RESEARCH COMMUNICATION

Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor

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A new receptor for oxidized low-density lipoprotein (LDL), lectin-like oxidized LDL receptor-1 (LOX-1), has recently been cloned from bovine endothelial cells and human lung. A limited tissue-distribution study suggested that the protein was mainly produced by the vascular endothelium. In the present study we demonstrate that LOX-1 is also expressed in macrophages, where it may function as a scavenger receptor. LOX-1 was not detected in undifferentiated THP-1 cells or in freshly isolated human blood monocytes. However, mature human monocyte-derived macrophages and differentiated THP-1 cells showed high levels

of LOX-1 transcripts. Consistent with these results, immunofluorescence staining and FACS analysis demonstrated that LOX-1 protein is expressed on the plasma membrane of macrophages. Western-blot analysis of membranes from macrophages (but not those from monocytes) identified a single band, with an apparent molecular mass of about 40 kDa, that displayed oxidized LDL-binding activity. These results suggest that differentiation induces the expression of LOX-1 in macrophages, where it may play a role as a scavenger receptor and/or a receptor for oxidized LDL.

INTRODUCTION

The unregulated uptake of oxidatively modified lipoproteins by macrophages and the accumulation of lipid-laden foam cells in the arterial intima are early events in atherogenesis [1–3]. Scavenger receptors expressed on the surface of macrophages appear to play a crucial role in the uptake of modified lipoproteins, resulting in foam cell formation [4]. The uptake of acetylated low-density lipoprotein (LDL) or oxidized LDL (OxLDL) was shown to be mediated in part by the acetylated LDL receptor [5,6], which subsequently cloned [7], characterized and renamed scavenger receptor A. However, the majority of OxLDL is internalized by receptors other than scavenger receptor A [8,9], and the rapidly expanding family of scavenger receptors now includes CD36 [10,11], macrophage scavenger receptors, resulting in a more effective clearance of OxLDL and enhancement of foam cell formation [20].

All known scavenger receptors are characterized by broad ligand-binding specificity for a diverse group of unrelated substances, including OxLDL and other modified lipoproteins, modified proteins, anionic phospholipids, anionic polysaccharides, poly- and oligonucleotides and apoptotic cells [17]. The primary function of scavenger receptors is the neutralization and destruction of foreign pathogens by receptor-mediated endocytosis and phagocytosis, crucial mechanisms in host defence. Although the clearance of OxLDL could be protective under some conditions, the excessive unregulated uptake of modified lipoproteins via scavenger receptors appears to accelerate atherosclerosis [18].

Circulating monocytes express low levels of scavenger receptors, but that expression is upregulated during differentiation into macrophages [19]. Oxidatively modified forms of LDL have many different biological effects and have been shown to induce

the expression of pro-inflammatory cytokines and adhesion molecules by cells of the arterial wall, which could accelerate atherogenesis. Modified forms of lipoproteins can also act directly on monocytes/macrophages. Both fully oxidized LDL (which is recognized by scavenger receptors but not by the LDL receptor) and minimally modified LDL (which is still recognized by the LDL receptor) can increase the expression of certain macrophage scavenger receptors, resulting in a more effective clearance of OxLDL and enhancement of foam cell formation [20].

Recently, a novel receptor for OxLDL, termed lectin-like OxLDL receptor (LOX-1), was cloned from bovine aortic endothelial cells and from human lung [21]. The cDNA encodes a protein of 270 (bovine) and 273 (human) amino acid residues, respectively, having no similarity to any of the known scavenger receptors. LOX-1 was shown to be expressed mainly in vascular endothelial cells, placenta and lung, but the function of LOX-1 in these cells remains uncertain.

The present study was undertaken to test whether LOX-1 is also expressed in macrophages, where it might function as a scavenger receptor.

EXPERIMENTAL

Lipoprotein preparation

Human LDL ($d = 1.019–1.063$) was isolated by preparative ultracentrifugation from fresh plasma collected in 1 mg/ml EDTA as described in [22]. Moderately oxidized LDL was prepared by incubating 2 mg/ml LDL with 2 μ M Cu²⁺ in EDTA-free PBS for 4 h at 37 °C. Fully oxidized LDL was prepared by incubating LDL (100 μ g/ml) in Ham's F-10 medium with 10 μ M Cu²⁺ for 24 h at 37 °C. The degree of oxidation was estimated

Abbreviations used: LDL, low-density lipoprotein; OxLDL, oxidized LDL; LOX-1, lectin-like OxLDL receptor; RT-PCR, reverse transcription PCR; TBARS, thiobarbituric acid-reactive substances; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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and moderately oxidized LDL contained about 10 nmol of thiobarbituric acid-reactive substances (TBARS)/mg of protein, estimated by a fluorimetric method [23], and displayed a relative electrophoretic mobility on agarose gel of 1.4 (native LDL, 1.0). Fully oxidized LDL contained about 55 nmol of TBARS/mg of protein and displayed a relative electrophoretic mobility of 3.3. Freshly isolated native LDL contained about 0.3 nmol of TBARS/mg of protein.

Cell culture

Human monocyte-derived THP-1 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 50 μ M β -mercaptoethanol, 100 μ g/ml streptomycin and 100 units/ml penicillin in a humidified cell incubator with 5 % CO₂ at 37 °C. THP-1 monocytes were differentiated by incubation with 100 nM PMA for 1–5 days. Human monocytes were isolated from freshly drawn blood by centrifugation through Nycoprep[®] (Nycomed Pharma AS), and plated with RPMI 1640 at 5×10^6 cells/60 mm culture dish. After 2 h of incubation, non-adherent cells were removed. To obtain differentiated macrophages, the freshly prepared monocytes were placed for 7 days in RPMI 1640 medium containing 10 % autologous serum and 100 μ g/ml of penicillin/streptomycin.

Analysis of LOX-1 expression by reverse transcription (RT) PCR

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure [24]. RNA was reverse transcribed using Superscript II (Life Technologies, Inc., Gaithersburg, MD, U.S.A.). The transcribed cDNA was then used for PCR amplification to estimate the expression of LOX-1, monocyte chemotactic protein-1 (MCP-1) receptor and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Two specific primers matching the published sequences [21,25,26] were used to identify and amplify LOX-1 (5'-GGCTGCTGCGACTCTAGG-3' and 5'-AGTGGGGCATCAAAGGAG-3'), MCP-1 receptor (5'-ATGCTGTCCACATCTCGTTCTCG-3' and 5'-TTATAAACCAGCCGAGACTTCCTG-3') and GAPDH (5'-TCGGAGTCAACGGATTGGTTCGTA-3' and 5'-ATGGAC-TGTGGTCATGAGTCCTTC-3'). The amplified transcripts were analysed by gel electrophoresis, and signal intensities of the bands with expected sizes (569 bp for LOX-1, 1083 bp for MCP-1 receptor, 521 bp for GAPDH) were quantified by densitometric scanning and compared with the internal standard GAPDH amplified under identical conditions. The identity of LOX-1 in monocytes was confirmed by DNA sequence analysis of full-length LOX-1 cDNA obtained by PCR amplification of reverse-transcribed RNA from THP-1 cells and human monocytes. A sequence comparison revealed that LOX-1 isolated from macrophages and endothelial cells was identical.

Cell membrane preparation and protein extraction

Cells were suspended in buffer A (50 mM Tris/HCl/150 mM NaCl/1 mM EDTA, pH 8) containing a cocktail of protease inhibitors, homogenized and, after removal of the crude nucleus membranes, were isolated by centrifugation at 100 000 *g* for 1 h. The pellet was re-suspended in buffer A, membrane proteins were extracted with 40 mM octyl glucoside (Sigma, St. Louis, MO, U.S.A.), separated by 12 % SDS/PAGE, transferred to nitrocellulose, and used for Western- and ligand-blotting experiments.

Generation of antibodies and Western-blot analysis

An antibody (LO-1) against a portion of the extracellular domain between amino acid residues 63 and 168 was generated. The corresponding cDNA fragment was amplified by PCR, subcloned into pGEX-2T vector (Pharmacia, Uppsala, Sweden), sequenced, and the protein was expressed in *Escherichia coli*. The fusion protein was isolated with glutathione-Sepharose 4B beads (Pharmacia) and used to generate antiserum in guinea pig. The antiserum LO-1 was used for Western-blot analysis in combination with ¹²⁵I-labelled goat anti-guinea pig IgG (Sigma).

Analysis of LOX-1 expression by immunocytochemistry and flow cytometry

For immunocytochemistry, the cells were fixed with 3 % paraformaldehyde, washed with PBS and incubated with LO-1 antiserum or pre-immune serum (both 1:20) for 1 h, followed by FITC-conjugated rabbit anti-guinea pig IgG. The washed cells were mounted on coverslips with mounting medium (Vectashield; Vector Laboratories Inc., Burlingame, CA, U.S.A.) and used for fluorescence microscopy (Microphot-FX; Nikon, Tokyo, Japan). For FACS analysis, 0.5×10^6 cells in 100 μ l of PBS, containing 0.1 % BSA and 0.01 % NaN₃, were incubated on ice for 20 min with either LO-1 or pre-immune serum (both 1:20), washed and incubated for another 20 min with 10 μ g/ml of FITC-conjugated rabbit anti-guinea pig IgG. After several washes the cells were immediately analysed by flow cytometry (Becton Dickinson, Mountain View, CA, U.S.A.).

Ligand blotting

Ligand-blotting analyses were performed as described previously [12,13]. Briefly, extracted membrane proteins were electrophoresed and transferred to nitrocellulose membranes as described above. Ligand blotting was carried out for 3 h at room temperature in buffer consisting of 50 mM Tris/HCl, 5 % BSA, 50 mM NaCl, 1 mM KI, 2 mM CaCl₂ (pH 8), using 10 μ g/ml OxLDL. Bound ligand was detected with mouse monoclonal IgG against human apolipoprotein B (MB47), a kind gift from L. Curtiss (Scripps Research Institute, La Jolla, CA, U.S.A.), followed by sheep anti-mouse IgG conjugated to alkaline phosphatase and the phosphatase substrates, 5-bromo-4-chloro-indoyl-3-yl phosphate and Nitro Blue Tetrazolium (Bio-Rad, Hercules, CA, U.S.A.).

RESULTS

We first tested for the expression of LOX-1 in monocytes and macrophages using RT-PCR. As shown in Figure 1, untreated THP-1 monocytes expressed only low levels of LOX-1 mRNA. Differentiation mediated by PMA induced a biphasic expression of LOX-1 message that reached a maximum at 48 h and then decreased. Cell differentiation was assessed by morphological changes and cell adhesion. THP-1 cells became adherent after 6 h of PMA treatment and after 24 h there was a change from the round shape of monocytes to spindle shapes typical of macrophages. The expression of GAPDH, included as an internal control, remained constant.

To demonstrate that LOX-1 expression was not peculiar to THP-1 monocytes, a line derived from human leukaemia cells, we isolated human blood monocytes and analysed LOX-1 expression in freshly isolated cells and then in cells that had been in culture for 8 days. As a marker for differentiation into macrophages, we used the MCP-1 receptor, which is expressed primarily in monocytes and T-lymphocytes but is absent in macrophages [27,28]. LOX-1 was not found in freshly isolated

Figure 1 Induction of LOX-1 expression during differentiation of THP-1 monocytes

THP-1 monocytes were differentiated into macrophages by incubation with PMA for the indicated times. Total RNA was isolated and LOX-1 transcripts were amplified by RT-PCR and estimated by agarose gel electrophoresis and staining with ethidium bromide as described in the Experimental section (upper panel). Primers were used to yield full-length LOX-1 cDNA of 822 bp. As internal standard and to control for equal loading, GAPDH was estimated under identical conditions (lower panel).

human blood monocytes but was expressed in mature macrophages (Figure 2), results similar to those in THP-1 cells. LOX-1 was not detected in lymphocytes and neutrophils.

To determine whether LOX-1 in macrophages and endothelial cells is transcribed from the same or different allele, we sequenced the full-length PCR products. The sequence comparison confirmed that the LOX-1 transcripts from human macrophages and endothelial cells were identical (results not shown).

To characterize the protein and to examine the surface expression, we generated an antiserum (LO-1) against a portion

Figure 3 Immunofluorescence staining of differentiated THP-1 macrophages

THP-1 monocytes were treated with 100 nM PMA for 3 days and fixed with paraformaldehyde. The expression of LOX-1 was determined using the antiserum LO-1 generated against the extracellular domain of LOX-1 (A), or the pre-immune serum (B), followed by FITC-conjugated secondary antibody.

of the putative extracellular domain of LOX-1 between amino acid residues 63 and 168. The antiserum specifically recognized LOX-1 protein on the plasma membrane of non-permeabilized intact THP-1 cells after differentiation induced by PMA (Figure 3). The surface expression of LOX-1 was further tested by flow

Figure 2 Expression of LOX-1 in human monocyte-derived macrophages

Human monocytes, monocyte-derived macrophages and a cell pool consisting of lymphocytes and neutrophils were prepared as described in the Experimental section. Total RNA was isolated and the transcripts for LOX-1 (left panel), GAPDH (middle panel) and MCP-1 receptor (right panel) were estimated by agarose gel electrophoresis as described in the Experimental section. Lane 1, human monocytes; lanes 2 and 3, human monocyte-derived macrophages; lane 4, lymphocytes and neutrophils.

Figure 4 FACS analysis of LOX-1 surface expression on intact THP-1 cells

Binding of anti-LOX-1 antiserum LO-1 (dashed line) or preimmune serum (unbroken line) to unstimulated THP-1 monocytes (A) or to THP-1 macrophages treated for 3 days with 100 nM PMA (B). The binding of the first antibody was determined using FITC-labelled secondary antibody.

cytometry. Untreated THP-1 monocytes showed minimal, if any, surface expression (Figure 4A). In contrast, PMA-treated THP-1 cells, which showed a large increase in LOX-1 transcript, clearly expressed LOX-1 protein on the plasma membrane (Figure 4B). Differentiation mediated by PMA resulted in a heterogeneous expression of LOX-1, and the presence of two cell populations with high and low levels of LOX-1 was suggested by the shape of the curves.

The functional role of LOX-1 was tested by Western- and ligand-blot analysis. Parallel with the increase in LOX-1 mRNA levels during differentiation, a single band with an estimated molecular mass of about 40 kDa was detected by Western-blot analysis in membrane extracts from PMA-treated THP-1 cells only, but not from untreated cells (Figure 5). This protein specifically recognized moderately oxidized LDL and this binding activity was absent in undifferentiated THP-1 monocytes. Neither fully oxidized LDL, containing about 55 nmol of TBARS/mg of protein, nor native LDL bound to LOX-1. This binding pattern was very similar to that reported for endothelial and lung LOX-1 [21]. The binding of moderately oxidized LDL was not inhibited by the antiserum (results not shown), indicating that the antibody does not block the ligand-binding site.

DISCUSSION

In this study we demonstrate that LOX-1 is expressed in macrophages and therefore may play a role as a scavenger receptor. Bovine and human LOX-1 were originally identified in vascular endothelial cells and in lung, respectively [21]. The bovine and human homologue show high sequence identity, but no relationship exists with any of the known macrophage scavenger receptors. LOX-1 binds, internalizes and degrades OxLDL, but it is uncertain whether this is its primary function in the vascular endothelium. The presence of several cytoplasmic phosphorylation sites implies a signalling function, and a recent report suggested the involvement of LOX-1 in endothelial dysfunction. The expression of LOX-1 was shown to be up-regulated in hypertensive rat aortas, which may be responsible

Figure 5 Western- and ligand-blot analyses of LOX-1 in THP-1 monocytes and macrophages

Proteins were extracted from membranes of unstimulated THP-1 monocytes and from THP-1 macrophages after 3 days of stimulation with 100 nM PMA, separated by SDS/PAGE, transferred to nitrocellulose and probed with either pre-immune serum or anti-LOX-1 antiserum LO-1. The ligand-binding activity was determined by ligand blotting with moderately oxidized LDL (10 nmol of TBARS/mg of protein), fully oxidized LDL (55 nmol of TBARS/mg of protein) and native LDL as described in the Experimental section. Lanes 1, 3 and 5, unstimulated THP-1 monocytes; lanes 2, 4, and 6–8, PMA-treated THP-1 macrophages. Arrow indicates LOX-1 protein.

for the reported OxLDL-induced decrease of endothelium-dependent vasodilatory responses [29,30].

The presence of LOX-1 in macrophages suggests an additional role as a scavenger receptor. LOX-1 was not found in circulating human blood monocytes or undifferentiated THP-1 cells, but was expressed in both cell types after differentiation, as with other scavenger receptors. The uptake of OxLDL by the macrophages recruited into the intima is mediated by scavenger receptors. Transfected Chinese hamster ovary cells expressing LOX-1 not only bound, but also internalized and degraded, moderately oxidized LDL [21], and LOX-1 may in this way contribute to the formation of macrophage-derived foam cells. The cDNA sequence of the macrophage LOX-1 was identical to that of endothelial LOX-1, suggesting that it is transcribed in both cell lines from the same gene.

The calculated mass of LOX-1 is about 31 kDa, but the apparent molecular mass estimated by Western-blot analysis of electrophoretically fractionated membrane extracts from human macrophages was about 40 kDa, suggesting that the protein is glycosylated. The estimated molecular mass of bovine LOX-1 was about 50 kDa. This difference may be in part the result of differences in glycosylation. The bovine protein contains four potential glycosylation sites, whereas only three are present in the human homologue. Further, the glycosylation pattern and chain length can vary depending on the cell type and thereby determine the apparent molecular mass of LOX-1 in endothelial cells and macrophages.

LOX-1 is structurally related to NKR-P1 of the C-type lectin family, a surface protein found on natural killer cells [31]. Although the physiological ligands have yet to be identified, it has been proposed that NKR-P1 may play a role in target-cell recognition and killer-cell activation [32]. It is possible that LOX-1 could have a similar role in target recognition by macrophages.

In summary, we have demonstrated that LOX-1 is not expressed in monocytes but is expressed in mature macrophages. The functional features of LOX-1 make it a candidate for an OxLDL receptor and scavenger receptor. Additional experiments are required to assess its physiological role *in vivo* and its relative importance as a scavenger receptor.

This work was supported by Grant HL56989-01 (SCOR in Molecular Medicine and Atherosclerosis) from the National Institutes of Health, U.S.A. H. Yoshida was supported in part by the National Defense Medical College, Saitama, Japan.

REFERENCES

- 1 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) *New Engl. J. Med.* **320**, 915–924

- 2 Gerrity, R. G. (1981) *Am. J. Pathol.* **103**, 181–190
- 3 Berliner, J. A., Navab, M., Fogelman, A. M., Frank, J. S., Demer, L. L., Edwards, P. A., Watson, A. D. and Lusis, A. J. (1995) *Circulation* **91**, 2488–2496
- 4 Brown, M. S. and Goldstein, J. L. (1983) *Annu. Rev. Biochem.* **52**, 223–261
- 5 Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337
- 6 Henriksen, T., Mahoney, E. M. and Steinberg, D. (1983) *Arteriosclerosis* **3**, 149–159
- 7 Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. (1990) *Nature (London)* **343**, 531–535
- 8 Sparrow, C. P., Parthasarathy, S. and Steinberg, D. (1989) *J. Biol. Chem.* **264**, 2599–2604
- 9 Loughheed, M., Lum, C. M., Ling, W. H., Suzuki, H., Kodama, T. and Steinbrecher, U. (1997) *J. Biol. Chem.* **272**, 12938–12944
- 10 Endemann, G., Stanton, L. W., Madden, K. S., Bryant, C. M., White, R. T. and Protter, A. A. (1993) *J. Biol. Chem.* **268**, 11811–11816
- 11 Nozaki, S., Kashiwagi, H., Yamashita, S., Nakagawa, T., Kostner, B., Tomiyama, Y., Nakata, A., Ishigami, M., Miyagawa, J. I., Kameda-Takemura, K., Kurata, Y. and Matsuzawa, Y. (1995) *J. Clin. Invest.* **96**, 1859–1865
- 12 Ramprasad, M. P., Fischer, W., Witztum, J. L., Sambrano, G. R., Quehenberger, O. and Steinberg, D. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9580–9584
- 13 Ramprasad, M. P., Terpstra, V., Kondratenko, N., Quehenberger, O. and Steinberg, D. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14833–14838
- 14 Van Velzen, A. G., da Silva, R. P., Gordon, S. and Van Berkel, T. J. C. (1997) *Biochem. J.* **322**, 411–415
- 15 Acton, S. L., Scherer, P. E., Lodish, H. F. and Krieger, M. (1994) *J. Biol. Chem.* **269**, 21003–21009
- 16 Murao, K., Terpstra, V., Green, S. R., Kondratenko, N., Steinberg, D. and Quehenberger, O. (1997) *J. Biol. Chem.* **272**, 17551–17557
- 17 Pearson, A. M. (1996) *Curr. Opin. Immunol.* **8**, 20–28
- 18 Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T. et al. (1997) *Nature (London)* **386**, 292–296
- 19 Geng, Y., Kodama, T. and Hansson, G. K. (1994) *Arterioscler. Thromb.* **14**, 798–806
- 20 Yoshida, H., Quehenberger, O., Kondratenko, N., Green, S. and Steinberg, D. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18**, 794–802
- 21 Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T. and Masaki, T. (1997) *Nature (London)* **386**, 73–77
- 22 Havel, R. J., Eder, H. A. and Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
- 23 Conti, M., Morand, P. C., Levillain, P. and Lemonnier, A. (1991) *Clin. Chem.* **37**, 1273–1275
- 24 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 25 Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J. and Coughlin, S. R. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2752–2756
- 26 Tokunaga, K., Nakamura, Y., Sakata, K., Fujimori, K., Ohkubo, M., Sawada, K. and Sakiyama, S. (1987) *Cancer Res.* **47**, 5616–5619
- 27 Tangirala, R. K., Murao, K. and Quehenberger, O. (1997) *J. Biol. Chem.* **272**, 8050–8056
- 28 Mackay, C. R. (1996) *J. Exp. Med.* **184**, 799–802
- 29 Nagase, M., Hirose, S., Sawamura, T., Masaki, T. and Fujita, T. (1997) *Biochem. Biophys. Res. Commun.* **237**, 496–498
- 30 Nagase, M., Hirose, S. and Fujita, T. (1998) *Biochem. J.* **330**, 1417–1422
- 31 Giorda, R., Rudert, W. A., Vavassori, C., Chambers, W. H., Hiserodt, J. C. and Trucco, M. (1990) *Science* **249**, 1298–1300
- 32 Ryan, J. C. and Seaman, W. E. (1997) *Immunol. Rev.* **155**, 79–89