 μmol/L chloroquine, a lysosomotropic agent, degradation was markedly inhibited while cell association increased by 70%. Chloroquine and other weak bases are known to increase the pH of endosomes and lysosomes and interfere

Apparent Binding Parameters of ^{125}I-Ki-M6 and ^{125}I-Ox-LDL to Human Macrophages

	K_d, nmol/L	B_{max}, fmol/mg Protein
^{125}I-Ki-M6	8.2±2.1	87±5
^{125}I-Ox-LDL	7.3±3.8	211±3

K_d indicates binding affinity; B_{max}, maximum specific binding capacity. Data for binding of ^{125}I-Ki-M6 and ^{125}I-Ox-LDL to human macrophages were fitted by using LIGAND software. Each value is the mean±SEM of three independent experiments.

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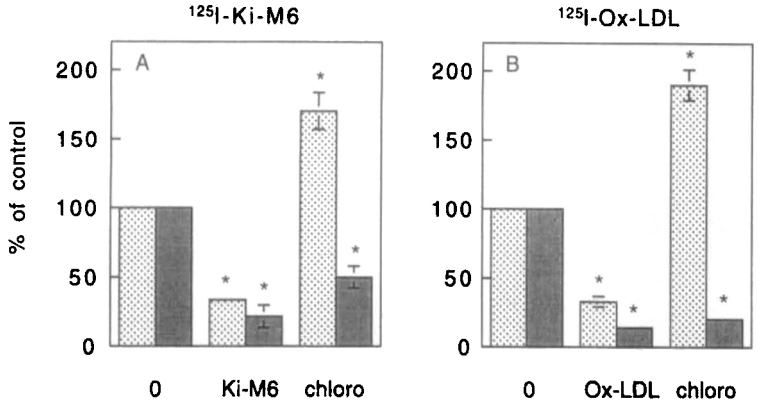

FIG 10. Cell association and degradation of ^{125}I–Ki-M6 and ^{125}I–Ox-LDL. Human macrophages cultured for 8 days were incubated at 37°C with 4 nmol/L ^{125}I–Ki-M6 in medium M199–2% human serum albumin alone or supplemented with either 20 nmol/L Ki-M6 or 100 μmol/L chloroquine. In the right panel, cells received 1.7 nmol/L (=5 μg/mL) ^{125}I–Ox-LDL in medium M199–2% human serum albumin alone or supplemented with either 33 nmol/L (=100 μg/mL) unlabeled Ox-LDL or 100 μmol/L chloroquine. After 4 hours, cell association (spotted bars) and degradation (dark bars) were measured as described in "Methods." The 100% values for ^{125}I–Ki-M6 association and degradation were 91±3 fmol/mg protein and 525±19 fmol/mg protein, respectively. For ^{125}I–Ox-LDL these values were 141±14 fmol/mg protein and 831±29 fmol/mg protein, respectively. Each value is the mean±SEM of three independent experiments. *$P<.05$ versus control by unpaired Student's t test.

with receptor recycling and ligand degradation.[43-45] Under identical conditions, a 20-fold excess of unlabeled Ox-LDL blocked both cell association and degradation of ^{125}I–Ox-LDL by 67% and 86%, respectively (Fig 10B). Similarly, chloroquine inhibited the degradation of ^{125}I–Ox-LDL by 80% while its association almost doubled. Taken together, these data strongly suggest that CD68 is expressed at the surface of human macrophages, with the potential to internalize substrates such as Ox-LDL from the extracellular space into the cells followed by lysosomal degradation.

Discussion

In this article, we have shown that human monocyte–derived macrophages express a ≈120-kD membrane protein that specifically binds Ox-LDL. In comparison, the binding of ^{125}I–Ac-LDL and ^{125}I-LDL to this protein was very weak. This protein might in fact correspond to an ≈120-kD membrane Ox-LDL binding protein previously found in phorbol myristate acetate–treated THP-1 cells.[18] We propose that human macrophage CD68 and this ≈120-kD Ox-LDL binding protein are identical on the basis of the following data: (1) Under our conditions, both Ox-LDL and Ki-M6, an anti-CD68 antibody, recognize almost exclusively one protein with the same molecular weight of ≈120 kD under reducing and nonreducing conditions. (2) CD68 expression and expression of the ≈120-kD Ox-LDL binding protein follow a similar time course during monocyte/macrophage differentiation. (3) CD68 and the Ox-LDL binding protein are glycoproteins whose apparent molecular weights are reduced to a similar extent after N-glycosidase F treatment. (4) CD68, probed with monoclonal antibody Ki-M6, and the ≈120-kD Ox-LDL binding protein are coprecipitated with EBM11, another anti-CD68 antibody. However, Ki-M6 and Ox-LDL would not share the same binding domain on the ≈120-kD protein, as evidenced by the absence of direct cross-competition between Ox-LDL and Ki-M6 for binding to this protein. It was also found that (1) Ki-M6 binds to cultured human macrophages in a saturable manner, (2) Ki-M6 binding is not mediated by Fc-receptors, and (3) Ki-M6 binding to human macrophages is coupled to internalization and lysosomal degradation. We have calculated that ≈5000 CD68 molecules are expressed on the surface of human macrophages. This compares with 4000 to 8000 CD68 molecules per cell, expressed at the surface of phorbol myristate acetate–treated THP-1 cells.[20] Consistently the total number of Ox-LDL binding sites at the surface of human monocyte–derived macrophages is ≈120 000, of which ≈4000 are selective Ox-LDL binding sites that do not bind Ac-LDL.[30] Assuming that one CD68 molecule binds one Ox-LDL particle, we may speculate that CD68 is expressed at the cell surface in amounts sufficient to account for the specific interaction with Ox-LDL. Binding of radiolabeled Ki-M6 to human macrophages was actively coupled to internalization and lysosomal degradation, thus showing that CD68 indeed has the potential to process ligands from the extracellular space into cells, followed by lysosomal degradation. Therefore, although a major part of CD68 would be intracellular,[20,40,41] small but significant amounts of the protein would be present at the cell surface, mediating the uptake and degradation of Ox-LDL as suggested recently for THP-1 cells.[20] Rapid recycling of CD68 between endosomes and the cell surface could then substantially contribute to the accumulation of Ox-LDL–derived cholesterol in macrophages.

CD68 expression and Ox-LDL binding activity increased during the initial phase of differentiation and declined to near-basal levels after prolonged differentiation. Eischen et al[46] demonstrated that CD68 expression measured with EBM11, Ki-M6, and Ki-M7 increased up to 7 days of differentiation but declined thereafter. Little is known about CD68 expression and scavenger receptor expression in human macrophages after prolonged culturing for up to 3 weeks. Geng et al[31] measured increasing scavenger receptor expression on human macrophages early in their differentiation until ≈3 days, whereas van Lenten et al[47] showed increased scavenger receptor expression until day 7 of differentiation. Maturation of macrophages is associated with a continuous change of antigen expression associated with functional alterations.[46] Accordingly, a decrease in CD68 expression and Ox-LDL binding activity could be the functional consequence of this maturation.

In resident murine peritoneal macrophages, macrosialin, the mouse homologue of CD68, is expressed at low levels in a glycosylated form that does not bind wheat germ agglutinin or peanut agglutinin. Upon stimulation of these cells with inflammatory agents, macrosialin is dramatically upregulated and differently "decorated" with N-linked and O-linked sugar residues as well as with poly-N-acetyllactosamine structures, resulting in a highly

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glycosylated protein that then binds the aforementioned lectins.[48] Such regulatory mechanisms of CD68 have not yet been examined in human macrophages, and it would be of interest to test whether intracellular CD68 can be translocated to the cell surface in response to cytokines or other stimuli, similar to the ≈95-kD protein that is upregulated in rabbit aortic foam cells compared with nonfoam cells.[18] The cDNA for CD68 was sequenced by Holness and Simmons[23] in the early 1990s. On the basis of sequence and domain homologies, CD68 belongs to the family of lamp/lgp proteins, which are involved in trafficking of vesicles between the plasma membrane and lysosomes.[49-51] The cytoplasmic tail of CD68 is relatively short but contains an amino acid sequence essential for lysosomal targeting, suggesting a role for CD68 in antigen presentation and processing, whereas the glycan residues of CD68 may serve as a protective sheet against the numerous proteases present in lysosomes.

In conclusion, our data strongly support a role for CD68 as a specific Ox-LDL binding protein in human macrophages expressed in part at the cell surface. Our observation is in line with the original proposal of Ramprasad et al,[15] that macrosialin is an Ox-LDL binding protein in mouse macrophages. A blocking anti-CD68 antibody would be very useful to determine to what extent CD68 contributes to recognition of Ox-LDL in intact cells and possibly to its internalization and degradation. Another aspect would be to assess whether CD68 expression is affected when cholesterol accumulates in human macrophages and to determine its role in the pathophysiological process of atherosclerosis.

Acknowledgments

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