Expression of lectin-like oxidized low density lipoprotein receptor-1 in human and murine macrophages: upregulated expression by TNF-α

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Abstract Uptake of oxidized low density lipoprotein (Ox-LDL) and subsequent foam cell transformation have been implicated in early atherogenesis. Although multiple molecules, including class A and B scavenger receptors, have been identified as Ox-LDL receptors, additional receptors may also be involved in this process. Here, we provide evidence that lectin-like Ox-LDL receptor-1 (LOX-1), a novel Ox-LDL receptor initially identified in vascular endothelial cells, is also expressed in macrophages in humans and mice. Expression of LOX-1 can be induced after macrophage-like differentiation in vitro in human peripheral blood monocytes and the related cell line THP-1 cells. Furthermore, LOX-1 expression can also be detected in resident peritoneal macrophages, and can be upregulated by an inflammatory cytokine TNF-α. These results suggest that LOX-1 in macrophages may play an important role in Ox-LDL uptake and subsequent foam cell formation in this cell type.

Key words: LOX-1; Oxidized low density lipoprotein; Macrophage; Tumor necrosis factor α; Atherosclerosis

1. Introduction

Accumulation of cholesterol-loaded foam cells derived from a monocyte-macrophage lineage in the arterial intima appears to be a hallmark and a key event in early atherogenesis [1]. Several lines of evidence suggested that oxidatively modified low density lipoprotein (Ox-LDL) may play crucial roles in atherogenesis. Ox-LDL can be avidly taken up by macrophages by receptor-mediated endocytosis, which subsequently transform them into foam cells [2–4]. Several different molecules, such as class A macrophage scavenger receptors [5,6], MARCO [7], Fc-γ RII [8], CD36 [9], scavenger receptor class B type I (SR-BI) [10] and CD68 (macrosialin) [11], have been identified as cell-surface receptors for atherogenic Ox-LDL; however, additional molecules specific for Ox-LDL may also be involved in Ox-LDL uptake and subsequent foam cell transformation in macrophages [12–15].

Recent studies in our laboratory have identified a novel class of Ox-LDL receptor, which has been designated lectin-like Ox-LDL receptor-1 (LOX-1) in vascular endothelial cells [16]. LOX-1 is a type II membrane glycoprotein with approximate molecular mass of 50 kDa. LOX-1 can bind, internalize, and proteolytically degrade Ox-LDL, but not significant amounts of acetylated LDL [17]. Expression of LOX-1 can be upregulated by tumor necrosis factor α (TNF-α), phorbol 12-myristate 13-acetate (PMA), and fluid shear stress, suggesting that LOX-1 expression in vivo in vascular endothelium may also be dynamically regulated by inflammatory and fluid mechanical stimuli [18,19].

In the present study, we explored whether LOX-1, a novel receptor for Ox-LDL identified in vascular endothelial cells, is also expressed in macrophages.

2. Materials and methods

2.1. Materials

RPMI-1640 medium and DMEM medium were obtained from Nissui, Japan. Fetal bovine serum (FBS) was purchased from Irvine Scientific, Santa Ana, CA. PMA was obtained from Sigma. Recombinant human TNF-α was purchased from Boehringer Mannheim. Mouse anti-bovine LOX-1 monoclonal antibody 5-2 (mAb 5-2; hybridoma supernatants) was prepared by immunization with a recombinant bovine LOX-1 extracellular domain produced by *Escherichia coli* as previously described. Rabbit anti-mouse LOX-1 polyclonal antibody (2-1) was prepared by immunization with synthetic peptides of mouse LOX-1 extracellular domain (amino acids 237–257). Murine anti-human CD68 monoclonal antibody (KP1) was purchased from Dako. Non-immune mouse IgG fraction was obtained from Funakoshi, Japan.

2.2. Cells

Human peripheral blood mononuclear cells were freshly isolated from blood of healthy volunteers by Ficoll density gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway), as well as buffy coats kindly provided by the Kyoto Red Cross Blood Center. Mononuclear cells were suspended in RPMI 1640 supplemented with 20% (v/v) autologous human serum. After 3 h of incubation, non-adherent cells were removed by washing with phosphate-buffered saline (PBS, 150 mM Nacl, 10 mM phosphate buffer, pH 7.4). Adherent cells were cultured as monocytes until used for experiments. Human monocytic cell line THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS. THP-1 cells were differentiated into adherent macrophages by treatment with $3–30\times10^{-8}$ M PMA for 3–4 days [20]. Mouse peritoneal macrophages were harvested from peritoneal lavage of female DDY mice which had been injected intraperitoneally with 2 ml of 3% thioglycollate broth (Difco) in PBS. Cells were suspended in DMEM containing 10% FBS at a density of 3×10^6 cells/ml and allowed to adhere for 3 h incubation. The cells were then washed with PBS to remove non-adherent cells and incubated overnight.

2.3. Northern blot analysis

10 μg of total cellular RNA extracted from cells using RNAzol B (Tel-Test, Friendswood, TX) was fractionated in formaldehyde-agarose (1.2%) gels and transferred onto maximum strength Nylon membranes (Schleicher and Schuell, Keene, NH). Membranes were hybridized with a 0.8 kb *Eco*RI fragment of human LOX-1 cDNA or a 0.8 kb *Bam*HI-*Hin*dIII fragment of mouse LOX-1 cDNA which had been labeled with [α-^{32}P]dCTP (Dupont-New England Nuclear) by random hexanuclide primers (DNA labeling Kit; Pharmacia). Blots were subsequently hybridized with radiolabeled human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA to control amounts of RNA

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loaded. Intensities of bands were quantified by BAS 2000 image analyser (Fuji Film, Japan)

2.4. Immunocytochemistry

Cells cultured in chamber slides were fixed with acetone/methanol (1:1) at −20°C for 5 min. After incubation with 0.1% BSA-PBS containing 2% horse serum for 30 min, cells were incubated with an anti-LOX-1 monoclonal antibody, an anti-human CD68 monoclonal antibody or non-immune mouse IgG for 20 min. Concentrations of the primary antibodies were optimized by preliminary experiments. After washing with 0.1% BSA-PBS three times, cells were incubated with biotinylated secondary antibodies (horse anti-mouse IgG) for 30 min and washed with 0.1% BSA-PBS twice and with PBS once. Endogenous peroxidase activity was then blocked by incubation with methanol containing 0.3% hydrogen peroxide for 30 min, after which avidin-biotin peroxidase complexes (ABC Elite kit, Vector Labs, Burlingame, CA) were added. Antibody binding was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB substrate kit, Vector Labs, Burlingame CA), and cells were then counterstained with Gill's hematoxylin.

2.5. Immunoblot analysis

Scraped cells were lysed in homogenization solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 μg/ml leupeptin, 1 mM aprotinin), and lysates were passed through 26-gauge needles 10 times. To prepare solubilized membrane proteins, lysates were centrifuged at 50 000 rpm at Beckman TLA 100.3 rotor for 30 min at 4°C. A fifth volume of 5×SDS sample buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.02% bromophenol blue) was added to the supernatants. After heating at 98°C for 5 min, samples were subjected to SDS-polyacrylamide gels (10% or 4–20% gradients) electrophoresis and electrotransferred onto PVDF membranes (PolyScreen, Dupont) using a semidry electrotransfer apparatus (Bio-Rad). After blocking non-specific binding with milk for 2 h, membranes were rinsed with TBS/0.1% Tween 20, incubated with anti-mouse LOX-1 polyclonal Ab 2-1 at a 1:500 dilution for 2 h and then washed three times. Protein-antibody complexes were detected using the Enhanced Chemiluminescence kit (Amersham). Protein concentration was determined with the Bradford reagent (BRL).

3. Results and discussion

Accumulation of macrophage-derived foam cells in arterial intima appears to be a hallmark of early atherogenesis. Although previous studies by several investigators have identified several molecules as receptors for Ox-LDL in macrophages, additional molecules may also been involved in foam cell transformation in this cell type. LOX-1 is a cell

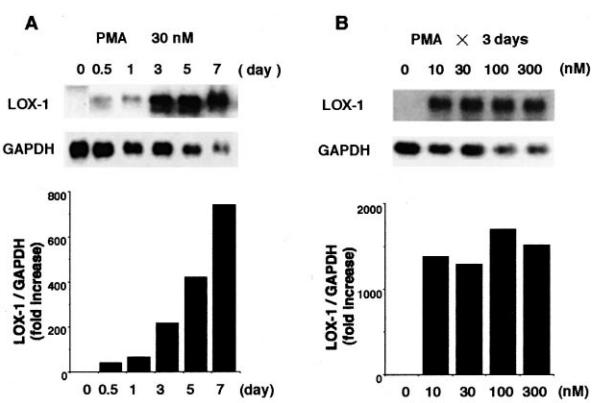

Fig. 1. Expression of LOX-1 mRNA during differentiation of THP-1 cells by PMA. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS in the presence of PMA. A: After incubation with 30 nM PMA for 12 h, 1, 3, 5, or 7 days, total RNA was isolated and subjected to Northern blot analysis. B: After incubation with the indicated concentrations of PMA for 3 days, total RNA was isolated and subjected to Northern blot analysis.

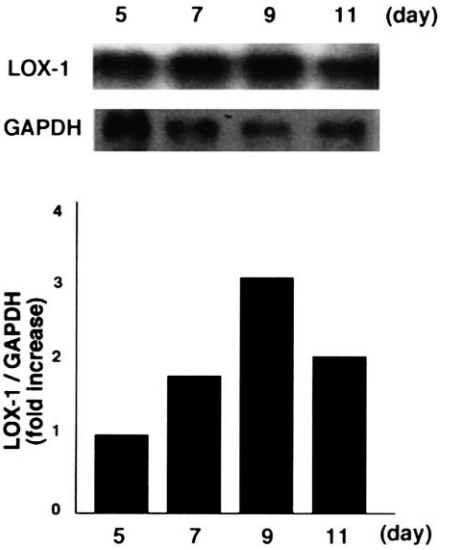

Fig. 2. Expression of LOX-1 mRNA in human monocyte-derived macrophages. Human peripheral blood monocytes were differentiated into macrophages by culturing in RPMI 1640 containing 20% autologous serum for the indicated time periods. Total cellular RNA was isolated on days 5, 7, 9, 11 and subsequently subjected to Northern blot analysis.

surface molecules identified as a receptor for Ox-LDL in vascular endothelial cells. In the present study, therefore, we sought to define to whether LOX-1 is also expressed in macrophages.

The human monocytic cell line THP-1 has been shown to be differentiated into macrophage-like cells after treatment with PMA [20,21]. Therefore, THP-1 cells were treated with PMA (30 nM) for the indicated time periods, and Northern blotting was performed to evaluate LOX-1 mRNA expression. As shown in Fig. 1A, LOX-1 mRNA was not detectable in untreated THP-1 cells. In PMA-treated THP-1 cells, in contrast, LOX-1 mRNA expression was time-dependently induced. LOX-1 mRNA was detectable as early as 12 h after PMA treatment, and remained expressed for at least 7 days. Fig. 1B shows concentration dependence in PMA-induced LOX-1 expression. PMA concentrations as low as 10 nM can induce expression of LOX-1 mRNA after 3 days (Fig. 1B). These results appear to be in parallel to the previous report which showed time- and concentration-dependent induction of class A scavenger receptors in PMA-treated THP-1 cells [20].

We further explored expression of LOX-1 mRNA in human monocyte-derived macrophages. Human peripheral blood monocytes were differentiated into macrophages after in vitro culture for several days, and Northern blot analysis was performed [22]. As shown in Fig. 2, significant expression of LOX-1 mRNA was found in these monocyte-derived macrophages. In freshly isolated human peripheral monocytes before cell culture LOX-1 mRNA expression was not detectable by RT-PCR analysis (data not shown). These results appear to be in parallel with those found in THP-1 cells.

To examine whether LOX-1 protein is also expressed in macrophages, immunocytochemistry using an anti-LOX-1 antibody was carried out. THP-1 cells were subjected to macrophage-like differentiation by PMA treatment for 3 days. Human monocyte-derived macrophages were obtained after in vitro cell culture for 10 days. In these cells that had under-

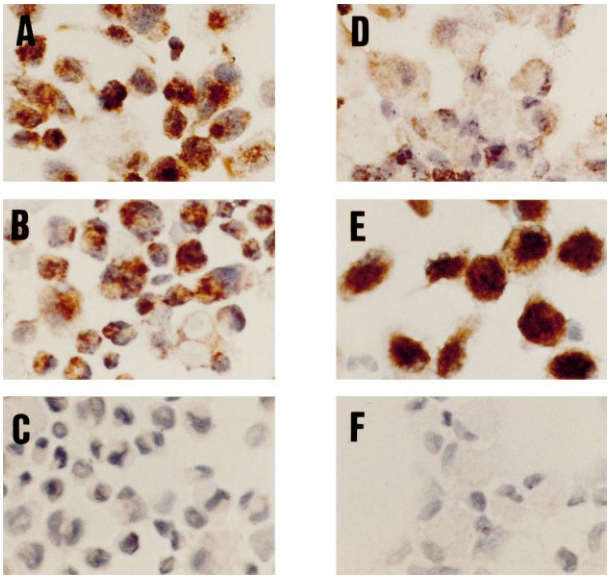

Fig. 3. Immunostaining for LOX-1 in differentiated THP-1 cells and human monocyte-derived macrophages. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS in the presence of 30 nM PMA for 3 days (A–C). Human peripheral blood monocytes were differentiated into macrophages by in vitro cell culture in RPMI 1640 containing 20% autologous serum for 10 days (D–F). Cells were then fixed with methanol/acetone and immunostaining was carried out using a mouse anti-LOX-1 mAb (A,D), mouse anti-CD68 mAb (B,E) or non-immune mouse IgG (C,F). Cells were counterstained with Gill's hematoxylin. ×400

gone macrophage-like differentiation, significant expression of LOX-1 as well as CD68, a marker for macrophages, was observed by immunostaining (Fig. 3).

In addition, murine peritoneal macrophages were harvested by peritoneal lavage, and expression of LOX-1 was evaluated. As shown in Fig. 4, expression of LOX-1 mRNA was detectable in murine peritoneal macrophages. Treatment with the inflammatory cytokine TNF-α significantly upregulated expression of LOX-1 mRNA in a time- and concentration-dependent manner. The upregulation of LOX-1 expression appears to be in parallel with our previous findings that LOX-1 expression can be upregulated by TNF-α in cultured vascular endothelial cells [18]. Immunoblot analysis also provided evidence that LOX-1 protein is similarly induced by TNF-α treatment for 24 h (Fig. 5).

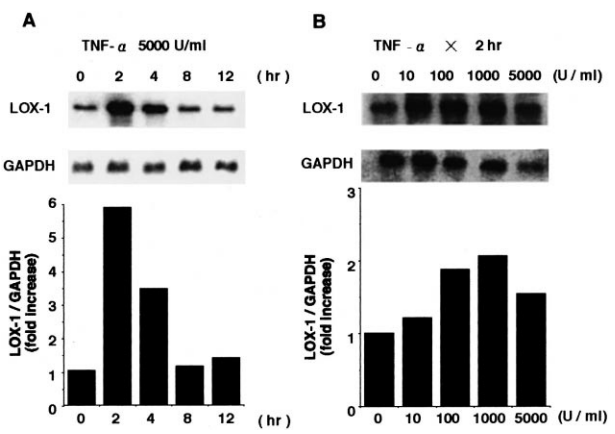

Fig. 4. Upregulated expression of LOX-1 mRNA by TNF-α in mouse peritoneal macrophages. After mouse peritoneal monocytes were cultured in DMEM supplemented with 10% FBS overnight, cells were incubated in the presence or absence of TNF-α. A: After stimulation with 5000 U/ml of TNF-α for 2, 4, 8, or 12 h, total RNA was isolated and subjected to Northern blot analysis. B: After stimulation with 10, 100, 1000, or 5000 U/ml of TNF-α for 2 h, total RNA was isolated and subjected to Northern blot analysis.

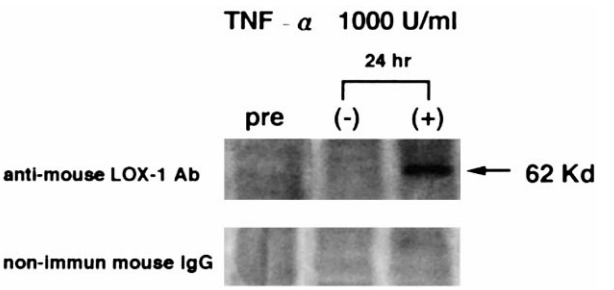

Fig. 5. Upregulated expression of LOX-1 by TNF-α in mouse peritoneal macrophages. After mouse peritoneal monocytes were cultured in DMEM supplemented with 10% FBS overnight, cells were incubated in the presence or absence of TNF-α. After 24 h, membrane protein was purified and subjected to Western blot analysis as described in Section 2. Anti-mouse LOX-1 polyclonal Ab was used at 1:250 dilution as first Ab. Non-immune mouse IgG was used at 10 μg/ml.

Uptake of Ox-LDL in macrophages and subsequent foam cell transformation appear to play key roles in atherogenesis. The present study provides evidence that LOX-1, a novel receptor for Ox-LDL which was originally identified in vascular endothelial cells, is also expressed in macrophages. Expression of LOX-1 in macrophages is also upregulated by the inflammatory cytokine TNF-α. Interestingly, expression of class A scavenger receptors has been shown to be downregulated by TNF-α in macrophages [23,24]. Since production of TNF-α in atherosclerotic lesions has been reported, LOX-1 may play more important roles in atherosclerotic lesions with inflammatory responses [25–27].

Our previous report has also shown that LOX-1 can support binding and engulfment of aged red blood cells and apoptotic cells [28]. LOX-1 in macrophages, therefore, may play important roles in the removal of these deteriorated cells.

In summary, this report provides evidence that LOX-1 is expressed in macrophages and upregulated by an inflammatory stimulus. Further studies relating to pathophysiological roles of LOX-1 in macrophages and other cell types in vivo may provide new insights into atherogenesis as well as inflammatory diseases.

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