Characterization of a receptor for oxidized low-density lipoproteins on rat Kupffer cells: similarity to macrosialin

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Rat liver Kupffer cell membranes contain a protein that recognizes specifically oxidized low-density lipoproteins (oxLDL). Visualization after blotting under reducing conditions indicates that the receptor is a monomeric protein, with an estimated molecular mass of 115–120 kDa. *N*-Glycosidase F and endoglycosidase F treatment resulted in a fall in estimated molecular mass of 24 and 11 kDa respectively, whereas *O*-glycosidase was ineffective. No effect on the extent of interaction with oxLDL was noticed, suggesting that glycans are not essential for ligand recognition. Using a polyclonal antibody to mouse macrosialin,

we visualized macrosialin on blot, and compared this glycoprotein with the oxLDL-binding protein. It appears that the two glycoproteins have a similar molecular mass and are comparably affected by treatment with the different glycosidases. Incubation with trypsin resulted in a reduction in the estimated molecular mass of about 25 kDa for both the oxLDL-binding protein and macrosialin. These results indicate that the oxLDL-binding protein and macrosialin are identical, suggesting a role for macrosialin in modified LDL catabolism.

INTRODUCTION

Evidence is accumulating that oxidized low-density lipoproteins (oxLDL) are important in the development of atherosclerosis. During the oxidation process, a number of chemical changes take place within the lipoprotein particle, including an increase in density, a decrease in content of esterified cholesterol, fragmentation of apolipoprotein B and an increase in negative charge, leading to increased electrophoretic mobility [1–3]. As a consequence, oxLDL are no longer recognized by the native LDL receptor, but instead interaction with the so-called scavenger receptor is induced. The acetyl-LDL receptor or scavenger receptor was first described by Goldstein et al. [4], and cloned and characterized by Kodama et al. [5,6]. It appeared to be a trimeric membrane glycoprotein with a molecular mass of 220 kDa. Its presence on macrophages is suggested to lead to the modified LDL-induced formation of foam cells.

It has become evident that there may exist more than one type of receptor that can recognize modified LDL. Stanton et al. [7] showed that FcγRII-B2, an Fc receptor, is able to bind oxLDL but not acetylated LDL (acLDL), and Endemann et al. [8] demonstrated interaction of oxLDL with CD36, a glycoprotein present on the surface of monocytes, platelets and endothelial cells. In addition, Acton et al. [9] reported the cloning of a new member of the CD36 family of membrane proteins, which is able to bind modified lipoproteins and native LDL and is designated scavenger receptor B1 (SR-B1). Sparrow et al. [10] and Otnad and colleagues [11] reported the existence of a plasma-membrane protein of 94–97 kDa on mouse peritoneal macrophages that is able to bind oxLDL but not acLDL, and which can also interact with oxidized red blood cells and phosphatidylserine liposomes [12,13]. Recently, this receptor was identified as macrosialin, the mouse homologue of CD68 [14].

Some years ago we speculated that rat liver Kupffer cells contain a specific receptor for the uptake of oxLDL, which is not able to bind acLDL [15]. Subsequently, a major binding protein with an estimated molecular mass of 95 kDa was visualized by

ligand blotting with ¹²⁵I-oxLDL [16]. The protein is present on Kupffer cells but absent from rat liver endothelial cells. Here we describe the further characterization of this oxLDL-binding membrane protein from Kupffer cells, and its similarity to macrosialin, a macrophage-specific highly glycosylated protein [17,18].

MATERIALS AND METHODS

Materials

The anti-macrosialin antibody is a Protein G-purified rabbit polyclonal antibody against macrosialin from mouse spleen. Rabbit anti-(apolipoprotein B) antibody was kindly provided by Dr. L. M. Havekes (TNO, Leiden, The Netherlands). ECL Western-blotting detection reagents, donkey anti-rabbit immunoglobulin peroxidase-linked antibody, rainbow-coloured protein molecular-mass markers and ¹²⁵I in NaOH were purchased from Amersham International (Amersham, Bucks., U.K.). Collagenase (type IV) and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *n*-Octyl glucoside, pepstatin and the different glycosidases were purchased from Boehringer-Mannheim (Mannheim, Germany). Suramin and Trasylol® [10000 kallikrein-inactivating units (KIU)/ml] were from Bayer (Leverkusen, Germany), and PMSF was obtained from Merck (Darmstadt, Germany). Leupeptin was purchased from the Peptide Institute (Osaka, Japan). Trypsin was obtained from Gibco-BRL (Breda, The Netherlands).

Isolation and oxidation of LDL

LDL (*d* = 1.019–1.063 g/ml) was isolated from plasma of healthy volunteers as described by Redgrave et al. [19]. Before oxidation, it was dialysed against PBS, pH 7.4, containing 10 μM EDTA. It (200 μg of protein/ml) was oxidized by incubation with 11 μM CuSO₄ in PBS for 20 h at 37 °C, the final EDTA concentration

Abbreviations used: oxLDL, oxidized low-density lipoproteins; acLDL, acetylated low-density lipoproteins; ECL, enhanced chemiluminescence.

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being 1 μ M. The oxidation was terminated by the addition of 0.2 mM EDTA. The degree of oxidation was tested on 0.75 % (w/v) agarose gels. For ligand-blotting experiments, oxLDL was radiolabelled with 125 I by the procedure of McFarlane [20] as modified by Bilheimer et al. [21].

Isolation and solubilization of membrane proteins from rat liver endothelial and Kupffer cells

Liver endothelial and Kupffer cells were isolated from male Wistar rats. Rats were anaesthetized by intraperitoneal injection of 25 mg of sodium pentobarbital. The liver was perfused for 9 min with 0.05 % (w/v) collagenase (type IV) at 37 °C by the method of Seglen [22], modified as described previously [23]. Endothelial and Kupffer cells were isolated at 4 °C by density-gradient centrifugation and centrifugal elutriation (3250 rev./min at 25 ml/min for endothelial cells and 70 ml/min for Kupffer cells). The purity of the cell preparations was checked by peroxidase staining (Kupffer cells > 80 % pure, endothelial cells > 95 % pure). The freshly isolated cells were homogenized by ultrasonication in buffer A (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl_2 , pH 7.5), containing a protease inhibitor mixture (1 mM PMSF, 100 KIU/ml Trasylol, 50 μ M suramin, 15 μ M pepstatin and 20 μ M leupeptin). The homogenate was centrifuged for 10 min at 500 *g*. The resulting supernatant was centrifuged for 2 h at 100 000 *g*, in order to isolate membranes. Membrane proteins were solubilized by stirring for 1 h in 20 mM sodium phosphate buffer (16.2 mM Na_2HPO_4 and 3.8 mM NaH_2PO_4 , pH 7.4), containing the protease inhibitor mixture and 40 mM *n*-octyl glucoside. Unsolubilized material was removed by centrifugation for 10 min at 16 000 *g*, and the protein concentration of the resulting supernatant was determined by the method of Lowry et al. [24], with BSA as standard.

Visualization of oxLDL-binding proteins and macrosialin by immunolabelling on blot

Solubilized membrane proteins were separated by SDS/PAGE (5 % gel) under non-reducing conditions, as described by Laemmli [25], and blotted on to nitrocellulose membranes. Immunolabelling was performed at room temperature. To visualize oxLDL-binding proteins, blots were blocked for 1 h in incubation buffer (150 mM NaCl, 10 mM Tris, 2 mM CaCl_2 , pH 7.6) containing 5 % (w/v) skimmed milk. The blots were next incubated for 70 min with oxLDL (8 μ g/ml) in incubation buffer containing 1 % skimmed milk. The blots were washed several times and incubated for 1 h with anti-(apolipoprotein B) antibody (1:1000) in incubation buffer (0.5 % milk). When indicated, blots were incubated with rabbit anti-macrosialin antibody (1:500) instead of oxLDL and anti-(apolipoprotein B) antibody to visualize macrosialin. After a wash, the blots were finally incubated with anti-rabbit immunoglobulin peroxidase-linked antibody (1:15000) for 1 h, and washed again for 90 min. The proteins were visualized by enhanced chemiluminescence (ECL) detection.

Ligand blotting

Blots containing 150 μ g of solubilized Kupffer cell membrane proteins per lane were blocked for 2 h in incubation buffer containing 5 % (w/v) skimmed milk. The blots were next incubated with 125 I-oxLDL (15 μ g/ml; specific radioactivity 139 c.p.m./ng of protein) for 2 h, and washed several times in incubation buffer (0.5 % milk). Autoradiograms were obtained by exposing the dried blots to Kodak XAR-5 films at -80 °C for 2 days.

Trypsin digestion of Kupffer cell membrane proteins

Solubilized Kupffer cell membrane proteins were dialysed against 20 mM sodium phosphate buffer, pH 7.4, at 4 °C to remove any protease inhibitors present. The protein (90 μ g) was then incubated with 0.75 mg of trypsin at 37 °C for 1 or 2 h, in a total incubation volume of 60 μ l. Trypsin digestion was terminated by heating the samples at 95 °C for 10 min. Then 10 μ g of the digested proteins was used to visualize the oxLDL-binding protein, and the remaining 80 μ g was used to visualize macrosialin on blot.

RESULTS AND DISCUSSION

Previously, by ligand blotting with 125 I-oxLDL, it was established that Kupffer cells express a specific binding protein for oxLDL [16]. We here describe a new blotting procedure, combining ligand blotting with immunolabelling. Blots are successively incubated with oxLDL, anti-(apolipoprotein B) antibody and anti-rabbit immunoglobulin peroxidase-linked antibody, and bands are visualized by ECL detection. A comparison of the two techniques showed that in both cases a major binding protein with an estimated molecular mass of 115–120 kDa is visualized (Figure 1, top). Whereas ligand blotting with 125 I-oxLDL requires about 150 μ g of solubilized membrane protein for visualization, the combination of ligand blotting with immunolabelling results in much higher sensitivity, so that 10 μ g of solubilized membrane protein is sufficient. When Kupffer cell membrane proteins are compared with those from endothelial cells, it is shown that the

Figure 1 Visualization of oxLDL-binding proteins on Kupffer and endothelial cell membranes by different labelling techniques

Kupffer and endothelial cell membrane proteins were isolated, separated by SDS/PAGE (5 % gel) and blotted on to nitrocellulose membranes. Top, OxLDL-binding proteins were visualized by either ligand blotting with 125 I-oxLDL (139 c.p.m./ng of protein; 150 μ g of solubilized Kupffer cell membrane protein per lane) or immunolabelling with oxLDL and an anti-(apolipoprotein B) antibody (10 μ g of solubilized Kupffer cell membrane protein per lane), as described in the Materials and methods section. Bottom, OxLDL-binding proteins on Kupffer and endothelial cell membranes were visualized on blot by immunolabelling (10 μ g of solubilized membrane protein per lane). KC, Kupffer cells; EC, endothelial cells. The molecular-mass standards are indicated on the left.

Figure 2 Effects of SDS, 2-mercaptoethanol and various glycosidases on the migration of oxLDL-binding proteins

Top, Kupffer cell membrane proteins were incubated with either 1% SDS or 0.4% SDS and 0.85% 2-mercaptoethanol (β -ME), and heated at 95 °C for 10 min. Subsequently, proteins were separated by SDS/PAGE, and blotted on to nitrocellulose membranes (10 μ g of protein per lane). The lane on the right contains untreated Kupffer cell membrane proteins as a control. Bottom, Kupffer cell membrane proteins were incubated with various glycosidases for 3 h at 37 °C, before SDS/PAGE and blotting. N-gly F, *N*-glycosidase F (0.4 unit/10 μ g of protein); endogly F, endoglycosidase F (0.2 unit/10 μ g of protein); O-gly, *O*-glycosidase (2 m-units/10 μ g of protein). OxLDL-binding proteins were visualized by immunolabelling, as described. The molecular-mass standards are indicated on the left.

binding protein is expressed only in Kupffer cells (Figure 1, bottom).

To characterize the oxLDL-binding protein further, membrane proteins were incubated with 1% (w/v) SDS alone or 0.4% (w/v) SDS and 0.85% (v/v) 2-mercaptoethanol, and heated for 10 min at 95 °C. After denaturation of the proteins by heating in the presence of 1% SDS, we were still able to visualize the oxLDL receptor, indicating that the tertiary structure of the receptor is not essential for ligand binding (Figure 2, top). In addition, although heating of proteins in the presence of 2-mercaptoethanol results in the reduction of disulphide bonds, it did not impair the binding of oxLDL to the receptor, and the molecular mass of the visualized band was not changed (Figure 2, top). This suggests that the oxLDL receptor is a monomeric protein. In most blots we also detected a faint band with an estimated molecular mass of 205–210 kDa. On heating of the proteins in the presence of 2-mercaptoethanol and/or SDS, this band disappeared, suggesting that it is a dimer of the oxLDL receptor.

Since many membrane proteins are known to contain carbohydrate groups, which may be involved in ligand binding, we explored the possible glycosylation of the Kupffer cell oxLDL receptor. Kupffer cell membrane proteins were incubated with different types of glycosidase before electrophoresis, and immunolabelling on blot was performed as described (Figure 2, bottom). Incubation of 10 μ g of Kupffer cell membrane protein with 0.4 unit of *N*-glycosidase F for 2 h resulted in a fall in the estimated molecular mass of about 24 kDa. Incubation for a longer period of time did not result in any further decrease in estimated molecular mass. Since *N*-glycosidase F is able to cleave most

Figure 3 Visualization of macrosialin and oxLDL-binding proteins on Kupffer cell membranes

Kupffer cell membrane proteins were separated by SDS/PAGE on a 5% (top) or 7.5% (bottom) gel. OxLDL-binding proteins were visualized on blot by immunolabelling, as described (10 μ g of Kupffer cell membrane protein per lane). Macrosialin was visualized on blot by successive incubation with polyclonal rabbit anti-macrosialin antibody (2 h) and anti-rabbit immunoglobulin peroxidase-linked antibody (1 h) (40 μ g of Kupffer cell membrane protein per lane). Bands were visualized by ECL detection. Molecular-mass standards are indicated on the left.

types of *N*-linked sugars, this indicates that the oxLDL receptor contains a considerable amount of *N*-glycans. The binding of oxLDL to the receptor appears to be unaffected, suggesting that the *N*-glycans are not required for ligand binding. When Kupffer cell membrane proteins were incubated with 0.2 unit of endoglycosidase F, the estimated molecular mass decreased by about 11 kDa. This indicates that some of the *N*-glycans are of the high-mannose and hybrid type. Again treatment of the membrane proteins with glycosidase did not impair ligand binding to the Kupffer cell receptor. Finally, membrane proteins were incubated with 2 m-units of *O*-glycosidase, which is able to cleave certain *O*-glycans. No decrease in estimated molecular mass was detected but, paradoxically, there seemed to be a slight increase in estimated molecular mass. Even after simultaneous incubation of membrane proteins with *O*-glycosidase and neuraminidase, to remove any blocking sialic acid residues, no change in migration was found (results not shown). Thus, under these conditions, no evidence for the presence of *O*-glycans was found. The migration of the faint band at 205–210 kDa also changed on incubation with the various glycosidases.

Recently, Ramprasad et al. [14] found evidence that the 94–97 kDa oxLDL receptor present on mouse peritoneal macrophages is identical with macrosialin. Macrosialin is the mouse homologue of human CD68, a heavily glycosylated protein present on macrophages. The oxLDL receptor on Kupffer cells is of comparable molecular mass and contains considerable amounts of *N*-glycans, so we decided to explore the possible similarities between the Kupffer cell oxLDL receptor and macrosialin. Macrosialin was visualized on blot by immunolabelling, using a polyclonal rabbit anti-macrosialin antibody and ECL detection. As shown in Figure 3 (top), after separation on 5% gels, a band was detected among the rat Kupffer cell membrane proteins, at an estimated molecular mass of 119 kDa. Thus macrosialin is present in rat Kupffer cells and appears to

Figure 4 Visualization of macrosialin on Kupffer and endothelial cell membranes

Kupffer and endothelial cell membrane proteins were isolated, separated by SDS/PAGE (5% gel) and blotted on to nitrocellulose membranes. Macrosialin was visualized on blot as described in the Materials and methods section (40 μ g of membrane protein per lane). KC, Kupffer cells; EC, endothelial cells. The molecular-mass standards are indicated on the left.

Figure 5 Comparison of the effects of various glycosidases on the migration of macrosialin and oxLDL-binding proteins from Kupffer cells

Solubilized Kupffer cell membrane proteins were incubated with *N*-glycosidase F (1 unit/50 μ g of protein), endoglycosidase F (0.75 unit/50 μ g of protein) or *O*-glycosidase (5 m-units/50 μ g of protein) for 3 h at 37 °C. Then 10 μ g of each incubation mixture was separated by SDS/PAGE and blotted on to nitrocellulose membrane. This blot was used to visualize the oxLDL-binding proteins by immunolabelling. The other 40 μ g of protein of each incubation mixture was used on blot to visualize macrosialin by immunolabelling with rabbit anti-macrosialin antibody. Molecular-mass standards are indicated on the left.

have the same molecular mass as the Kupffer cell oxLDL receptor. Since it is known that the migration of heavily glycosylated proteins on polyacrylamide gels is sometimes anomalous [18], we compared the oxLDL-binding protein and macrosialin on 7.5% gels (Figure 3, bottom). On these gels also the oxLDL-binding protein and macrosialin appear to have a comparable molecular mass, both migrating slightly above the 97.4 kDa molecular-mass standard.

Macrosialin is known for its macrophage-restricted expression [17,18], so we checked for the absence of macrosialin from liver endothelial cells. Indeed, as shown in Figure 4, macrosialin can be detected only in Kupffer cells, and not in endothelial cells, as is also the case for the oxLDL-binding protein.

To obtain more data on the possible similarities between the oxLDL receptor and macrosialin, we compared the migration of macrosialin and the oxLDL receptor after simultaneous glycosidase treatment. Kupffer cell membrane proteins were treated with various glycosidases, and both macrosialin and the oxLDL receptor were visualized on blot. The results are shown in Figure 5. Macrosialin exhibited comparable changes in estimated molecular mass after the glycosidase treatments with those in the oxLDL receptor. Incubation with *N*-glycosidase F

Figure 6 Comparison of the effect of trypsin digestion on the migration of macrosialin and oxLDL-binding proteins from Kupffer cells

Solubilized Kupffer cell membrane proteins were incubated with trypsin (0.75 mg/90 μ g of protein) for 1 or 2 h, at 37 °C, as indicated. Then 10 μ g of protein of each incubation mixture was used to visualize oxLDL-binding proteins by immunolabelling on blot, and the remaining 80 μ g of protein was used to visualize macrosialin, as described in the Materials and methods section. Molecular-mass standards are indicated on the left.

and endoglycosidase F resulted in a clear decrease in molecular mass, and incubation with *O*-glycosidase caused a slight decrease in relative mobility. Thus the oxLDL receptor and macrosialin have a comparable carbohydrate composition.

Further evidence for the similarity between the oxLDL receptor and macrosialin was obtained by comparing the sensitivity of the glycoproteins to digestion by trypsin. Kupffer cell membrane proteins were incubated with trypsin for 1 or 2 h, after which the oxLDL-binding protein and macrosialin were visualized on blot. As shown in Figure 6, incubation with trypsin resulted in the appearance of a fragment of lower molecular mass than the control for both the oxLDL receptor and macrosialin. The fragment is reduced in mass by about 25 kDa, and still able to bind oxLDL and the anti-macrosialin antibody, although less efficiently. There is no clear difference between the 1 h and the 2 h incubation for either glycoprotein, so that not only the size of the new fragment, but also the rate of trypsin digestion seems to be comparable.

As is most evident in Figure 4, the anti-macrosialin antibody also stained a faint band at about 205–210 kDa, which was also present after visualization of the oxLDL receptor. This confirms the hypothesis that macrosialin and the Kupffer cell oxLDL receptor are identical, and it suggests that this band indeed represents a dimer of the oxLDL receptor.

We also made a major effort to immunoprecipitate rat macrosialin with the available antibody, as was done earlier with macrosialin from mouse macrophages [17]. It appeared that the affinity of the antibody for rat macrosialin is insufficient to allow immunoprecipitation. However, on the basis of a similar molecular mass under various conditions, the specific localization in Kupffer cells, a similar sensitivity to *N*-glycosidase F, endoglycosidase F and *O*-glycosidase, and a comparable sensitivity to digestion by trypsin, we conclude that the most likely candidate for the oxLDL-specific binding protein on Kupffer cells is macrosialin.

Our conclusion that the Kupffer cell oxLDL receptor and macrosialin are identical implies that the oxLDL receptor on mouse peritoneal macrophages, which was described by Steinberg and co-workers [11,14], is the same receptor as the Kupffer cell oxLDL receptor described in this paper. The difference in molecular mass (94–97 kDa and 115–120 kDa respectively) between the two could then be explained by differences in glycosylation, since Rabinowitz and Gordon [17,18] showed that

glycosylation of macrosialin can differ between different macrophage populations, and is dependent on the state of cell stimulation. Furthermore, we previously estimated a molecular mass of 95 kDa for the Kupffer cell oxLDL receptor [16], whereas under the present conditions a molecular mass of 115–120 kDa is observed. Also for macrosialin, a broad band with a molecular mass ranging from 89 to 115 kDa has been reported.

The predominant intracellular localization of macrosialin in late endosomes [26] contradicts its possible role as an oxLDL receptor. However, it is possible that macrosialin exhibits very rapid translocation between intracellular membranes and the plasma membrane, so that a small amount of macrosialin on the plasma membrane is sufficient for the rapid uptake of oxLDL from the blood by Kupffer cells [15].

We thank Dr. L. M. Havekes (TNO, Leiden, The Netherlands) for providing rabbit anti-(apolipoprotein B) antibody. This work was financially supported by the Netherlands Heart Foundation (NHS) and the Netherlands Organization for Scientific Research (NWO).

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